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I. MILK—ITS MILK SUGAR, CONDUCTIVITY AND DEPRESSION OF FREEZING POINT.

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(*Received Nov. 18th, 1913.*)

In this paper the results of the application of measurements of the electrical conductivity, percentage of milk sugar and depression of freezing point will be discussed chiefly from the standpoint of the physiology of milk.

In fact the measurements were obtained rather as the basis from which to draw certain deductions and interpretations, than as data for the identification and characterisation of different milks.

This latter work has already been accomplished and it is only necessary to give a brief survey of it in so far as it bears upon the subject matter of this paper.

Depression of freezing point.

The average depression of freezing point of cows' milk as found by 10 independent workers [Koeppe, Sommerfeld, 1909, p. 147] lies between 0·552° and 0·572°. In a recent paper Pins [1912] gives results of a large number of determinations of the depression of freezing point of the milk of 40 different cows, in which he finds that the largest proportion of the values obtained lie between 0·556° and 0·560°.

In the milk of sick animals higher values are often obtained [Schnorf, 1905, Pins, 1912].

The constancy of the depression of freezing point in normal milk is insisted upon by Guiraud and Lasserre [1904]. Parmentier [1903] points to the constancy of Δ even in milk from inflamed quarters of the udder. Schnorf [1905] draws attention to the constancy of the conditions determining the osmotic pressure of the milks from different quarters of the udder, and notes that the two fore-quarters and two hind-quarters go together.

H. Dreser [1892], in discussing the osmotic condition of different body fluids, lays stress on the closeness of the depression of freezing point of milk ($\Delta = 0{\cdot}57$) to that of the blood ($\Delta = 0{\cdot}58$ to $0{\cdot}59$). This osmotic equilibrium between the blood and milk is again emphasised by J. Winter [1895].

Rennet coagulation of milk has no effect on the depression of freezing point according to Hotz [1902] but boiling the milk slightly diminishes it.

The depression of freezing point of human milk shows greater individual variations than that of cows' milk, and Koeppe believes this to be due to variations in the salt content of the food. Strauss [1900] however denies that alterations in the food affect the depression of freezing point of the milk.

The following values have been given for human milk:

Koeppe	Δ 0·495 to 0·630
Barthe [1904]	Δ 0·590 to 0·610
Villejean [1905]	Δ 0·535 to 0·615
Grassi [1906]	Δ 0·540 to 0·740

Electrical conductivity.

In contrast with the relative constancy of the depression of freezing point of the milk of mammals, the specific conductivity shows greater variations, although under normal conditions the value for the conductivity in any one species does not vary to any large extent.

The following averages are given by different authors:

Cows' milk.	
Koeppe [Sommerfeld, 1909, p. 149]	K at 25° C. = 0·0043 – 0·0056
Lehnert [1897] and Koeppe [1898]	,, ,, = 0·00487 – 0·00551
Binaghi [1910]	,, ,, = 0·00494 – 0·00517
Schnorf [1905]	,, ,, = 0·00485
Goats' milk.	
Binaghi [1910]	,, ,, = 0·00470 – 0·00499
Human milk.	K at 18° C. = 0·00149 – 0·00843

Friedrich Petersen [1904], examining cows' milk, notes that the first drawn portions have a lower electrical resistance (higher conductivity) than the last drawn portions.

He notes that the resistance diminishes (i.e. conductivity increases) at the end of lactation. Also, in colostrum the resistance is less. He finds no proportionality between specific gravity and electrical resistance or total solids and resistance, but mentions a general proportionality to the ash though there is no direct relationship.

H. Hotz [1902] notes that milk whey has a higher conductivity than milk itself, due to removal of the caseinogen. C. Schnorf in his book

mentions a rise of 10–17 % in the conductivity when the caseinogen is removed by rennet. The caseinogen mechanically obstructs the carriage of electricity by the moving ions.

Differences in the conductivity of milk from different quarters are assigned to different amounts of non-electrolytes.

In small milk yields the conductivity is higher, and he notes the high conductivity of colostrum except in the very early stages, where the large amount of protein depresses the conductivity.

He also mentions the uniformly high values obtained for the conductivity in udder inflammations.

A paper by Bugarsky and Tangl [1898] will be referred to later on in connection with the effect of protein upon conductivity. They find 1 gram of protein per litre to depress the conductivity 0·25 %. The experiments were conducted:

(*a*) By dialysing blood serum for two months till free from salts, and using this serum concentrated as a means of adding different amounts of protein to 0·8 % salt solution.

(*b*) By dialysing 15 to 25 c.c. blood serum against 150 to 250 c.c. water until equilibrium was established and then noting the higher conductivity of the fluid outside the dialysing tube as compared with that inside the tube, and estimating the percentage of protein in the latter fluid.

Milk sugar.

The sugar from the milk of the cow, sheep, goat, mare, ass, dog and woman is lactose [Déniges, Bonmartini, Sommerfeld, 1909, p. 193]. The existence of a different sugar has from time to time been assumed owing to discrepancies between the reduction and polarimetric methods of estimation.

Scheibe [1901] has however shown that making due allowance for errors in both methods the results obtained do not differ. Consequently the conclusions of Schmöger, V. Raumer and Spath, and Landolph [Sommerfeld, 1909, p. 194] that another sugar is present in addition to lactose are unfounded. That the milk sugar is diminished in amount in milk from inflamed quarters of the udder is well known [J. Bongert, Sommerfeld, 1909, p. 555].

Methods employed.

Depression of freezing point. The Beckmann apparatus was used with an additional outer jacket and a mechanical stirrer. The determinations were made upon milk samples from which the greater part of the fat had been

removed by centrifugalisation. The experimental work was carried out as advised in a paper by Dekhuyzen.

Electrical conductivity. Kohlrausch's method was employed. It is very simple and can be very quickly carried out. For these reasons it is specially suited to the examination of milk.

The Wheatstone's bridge consisted of a wire wound on a cylindrical drum, with a sliding contact, operated by revolving the drum. The electrodes of the conductivity cell were replatinised at intervals and the cell standardised from time to time with a specially prepared solution of potassium chloride. All determinations were made at exactly 25° C. For all the measurements to be quoted in the present paper the cream was separated from the milk before the determinations were made.

Milk Sugar. The method selected was to determine the optical rotation of the filtrate after precipitating the milk proteins with Wiley's acid mercuric nitrate solution. This solution is made by dissolving mercury in twice its weight of nitric acid of specific gravity 1·42 and adding an equal volume of water after solution is completed.

In the case of cows' milk 3 c.c. of the reagent are necessary per 100 c.c. of milk, but in the milk of smaller animals with 7 % protein or more 6 or 7 c.c. are necessary. It had to be assumed that the volume of precipitate formed was equivalent to the volume of Wiley's reagent added. Undoubtedly this assumption was not always correct and therefore slight experimental errors are present in the estimations, and milks from different species of animals do not show strictly comparable sugar determinations. This criticism does not, however, apply to the milks of the same animal, and here the sugar estimations are relatively to one another highly accurate.

Milk samples always had the fat removed before being used, and in the case of small amounts of milk very rich in protein (cat and dog) the samples were diluted with an equal volume of water before being precipitated by the Wiley's reagent. A Schmidt and Haensch triple field polarimeter was the instrument employed for taking the rotations.

The inverse proportionality of milk sugar and salts.

The substances in milk which are chiefly responsible for its osmotic pressure are the milk sugar and soluble salts. The osmotic pressure of milk is, however, dictated by the osmotic condition of the blood, or in other words the conditions governing the osmotic pressure of the milk (as measured by the depression of freezing point) are to be sought in a fluid of fairly uniform

composition, whose osmotic condition is kept constant by the action of the kidneys.

This explains the constancy of the depression of freezing point in milk, and it follows that the substances in milk chiefly responsible for this physical manifestation cannot vary independently, but must be inter-related.

If the milk sugar is high the salts must be low, otherwise the osmotic pressure would be unusually high, and conversely, if the milk sugar is low the salts must be high or the osmotic pressure would be lower than normal.

Tables giving collected analyses of the milk of different species of animals, such as the following table quoted from Droop Richmond's *Dairy Chemistry*, do, as a rough generalisation, show an inverse proportionality between milk sugar and salts.

Animal	Sugar	Ash
Cow	4·75	0·75
Goat	4·22	0·76
Ewe	4·28	0·97
Buffalo	4·7	0·90
Woman	6·8	0·20
Mare	6·89	0·30
Ass	6·50	0·46
Mule	4·80	0·38
Bitch	3·09	0·73
Cat	4·91	0·58
Rabbit	1·95	2·56
Llama	5·60	0·80

That the relationship is not exhibited in a closer degree than is shown by these tables is due to the fact that the analyses of ash give no information as to the relative amounts of soluble and insoluble salts. In cows' milk, for example, much of the calcium phosphate (probably two-thirds of it) is in a state of colloidal suspension. This practically contributes nothing to the osmotic pressure and so should be deducted from the ash analyses to derive an estimate of the soluble salts. It is not, then, the total ash or salt content of the milk which is to vary inversely to the milk sugar, but the soluble ash, which alone appreciably contributes to the osmotic pressure.

The insoluble salts may be regarded as inert and it may be pointed out that their presence is probably a device of nature to get beyond the limitations imposed by the necessity of adjusting the osmotic pressure of the milk upon that of the blood. Colloidal calcium phosphate permits of a high calcium content of cows' milk without unduly reducing other soluble constituents such as the sugar and salts of sodium and potassium.

Undoubtedly also the limitation of an adjusted osmotic pressure has led to the fixing of a disaccharide as the sugar of the milk, which weight for

weight has only half the osmotic effect of the monosaccharide, dextrose, present in the blood.

The sugar and soluble ash then should vary inversely in any two milks whose salts are the same in character and whose osmotic pressures are equal.

Now in the case of soluble salts their osmotic effects are dependent upon their ionisation, and as the electrical conductivity of a salt solution is dependent upon the same factor, we have in the conductivity of milk a very close indication of the osmotic value of the soluble milk salts.

Putting this reasoning to the test of experiment it ought to be shown that conductivity and milk sugar vary inversely.

There are of course disturbing factors, some of which will be discussed later, but whereas it is only a very rough generalisation that ash and sugar are reciprocating, it can be shown that the inverse relationship between sugar and electrical conductivity is practically a law.

It is seen best of all in milk from one animal taken at the same milking, but from different teats. In the case of a cow it is possible to obtain four different milks from the four distinct quarters of the udder. If obtained at the one milking they will have within the limits of experimental error the same depressions of freezing point.

Here are the figures for the milk sugar and conductivity as estimated in the milk after separation of the fat.

TABLE I.

Connection between the conductivity of milk and the percentage of lactose.

Milk from the four different quarters of the cow's udder.

	Quarter	K	Percentage of lactose
Series I	Left anterior	0·00596	5·41
	Right ,,	0·00613	5·30
	Left posterior	0·00630	5·20
	Right ,,	0·00641	5·13
Series II	Left anterior	0·00500	5·70
	Right ,,	0·00502	5·68
	Left posterior	0·00506	5·61
	Right ,,	0·00512	5·58
Series III	Left anterior Right ,,	0·00527	5·74
	Left posterior Right ,,	0·00531	5·68
Series IV	Left anterior	0·00544	5·73
	Right ,,	0·00545	5·64
	Left posterior	0·00557	5·55
	Right ,,	0·00555	5·59

It is seen that milk sugar and conductivity vary inversely, that in every series the milk from the anterior quarters has more milk sugar than the milk from the posterior quarters but a lower conductivity.

The same relationship is also shown in the case of human milk.

TABLE II.

Human milk.

Individual	*K*	Percentage lactose	Remarks
A	0·00259	6·85	Left breast.
	0·00263	6·24	Right ,,
B	0·00290	5·18	Left ,,
	0·00262	5·41	Right ,,

Note. These samples of milk were obtained from women who for forty-eight hours previously had practised nursing their baby from both breasts. Two breast pumps were used and applied simultaneously one on each breast. We found that the practice at the Women's Hospital, Melbourne (from which institution most of our milk samples were obtained), was to nurse from only one breast at a time, saving the other breast for the next meal. This quite prevented samples taken simultaneously from right and left breasts showing the reciprocity of the above table, for the milk in each breast is not then secreted under comparable conditions of the blood.

In subsequent tables (see pp. 11, 12, 13) showing the composition of the milk of various species of animal, the sow, ass, mare, llama and goat, the inverse proportionality between conductivity and sugar is generally in evidence.

It has been our universal experience (which is in agreement with that of Guiraud and Lasserre, Parmentier and others) that the depression of freezing point of abnormal milk is not different from that of normal milk. One mammary gland may be giving a pathological fluid without the slightest resemblance to true milk yet with an identical depression of freezing point to the normal milk from the other gland (goat) or other quarters of the udder (cow).

In comparing the milk from an abnormal and normal quarter in the same animal the inverse proportionality of conductivity and sugar should be very apparent.

The method employed to disturb the secretion of one of the quarters was to return normal milk, guarding carefully against outside bacterial infection, and taking precautions to maintain the re-injected milk at body temperature.

Table III gives the results of two such experiments, and the figures showing the gradual recovery of the experimental quarter bear out the inverse relationship of milk sugar content and conductivity value very forcibly.

From the records below it will be seen that the pathological milk shows at first a high conductivity and a very low percentage of milk sugar, but as recovery of the quarter takes place, the conductivity gradually becomes lower, and the sugar percentage rises again towards its normal figure.

In Experiment II the ash in a sample of milk from the affected left anterior quarter was compared with the ash from the corresponding normal quarter—the right anterior quarter.

It will be seen that though the total ash is only slightly higher in the pathological milk, the difference between the proportions of soluble and insoluble ash is most marked as compared with the distribution in the ash of normal milk.

TABLE III.

Experiment I. 23/2/12.

On Friday morning February 23, 1912, 300 c.c. of milk were milked from the left anterior quarter of the cow's udder.

A silver teat cannula was then inserted and another 400 c.c. drawn off into a bulb condenser as receiving vessel. Water at 40° was flowing through the outer jacket of the condenser. The cannula was connected to the condenser by spiral flexible metal tubing wrapped in cotton wool. The whole apparatus had been carefully sterilised prior to use in an autoclave.

By raising the condenser approximately 350 c.c. of the 400 c.c. withdrawn were made to flow gently back into the same quarter of the udder, the cannula never having been withdrawn.

This operation is a sure method of obtaining a temporary upset in the quarter of the udder so handled, the other quarters remaining unaffected.

	Quarter	K	Percentage of lactose
23/2/12 (morning)	**Left anterior**	**0·00557**	**5·52**
	Right posterior	0·00566	5·52
23/2/12 (afternoon)	**Left anterior**	**0·01259**	**0·65**
	Right anterior	0·00581	5·65
	Left posterior	0·00589	5·57
	Right posterior	0·00592	5·62
24/2/12 (afternoon)	**Left anterior**	**0·00718**	
25/2/12 (morning)	**Left anterior**	**0·00707**	**4·13**
	Right anterior	0·00567	5·37
26/2/12 (morning)	**Left anterior**	**0·00635**	**4·40**
	Mixed sample from the 3 other quarters	0·00531	5·30
27/2/12 (morning)	**Left anterior**	**0·00612**	**5·41**
	Right anterior	0·00525	5·90
	Left posterior	0·00542	5·85
	Right posterior	0·00539	5·87
28/2/12 (morning)	**Left anterior**	**0·00579**	**5·11**
	Mixed sample from the 3 other quarters	0·00539	5·33

Note. The experimental quarter is indicated by heavy type.

Experiment II. 5/3/12.

On Tuesday morning March 5th, 1912, about 200 c.c. of milk were milked from the left anterior quarter and then 300 c.c. were withdrawn through a sterilised teat cannula into a bulb condenser as in Experiment I.

Of these 300 c.c. approximately 250 c.c. were returned. The whole proceeding was neatly and cleanly carried out. The disturbance which followed was profound.

	Quarter	K	% Lactose	Δ	% Sol. ash	% Insol. ash
5/3/12 (evening)	**Left anterior**	**0·0114**	**1·50**	**0·580**	**0·615**	**0·44**
	Right anterior	0·00569	5·40	0·575	0·285	0·625
6/3/12 (morning)	Not enough milk obtainable from left anterior quarter to take either conductivity or rotation.					
7/3/12 (morning)	**Left anterior**	**0·00734**	**2·86**	—	—	—
	Right anterior	0·00535	5·72	—	—	—
8/3/12 (morning)	**Left anterior**	**0·00699**	**4·18**	**0·545**	**0·352**	**0·585**
	Right anterior	0·00535	5·36	0·543	0·250	0·617
	Left posterior	0·00536	5·32	0·540	—	—
	Right posterior	0·00536	—	0·545	—	—
9/3/12 (morning)	**Left anterior**	**0·00616**	**4·81**	—	—	—
	Right anterior	0·00520	5·44	—	—	—
11/3/12 (morning)	**Left anterior**	**0·00551**	**5·28**	—	—	—
	Right anterior	0·00521	5·48	—	—	—

In the first set of analyses (5/3/12 evening), when the conductivity of the milk from the left anterior quarter was very high, the amount of soluble ash was very much greater than normal and the amount of insoluble ash considerably less than normal.

The same differences between the pathological and normal milks are still apparent on the morning of 8/3/12 but in a less degree, indicating the return of the left anterior quarter of the udder to a normal condition. It is interesting to note that in a pathological milk there may be the paradox of a diminished percentage of sugar and ash with an unaltered depression of freezing point. In the ordinary determinations of ash however, no distinction is made between the soluble and insoluble portions and moreover the heat employed in getting rid of the carbon causes the volatilisation of some of the chlorides.

The inverse proportionality between milk sugar and electrical conductivity may be shown by comparing the milks of different animals, and the manner in which the results obtained conform to theoretical considerations is, in view of the many disturbing factors, exceedingly satisfactory. The experimental difficulties are to be placed first, for many of the animals being unused to handling about the udders, had to be educated to give milk. Generally it meant establishing a habit, whereby the young were separated from the parent, and only admitted at stated times for their feed. When milk samples were required the young were allowed to start the flow of

milk, and then removed. In many cases this meant a wet teat, and with the sow the young pigs leave the teats covered with a very slimy mucous.

With the smaller animals it was particularly difficult to get samples large enough for the determinations of freezing point and milk sugar content, and the cat, bitch and kangaroo had to be kept apart from their young for longer periods than could be called natural.

As already mentioned actual methods of analysis were difficult to keep free from small errors in the determination of milk sugar, chiefly on account of the varying protein contents of the milk.

Also the large amounts of protein in the milks of the smaller animals would undoubtedly depress the conductivity values, as will be shown later in discussing the influence of colloids on the conductivity of milk.

But apart from experimental difficulties and errors of analysis, there is the added complication that the milks of different animals and even of the same animal have a varying depression of freezing point. Strictly to compare two different milks with a view to showing that the milk with the higher milk sugar percentage will have the lower conductivity, it is necessary that both should have the same osmotic pressure, i.e. the same depression of freezing point.

Again many milk samples we obtained were abnormal, for the most part because the milk was still in the colostrum period or because the lactation was practically at an end.

The analyses of these samples have been discarded and the averages of the other analyses we have made taken for each individual species.

In some cases, e.g. sow, llama, kangaroo, ass, only one animal was available, but we have only used the results of our own analyses because we know that the conductivity, milk sugar and depression of freezing point determinations were all carried through on the one sample.

Collecting together the averages representing milk sugar and conductivity the following table is obtained:—

TABLE IV.

Animal	K in ascending values	K corrected	Milk sugar % in descending values	Animal
Mare	0·00208	0·00231	7·52	Mare
Ass	0·00247	0·00276	7·37	Ass
Woman	0·00252	0·00281	6·40	Woman
Sow	0·00375	0·00477	6·11	Sow
Goat	0·00499	0·00595	6·05	Goat
Cow	0·00503	0·00587	5·72	Cow
Cat	0·00537	0·00712	5·24	Cat
Bitch	0·00538	0·00742	5·17	Bitch

The only two milks out of order and therefore omitted from this table are those of the llama and kangaroo. Their non-agreement can be accounted for by abnormal depressions of freezing point.

Thus, for the llama $\Delta = 0{\cdot}600°$ which is exceptionally high;
for the kangaroo $\Delta = 0{\cdot}515°$ which is lower than with other animals.

The corresponding values for these animals are

	K	*K* corrected	Milk sugar
Llama	0·00395	0·00465	6·95
Kangaroo	0·00494	—	2·66

The corrections for *K* have been made by taking the average figures for the percentage of protein in the milks of the various species of animals concerned as published by Droop Richmond in his *Dairy Chemistry*, p. 323, and adding 2·76 % of the conductivity for each 1 % protein in the milk.

The corrections practically leave the table unaffected. They might justify actually reversing the positions of cow and goat in the left-hand list, just as they justify placing the llama ahead of the sow. With the corrected *K*, the llama is most definitely brought more into line with the generalisation established by the table and is far from being a glaring exception, such as the kangaroo remains[1].

It is apparent from the figures that the reciprocal relation between milk sugar and conductivity holds closely in a survey of the milk of different animals in spite of the disturbing factors previously discussed.

The following tables give our analyses for milks of the different animals we have investigated and from these tables the averages used in Table IV were compiled.

Date	No.	*K*	Percentage lactose	Depression of freezing point	Remarks
Human Milk.					
23/3/11	1	0·00327	—	—	Colostrum.
19/3/12	2	0·00391	8·48	—	,,
25/3/12	3	0·00959	0·57–0·76	—	Pathological.
4/4/12	4	0·00321	5·5	—	Colostrum.
,,	5	0·00214	6·78	—	Normal.
17/4/12	6	0·00234	6·23	—	,,
27/4/12	7	0·00230	6·81	—	,,
,,	8	0·00221	6·81	—	
,,	9	0·00229	7·20	0·560	
,,	10	0·00223	6·45	0·550	
22/7/12	11	0·00216	6·66	—	
26/7/12	12	0·00207	6·80	—	
14/8/12	13	0·00243	6·50	—	
21/10/12	14	0·00274	5·14	—	
25/10/12	15	0·00264	5·79	—	
17/2/13	16	0·00300	5·70	0·545	
,,	17	0·00313	5·18	0·530	
,,	18	0·00256	5·63	0·550	

[1] This animal was in the later months of lactation, its young one spending most of its time out of the pouch.

Date	No.	K	Percentage lactose	Depression of freezing point	Remarks
17/2/13	19	0·00278	5·04	0·535	
24/2/13	20	0·00269	—	—	
,,	21	0·00260	—	—	
,,	22	0·00298	—	—	
,,	23	0·00305	—	—	
6/3/13	24	0·00290	6·19	0·530	
,,	25	0·00294	6·50	0·535	
,,	26	0·00258	6·66	0·540	
,,	27	0·00263	6·23	0·542	
Milk of the Mare.					
25/9/12	1	0·00982	2·33	0·589	12 months after foaling.
15/10/12 } same	2	0·00395	6·55	0·561	2 days ditto.
4/12/12 } animal	3	0·00214	7·49	0·560	2 months ditto.
6/1/13	4	0·00203	7·56	—	2 months ditto.
Milk of the Goat.					
15/5/12	1	0·00433	—	0·590	
,,	2	0·00431	6·28	0·580	
16/5/12	3	0·00467	—	0·577	
18/5/12	4	0·00459	6·00	0·575	
16/7/12	5	0·00536	5·98	—	
14/5/13	6	0·00567	5·39	—	Right side.
14/5/13	7	0·00541	6·15	—	Left side.
,,	8	0·00554	6·08	—	Mixed sample.
15/5/13	9	0·00493	6·19	0·590	,,
16/5/13	10	0·00517	6·13	0·563	,,
19/5/13	11	0·00497	6·21	0·564	,,
Milk of the Ass.	All samples obtained from the same animal.				
11/1/13	1	0·00249	7·35	0·513	
16/1/13	2	0·00246	7·38	0·535	
20/2/13	3	0·00794	4·28	0·564	Obviously pathological.
,,	4	0·00888	—	—	
,,	5	0·01136	2·09	0·564	
,,	6	0·01138	1·25	—	
Milk of the Sow.	All samples obtained from the same animal. Littered 1/1/13.				
9/1/13	1	0·00469	4·30	—	
10/1/13	2	0·00366	6·24	0·573	
14/1/13	3	0·00378	6·15	0·565	
16/1/13	4	0·00382	5·98	0·580	
Milk of the Llama.	All samples obtained from the same animal, 4th–5th month of lactation.				
21/1/13	1	0·00324	7·51	0·605	
20/2/13	2	0·00445	6·70	0·602	
,,	3	0·00417	6·96	0·589	
Milk of the Cat.	Both samples obtained from same animal.				
15/3/12	1	0·00557	5·33	—	Both samples were small in amount & obtained with difficulty. Fat was separated before any determination was made.
22/3/12	2	0·00518	5·14	—	

Date	No.	K	Percentage lactose	Depression of freezing point	Remarks
Milk of Bitch.					
3/4/12	1	0·00611	6·2	—	All samples obtained with much difficulty.
16/7/12	2	0·00425	not obtainable		
20/7/12	3	0·00465	4·15	—	
27/8/12	4	0·00421	—	0·573	
Milk of Kangaroo.	Both samples from one animal.		Late stage of lactation.		
27/1/13	1	0·00409	2·28	0·520	Very small sample.
8/2/13	2	0·00494	2·66	0·515	Good sample.

First and last portions of milk.

It is generally known that differences exist between the first milk drawn off from the mammary gland and the last milk. The latter for instance is very rich in fat, the former poor in fat. One of the many suggestions put forward to account for this is that the first and last milks are secreted under different conditions. This explanation has probably a great deal of truth in it, for not only is there the marked variation in fat content, but the early and late portions may show differences in depression of freezing point, milk sugar, and conductivity. The fact that late samples from the mammary gland in cows' milk generally have a greater depression of freezing point than the early samples was noted by Pins.

With regard to the electrical conductivity, it is sometimes higher in the first portions milked and sometimes in the last portions. In the case of full milk from the cow the conductivity of the last portions is invariably lower owing to the larger fat content of the end milk. But if the milk be separated before measuring the conductivity then definite exceptions to the above rule are found.

The following table gives the results obtained with a single cow and with first and last portions of milk from different quarters of the udder.

TABLE V.

Conductivity of first and last portions of milk, or strippings, of cow.

	Quarter	K (first portion)	K (last portion)
Series I	Left anterior	0·00548	0·00554
	Left posterior	0·00543	0·00548
Series II	Left anterior	0·00611	0·00582
	Right ,,	0·00621	0·00606
	Left posterior	0·00629	0·00631
	Right ,,	0·00636	0·00647
Series III	Right anterior	0·00508	0·00514
	Left posterior	0·00514	0·00512
	Right ,,	0·00522	0·00520
Series IV	Left anterior	0·00496	0·00496
	Right ,,	0·00493	0·00505

The samples taken were approximately 100 c.c. in volume. The anterior quarters were yielding about 900—1100 c.c. the posterior quarters 1200—1400 c.c. of milk.

With human milk the conductivity in the later samples was invariably higher than in the earlier samples.

TABLE VI.

Human milk.

Individual	Early sample	Late sample
A	0·00300	0·00313
B	0·00256	0·00278
C	0·00298	0·00305
D	0·00269	0·00260
E	0·00290	0·00294
F	0·00258	0·00263

The milk sugar also varies between first and last samples as shown in Table VII.

TABLE VII.

In the cow	First portion	Last portion
Left anterior quarter	5·53 % sugar	5·29 % sugar
Right ,, ,,	5·38 ,,	5·04 ,,
Left posterior ,,	5·35 ,,	5·27 ,,
Right ,, ,,	5·22 ,,	5·04 ,,

These differences in the depression of freezing point, conductivity and milk sugar content of first and last samples of milk taken from a single quarter, or gland, all bear out the assumption that the first and last portions are secreted under different conditions. In fact everything points to:

(1) A steady slight secretion between the intervals of milking or suckling, and

(2) a reflex further secretion produced as the result of the stimuli applied to the teat and gland.

In the cow (1) is relatively important and the animal comes to the milking shed with a great deal of milk already secreted. In other animals (1) is almost absent and only after the efforts of the young is the milk flow established as a result of a nervous reflex. We may mention the cat, bitch, sow and llama in this connection.

In women an intermediate condition is usually met with, there being some milk present in the breast at the time of suckling. The greater portion however is usually secreted under the reflex, initiated by the child. A child may occasionally take the milk already secreted and then have to work for as long as 10 to 15 minutes to establish the reflex flow. In such cases there is the danger that the mother may remove her child from the breast without the reflex flow of milk having been established.

To strengthen the view that milk may be secreted under different conditions, examinations of milk, obtained before and after the putting of the child to the breast, were made with different women. We have to express our very sincerest thanks to Dr A. W. Robertson, Honorary Physician of the Women's Hospital, Melbourne, for his kindness in procuring us these samples. The samples were taken under the supervision of one of us, and in every case the strictest precautions were observed.

Invariably the putting of the child to the breast changed the character of the milk. Some of the figures for milk sugar and conductivity have already been quoted separately.

The results are collected together in the following table:

Human milk.

First and last portions contrasted.

	First portion (before child put to breast)			*Last portion* (after child put to breast)		
Individual	*K*	Percentage lactose	Δ	*K*	Percentage lactose	Δ
A	0·00300	5·70	0·545°	0·00313	5·18	0·530°
,,	0·00256	5·63	0·550	0·00278	5·04	0·535
B	0·00269	—	—	0·00260	—	—
C	0·00298	—	—	0·00305	—	—
D	0·00290	6·19	0·530	0·00294	6·50	0·535
E	0·00258	6·66	0·540	0·00263	6·23	0·542

There is no rule as to the effect produced by putting the child to the breast. The depression of freezing point, milk sugar percentage and conductivity may either be diminished or increased.

It is certainly not merely a question of first and last portions as such, but of two milks secreted at different times. Being secreted at different times they would undoubtedly be influenced by the variations occurring in the blood, due to the meals, drinking and varying activity of the kidneys and glands of the skin.

The first and last portions with the child placed at the breast between the taking of samples is simply the best manner of showing the difference between the milk already secreted and that poured forth as the response to the stimulus of suckling.

In the following experiment the influence of the child is apparent though only the first samples were taken, April 26th, 1913—evening.

A sample of milk was taken from the left breast and the baby then applied to this breast. There resulted a reflex flow of milk in both breasts and milk came dripping away from the right nipple. A sample was now taken from the right breast.

Evening	*Right* (child being on left breast)	$K = 0{\cdot}00229$	Sugar 7·20 %
	Left (before child applied)	$K = 0{\cdot}00230$	,, 6·82 %

The next morning a sample was taken from the right breast, the child then put to this breast and a sample now taken from the left side.

Morning	*Right* (before child applied)	$K = 0{\cdot}00223$	Sugar 6·46 %
	Left (child being on right breast)	$K = 0{\cdot}00221$	,, 6·82 %

In this experiment right morning, and left evening, are comparable as the samples were taken before bringing the child to its mother. The bringing of the child and subsequent taking of samples from the left breast in the morning, and the right breast in the evening in both cases led to a milk with slightly lowered conductivity and definitely increased milk sugar from 6·46 % to 6·82 % in the morning and 6·82 % to 7·20 % in the evening.

In evaluating the figures in this experiment allowance must be made in comparing morning milk with evening milk, for the generally higher conductivity and milk sugar content of evening milk, as compared with morning milk. The following analyses of the milk from another woman are typical of these morning and evening differences.

Morning and evening samples of milk.

Series III. Human milk (one individual).

	Morning			Evening			
	K	Percentage lactose	Δ		K	Percentage lactose	Δ
Right breast	0·00295	5·14	0·610°	Right breast	0·00333	5·35	0·700°
Left breast	0·00289	5·06	—	Left breast	0·00323	5·25	—

Morning and evening milk.

Cows' milk. With regard to morning and evening samples of milk it seems impossible to state any definite rule, although a large number of determinations have been made, but as will be seen later, it is probable that external climatic conditions, as well as the food factor referred to previously, affect the conductivity.

In the first series taken, where the milk of a single cow was employed, six morning samples taken at varying intervals were contrasted with six evening samples taken on the same days.

In this series four times was the morning conductivity greater than the evening and twice the evening was greater than the morning.

In the next series on 15 different days, evening and morning samples of milk were obtained from the Willsmere Co. These samples were taken from large mixed quantities (150 to 500 qts.) of milk from a special dairy herd (Holstein) and, in this series, of the 30 samples examined, in two instances

morning and evening conductivity were identical, ten times the evening conductivity was greater, while three times the morning conductivity was greater though only pronouncedly so in one case.

Conductivity of morning and evening samples of milk.

		Date	K (morning)	K (evening)
Series I.	**1 cow.**	16/10/11	0·00611	0·00584
		18/10/11	0·00613	0·00641
		19/10/11	0·00652	0·00644
		24/11/11	0·00633	0·00628
		19/ 2/12	0·00516	0·00504
		21/ 2/12	0·00501	0·00527
		23/ 2/12	0·00509	0·00531
Series II.	**Willsmere samples, from 150 to 500 qts.**	5/12/12	0·00554	0·00563
		6/12/12	0·00555	0·00554
		9/12/12	0·00562	0·00562
		10/12/12	0·00556	0·00562
		11/12/12	0·00554	0·00555
		12/12/12	0·00549	0·00557
		16/12/12	0·00568	0·00566
		18/12/12	0·00573	0·00559
		17/ 2/13	0·00587	0·00593
		18/ 2/13	0·00562	0·00593
		19/ 2/13	0·00568	0·00585
		20/ 2/13	0·00562	0·00573
		27/ 2/13	0·00555	0·00599
		3/ 3/13	0·00573	0·00582
		4/ 3/13	0·00575	0·00575

These irregularities in the electrical conductivities of morning and evening milk are to be sought in the conditions under which the animals live during the hot months of the year in Victoria.

They feed by night, as well as by day, upon grass which is burnt dry. They receive their chief water supply when brought to the sheds to be milked, and lastly, with a very changeable climate, a given night may be hotter than the following day.

A regular diurnal periodicity in the concentration of the animals' blood (and consequently in the concentration of their milk) is therefore hardly to be expected.

In contrast to this, women's milk appears to be regularly more concentrated in the evening. This may be assigned to the regular daily eating and activity which, following the night's abstinence and rest, produce a rise in the osmotic concentration of the blood.

THE DEPRESSION OF THE CONDUCTIVITY BY THE PROTEINS OF THE MILK.

The effect of colloids simultaneously present in solution in water with inorganic salts may be postulated as diminishing the conductivity which the salts alone in water would otherwise show.

In the case of milk, freed as far as possible from fat globules, the chief source of obstruction would undoubtedly be the colloidal particles of protein.

The richer a separated milk in protein the greater probably the depression of the conductivity, so that in comparing the milks of different species of animals, the conductivity of each could not necessarily be taken as a quantitative indication of soluble ionised salts without a correction being made.

For instance in mares' milk the protein averages about 1·5 % and probably has only a small influence on the conductivity of what may be termed the "protein free whey."

On the other hand there is every reason to think that in the milk of smaller animals such as the dog and cat, with their high protein content (quoted as high as 11·15 %), the conductivity of the protein free whey would be distinctly greater than that of the fat free milk.

The presence of the protein will diminish the power of the ions to transport electricity by reducing their available free paths; also perhaps the conductivity may be affected by the protein diminishing the ionisation of the salts.

In order to determine the extent to which the protein in cows' milk depresses the electrical conductivity, the following experimental methods were employed.

The milk was obtained directly from a cow on the premises and the cream separated from it as quickly as possible. One portion of the separated milk was weighed and then boiled for one hour in a large flask under a reflux condenser. After cooling, the milk was reweighed, and the very small loss of water made good by adding a special distilled water.

The boiled and raw milks were then placed in two large beakers and in each were suspended dialysing tubes of peritoneal membrane. Each tube contained a measured volume (not exceeding 50 c.c.) of specially distilled water ($K = 0{\cdot}00007$).

Toluene was added to the milk in each beaker (in all the earlier experiments), and when the weather was warm the beakers were placed in an ice chest at a temperature of about 12° C. At intervals 5 c.c. samples were measured from the dialysing tubes and from the surrounding milk.

In accordance with physico-chemical laws, after a sufficient length of time,

equilibrium should be established between the milk outside the membrane and the water inside.

The membranes in all later experiments were the prepared peritoneal sacs from the appendices of sheep, known technically as "*Fischblasen-condom*" and here referred to as "peritoneal membranes." They were apparently perfect in action and much to be preferred to parchment for this purpose.

We quote however earlier results obtained with parchment as the effects observed are the same as with the peritoneal membranes.

Experiment 1. 26/10/11–28/10/11.

A. 630 c.c. *raw* separated milk plus 15 c.c. toluene: 25 c.c. pure distilled water dialysed against it for 2 days. Less than 5 c.c. of a yellow-green faintly opalescent fluid recovered from the *parchment* dialysing tube.

B. 630 c.c. *boiled* separated milk (1 hour's boiling) treated as in A. 5 c.c. clear yellow-green fluid recovered from the parchment.

A. *Raw milk.*		B. *Boiled milk.*	
Before dialysis	$K=0{\cdot}00518$	Before dialysis began	$K=0{\cdot}00514$
After ,,	$K=0{\cdot}00507$	After ,,	$K=0{\cdot}00503$
Water & salts from parchment	$K=0{\cdot}00570$	Water & salts	$K=0{\cdot}00577$

Experiment 2. 3 p.m. 27/2/13–8 p.m. 1/3/13.

A. 1250 c.c. fresh separated milk dialysed against two tubes of peritoneal membrane each containing 50 c.c. conductivity water. 15 c.c. toluene were added to milk as preservative. Vessel placed in ice chest at 12° C. Evaporation prevented by parchment cover placed on mouth of beaker.

B. 1300 c.c. fresh separated milk *boiled for one hour* under reflux condenser, then dialysed against two peritoneal tubes each containing 50 c.c. conductivity water.

Other details as in A.

27/2/13. *Before dialysis.*

A.	Raw milk and toluene	$K=0{\cdot}00500$
	Conductivity water	$K=0{\cdot}00007$
B.	Boiled milk and toluene	$K=0{\cdot}00498$
	Conductivity water	$K=0{\cdot}00007$

Dialysis commenced 3 p.m. 27/2/13.

Date	Time	No.	Raw milk	Boiled milk	Dialysate Tube 1	Dialysate Tube 2
28/2/13	12 noon	A	$K=0{\cdot}00492$	—	$K=0{\cdot}00494$	$K=0{\cdot}00478$
		B	—	$K=0{\cdot}00496$	$K=0{\cdot}00460$	$K=0{\cdot}00440$
28/2/13	6.30 p.m.	A	$K=0{\cdot}00486$	—	$K=0{\cdot}00496$	$K=0{\cdot}00490$
		B	—	$K=0{\cdot}00486$	$K=0{\cdot}00484$	$K=0{\cdot}00470$
1/3/13	12 noon	A	$K=0{\cdot}00488$	—	$K=0{\cdot}00502$	$K=0{\cdot}00508$
		B	—	$K=0{\cdot}00492$	$K=0{\cdot}00498$	$K=0{\cdot}00494$
1/3/13	8 p.m.	A	$K=0{\cdot}00488$	—	$K=0{\cdot}00512$	$K=0{\cdot}00514$
		B	—	$K=0{\cdot}00486$	$K=0{\cdot}00514$	$K=0{\cdot}00512$

In both experiments quoted it was found that after two days' dialysis the conductivity of the liquid from the dialysing tubes was greater than that of the surrounding milk.

Boiling the milk has apparently no influence on the end equilibrium.

The second experiment suggested a slower course in the establishment of equilibrium in the case of boiled milk and it might be that some fixation of soluble dialysable salts had occurred as the result of the boiling, the change being slowly reversible. It was realised however that diffusion processes and the three occasions on which the milk was disturbed in taking samples, would be responsible for the rate of dialysis.

Consequently in the experiments which follow, performed in mid-winter, stirrers driven by an electrical motor kept the milk in continuous movement during the whole course of the dialysis. Further, the percentages of fat and proteins in the milk employed were directly estimated and gravimetric estimations of ash in the dialysate were made. As will be seen from the records which follow there is no reason to assign any special importance to the slower establishment of equilibrium to which attention was called in Experiment 2.

Experiment 3. 9/6/13.

1600 c.c. fresh separated milk (from one cow) and 5 c.c. toluene were dialysed against 50 c.c. conductivity water. The beaker containing the milk and the dialysing tube was placed in a water bath, which during the course of the experiment maintained an average temperature of 8° C. The milk was kept in constant movement by means of an electrical stirrer, and a filter paper, kept slightly moistened, was arranged over the top of the beaker to check evaporation.

An estimation of the fat by Babcock's method gave the content 0·025 % and from a Kjeldahl determination of the nitrogen the protein was found to be 4·35 %, after correcting for dialysable nitrogen and dilution by 35 c.c. water.

5 c.c. samples were removed at intervals from the milk and the dialysing tube.

Experiment set up 12.30 p.m. 9/6/13.

Date	Time	Milk	Dialysate
9/6/13	12.30 p.m.	$K=0{\cdot}00747$	—
10/6/13	9.30 a.m.	$K=0{\cdot}00736$	$K=0{\cdot}00843$
10/6/13	4.30 p.m.	$K=0{\cdot}00740$	$K=0{\cdot}00843$
11/6/13	10.15 a.m.	$K=0{\cdot}00740$	$K=0{\cdot}00839$

In this experiment equilibrium appears to have been attained at 4.30 p.m. 10/6/13.

Averaging the figures with those obtained at 10.15 a.m. 11/6/13, gives

Milk	$K=0{\cdot}00740$
Dialysate	$K=0{\cdot}00841$

The differences between these figures, 0·00101, stated as percentage depression of the conductivity gives :—

$$\frac{0{\cdot}00101}{0{\cdot}00841}\times 100 \text{ or } 12{\cdot}01\ \%.$$

This 12·01 % depression of the conductivity is produced by 4·35 protein in the milk, or 1 % protein is equivalent to a depression of 2·77 % of the conductivity.

Experiment 4 *a*.

The milk used in this experiment was obtained from a cow in the third month of lactation. Immediately after separation a portion of it was taken and boiled for 45 minutes, the remaining part being kept in the meantime at a fairly low temperature. The two beakers were subsequently placed in a water bath at 9° C. and the milk was stirred as in the previous experiment, but no toluene was added.

A Babcock determination showed 0·1 % fat in the separated milk and a nitrogen estimation by Kjeldahl's method fixed the protein content at 3·07 % after correcting for dialysable nitrogen and dilution with 10 c.c. water.

A. 1100 c.c. fresh separated milk dialysed against 25 c.c. pure distilled water.

B. 1100 c.c. separated milk boiled for 45 minutes under reflux condenser, then dialysed against 25 c.c. pure distilled water.

Before dialysis.

13/6/13. 6 p.m.	A. Raw milk	$K=0{\cdot}00491$
	B. Boiled milk	$K=0{\cdot}00487$

Dialysis commenced 6 p.m. 13/6/13.

		Raw			Boiled		
Date	Time	No.	Milk	Dialysate	No.	Milk	Dialysate
13/6/13	10.45 p.m.	A	$K=0{\cdot}00489$	$K=0{\cdot}00496$	B	$K=0{\cdot}00483$	$K=0{\cdot}00508$
14/6/13	10.20 a.m.	A	$K=0{\cdot}00484$	$K=0{\cdot}00529$	B	$K=0{\cdot}00485$	$K=0{\cdot}00527$
14/6/13	4 p.m.	A	$K=0{\cdot}00478$	$K=0{\cdot}00526$	B	$K=0{\cdot}00480$	$K=0{\cdot}00523$

4 c.c. of each dialysate were taken and the amount of ash estimated. The ash of the dialysate from the raw milk amounted to 0·0175 g. and that from boiled milk to 0·0180 g.

In this experiment equilibrium is established at 10.20 a.m. 14/6/13.

Also there is no essential difference between the results for the raw and boiled milks. Consequently the average of the conductivities for the last four milks (two raw and two boiled) and for the last four dialysates may be used to calculate the depression due to the protein.

Average for milk	$K=0{\cdot}00482$
Average for dialysates	$K=0{\cdot}00526$

Hence conductivity is depressed 8·365 % for 3·07 % protein or 1 % protein depresses the conductivity 2·73 %.

Experiment 4 *b*. 20th and 21st July, 1913.

1000 c.c. separated milk (morning milk from several cows). Fat 0·25 %; $K=0{\cdot}00541$. No toluene added. Two dialysing tubes each with 20 c.c. conductivity water. Stirrer used throughout. Commenced 2 p.m. 20th July, 1913. Temperature 10·5°.

21/7/13.	11.30 a.m.	Milk	$K=0{\cdot}00528$
		Dialysate I	$K=0{\cdot}00577$
		II	$K=0{\cdot}00579$
	2.30 p.m.	Milk	$K=0{\cdot}00528$
		Dialysate I	$K=0{\cdot}00577$
		II	$K=0{\cdot}00578$

Results of Kjeldahl determinations.

5 c.c. of milk at end of experiment = 18·9 c.c. $N/10$,
5 c.c. dialysate I at end of experiment = 0·6 c.c. $N/10$,
5 c.c. dialysate II at end of experiment = 0·9 c.c. $N/10$,
Blank determination = 0·00.

Hence 18 c.c. $N/10$ represents protein nitrogen of milk = 3·12 % protein.

K is depressed 8·65 % by this amount of protein or 1 % protein depresses the conductivity 2·77 %.

Experiment 5. 21/6/13.

The milk used in this experiment was bulk milk obtained from a dairy. The same experimental detail was observed as in the previous experiments, but in this case the separation of the cream from the milk was by no means complete. No toluene used.

A. 2000 c.c. fresh separated milk dialysed against 40 c.c. conductivity water.

B. 2000 c.c. fresh separated milk, boiled for one hour under reflux condenser then dialysed against 40 c.c. conductivity water

21/6/13. 12.30 p.m. A. Raw milk $K=0{\cdot}00527$
B. Boiled milk $K=0{\cdot}00514$

Dialysis commenced 12.30 p.m.

		Raw			Boiled		
Date	Time		Milk	Dialysate		Milk	Dialysate
21/6/13	2 p.m.	A	$K=0{\cdot}00522$	$K=0{\cdot}00447$	B	$K=0{\cdot}00511$	$K=0{\cdot}00451$
21/6/13	4 p.m.	A	$K=0{\cdot}00519$	$K=0{\cdot}00540$	B	$K=0{\cdot}00509$	$K=0{\cdot}00564$
21/6/13	5 p.m.	A	—	$K=0{\cdot}00563$	B	—	$K=0{\cdot}00564$
21/6/13	7.30 p.m.	A	$K=0{\cdot}00522$	$K=0{\cdot}00578$	B	$K=0{\cdot}00512$	$K=0{\cdot}00570$

At the conclusion of the dialysis, 10 c.c. of each dialysate were taken and evaporated to dryness and ashed. The amount of ash from 10 c.c. of the dialysate from raw milk was 0·052 g. and exactly the same amount was given by the dialysate from the boiled milk.

The ash was then dissolved in dilute hydrochloric and 0·5 c.c. of a sodium acetate solution added; ammonia was added till the reaction was alkaline and then 1 c.c. saturated solution of ammonium oxalate. The mixture was kept at 100° for ten minutes, then the oxalate precipitate transferred to a filter paper and well washed with dilute acetic acid and distilled water. Paper and precipitate were dried at 100° and ashed over a low flame. The ashes at this stage weighed.

(1) From boiled milk dialysate 0·0187 g.
(2) From raw milk dialysate 0·0150 g.

In both cases the ash was moistened with 25 % sulphuric acid, carefully dried and at first gently then strongly heated.

(1) From boiled milk dialysate 0·0165 g.
(2) From raw milk dialysate 0·0150 g.

Discussion of dialysis experiments.

The experiments all show the same qualitative results, but owing to absence of analyses for the milk as actually used in Experiments 1 and 2 and also to lack of evidence that equilibrium had definitely been reached, it is only the later experiments which can be used for obtaining an accurate quantitative evaluation of the depressant action of protein on the conductivity of milk.

It is most interesting to compare these values (average 2·76 %) with those of Bugarsky and Tangl obtained for the proteins of blood serum (2·5 % depression of conductivity per 1 % protein).

We feel that we may claim for Experiment 4 *a* and *b* an almost perfect technique. Not even a preservative agent such as toluene was necessary.

Also Experiments 3 and 4*a* give uniform results with two totally different milks. In Experiment 3 milk very rich in protein was used from a cow at the end of lactation (8th month). In Experiment 4*a* the milk was from a cow in the third month of lactation. Experiment 5 was with market milk, which having previously creamed repeatedly clogged the separator. Owing to the large amount of fat present the experiment cannot be used to evaluate the depressant action of the protein upon the conductivity.

Analyses of ash show that the dialysates from raw and boiled milk not only contain the same amounts of ionised salts, as shown by their equal conductivities, but also the same amounts of total ash, and of calcium. Söldner's statement [1888] that boiling milk renders a great portion of the calcium insoluble, a statement which has often been used to explain the absence of rennet coagulation in boiled milk, is incorrect in the strict sense. No calcium which is in true molecular solution can become insoluble or results, such as those just quoted, would not be obtained. The chief effect of boiling milk is a physical alteration of the colloids. Probably an insoluble irreversible calcium caseinogenate is formed which mechanically fixes colloidal calcium phosphate.

Such an explanation will account for Söldner's observation that with boiled milk less calcium will pass a porous clay filter than is the case with raw milk.

Daily fluctuations in conductivity of milk associated with varying climatic conditions.

During the summer months in Victoria rapid changes from hot dry weather to a period of coldness and rain are experienced from time to time, and the following observations which extended over portions of the months of November, December, February and March include well marked extremes of summer temperature.

The milk used in this series of conductivity determinations was a special supply from the Syme Model Farm, Gisborne, Victoria. This farm possesses modern refrigerating machinery. The milk is immediately put over the coolers and sent packed with ice by motor to Melbourne. The conductivity changes are therefore not due to bacterial changes in the milk but to climatic influences affecting the cows. As soon as the supply arrived at the Melbourne depot a composite sample from 3–10 cans, each containing 50 quarts, was carefully collected by the Dairy Expert of the Willsmere Certified Milk Company and our thanks are due to him and to the Company for obtaining these samples for us.

The weather reports quoted in the following table are abridged from the daily bulletins published in the *Argus*; the figures for humidity and the dry bulb temperature as recorded by the Melbourne Observatory were obtained from the same source.

Date	Weather report	Relative humidity %	Dry bulb temperature, °F. Max.	Min.	Mean	*K*
27/11/12	Fine, warm, pleasant	57·3	77·4	43·2	60·3	0·00578
28/11/12	Hot, dry, north winds	37·6	83·6	53·7	68·6	0·00573
29/11/12	Small fall of rain, followed by fine, pleasant day, light cool winds	59·6	67·3	57·9	62·6	0·00562
1/12/12	Light steady rain, sultry conditions ...	74·3	73·9	61·1	67·5	0·00570
3/12/12	Tropical downpour, severe thunderstorm	74·3	75·0	49·0	62·0	0·00557
4/12/12	Dull cloudy morning, followed by fine pleasant day, light southerly wind	63·0	70·3	53·0	61·6	0·00554
5/12/12	Cloudy, sultry generally	49·0	84·7	51·7	68·2	0·00555
6/12/12	Thunderstorms, followed by squally showery weather	56·3	68·8	56·4	62·6	0·00554
8/12/12	Unsettled, showery, "unusual weather"	63·0	67·3	47·3	57·3	0·00562
9/12/12	Cloudy but fine at first, gradually becoming windy and threatening	60·6	67·7	54·2	60·9	0·00556
10/12/12	Weather very disturbed, stormy ...	61·3	62·3	50·7	56·5	0·00554
11/12/12	Cool, showery	61·0	57·6	44·2	50·9	0·00549
15/12/12	Weather becoming finer, max. temp. higher than any recorded since 6th Dec.	56·6	70·2	47·5	58·8	0·00568
17/12/12	Hot, slightly cloudy, light breezes ...	52·3	83·7	59·3	71·5	0·00573
12/ 2/13	Fine, light variable winds and little rain	61·0	82·8	55·2	69·0	0·00585
16/ 2/13	Close, sultry	74·6	74·8	64·5	69·6	0·00587
17/ 2/13	Fine, warm, sultry	57·3	85·0	60·8	72·9	0·00562
18/ 2/13	Fine, unpleasant strong westerly winds	43·6	78·0	60·3	69·1	0·00568
19/ 2/13	Fine, after a cloudy threatening morning	49·3	73·0	57·5	65·2	0·00562
20/ 2/13	Cloudy & dull at first, afterwards bright	59·3	66·6	57·4	62·0	0·00559
24/ 2/13	Northerly winds at first, followed by light sea breezes. Very hot	44·3	95·0	59·9	77·4	0·00573
26/ 2/13	Cloudy, sultry, thundery	69·0	71·0	62·0	66·5	0·00555
27/ 2/13	Bright, warm, sea breezes	68·3	84·0	60·0	72·0	0·00577
2/ 3/13	Fine, clear, hot	48·6	93·4	48·5	70·9	0·00573
3/ 3/13	Warm night followed by dull sultry day. Thunderstorm	51·6	95·3	70·2	82·6	0·00575
4/ 3/13	Oppressive weather, high humidity, small amount of rain	81·3	76·5	64·9	70·7	0·00551
6/ 3/13	Cold with squally southerly wind, at times showery	68·3	62·8	55·0	58·9	0·00562

By reference to the table it will be seen that on November 27, 28, 29 and December 1, warm weather was experienced and the average conductivity obtained for the milk during that time = 0·00570.

On December 3 there was a large fall of rain and a lowered conductivity recorded = 0·00557.

On December 5 a quick cold change followed a hot sultry day and from that date until December 11 there was a period of cold, showery stormy weather and the average conductivity during this time = 0·00555.

On December 15 and 17 the weather was becoming finer with rising temperatures and the mean conductivity for these two days = 0·00576.

On various days between February 15 and 19, both inclusive, fairly high temperatures were recorded and the average conductivity for 5 days = 0·00572.

On February 24 and 27 and March 2 and 3 the dry bulb temperatures were high and the average conductivity = 0·00574.

February 26 was cloudy and thundery with moderate temperatures and $K = 0{\cdot}00555$.

The weather of March 4 was very oppressive with a high humidity and $K = 0{\cdot}00551$.

By March 6 the weather was colder and squally and the conductivity was found = 0·00562.

It will thus be seen that as a generalisation it may be stated that hot dry weather causes the conductivity to become higher, while cold, wet or very humid weather has the reverse effect.

The value given for the relative humidity is the mean of three determinations made at 9 a.m., 3 p.m. and 9 p.m. respectively.

The maximum and minimum dry bulb temperatures are those recorded during twenty-four hours.

The conductivities quoted in the table are all for early morning samples of milk and placed against them are the climatic details of the *previous* day. The conductivities obtained ranged from 0·00549–0·00587.

Summary.

(1) In milks secreted from different quarters of the cow's udder and from the right and left breasts in women, the electrical conductivity and percentage of milk sugar show a strict reciprocity provided that the secretion of the milk samples corresponds to the same period. In this case the milk samples are secreted against the osmotic pressure of the blood with its variation over that period. They will all have the same osmotic pressure and if in one sample the sugar is higher than in another, then the electrical conductivity will be lower.

(2) The reciprocity of milk sugar content and electrical conductivity is well seen in the milks from a pathological gland which is slowly recovering and becoming normal.

(3) In a comparison of the milks of different species of animals the reciprocity between milk sugar and electrical conductivity is evident.

(4) It is shown that the milk secreted under the stimulus of removal (milking or natural suckling) differs in character from that secreted previously. The contention is that the condition of the blood has not kept absolutely constant, and that the reflex milk is secreted against a slightly different blood from that against which the previously formed milk was secreted.

(5) Morning and evening samples of cows' milk have been compared. The evening milk generally has the higher conductivity but exceptions exist.

(6) The exact effect of the proteins of cows' milk in diminishing the electrical conductivity has been estimated, the value found being a diminution of 2·76 % of the conductivity for every 1 % of protein in the milk.

(7) The dialysis experiments employed for determining this effect showed no difference between raw fresh milk, and the same boiled for one hour. Also there was no evidence that boiling has any effect on soluble calcium salts in a state of ionisation.

(8) The effect of climatic changes upon a Holstein herd of cows has been studied. The generalisation holds that hot dry weather increases the electrical conductivity of the milk, whilst wet or cold weather diminishes it. The climatic conditions affect the cows and so indirectly their milk.

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II. OSMOTIC PHENOMENA OF YOLK OF EGG.

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The following short research arose from an attempt to employ yolks of eggs as models illustrating the osmotic behaviour of red corpuscles. An unbroken yolk immersed in distilled water slowly swells, the contents become cloudy and ultimately the membrane bursts. If another yolk be placed in 0·9 % sodium chloride no change is observable. A third yolk floated on glycerol will shrink and display marked corrugation. So far there is close parallelism and these three experiments can be used for class demonstration. With strong salt solution however and with organic solvents the behaviour of the yolk is widely different from that of corpuscles, as might in part be inferred from the sclero-proteid nature of the vitellin membrane.

If the yolk be floated on to 10 % NaCl, or greater concentrations, there is a marked swelling and not the shrinking which one might expect. As is well known the chief protein, or lecithoprotein, of yolk is soluble in strong saline. When dissolved it permeates either not at all or with great difficulty through the vitellin membrane and so conditions an osmotic effect. A series of 2M. solutions of NaCl, $CaCl_2$, $MgCl_2$ and $MgSO_4$ all gave this swelling effect. With the sulphate solution the rate of distension was slightly greater. If a 50 % $CaCl_2$ solution or a higher concentration be employed, shrinking can nevertheless be seen owing to the rapid extraction of water.

With the following solvents more or less interesting effects can be observed.

(1) Ether. The yolk sinks and slowly swells. About the end of the second day an accumulation of ether, deeply pigmented but transparent, may be observed in the upper part of the yolk. This ethereal solution increases in volume and produces a doming of the yolk. In some cases the latebra is beautifully displayed in the form of a tent attached to the vitellin membrane at the cicatricula. Eventually the membrane bursts liberating the contents.

But as long as the membrane is intact the ether outside is unstained and indeed does not contain even a trace of solid matter. The dissolved substance is therefore imprisoned in the yolk and exerts its osmotic effect. If the yolk be placed in ether which has for some time been shaken up with broken yolk and separated from this, the swelling may be completely absent and no collection of ethereal solution may be observed.

(2) Chloroform. The yolk floats on the fluid but the sequence of events is very similar to that with ether except that the growing volume of coloured chloroform is found at the bottom of the yolk. So long as the membrane is intact the outside chloroform is unpigmented.

(3) Carbon disulphide. In every particular the action of this solvent is similar to that of chloroform.

(4) Alcohol. The yolk which sinks in this fluid does not swell and very soon the outside fluid is seen to be pigmented, though the membrane, as far as the eye can judge, is intact. Apparently the alcoholic solution can pass through the membrane just as alcoholic solutions of soap can diffuse through parchment paper; hence no osmotic effect is obtained.

(5) Petroleum ether. The yolk sinks in this but does not change. There is no extraction of pigment nor accumulation of solution inside. If broken yolk be shaken up with this solvent nothing but a mere trace of fatty matter is dissolved. The absence of osmotic effects is due therefore to an absence of solution. Boiling petroleum ether can however exert a solvent action on some yolk constituents and an unbroken yolk placed in this under a reflux condenser will show swelling and formation of a globule of solution under the membrane.

(6) Benzene. This acts in a manner almost identical with that of petroleum ether.

(7) Acetone. There is a gradual extraction of colour from the yolk; prolonged action may give a slight globule. If boiling acetone be employed the extraction of pigment proceeds more rapidly.

(8) Olive oil. No change is observable.

(9) Isotonic urea solution. The yolk swells fairly quickly and a globule is formed on the top as in ether. If a 4 % solution of urea in 0·9 % NaCl be employed there is no effect. Urea solution can therefore be added to those mentioned at the outset of the paper as giving effects similar to those observable with red blood corpuscles.

III. CASEINOGEN AND CASEIN.

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(*Received November 29th, 1913.*)

The question of the chemical identity of caseinogen and casein and with it, of the nature of rennet action, still remains open in spite of many attempts at a solution. Hammarsten suggested that by the action of rennet, caseinogen was hydrolysed to form two new proteins, casein and whey-protein. Köster [1881] analysed these proteins and found that casein contained somewhat more nitrogen (15·84 %) than caseinogen and whey-protein much less (13·1–13·6 %). On the other hand it has been suggested that rennet action is either purely physical or is only concerned with the inorganic constituents of milk and that casein is chemically identical with caseinogen. During the progress of this work a new suggestion has been put forward by van Slyke and Bosworth [1913], according to whom caseinogen is split by rennet into two molecules of casein.

If Hammarsten's theory is correct, it should be possible to find some chemical differences between caseinogen and casein. Elementary analyses have yielded the following results:—

Caseinogen	C	H	N	S	P
Makris [1876]	53·02	7·42	14·20	—	—
Hammarsten [1883–1885]	52·96	7·05	15·65	0·758	0·347
Chittenden & Painter [1887]	53·3	7·07	15·91	0·82	0·84–0·89
Lehmann & Hempel [1894]	54·0	7·04	15·6	0·771	0·847
Ellenberger [1902]	53·07	7·13	15·64	0·76	0·8
Laqueur & Sackur [1903]	—	—	15·45	0·757	0·772
Burow [1905]	52·825	7·095	15·64	0·725	0·808
Tangl [1908]	52·69	6·81	15·65	0·832	0·877
van Slyke & Bosworth [1913]	53·50	7·13	15·80	0·72	0·71
Mean	53·17	7·09	15·67	0·768	0·82
Casein					
Köster [1881]	—	—	15·84	—	—
Rose & Schulze [1885]	53·94	7·14	15·14	1·01	—
Raudnitz [1904]	—	—	15·5	—	0·7–0·88
Kikkoji [1909]	—	—	—	—	0·85–0·87
van Slyke & Bosworth [1913]	53·50	7·26	15·80	0·72	0·71
Mean	53·72	7·20	15·57	0·87	0·79

The only reliable analysis of casein is that of van Slyke and Bosworth [1913] who obtained identical results for caseinogen and casein. They have also obtained somewhat more sulphur than phosphorus, which is in agreement with the supposition that there are equal numbers of atoms of these two elements in the molecules of these proteins.

The object of the following investigation was to determine the difference, if any, between caseinogen and casein both in elementary composition and in Hausmann numbers.

Most of the nitrogen estimations quoted above have been carried out by Kjeldahl's method and are thus inclined to be too low. The author has made estimations both by Kjeldahl's and by Dumas' method. Sulphur estimations are usually carried out by one of the various fusion methods described in the literature. These all lead to the precipitation of barium sulphate in the presence of comparatively excessive amounts of alkali salts, usually chlorides. It has however been shown by Allen and Johnston [1910] and Johnston and Adams [1911] that the presence of even relatively small amounts of such salts destroys the accuracy of sulphate estimations. This source of error has been avoided in the analyses described below by adopting a slight modification of Carius' method.

The following mean results were obtained for caseinogen and casein:—

	Caseinogen %	Casein %
C	53·20	53·05
H	7·09	7·03
N (Dumas)	15·63	15·81
N (Kjeldahl)	15·61	15·62
S	1·015	1·009
P	0·731	0·809

The results for sulphur are higher than have been previously obtained for caseinogen. It will be seen above that Rose and Schulze [1885] obtained 1·01 % sulphur in casein in agreement with the author's result. The sulphur contents of caseinogen and casein appear to be identical, but casein apparently contains more phosphorus than caseinogen. The difference is however not sufficient to warrant the supposition that the two proteins are chemically different.

As will be seen from the following figures the Hausmann numbers for the two proteins are also too close to establish any definite difference.

	Caseinogen % total N	Casein % total N
Ammoniacal N	10·23	10·31
Melanin N	1·53	1·66
Diamino N	22·94	24·03
Monamino N	65·31	63·90
	100·01	99·90

Osborne and Harris [1903] obtained from caseinogen

	% total N
Ammoniacal N	10·31
Melanin N	1·34
Diamino N	22·34
Monamino N	66·01
	100·00

with which the author's results are in substantial agreement.

Experimental part.

Elementary analysis of caseinogen and casein.

The caseinogen used was Kahlbaum's "Casein nach Hammarsten" carefully freed from fat by prolonged extraction in a Soxhlet apparatus with ether.

The casein was prepared from milk by the action of rennet and purified by Hammarsten's method. The last traces of fat were removed as above. Two specimens were separately prepared and analysed.

Before analysis the samples were allowed to stand at least 16 hours exposed to the air of the balance room, and when each portion was weighed out for an estimation a second portion was taken and dried to constant weight at 40° in vacuum over P_2O_5 and the results corrected for the percentage of water thus found. It was found impossible to handle the very hygroscopic dry proteins with any certainty of accuracy. The ash was also estimated and allowed for. All the results are calculated for the ash-free dry proteins.

Carbon and hydrogen estimations were carried out in an ordinary combustion tube filled with alternate layers of copper oxide and lead chromate. The substance in the boat was covered with a mixture of lead chromate and potassium bichromate.

Nitrogen estimations were carried out both by Kjeldahl's and by Dumas' method.

Sulphur was estimated by the following modification of the Carius method. About 0·5 grm. of the protein was heated in a sealed tube with 7–8 cc. fuming nitric acid for two days at 300°. After opening the tube the contents were diluted and filtered and the filtrate treated with a slight excess of barium chloride over that required to combine with the sulphuric and phosphoric acids. No precipitate was obtained. The solution was evaporated to dryness, a few cc. of concentrated hydrochloric acid added and again evaporated to dryness completely to remove the nitric acid. The residue

was taken up with 50 cc. of water and 2·2 or 4·5 cc. of dilute (6·5 %) hydrochloric acid added. The barium sulphate was thus obtained in a granular condition. It was collected on a layer of "$BaSO_4$ asbestos" in a Gooch crucible, washed and dried in an air oven at 110–120° to constant weight. From Allen and Johnston's [1910] results the following corrections were made for the solubility of barium sulphate :—

2·2 cc. dilute HCl add 0·5 mgm. $BaSO_4$.
4·5 cc. dilute HCl add 0·6 mgm. $BaSO_4$.

Phosphorus was estimated in the filtrate from $BaSO_4$ by Gregerson's [1907] modification of Neumann's [1900] method.

Caseinogen.

Ash $\left\{\begin{matrix}0{\cdot}5764\\0{\cdot}5750\end{matrix}\right\}$ = 0·57 % except where otherwise stated.

C & H 0·2118 g. containing 10·30 % H_2O; 0·3690 g. CO_2; 0·1403 g. H_2O.
0·2214 g. ,, 6·54 % H_2O; 0·4010 g. CO_2; 0·1492 g. H_2O.
0·2415 g. ,, 6·54 % H_2O; 0·4377 g. CO_2; 0·1549 g. H_2O.

N (Dumas) 0·2227 g. containing 6·54 % H_2O; 27·2 cc. nitrogen over 50 % KOH at 15·5° C. and 760 mm.

N (Kjeldahl) Ash = $\left\{\begin{matrix}0{\cdot}32\\0{\cdot}20\end{matrix}\right\}$ = 0·26 %; H_2O = 11·30 %.
0·2510 g. required 24·63 cc. N/10 H_2SO_4.
0·2494 g. ,, 24·72 cc. ,,
0·2507 g. ,, 24·66 cc. ,,
0·2493 g. ,, 24·43 cc. ,,

S 0·5008 g. containing 7·69 % H_2O; 0·0332 g. $BaSO_4$.
0·5055 g. ,, 7·69 % H_2O; 0·0354 g. $BaSO_4$.

P 0·4936 g. containing 7·69 % H_2O required 29·61 cc. N/10 NaOH.
0·5055 g. ,, 7·69 % H_2O ,, 30·71 cc. ,,

Ash-free dry caseinogen :		Mean
C = 53·25, 53·15, 53·20	... ...	53·20 %
H = 7·03, 7·32, 6·93	... ...	7·09
N (Dumas) = 15·63	... ...	15·63
N (Kjeldahl) = 15·54, 15·76, 15·61, 15·54		15·61
S = 0·992, 1·048	... ...	1·015
P = 0·727, 0·734	... ...	0·731

Casein (Prep. 1).

Ash = 0·31 % of dry casein.

C & H 0·2408 g. containing 11·75 % water; 0·4133 g. CO_2; 0·1576 g. H_2O.
0·2040 g. ,, 11·70 % water; 0·3493 g. CO_2; 0·1390 g. H_2O.

N (Dumas) 0·2792 g. containing 11·75 % water; 33·6 cc. nitrogen over water at 12° C. and 742 mm.
0·3342 g. containing 11·70 % water; 40·0 cc. nitrogen over water at 15·3° C. and 749 mm.

S 0·5063 g. containing 11·75 % water; 0·0323 g. $BaSO_4$.
0·4939 g. ,, 10·09 % water; 0·0295 g. $BaSO_4$.
0·4250 g. dry casein; 0·0312 g. $BaSO_4$.

P 0·5063 g. containing 11·75 % water required 33·33 cc. N/10 NaOH.
0·4939 g. ,, 10·09 % ,, ,, 32·30 cc. N/10 NaOH.

Dry ash-free casein (Prep. I):

	Mean
C = 53·21, 53·06	53·14 %
H = 6·82, 7·17...	7·00
N (Dumas) = 15·85, 15·68	15·77
S = 0·999, (0·919), 1·008	1·004
P = 0·829, 0·809	0·819

Casein (Prep. II).

Ash = 0·45 % of dry casein.

C & H 0·2268 g. containing 11·95 % water; 0·3867 g. CO_2; 0·1539 g. H_2O.
0·2127 g. ,, 11·94 % water; 0·3616 g. CO_2; 0·1413 g. H_2O.

N (Dumas) 0·2487 g. containing 11·95 % water; 30·1 cc. nitrogen over water at 17·2° C. and 754 mm.
0·2886 g. containing 11·94 % water; 33·7 cc. nitrogen over 50 % potash at 15·7° C. and 756 mm.

S 0·5318 g. containing 11·94 % water; 0·0340 g. $BaSO_4$.
0·5154 g. ,, 11·94 % water; 0·0337 g. $BaSO_4$.

P 0·5318 g. containing 11·94 % water required 34·05 cc. N/10 NaOH.
0·5154 g. ,, 11·94 % ,, ,, 32·08 cc. ,,

Dry ash-free casein (Prep. II):

	Mean
C = 53·02, 52·88	52·95 %
H = 7·14, 6·95...	7·05
N (Dumas) = 15·88, 15·81	15·85
S = 1·002, 1·024	1·013
P = 0·810, 0·787	0·799

Kjeldahl nitrogen estimations of casein were carried out with a third specimen prepared by the same method.

Ash = 1·03 % in dry casein.

Water = $\left\{\begin{matrix}11{\cdot}41\\11{\cdot}46\end{matrix}\right\}$ = 11·44 %.

0·2478 g. required 24·13 cc. N/10 H_2SO_4.
0·2517 g. ,, 24·61 cc. ,,

N = 15·59, 15·65. Mean = 15·62 %.

The mean compositions of caseinogen and casein are thus :—

	Caseinogen %	Casein %
C	53·20	53·05
H	7·09	7·03
N (Dumas)	15·63	15·81
N (Kjeldahl)	15·61	15·62
S	1·015	1 009
P	0·731	0·809

Hausmann Numbers.

The caseinogen used was Kahlbaum's "Casein nach Hammarsten." The ash and water were estimated and allowed for as above.

Three samples of casein were used.

(1) Prepared by the action of rennet on a solution of caseinogen in disodium hydrogen phosphate. The casein was precipitated by calcium chloride and purified by Hammarsten's method.

(2) and (3) Prepared by the action of rennet on a solution of caseinogen in the minimum amount of NaOH. The rennet was destroyed by heating momentarily to 90° C. with steam and the casein precipitated with glacial acetic acid. It was purified by Hammarsten's method. At each purification after the first a portion was washed with alcohol and ether and not further purified. In this way four fractions were obtained.

From 110 g. air-dry (about 100 g. dry) caseinogen were obtained:—

	Prep. (2)	Prep. (3)
	g.	g.
Fraction *a*	12	13
b	11	16
c	13	12
d	14	11
	50	52

The fractions (2) *a*, (2) *d*, (3) *b* and (3) *d* were used for Hausmann numbers.

The Hausmann numbers were estimated by the method given by Röhmann [1908]. Usually the diamino nitrogen was estimated in the phosphotungstic acid precipitate and the monamino nitrogen in the filtrate from this, in some cases only one of these two was estimated and the other obtained by difference. Figures thus obtained by difference are given below in brackets. The results are given in percentages of the total nitrogen obtained by addition of the four separate nitrogen percentages.

Caseinogen.

1st Series.

	(1)	(2)	(3)	(4)	Mean
Ammoniacal N	10·52	10·64	10·06	9·73	10·24
Melanin N	1·79	0·58	2·07	1·57	1·50
Diamino N	(23·07)	(26·19)	20·00	20·92	22·55
Monamino N	64·62	62·59	67·87	67·78	65·72
					100·01

2nd Series.

	(5)	(6)	(7)	(8)	(9)	Mean
Ammoniacal N	10·03	10·32	10·35	10·26	10·17	10·23
Melanin N	1·34	1·55	1·57	1·59	1·71	1·55
Diamino N	23·28	22·66	23·77	22·98	23·56	23·25
Monamino N	65·35	65·48	64·31	65·23	64·57	64·99
						100·02

The second series is the more reliable.

Casein (*Prep.* (1)).

	(1)	(2)	(3)	(4)	Mean
Ammoniacal N	9·92	10·38	10·26	10·51	10·27
Melanin N	1·50	1·57	1·63	1·37	1·52
Diamino N	(23·89)	(25·59)	24·05	(23·96)	24·37
Monamino N	64·69	62·46	64·07	64·16	63·85
					100·01

Casein (*Prep.* 2 *a*).

	(1)	(2)	(3)	Mean
Ammoniacal N	10·78	10·38	9·87	10·34
Melanin N	1·46	1·43	1·49	1·46
Diamino N	23·94	25·14	22·18	23·75
Monamino N	63·82	63·05	66·48	64·45
				100·00

Casein (*Prep.* 2 *d*).

	(1)	(2)	(3)	(4)	Mean
Ammoniacal N	9·91	10·31	10·17	10·22	10·13
Melanin N	1·66	—	2·28	2·14	2·01
Diamino N	—	—	25·30	(23·40)	24·35
Monamino N	—	62·99	62·24	64·24	63·24
					99·73

Casein (*Prep.* 3 *b*).

	(1)	(2)	(3)	(4)	Mean
Ammoniacal N	10·42	10·29	10·55	10·69	10·49
Melanin N	1·59	2·00	1·51	1·64	1·69
Diamino N	(28·06)	(20·67)	24·66	(24·97)	24·60
Monamino N	59·92	67·04	(63·28)	62·70	63·28
					100·02

Casein (*Prep.* 3 *d*).

	(1)	(2)	Mean
Ammoniacal N	10·65	10·00	10·33
Melanin N	1·65	1·70	1·68
Diamino N	22·47	22·11	22·29
Monamino N	65·23	66·19	65·71
			100·01

The mean of all the results gives the following values for caseinogen and casein :—

	Caseinogen	Casein
Ammoniacal N	10·23	10·31
Melanin N	1·53	1·66
Diamino N	22·94	24·03
Monamino N	65·31	63·90
	100·01	99·90

Conclusions.

The difference between caseinogen and casein is thus scarcely appreciable. If for caseinogen the second series alone is taken, the difference is still less than that given above. No difference could be established between the different fractions of casein.

In conclusion I wish to thank the University Colston Research Committee for a grant which defrayed most of the expenses of this work.

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IV. A CONTRIBUTION TO THE STUDY OF A PROTEOLYTIC ORGANISM.

By JOHN MALCOLM DRUMMOND.

From the Frankland Laboratory, Manchester University.

(*Received December 22nd, 1913.*)

During a recent investigation of the bacterial population of a number of sewage sludges, the author isolated an organism which possessed the property of causing the rapid liquefaction of gelatin.

It was thought that a more detailed examination of this organism and its mode of action might prove of interest. Accordingly a pure culture on gelatin peptone bouillon was obtained in the usual way by the method of repeated "plating" and the characteristics of the organism were investigated.

On gelatin plates slightly greyish colonies develop in from 24 to 36 hours. These colonies are round with whitish opaque centres and the plate is rapidly liquefied.

Gelatin peptone bouillon (*G.P.B.*). A cup of liquefied medium appears within 48 hours and a funnel-shaped growth can be observed along the track of the needle. Liquefaction continues downwards, finally yielding an amber coloured viscous liquid which in old cultures darkens in colour and becomes more mobile. A dense white deposit forms at the bottom of the tube leaving the supernatant liquid transparent. A putrefactive odour is noticed which disappears as the age of the culture increases.

Glucose gelatin peptone bouillon. In a shake culture, gas production occurs and later liquefaction sets in on the surface of the gelatin.

Nitrate peptone bouillon. Nitrate is reduced to nitrite.

Ammonia is produced in peptone cultures.

Potato. A brownish growth with little tendency to spread.

Agar peptone bouillon. A white fern-shaped growth spreading along the track of the needle.

Milk. Coagulation followed by alkalinity. The characteristic reduction of neutral red and gas evolution occur. The organism is, therefore, a member

of the Proteus or intestinal group and the laboratory number D4 was assigned to it. Microscopically the organism is a short broad bacillus of dimensions $1{\cdot}4 \times 0{\cdot}6\ \mu$. Its length, however, varies somewhat. It can exist singly but generally forms chains often of considerable length. It is motile, does not form spores and in old cultures usually takes up an involution form.

To ascertain whether the proteolytic action of the organism was due to the presence of an extracellular enzyme the following experiments were carried out. A short stab culture was made in nutrient gelatin and the organism allowed to develop until about one-fourth of the gelatin was liquefied. To this was added in one case toluene and in another a few drops of water saturated with thymol, and the tubes incubated at 21°. Liquefaction continued, and since the action of most organisms is inhibited by thymol and toluene it follows that a proteolytic enzyme was probably secreted by the bacillus.

In other experiments a large quantity of nutrient gelatin was liquefied by inoculation with the bacillus and the products submitted to filtration through a Chamberland filter candle. By this means an absolutely sterile filtrate was obtained. This was checked by plating: no colonies developed and microscopical examination of the filtrate failed to show the presence of any bacteria.

With this filtrate experiments according to the method described by Fuhrmann were made.

100 grams of gelatin were dissolved in 1 litre of water saturated with thymol. A little very finely powdered cinnabar was then added and the mixture thoroughly shaken.

15 to 20 cc. of this mixture were poured into each of a number of tubes, which were placed in a sloping position and a jet of cold water allowed to flow over them until the gelatin began to set. They were then kept in an upright position and the gelatin allowed to solidify completely; the cinnabar becomes evenly distributed through the gelatin.

The sterile filtrate was then poured into these tubes in sufficient quantity to cover the top of the gelatin slope, a little thymol water being added to prevent the growth of moulds etc.

Liquefaction set in readily and its progress could be followed by the gradual disappearance of the red gelatin slope, the cinnabar as liberated collecting at the junction of the enzyme solution and the solid gelatin. To a portion of the sterile filtrate absolute alcohol was added and the precipitate obtained filtered off.

After drying *in vacuo* at 40° to remove alcohol, this precipitate was

redissolved in sterile water containing thymol and the above experiments repeated with similar results.

Two tubes of gelatin peptone bouillon were next taken and the medium melted. To one was added a little of the precipitate obtained as above and a few drops of thymol water, while the second tube was kept without additions, to act as a check. Both tubes were incubated at 37° for 24 hours and then placed in ice when the check tube of gelatin at once solidified while that containing the precipitate remained liquid after standing in ice for over one hour.

Thus the precipitate contained the proteolytic enzyme, and while its action was rapid at 37° and less so at temperatures below this, no optimum could be obtained since the gelatin was liquid at 27° and consequently no rate of action could be determined above that temperature.

These experiments, therefore, prove that the power of causing the liquefaction of gelatin, exhibited by the organism, is due to the presence of an extracellular proteolytic enzyme.

It had been observed in a previous experiment that upon standing in the presence of the organism, the enzyme contained in the products of liquefaction of nutrient gelatin was destroyed, whereas the sterile enzyme solution retained its activity, at any rate for several days. It was proposed to endeavour to determine when the enzyme first makes its appearance and when it is destroyed and a number of experiments were made with this end in view.

Several tubes of the following description were made. A piece of narrow glass tubing about 2 inches long and $\frac{1}{10}$ inch internal diameter was fused to a thin glass rod 4 inches in length. The tube portion was graduated in millimetres and filled completely with thymol gelatin containing a small quantity of cinnabar. Before each tube was filled it was gauged by inserting a wooden peg in the open end. By this means it was ensured that the same surface area of gelatin would be in contact with the solution in every case and thus comparable results be obtained.

The rod attached to this tube was passed through a cork of such a size as to fit a number of culture tubes. Cultures were made in peptone water, inoculation being made by loop from an old culture of D4 in gelatin peptone bouillon, and after 24 hours' incubation at 37° toluene was added to one tube, thoroughly shaken and one of the above described tubes introduced so that about 2 millimetres of the gelatin tube were below the surface of the peptone water. 72 hours after inoculation a second tube was treated in a similar manner and also other tubes, 192 hours and 888 hours respectively after inoculation.

Any liquefaction of the gelatin that might occur would be due to the presence of the enzyme and the rate would be proportional to the amount of enzyme present.

The limit to which liquefaction had proceeded could easily be read on the graduated scale, a sharp line being discernible between the solid and liquid gelatin; the former being red and opaque when viewed by reflected light and the latter clear and colourless. The results of one set of experiments are given below.

(i)	24 hours	culture,	liquefaction	after 20 days	= 0·0 mm.
(ii)	72 ,,	,,	,,	,,	= 8·8 ,,
(iii)	192 ,,	,,	,,	,,	= 22·5 ,,
(iv)	888 ,,	,,	,,	,,	= 16·4 ,,

The enzyme was not secreted at once but increased in amount as the age of the culture increased up to a certain point after which it was slowly destroyed.

This disappearance of the enzyme also occurred when the organism was cultured in peptone bouillon.

(v)	48 hours	culture,	liquefaction	after 30 days	= 44·0 mm.
(vi)	49 days	,,	,,	,,	,, = 6·8 ,,

From the foregoing experiments it is seen that the enzyme was secreted when the organism was grown upon nutrient gelatin, peptone bouillon and peptone water. It is therefore conceivable that the enzyme is produced in the ordinary metabolism of the bacillus and regardless of the medium upon which it is cultivated. To test the accuracy of this hypothesis cultures were made in sterile egg albumin in water and also in gelatin containing a few inorganic salts. In the latter no liquefaction occurred and in the experiment with egg albumin no enzyme was found to be present (as measured by the thymol gelatin method), although when examined microscopically a number of the bacilli were found in a normal condition, and when cultured on nutrient gelatin rapidly liquefied the medium. It therefore appears that the presence of a peptone is essential to the production of the enzyme, which may be a combination of a substance secreted by the organism with a peptone. This enzyme thus formed then attacks the protein present, peptonising it and so converting it into a form suitable for the production of still more enzyme, thereby increasing the action.

If this is the case it might be expected that the higher the protein present the more enzyme would be produced provided the action could be started.

The following results corroborate this, the figures given being the amount of liquefaction which occurred after 30 days.

(*a*) Peptone water = 18 mm.
(*b*) Peptone broth = 44 ,,
(*c*) Nutrient gelatin G.P.B. = 93·6 ,,

Experiments were made to determine whether the enzyme possessed a pepsin or trypsin character and also to ascertain whether coagulated egg albumin was attacked by the sterile filtrate obtained from a culture in egg albumin although it had no action upon gelatin.

The tubes described below were therefore made up, a strip of coagulated egg albumin being introduced into each.

1. 2 cc. sterile filtrate from G.B.P. culture + 3 cc. thymol water.
2. 2 cc. ,, ,, ,, ,, +2 cc. ,, ,, +1 cc. 1 % sodium carbonate solution.
3. 2 cc. sterile filtrate from G.B.P. culture + 2 cc. thymol water + 1 cc. 0·2 % hydrochloric acid solution.
4. 4 cc. sterile filtrate from egg albumin culture + 2 cc. thymol water.
5. 4 cc. ,, ,, ,, ,, ,, +1 cc. ,, ,, +1 cc. 1 % sodium carbonate solution.
6. 4 cc. sterile filtrate from egg albumin culture + 1 cc. thymol water + 1 cc. 0·2 % hydrochloric acid solution.

After several weeks considerable decomposition had occurred in 1, 2 and 3, and to a similar extent in each case, but no change in the albumin could be detected in 4, 5 or 6.

Therefore no enzyme is secreted when the organism is grown upon egg albumin and the enzyme from nutrient gelatin cultures can act in both acid and alkaline solutions.

A number of experiments were made in order to obtain data characteristic of the enzyme which could be used for purposes of identification.

Thus the rate of liquefaction of gelatin by the enzyme (obtained from a culture in nutrient gelatin) was determined in presence of six typical acids, the amount of each present in an individual tube varying from 0·1 % to 1 % of the total contents.

Certain precautions were taken to ensure strictly comparable results and a number of curves were obtained.

It was found that the rate of liquefaction was inhibited by every acid under observation but that the retardation varied both with the acid present and the concentration. A rise in concentration of the foreign substance invariably produced a decrease in the rate at which liquefaction proceeded, but for a given rise in concentration the amount of retardation was not necessarily the same for every compound; e.g. an increase in concentration of

acetic acid from 0·1 % to 0·3 % merely slowed down the rate whereas a similar increase in concentration of hydrochloric acid was sufficient to arrest liquefaction altogether.

That the constitution of the acid present produces an effect upon the rate of liquefaction is shown by the fact that acetic and lactic acids produced the least and an approximately equal decrease in the rate of action. Tartaric and citric occupy an intermediate position while oxalic and hydrochloric acids exercised the greatest action. In the observations under discussion 0·6 % of oxalic acid arrested all liquefaction while 0·2 % of hydrochloric acid reduced the rate of liquefaction by 80 % and 0·3 % was entirely prohibitive.

It is probable also that the degree of dissociation of the acids has a considerable effect on the proteolytic activity of the enzyme, an increase in the concentration of the hydrogen ion exercising a prohibitive action.

This view is borne out by the position of the acids when placed in the order of the effect they have upon the rate of liquefaction, viz. hydrochloric, oxalic, citric, tartaric, acetic and lactic.

It is hoped that further work may be done on this subject, more especially with regard to the action of the bacillus upon blood serum and other albumins.

The author desires to thank Dr G. J. Fowler for his invaluable help and suggestions throughout the course of the work.

V. ACETYLCHOLINE, A NEW ACTIVE PRINCIPLE OF ERGOT.

By ARTHUR JAMES EWINS.

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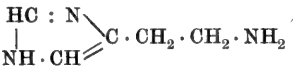
(*Received January 7th, 1914.*)

Investigations carried out during the past few years have resulted in the isolation from ergot of several active principles, the presence of which adequately accounts for those actions of the drug which have been regarded as specially related to its therapeutic effects, each type of action having been advocated by one observer or another as a basis for its physiological standardisation.

The principles in question are (1) the alkaloid ergotoxine $C_{35}H_{41}O_6N_5$ (the hydroergotinine of Kraft [1906]) which was isolated by Barger and Carr [1907] and further investigated by Barger and Ewins [1910]; (2) *p.* hydroxyphenylethylamine, $OH.C_6H_4.CH_2.CH_2.NH_2$ [Barger 1909; Barger and Dale 1909], and (3) β-iminazolylethylamine



[Barger and Dale 1910], the last two being amines derived respectively from the amino-acids tyrosine and histidine by decarboxylation.

In addition, however, to those of apparent therapeutic importance, certain other effects are shown in a more or less marked degree by all samples of the drug and by some with marked intensity. These effects have been familiar to the pharmacological workers in these laboratories for some years, but apparently have not been the subject of exact description or investigation. Conspicuous among these is an inhibitor effect on the heart, suggesting an intense though curiously evanescent muscarine action. Finding that the prominence of this effect in the action of different specimens of ergot ran closely parallel with their stimulant action on intestinal muscle, and that both effects were abolished by atropine, Dr Dale was led to suspect the presence in ergot of a principle producing both these effects. He was also able to

devise a convenient method for its physiological estimation by the use of a loop of rabbit's intestine isolated according to Magnus's method. When an opportunity recently occurred of obtaining an adequate supply of a preparation which exhibited this type of action with marked intensity, he suggested to me that an attempt should be made to identify the supposed new principle, and has followed the successive steps of its isolation with the physiological control. This paper deals with the chemical procedure by which the principle in question was isolated and identified. The details of its action will be described elsewhere by Dale.

The physiological effects described above resemble somewhat closely those described for muscarine, especially as the action in both cases is completely abolished by small amounts of atropine. On other grounds also it appeared not improbable that ergot might contain muscarine, since choline has long been recognised as one of its constituents [Brieger 1887], while Böhm [1885] had shown many years ago that fungi other than *Amanita muscaria* contain muscarine. Preliminary experiments carried out with a view to the isolation of the base, lent further support to this idea, since it was always found associated with the choline fraction. Thus it was completely precipitated from alcoholic solution, and partially precipitated from concentrated aqueous solution by mercuric chloride, and when the extract was fractionally precipitated by means of silver and baryta (Kutscher's method) only the last (choline) fraction contained any of the active base with which we were concerned.

The actual isolation of the active base was finally accomplished by the method which is set out in detail in the experimental portion of this paper. In the first instance there was obtained only a very small quantity of a crystalline platinichloride; 2·8 milligrams in all. The base from this was found to be extremely active in producing the physiological effects with which we were concerned. Such chemical comparison as was possible with this minute quantity of material tended to support the identity of the unknown base with muscarine. The melting point, for example, was practically the same as that of synthetic muscarine platinichloride. A physiological comparison made by Dale with both natural and synthetic muscarines[1] showed however that the new base could not be either of these.

The clue to its identity was furnished by the observation, made repeatedly during the process of its isolation, that it was very susceptible to the action of alkali. In fact a dilute solution of the active base, if made distinctly alkaline with caustic soda at the ordinary temperature and neutralised again

[1] For the former we are indebted to the kindness of Dr O. Rosenheim.

almost immediately, loses nearly all its original activity. This fact, and its constant association with choline, suggested that it might be a choline ester. This was the more probable since Hunt and Taveau [1911] had already described a number of choline derivatives, many of which were physiologically considerably more active than choline itself. Of these derivatives acetylcholine had been indicated as one of the most active bases, and was also on general grounds the most likely to occur naturally. A small quantity of acetylcholine was therefore prepared by the method originally described by Nothnagel [1894]. Comparison of the physiological action of this base with that isolated from the extract of ergot showed that it was qualitatively and quantitatively the same. Further, by working up a larger quantity of the ergot extract, there was obtained sufficient of the crystalline platinichloride of the active base, to establish beyond doubt, by melting point and analysis, its identity as acetylcholine.

Acetylcholine therefore exists in ergot and is the base responsible for the physiological action described at the commencement of this paper. That it occurs as such in the original ergot grains, is shown by the fact that a fresh extract made by boiling the drug with dilute alcohol, produces the effects shown by the extract prepared according to the directions of the British Pharmacopoeia. The presence of acetylcholine is consequently not due to fermentative or other changes taking place during the preparation of the extract.

Experimental.

With the help of the physiological control above mentioned the following method was ultimately adopted and led to the isolation of the active base.

The preparation available for investigation was a liquid extract, prepared according to the directions of the British Pharmacopoeia. 1600 cc. of this extract were concentrated under reduced pressure on the boiling water-bath to remove the alcohol. The residual syrupy liquid was diluted with water to 480 cc. and aqueous mercuric chloride added until no further precipitation occurred. The amount required was 1100 cc. of a saturated aqueous solution. The precipitate was filtered off, washed with water and, since it was found to be almost physiologically inactive, discarded. From the filtrate and washings the slight excess of mercury was removed as sulphide, and the excess of sulphuretted hydrogen by means of a current of air. The solution was then neutralised with sodium carbonate and concentrated on the boiling water-bath

under reduced pressure to a thin syrup. This was poured into strong alcohol (92–95%) and allowed to stand for a few hours. The gummy precipitate was filtered off, washed with alcohol, and, being found to be practically inactive, discarded. The alcoholic filtrate and washings were taken to dryness and the residue dissolved in a small quantity of pure methyl alcohol, in which it was almost completely soluble. The methyl alcoholic solution was again precipitated by the addition of four or five volumes of absolute alcohol. The precipitate was filtered off, the filtrate evaporated to dryness, and the residue completely extracted with small quantities of absolute ethyl alcohol. If necessary, precipitation by means of excess of alcohol was repeated, until the alcoholic solution obtained gave no further precipitate on addition of a large volume of alcohol.

To the alcoholic solution (240 cc.) so obtained alcoholic mercuric chloride (560 cc.) was added until no further precipitate was produced. The mixture was allowed to stand over night. The precipitate, which contained practically the whole of the active base originally present in the alcoholic solution, was filtered, washed with alcohol, and then extracted four times with boiling water, 150 cc. of water being used for each extraction. The portion of the mercury precipitate insoluble in hot water was found to be inactive and was therefore discarded. The hot aqueous extract on cooling deposited a further small quantity of precipitate which was very slightly active and was neglected. The clear, light yellow filtrate was then concentrated *in vacuo* on the water-bath. A precipitate, which was for the most part crystalline, soon commenced to separate. Concentration was continued until the volume was about 50 cc. The solution was then cooled and the precipitated mercuric chloride compound filtered off, when there was obtained 17 grams of a mixture consisting for the most part of the mercuric chloride compounds of choline and the active base. The salt was finely ground in a mortar, suspended in about 200 cc. of water, and decomposed by sulphuretted hydrogen. The mercuric sulphide was filtered off, re-suspended in water, and again treated with sulphuretted hydrogen, the process being repeated until decomposition was complete. The combined filtrates and washings were freed from sulphuretted hydrogen by a current of air, and the strongly acid solution treated with freshly precipitated silver carbonate until free from chlorine ions. The slight excess of silver in solution as carbonate was removed as sulphide and excess of sulphuretted hydrogen again removed by air. The slightly alkaline solution was then neutralised with tartaric acid, and a further amount of the latter, equal to that required for neutralisation, was added. The solution was then taken to dryness *in vacuo* on the water-bath at 60°–70°, and the residue

completely extracted with absolute alcohol. The alcoholic solution was concentrated to small volume (15 cc.) and allowed to stand. After about 18 hours the acid tartrate of choline which had separated[1] was filtered off, and, when dry, weighed 0·3 gram. It was identified as choline by:—

(*a*) the mercuric chloride compound m.p. 249–250°,

(*b*) the aurichloride m.p. 261–262°,

(*c*) the platinichloride m.p. 245°.

An analysis of the aurichloride gave the following result:

0·1625 gave 0·0722 Au. Au = 44·43 per cent.
Calculated for $C_5H_{14}ONCl$. $AuCl_3$. Au = 44·47 per cent.

The alcoholic filtrate from the choline tartrate was next treated with an alcoholic solution of platinum chloride until no further precipitate was produced. The precipitated platinichlorides were filtered off and dried. The weight obtained was 1·3 grams. A small quantity of this platinichloride was decomposed by evaporating its aqueous solution to dryness with an excess of potassium chloride, extracting with absolute alcohol, evaporating off the alcohol, and dissolving the residue in a little water. This solution when tested physiologically was extremely active and it was found that practically the whole of the active base had been precipitated, as platinichloride. In order to separate the platinichloride of the active base from that of choline, which was still present, the main portion of the platinichlorides was treated with 2 cc. of boiling water in which it completely dissolved. The solution was then cooled to about 35°, when a small quantity of imperfectly formed polyhedra separated, which were quite different in appearance from the platinichloride of choline, which at the temperature indicated remained completely in solution. The crystals were filtered off, washed with a little cold water and dried at 100°. There was thus obtained 0·205 gram of a platinichloride melting at 253–254°. On decomposing a few milligrams by the method already described, the solution of the free base obtained was qualitatively and quantitatively indistinguishable in physiological action from a specimen of acetylcholine prepared by Nothnagel's method.

The platinichloride was recrystallised from a little hot water. It is considerably less soluble in water than choline platinichloride. On recrystallising, however, a certain amount of hydrolysis occurs, as was pointed out by Hunt and Taveau [1911]. On cooling there was obtained 0·060 gram of platinichloride, which in solubility, crystalline form, and melting point

[1] This method was employed by Honda [1911] for the separation of choline for muscarine.

(256–257°) was identical with that of acetylcholine prepared from choline by the action of acetylchloride.

Analysis gave the following results:—

0·0383 g. gave 0·0109 g. Pt.	Pt = 28·4 per cent.
Calculated for acetylcholine platinichloride $(C_7H_{16}O_2NCl)_2PtCl_4$.	Pt = 27·8 per cent.

Summary.

An active principle of ergot, recognisable by its inhibitor action on the heart and its stimulant action on intestinal muscle, has been identified as acetylcholine.

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VI. ON THE BEHAVIOUR OF TRYPSIN IN THE PRESENCE OF A SPECIFIC PRECIPITATE.

By AGNES ELLEN PORTER, *Carnegie Research Scholar.*

From the Royal College of Physicians Laboratory, Edinburgh.

(*Received Dec. 17th, 1913.*)

In a former paper on amylase [1913], I discussed the possibility of finding a substitute for complement in the recognition of antibody as well as the advantages which would follow if such a substitute were found.

Certain animal ferments are rather similar to complement in their general behaviour, each being a hydrolyst of a particular substrate by which it is first absorbed. Each is also readily taken up by different substances such as collodion and gelatin membranes [Porter 1909, 1910, Pribram 1910], or even inorganic precipitates [Hailer 1908, p. 283]. Pepsin absorbed into egg-white [Dauwe 1905, p. 426], or pepsin, ptyalin etc. into collodion [Porter 1910, p. 382] can be recovered in a small degree by a solution of the particular substance which they digest. Pepsin absorbed into collodion was however unable to act on a solid protein suspended in water [Porter 1910, p. 379]. As it is only by producing digestion that these ferments are recognised,—through the introduction of their particular substrate,—this possibility of partial recovery presents the chief difficulty in the use of ferments as substitutes for complement.

The first attempt to absorb a ferment by means of a specific precipitate was made by Hailer [1908, p. 280]. His choice of rennet was unfortunate, as it loses hardly at all on dilution, being active at a dilution of 1 in 340,000 [Porter 1911, p. 394]. Still, though unsuccessful at stronger concentrations, his results do show with diluted rennet that some absorption had taken place. Ptyalin [Porter 1913, p. 601] displays only a very slight affinity for a precipitate of this nature, while taka diastase shows itself quite indifferent.

Ferments may be divided into two classes in respect to their behaviour with serum. In the one class are the proteolytic ferments which combine with serum proteins in the dissolved condition so firmly that they are inhibited by normal serum. On the other hand non-proteolytic ferments, the action of many of which is accelerated by serum, form much less stable compounds

with precipitated serum proteins. In search of a suitable ferment, I treated a number of ferments by adding diluted serum and saturating with carbon dioxide. Next morning the precipitate was removed, after centrifuging, and the upper fluid tested for ferment. Emulsin and lipase were unaffected by this treatment, except that insoluble lipase is removed. On turning to proteolytic ferments, pepsin was found less influenced by the globulin precipitate than might have been expected, possibly because the acidity of the carbonic acid enabled it to produce some hydrolysis. A rennet was accidentally examined in the form of a mere trace in a sample of trypsin for which I have to thank Dr Cramer. After precipitation with carbon dioxide the upper fluid differed from the control in not being able to curdle milk at 56°, although it increased its viscosity figure.

Trypsin alone, of all the ferments which I have studied, is absolutely absorbed by a diluted serum precipitated with carbon dioxide, while it is only partly absorbed by the same dilution of the same serum before precipitation. If the upper fluid is left in contact with the precipitate only for one night, and then removed from it, it will be found by every test applied to it devoid of trypsin.

Method.

A trypsin solution was made by dissolving Armour's pancreatin in water. The powder did not perfectly dissolve in the proportion of 1 gram to 100 cc., so that after standing for a night in the cold room, the upper fluid was separated off. The solution was kept in the cold room. Several methods were employed of testing tryptic action:

(1) by dissolving coagulated serum contained in Mett's tubes, as recommended by Dauwe [1905] in the case of pepsin,

(2) by dissolving fibrin,

(3) by forming hollows on serum plates, as recommended by Mueller and Jochmann [1906],

(4) by rendering dilute caseinogen incapable of being precipitated by 0·25 % acetic acid,

(5) by lowering the viscosity of caseinogen according to Golla [quoted from Eve 1910].

The most delicate and rapid method is undoubtedly the digestion of caseinogen. This cannot be tested in the presence of serum by means of the ordinary method of slight acidulation, as the globulins of the serum are as easily precipitated by acid as is the caseinogen itself. The viscosity method of Golla, as described by Eve [1910], is very simple and accurate, very small

differences indeed being estimated with the help of a stop-watch. This method presents a very severe test of trypsin absorption because, as I have pointed out, an absorbed ferment is much more easily reached by a solution of its corresponding "substrate" (the specific substance on which it acts) than where this substrate is in solid form, suspended in a medium which is in contact with the absorbed ferment. The form of caseinogen used was 2, 3, and 4 % casumen, a soluble preparation of caseinogen (that is to say easily soluble with heat and shaking), which was made up fresh on each occasion. Digestion was carried out at 56° to prevent bacterial action. The method of using serum tubes was found rather slow, but fibrin was a good deal used, being either digested in the presence of the serum precipitate or else treated with it and afterwards washed and transferred to an alkali. Digestion in this case was carried on at 37° under toluene. The method is somewhat rough so that fine differences are not distinguishable, but it is quite serviceable.

Trypsin in serum precipitated with CO_2.

A. *Examination of upper fluid alone.*

The following solutions were made up.

(1) To 3 cc. ox serum were added 15 cc. trypsin solution and 12 cc. water (i.e. 50 % trypsin, and 10 % serum).
(2) A control trypsin solution 50 % in water.
(3) N/10 NaOH containing 1·6 % NaCl, called NaCl—NaOH.
(4) 10 % ox serum in NaCl—NaOH.
(5) N/5 Na_2CO_3.
(6) 10 % ox serum in N/5 Na_2CO_3 (containing no NaCl as this rendered the serum too cloudy).

No. 1, the mixture containing trypsin and serum, was saturated with CO_2, and left over night in the cold room. Next morning the upper fluid was separated from the precipitate after centrifuging.

This upper fluid was then tested for trypsin by the following methods:

With Fibrin.

		5 hrs.	1 day	2 days
(1)	0·75 cc. control trypsin + 0·75 cc. NaCl—NaOH	—	gone	—
(2)	,, ,, ,, + ,, serum—NaCl—NaOH ...	—	0·5	—
(3)	,, ,, ,, + ,, serum—Na_2CO_3	0·5	—	0·75
(4)	,, absorbed trypsin upper fluid + 0·75 cc. NaCl—NaOH ...	—	0	—
(5)	,, ,, ,, ,, + ,, Na_2CO_3	0	—	0

Fibrin in NaOH and Na_2CO_3 also negative, except of course for swelling.

With Mett's Tubes (Serum).

Absorbed serum with Na_2CO_3 or NaCl and Mett's tubes was negative after a week, while control trypsin with serum under similar conditions was partly digested in a day or two.

With Serum Plates.

		24 hrs. at 56°
(1)	Equal parts control trypsin + 10 % serum in NaCl—NaOH	Faint hollow
(2)	,, absorbed trypsin upper fluid + NaCl—NaOH	Negative

With the Viscosity Test.

(Each contained 5 cc. 2 % casumen; 10 hours at 56°.)	Mins.	Secs.
(1) 0·75 cc. control trypsin + 0·75 cc. 10 % serum—NaCl—NaOH	1	30
(2) ,, ,, ,, + ,, ,, serum—Na_2CO_3 ...	1	25
(3) ,, absorbed trypsin upper fluid + 0·75 cc. NaCl—NaOH...	1	46
(4) ,, ,, ,, ,, + ,, Na_2CO_3 ...	1	38
(5) 1·5 cc. H_2O	1	43

With Caseinogen (tested by acid).

Serum containing globulins cannot be used with this test, so very dilute trypsin was substituted for trypsin serum.

(Each contained 2 cc. 0·1 % pure caseinogen, 24 hours at room temperature.)

	Precipitate with 0·25 % acetic
(1) 0·75 cc. 1·25 % trypsin + 0·75 cc. Na_2CO_3	0
(2) ,, 50 % absorbed trypsin upper fluid + 0·75 cc. Na_2CO_3	+

By the above tests it is clear that trypsin is only partially absorbed by 1/10 normal serum, but is wholly absorbed by the same strength of serum which has been precipitated by saturation with carbon dioxide. That is to say, if the precipitate bearing the trypsin is removed out of the serum the remaining fluid is found to be without trypsin.

It is however necessary to discover whether the absorbed ferment is so fixed that it cannot be recovered by the addition of more protein, especially in solution and in an alkaline medium.

B. *Examination of upper fluid plus precipitate.*

The following mixtures were made, in water, and all precipitated by saturation with CO_2.

(1) Ox serum 10 %, trypsin solution 50 %.
(2) ,, 5 %, ,, ,, ,,
(3) ,, 2·5 %, ,, ,, ,,
(4) ,, 1·25 %, ,, ,, ,,
(5) No serum ,, ,, ,,

Next day each mixture was shaken up, and the whole, containing the precipitated globulin, was tested for trypsin by the following methods:

With Fibrin.

(In this case the alkali was added to the precipitate before the fibrin.)	Time, over night at 37°. Result
(1) 0·75 cc. control trypsin + 0·75 cc. 10 % serum in Na_2CO_3	0·5 dissolved
(2) ,, ,, ,, + ,, 5 % ,, ,,	all ,,
(3) ,, ,, ,, + ,, 2·5 % ,, ,,	,, ,,
(4) ,, ,, ,, + ,, 1·25 % ,, ,,	,, ,,
(5) ,, ,, ,, + ,, Na_2CO_3	,, ,,
(6) ,, absorbed trypsin (10 % serum) + 0·75 cc. Na_2CO_3	Slightly ,,
(7) ,, ,, ,, (5 % ,,) + ,, ,,	Almost ,,
(8) ,, ,, ,, (2·5 % ,,) + ,, ,,	Dissolved
(9) ,, ,, ,, (1·25 % ,,) + ,, ,,	,,
(10) ,, H_2O + ,, ,,	0

If the fibrin be added to the precipitated serum mixture while it is neutral, and after an hour removed, washed and placed in an alkaline solution, less of the ferment is recovered.

		Next day
(1)	0·75 cc. control trypsin + 0·75 cc. 10 % serum in NaCl	0·5 dissolved
(2)	,, absorbed ,, + ,, 1·6 % NaCl	0

With the Viscosity Test.

(Each + 5 cc. 3 % casumen, containing 20 % N/5 Na_2CO_3.) Time 4 hours at 56°, then room temperature for 24 hours.

		Hours 4 min.	4 secs.	24 min.	24 secs.	
(1)	0·75 cc. control trypsin + 0·75 cc. 10 % serum in Na_2CO_3	1	42	1	42	Time in Viscosimeter
(2)	,, ,, ,, + ,, 5 % ,, ,,	1	36	1	36	
(3)	,, ,, ,, + ,, 2·5 % ,, ,,	1	37	1	37	
(4)	,, ,, ,, + ,, 1·25 % ,, ,,	1	32	1	32	
(5)	,, ,, ,, + ,, Na_2CO_3	1	30	1	27	
(6)	,, absorbed trypsin (10 % serum) + 0·75 cc. Na_2CO_3	1	47	1	41	
(7)	,, ,, ,, (5 % ,,) + ,, ,,	1	47	1	45	
(8)	,, ,, ,, (2·5 % ,,) + ,, ,,	1	45	1	41	
(9)	,, ,, ,, (1·25 % ,,) + ,, ,,	1	40	1	37	
(10)	,, H_2O + ,, ,,	1	56			

The above experiments show clearly that some trypsin can free itself from the serum precipitate and enter the casumen or fibrin. Still this tendency to recovery is not sufficient to obliterate the difference of the effects of original and precipitated serum upon trypsin, which is still marked enough to permit an expectation that a similar difference may be found in the case of a specific precipitate.

Trypsin with a specific precipitate.

In this case more protein was present,—in the form of antigen,—than in the last, where the influence of serum alone was under investigation. The antigen used was egg-white. The extent to which egg-white absorbs trypsin was next investigated, both with fibrin and by the viscosity test. To 0·15 cc. normal ox serum, or 0·15 cc. saline solution, 0·15 cc. egg-white, in dilutions of from 1/10 upward, was added, followed by 0·75 cc. trypsin solution and 0·75 cc. N/5 Na_2CO_3, and after an hour's contact, fibrin, or 5 cc. 3 % casumen. At these dilutions egg-white exerted no visible influence upon the results, other than by its presence diluting the serum by one-half, and so lessening its inhibitive effect.

Serum was obtained from rabbits which had been treated as described on p. 55, and used in the following condition:

(1) Thrice injected, precipitation at 1/10,000 (absorption of trypsin positive).
(2) Thrice injected, precipitation negative (absorption negative).
(3) Once injected, precipitation at 1/1000.
Twice injected, precipitation not tested higher.
Four times injected, precipitation at 1/10,000 (absorption in all positive).
(4) Normal (absorption negative).
(5) Normal (absorption negative).
(6) Normal (absorption negative).
(7) Normal (absorption negative).
Twice injected, precipitation at 1/5000.
Thrice injected, precipitation at 1/5000, not tested higher (absorption in both positive).
(8) Normal (absorption negative).

Experiment I.

Rabbit serum 1, to each tube added 0·75 cc. trypsin solution + 0·75 cc. Na_2CO_3 N/5, after 0·5 hour 5 cc. 3 % casumen. Time 2 hours at 56°.

							Viscosity min.	secs.
(1)	0·15 cc.	Immune	serum	1 + 0·15 cc.	1/10	egg-white	1	45
(2)	,,	,,	,,	+ ,,	1/100	,,	1	46
(3)	,,	,,	,,	+ ,,	1/250	,,	1	46
(4)	,,	,,	,,	+ ,,	1/500	,,	1	46
(5)	,,	,,	,,	+ ,,	1/1000	,,	1	40
(6)	,,	,,	,,	+ ,,	1/2500	,,	1	40
(7)	,,	,,	,,	+ ,,	1/5000	,,	1	40
(8)	,,	,,	,,	+ ,,	NaCl		1	40

(All later viscosity experiments from this point were tested with a stop-watch.)

Experiment II.

Rabbit 3 injected once, positive, Rabbit 2 injected thrice, negative, and Rabbit 4 normal. To each tube added 0·75 cc. trypsin solution, after 3 hours added 5 cc. 3 % casumen and 0·75 cc. N/5 Na_2CO_3. Digestion at 56° 0·75 hour, then 0° over night.

							Viscosity min.	secs.
(1)	0·15 cc.	Immune	serum	3 + 0·15 cc.	1/10	egg-white	1	32·5
(2)	,,	,,	,,	3 + ,,	1/100	,,	1	31
(3)	,,	,,	,,	3 + ,,	1/250	,,	1	31
(4)	,,	,,	,,	3 + ,,	NaCl		1	30·5
(5)	,,	,,	,,	2 + ,,	1/10	,,	1	31·5
(6)	,,	,,	,,	2 + ,,	1/100	,,	1	31·5
(7)	,,	,,	,,	2 + ,,	1/250	,,	1	31
(8)	,,	,,	,,	2 + ,,	NaCl		1	31·5
(9)	,,	Normal	,,	4 + ,,	1/10	,,	1	29·5
(10)	,,	,,	,,	4 + ,,	1/100	,,	1	29
(11)	,,	,,	,,	4 + ,,	1/250	,,	1	29
(12)	,,	,,	,,	4 + ,,	NaCl		1	29

Experiment III.

Rabbit 3 twice injected, Rabbits 5, 6, 7 and 8, all normal. Each tube received 0·75 cc. trypsin solution, after half an hour's contact 5 cc. 4 % casumen and 0·5 cc. Na_2CO_3 were added. Digestion at 56° for ½ hour, then cold room till next day.

								Viscosity min.	secs.
(1)	0·15 cc.	Immune	serum	3+	0·15 cc.	1/10	egg-white	1	40
(2)	,,	,,	,,	3+	,,	1/100	,,	1	40
(3)	,,	,,	,,	3+	,,	1/500	,,	1	40
(4)	,,	,,	,,	3+	,,	1/1000	,,	1	39
(5)	,,	,,	,,	3+	,,	NaCl		1	36
(6)	,,	Normal	,,	5+	,,	1/10	,,	1	37·5
(7)	,,	,,	,,	5+	,,	1/100	,,	1	37·5
(8)	,,	,,	,,	5+	,,	1/500	,,	1	38
(9)	,,	,,	,,	5+	,,	NaCl		1	37·5
(10)	,,	,,	,,	6+	,,	1/10	,,	1	40
(11)	,,	,,	,,	6+	,,	1/100	,,	1	40
(12)	,,	,,	,,	6+	,,	1/500	,,	1	41
(13)	,,	,,	,,	6+	,,	NaCl		1	40
(14)	,,	,,	,,	7+	,,	1/10	,,	1	40·5
(15)	,,	,,	,,	7+	,,	1/100	,,	1	40·5
(16)	,,	,,	,,	7+	,,	1/500	,,	1	40
(17)	,,	,,	,,	7+	,,	NaCl		1	40
(18)	,,	,,	,,	8+	,,	1/10	,,	1	39
(19)	,,	,,	,,	8+	,,	1/100	,,	1	38·5
(20)	,,	,,	,,	8+	,,	1/500	,,	1	38·5
(21)	,,	,,	,,	8+	,,	NaCl		1	39·5

Experiment IV.

Rabbit 7, twice injected. Each tube received 0·75 cc. trypsin solution, after ¼ hour 5 cc. 3 % casumen and 0·75 cc. N/5 Na_2CO_3. Digestion at 56° for 3 hours.

								Viscosity min.	secs.
(1)	0·15 cc.	Immune	serum	7+	0·15 cc.	1/10	egg-white	1	32
(2)	,,	,,	,,	7+	,,	1/500	,,	1	32
(3)	,,	,,	,,	7+	,,	1/1000	,,	1	32
(4)	,,	,,	,,	7+	,,	1/5000	,,	1	31·5
(5)	,,	,,	,,	7+	,,	NaCl		1	28·5
(6)	,,	,,	,,	7+	,,	,,		1	28·5

Experiment V.

Comparison of some rabbits' sera with fibrin. Immune sera 3 and 7, both twice injected, normal sera, 4, 5, 6 and 7. Each tube received 0·75 cc. trypsin solution, after a night's contact in the cold room, fibrin was added to each mixture next morning, and after half an hour removed, washed, and placed in N/5 Na_2CO_3 at 37° over night.

								Days 1	3
(1)	0·15 cc.	Immune	serum	3+	0·15 cc.	1/10	egg-white	Frayed	Almost gone
(2)	,,	,,	,,	3+	,,	NaCl		Gone	Gone
(3)	,,	Normal	,,	4+	,,	1/10	,,	Frayed	,,
(4)	,,	,,	,,	4+	,,	NaCl		,,	,,
(5)	,,	,,	,,	5+	,,	1/10	,,	,,	Almost gone
(6)	,,	,,	,,	5+	,,	NaCl		,,	Gone
(7)	,,	,,	,,	6+	,,	1/10	,,	,,	Frayed
(8)	,,	,,	,,	6+	,,	NaCl		,,	,,
(9)	,,	,,	,,	7+	,,	1/10	,,	,,	,,
(10)	,,	,,	,,	7+	,,	NaCl		,,	,,
(11)	,,	Immune	,,	7+	,,	1/10	,,	Untouched	Untouched
(12)	,,	,,	,,	7+	,,	NaCl		Frayed	Frayed

Experiment VI.

Immune sera 3 and 7, and normal serum 7, were tested with fibrin and casumen. Rabbit 3 had been injected four times, Rabbit 7 thrice.

With Fibrin.

Hitherto the trypsin had been added to a mixture of serum and egg-white. Here 0·75 cc. trypsin solution was added to serum, and the egg-white added immediately afterwards. After three hours of contact fibrin was immersed for 0·75 hour and then washed and placed in N/5 Na_2CO_3, covered with toluene, at 37° over night.

								Days 1	Days 2
(1)	0·15 cc.	Immune	serum	3+	0·15 cc.	1/10	egg-white	Whole	Whole
(2)	,,	,,	,,	3+	,,	1/100	,,	Gone	Gone
(3)	,,	,,	,,	3+	,,	1/500	,,	,,	,,
(4)	,,	,,	,,	3+	,,	NaCl		,,	,,
(5)	,,	,,	,,	7+	,,	1/10	,,	Whole	Whole
(6)	,,	,,	,,	7+	,,	1/100	,,	0·5 gone	Gone
(7)	,,	,,	,,	7+	,,	1/500	,,	,,	,,
(8)	,,	,,	,,	7+	,,	NaCl		Gone	,,
(9)	,,	Normal	,,	7+	,,	1/10	,,	Almost gone	,,
(10)	,,	,,	,,	7+	,,	1/100	,,	Gone	,,
(11)	,,	,,	,,	7+	,,	NaCl		Almost gone	,,

With Casumen.

To 0·15 cc. of serum were added 0·75 cc. trypsin solution and 0·15 cc. of egg-white. After three hours 5 cc. 3 % casumen and 0·75 cc. N/5 Na_2CO_3 were added. Digestion at 56° for 0·75 hour, then all were transferred to the cold room for the night, and their viscosity determined next morning.

								Min.	Secs.
(1)	0·15 cc.	Immune	serum	3+	0·15 cc.	1/10	egg-white	1	30
(2)	,,	,,	,,	3+	,,	1/100	,,	1	29
(3)	,,	,,	,,	3+	,,	1/500	,,	1	29
(4)	,,	,,	,,	3+	,,	NaCl		1	28
(5)	,,	,,	,,	7+	,,	1/10	,,	1	32·2
(6)	,,	,,	,,	7+	,,	1/100	,,	1	31·4
(7)	,,	,,	,,	7+	,,	1/500	,,	1	29
(8)	,,	,,	,,	7+	,,	NaCl		1	28
(9)	,,	Normal	,,	7+	,,	1/10	,,	1	32
(10)	,,	,,	,,	7+	,,	1/100	,,	1	32·4
(11)	,,	,,	,,	7+	,,	NaCl		1	32·2

The above results show clearly that trypsin can be inhibited by a specific precipitate in a greater degree than by immune serum alone or by normal serum with antigen. This result is, however, only relative. Trypsin is partly inhibited by untreated serum, and it is not wholly inactivated by precipitated serum. Judging from the analogy of the carbonic acid precipitate where trypsin is wholly absorbed, but is in part recoverable, a total inhibition in the presence of a specific precipitate could hardly have been expected. A similar recovery of trypsin—from the antitrypsin of Weinland—has been described by Dastre and Stassano [1903]. Time, which would favour fixation, or inactivation, of absorbed trypsin through aeration [Levy 1905],

also favours the recovery of trypsin from antitrypsin (Dastre and Stassano), a tendency which can also be seen in the above results. The recovery of trypsin is therefore an unfavourable factor which cannot be entirely avoided. It can be diminished by using a solid protein as a test for the ferment, especially in a neutral or slightly acid medium, to avoid tryptic action, as any such action would produce a soluble protein which could combine with the absorbed trypsin. After contact with the serum-trypsin mixture, the solid protein would be removed from the sphere of action of the precipitate into an alkaline medium suitable for promoting the action of any trypsin which it might have taken up. If such precautions are taken—and even without these precautions—trypsin-absorption might well be used in the recognition of antibody. At least, as far as my experience goes, trypsin is the most suitable ferment for the purpose.

Conclusions.

Trypsin is only partially inhibited in the presence of 10 % ox serum.

It is however completely bound by the precipitate produced in 10 % ox serum by saturation with carbon dioxide and if this precipitate be removed the trypsin is completely removed with it.

The absorbed ferment is partly recoverable from the precipitate by protein, especially in solution.

This recovery is not however sufficient to obliterate the difference between the inhibition exerted by 10 % untreated ox serum and that by 10 % precipitated ox serum.

In the presence of a specific precipitate (immune rabbit serum and egg-white) trypsin is more inhibited than by immune serum alone or by normal serum and egg-white. This is especially the case where free trypsin is tested for by a solid protein, but also occurs if the test is carried out with protein in solution.

I wish to thank Professor Ritchie for his kindly interest and advice.

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VII. THE VISCOSITY OF SOME PROTEIN SOLUTIONS.

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(2 figures.)

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In the following work the same general methods were employed as in that already published by Martin and Chick [1912] upon the viscosity of solutions of caseinogen.

In the present instance an investigation was made of the influence of (1) concentration of protein, and (2) temperature, upon the viscosity of solutions of pure crystallised egg- and (horse) serum-albumin. For purposes of comparison, a few experiments were also made with whole serum, in some cases concentrated in vacuo in order to yield material of high protein content. The viscosity, as in the previous work, was determined by measuring the time of flow in an Ostwald viscosimeter, the figures obtained being relative to the time taken under similar conditions by pure water, which is expressed by unity. The concentrations throughout are expressed as grams per 100 grams of solution.

Egg-albumin.

The crystals of egg-albumin were obtained from egg-white by the method of Hopkins and Pinkus [1898]. The material was twice recrystallised, and then dialysed for two or three weeks against distilled water, till it contained an insignificant trace of ammonium sulphate. As considerable dilution took place during dialysis, it was necessary to concentrate the material in order to obtain the high concentrations necessary for the experiments. This was done by allowing the solution to evaporate at room temperature in vacuo over sulphuric acid, material being thus obtained which contained 28 % by weight of egg-albumin. The protein-content in this and other cases was determined by boiling a weighed quantity of the solution, after dilution and acidification, and weighing the coagulum on a weighed filter, after drying at 110°.

TABLE I.

Influence of concentration of the protein upon the viscosity of solutions of pure egg-albumin (crystallised).

Temperature 25·2° C. Time of flow in viscosimeter for water 47·8 seconds.

Concentration of protein, %	Mean time of flow in viscosimeter, secs.	Density of the solution (H_2O at 25° C. = 1)	Coefficient of viscosity ($H_2O = 1$)
28·15	441·8	1·0805	9·99
26·83	367·4	1·0775	8·30
24·33	257·2	1·0693	5·81
20·12	159·2	1·0566	3·60
14·53	97·9	1·0402	2·21
8·877	69·4	1·0242	1·57
3·016	53·8	1·0083	1·22

Influence of concentration of Protein. In Table I are given the details of an experiment showing the influence of concentration upon the viscosity coefficient in the case of this protein. The same results are shown graphically in curve (*a*) of Fig. 1. Solutions containing different proportions by weight of protein were obtained by dilution of the concentrated material described above. In order to apply the necessary correction in calculating the coefficient of viscosity, the density of the solutions was directly determined by weighing a known volume in a pyknometer.

In the case of the weaker solutions, the viscosity remained low, increasing but slightly with increasing concentration of protein and only reaching a value equal to twice that of distilled water at a concentration of protein equal to about 13 %. This is well seen in the curve in Fig. 1, where, up to a concentration of about 9 %, the slope is very slight, in fact the relation between viscosity and concentration of protein approximates to that obtaining in solutions of non-colloidal material, and might satisfactorily be expressed by a straight line. At higher concentrations the curvature becomes increasingly greater until, at a concentration of 28 % protein, the viscosity reaches the figure of nearly 10.

The curve expressing the relation between protein concentration and viscosity in case of solutions of egg-albumin is of the same general type as that obtained for caseinogen [Chick and Martin 1912]; in the case of the latter, however, a comparatively low concentration (7 to 8 %) produced a viscosity equal to the value obtained with 28 % egg-albumin. According to the hypothesis of Hatschek [1910, 1911, 1912] the difference between these two proteins in regard to their viscosity should be attributed to the fact that in a solution of egg-albumin much less water is appropriated

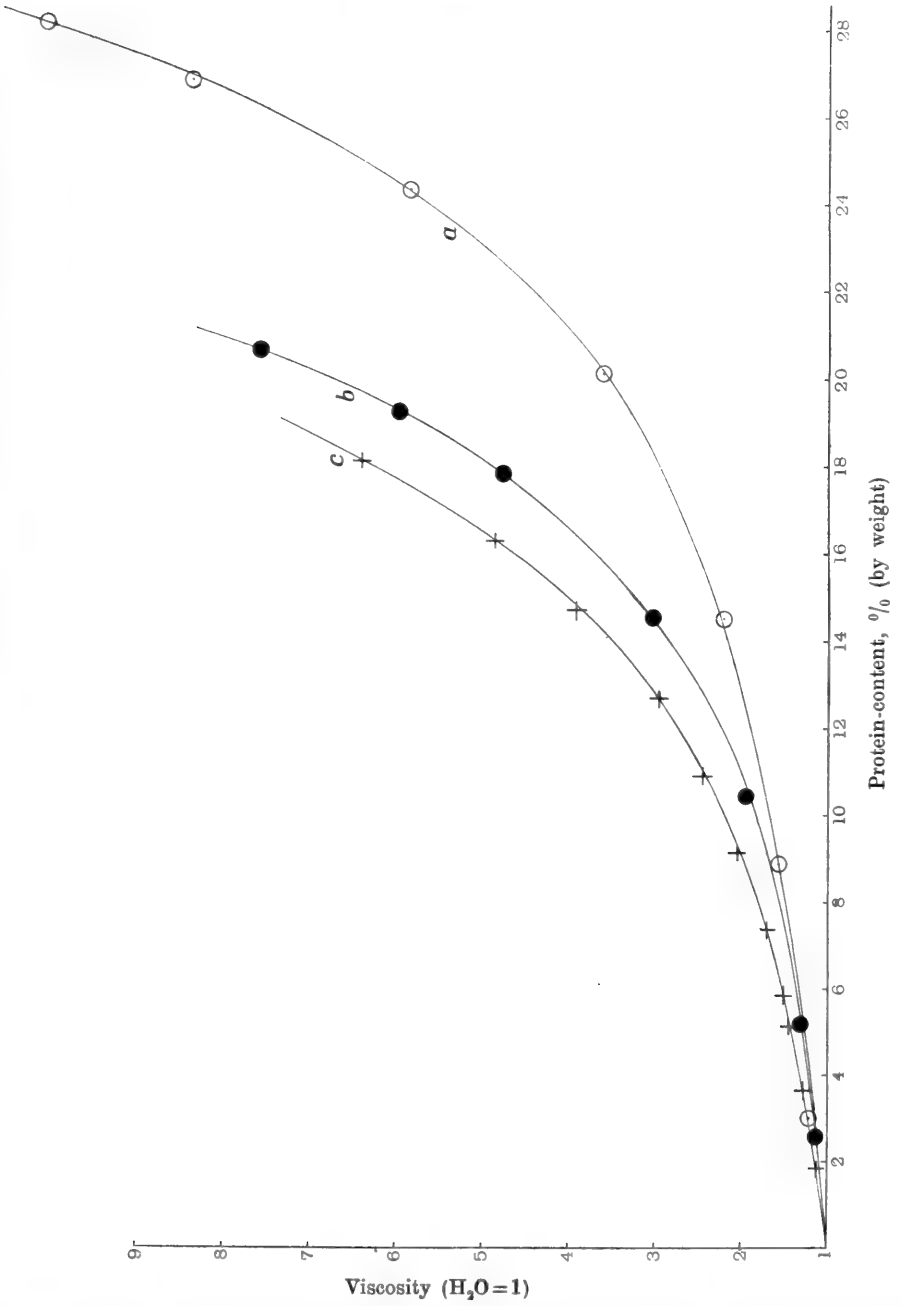

Fig. 1. Influence of protein-concentration upon the viscosity of solutions of various proteins.

Curve (*a*) —○— = pure egg-albumin.
,, (*b*) —●— = pure serum-albumin (horse).
,, (*c*) —+— = whole serum, at protein-concentrations above and below the normal; original serum contained 7·3 % protein.

by the protein phase than is the case with caseinogen. Consequently, in the former case, much higher concentration of protein is necessary to yield the high viscosity characteristic of the condition in which the aggregates of the disperse phase are approaching contact with one another. In a later communication it is proposed to discuss this theory as regards both egg-albumin and other proteins in greater detail.

Influence of Temperature. The variation of temperature was necessarily limited, the maximum range being from 0° to about 40°. Higher temperatures could not be employed without danger of the protein becoming "denaturated."

In order to make the necessary comparison with the time of flow taken by water, experiments were made over the same range of temperature with an equal total volume of distilled water in each of the two viscosimeters employed in Exp. 1 and Exps. 2, 3 and 4 respectively. Smoothed curves

TABLE II.

Influence of temperature upon the viscosity of solutions of pure egg-albumin (crystallised) of varying concentration.

Exp. No.	Protein content, %	Density at 25° C. (H_2O at 25° C. = 1)	Temperature, °C.	Mean time of flow in viscosimeter, seconds: Albumin solution	Mean time of flow in viscosimeter, seconds: Distilled water (from curve)	Coefficient of viscosity (H_2O at the same temperature = 1)
1	7·04	1·0192	2·8	136·8	101·8	1·37
			8·3	115·0	86·0	1·36
			15·2	95·0	70·3	1·38
			25·0	74·2	55·5	1·36
			32·3	63·0	47·9	1·34
			42·1	52·1	40·5	1·31
2	14·6	1·0404	2·8	179·2	86·2	2·16
			8·6	147·4	72·1	2·13
			14·7	123·4	61·5	2·09
			15·1	123·0	61·0	2·10
			25·0	94·0	48·0	2·04
			33·1	79·5	41·4	2·00
			42·9	64·2	34·5	1·94
3	20·1	1·0566	0	368·3	96·4	4·04
			8·0	269·7	73·3	3·89
			17·0	202·3	58·0	3·68
			25·4	161·4	47·6	3·58
			33·0	132·1	41·4	3·37
			41·6	109·5	35·4	3·27
4	28·15	1·0805	0·6	1170·2	93·8	13·48
			7·8	828·6	73·8	12·13
			15·6	614·1	60·1	11·04
			25·4	440·9	47·6	10·01
			33·9	344·9	40·8	9·13
			41·9	283·7	35·1	8·73

were drawn through the experimental points and from these the time of flow corresponding to any intermediate temperature could be read off. The values given in the 5th column of Table II were obtained in this way and, with the help of these, the coefficients of viscosity in the 7th column were calculated.

In case of the weaker solutions (from 7 % up to 20 %) the influence of temperature upon the viscosity was comparatively trivial, that is to say, the phenomenon was of the same order as that obtaining in the case of distilled water. A 7 % solution of egg-albumin, with viscosity of 1·3, behaved on heating as pure water containing a crystalloid in solution. Even with a 20 % solution, the viscosity relative to water was only about 20 % greater

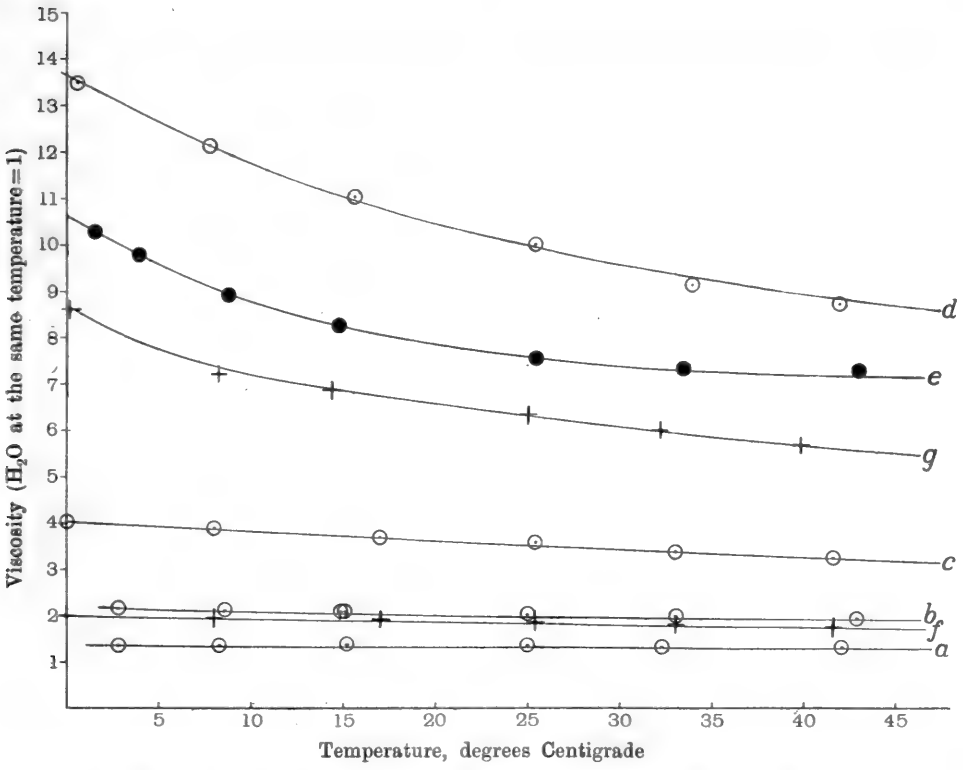

Fig. 2. Influence of temperature upon the viscosity of solutions of various proteins with differing protein-content.

—⊙— = egg-albumin: curve *a*, protein content = 7·0 %.
,, *b*, ,, ,, = 14·6 ,,
,, *c*, ,, ,, = 20·1 ,,
,, *d*, ,, ,, = 28·1 ,,
—●— = serum protein: curve *e*, ,, ,, = 20·6 ,,
—+— = whole serum: curve *f*, ,, ,, = 7·0 ,,
,, *g*, ,, ,, = 18·1 ,,

at 0° than at 41·6°, and not until the concentration of albumin reached the high figure of 28 %, was temperature found to have any specially marked influence.

These facts are well expressed in the four curves *a*, *b*, *c* and *d* in Fig. 2, where viscosity (relative to water at the same temperature) is plotted as ordinate against temperature as abscissa.

Influence of Ammonium Sulphate upon Viscosity of Crystallised Egg-albumin. It has been shown [Chick and Martin 1913, 2; Spiro 1904] that "precipitation" of egg-albumin by ammonium sulphate is a phase-separation and that a definite proportion of both salt and water is associated with the egg-albumin in the "precipitate." Arguing from analogy, it is exceedingly probable that crystallisation of proteins from strong solutions of ammonium sulphate is of a similar character.

In order to ascertain whether such hypothetical association with ammonium sulphate had any influence on the viscosity of solutions, strictly comparable experiments were made, both before and after dialysis. Dabrowski [1912], on the basis of a comparison between the different rates of diffusion obtained with the two kinds of material, came to the conclusion that aggregates formed in solution by undialysed crystals of egg-albumin were much smaller, nearly one-sixth the size of those of dialysed albumin. It therefore seemed possible that such a fundamental difference in the type of solution might also be expressed by some significant change in viscosity.

The results obtained were, however, entirely negative (see Table III). Solutions were made of exactly similar protein content, 6·98 %, in the one

TABLE III.

Viscosity of crystallised egg-albumin.

(*a*) Before, and (*b*) after dialysis.

Exp.	Protein content, %	Ammonium sulphate, %	Temperature, °C.	Mean time of flow in viscosimeter, seconds: Albumin solution	Mean time of flow in viscosimeter, seconds: 3·51 % $(NH_4)_2SO_4$ solution in exp. (*a*) and H_2O in exp. (*b*)	Coefficient of viscosity ($(NH_4)_2SO_4$ solution in exp. (*a*) and H_2O in exp. (*b*) = 1)
(*a*)	6·98[1]	3·51	0·6	145·9	109·9	1·35
	,,	,,	25·5	75·9	58·8	1·31
	,,	,,	45·1	51·4	39·8	1·32
(*b*)	6·98[1]	—	1·0	145·4	109·0	1·36
	,,	—	25·4	74·5	57·0	1·33
	,,	—	45·1	49·2	38·3	1·31

[1] Density of the solution taken as 1·019 for the purpose of calculating viscosity coefficient.

case from undialysed crystals, with their ammonium sulphate (= 3·51 %), and in the other from dialysed material. In the former case viscosity was determined relative to pure water and in the latter case in relation to a 3·51 % solution of ammonium sulphate. Comparison was instituted at three different temperatures. Temperature was not found to have any unusual influence on viscosity, nor was any difference traced between the two types of solution. At the same time it must be admitted that the concentration of protein employed (7 %) was rather low, and it would be well to repeat the experiment with stronger solutions.

Serum-albumin (horse).

A series of experiments similar to those with egg-albumin were also carried out with pure serum-albumin. The protein in this case was also crystallised in presence of ammonium sulphate according to the method of Hopkins and Pinkus [1898] and, after recrystallisation, dialysed against distilled water in presence of toluene for some weeks. A solution was obtained, containing 20·65 % protein and traces only of ammonium sulphate.

Influence of Concentration of Protein. The density of serum-albumin solutions has been shown to bear a linear relation to the concentration of protein [Chick and Martin 1913, 1]. In order, therefore, to apply the appropriate correction in calculating the coefficient of viscosity it was only necessary to make direct determinations in a few cases. The straight line could then be drawn expressing the relation of density to protein content and the other values of density required be obtained by interpolation; such values are marked with an asterisk in Table IV.

TABLE IV.

Influence of concentration of the protein upon the viscosity of pure serum-albumin (crystallised).

Temperature 25·4° C. Time of flow in viscosimeter for water 55 seconds.

Concentration of protein, %	Mean time of flow in viscosimeter, secs.	Density of the solution (H_2O at 25° C. = 1)	Coefficient of viscosity, $H_2O = 1$
20·65	391·4	1·0593	7·54
19·24	310·2	1·0549*	5·95
17·85	249·2	1·0509*	4·76
14·54	159·8	1·0412	3·02
10·45	104·3	1·0296	1·95
5·19	71·3	1·0153	1·32
2·59	61·6	1·0075	1·13

* Interpolated values.

The experiments showing the influence of protein content on viscosity are detailed in Table IV and graphically expressed in Fig. 1, curve (*b*), where viscosity as ordinate is plotted against concentration of protein as abscissa.

The results show great similarity with those obtained for egg-albumin. In Fig. 1, curves (*a*) and (*b*) run closely together at first. As the concentration of protein increases, however, it is seen that serum-albumin has a much higher viscosity than egg-albumin.

Influence of Temperature. The influence of temperature was investigated in one experiment only, of which details are given in Table V, and graphically expressed in curve (*e*) Fig. 2. This experiment was made with the most concentrated solution available, containing 20·1 % protein by weight. The viscosity relative to water was not only much greater than that of egg-albumin of similar strength (see Exp. 3, Table II), but showed a much greater change with alteration of temperature, viz. from 7·3 at 43° to 10·3 at 1·6° (compare Table V with Exp. 3, Table II).

TABLE V.

Influence of temperature upon the viscosity of pure serum-albumin (crystallised).

Protein-content = 20·65 %. Density = 1·0593 (at 25° C.).

Temperature, °C.	Mean time of flow in viscosimeter, seconds		Coefficient of viscosity (H_2O at the same temperature = 1)
	Albumin solution	Distilled water (from curve)	
1·6	1026·0	105·6	10·29
3·95	906·2	98·2	9·77
8·8	713·2	84·7	8·92
14·8	553·8	71·0	8·26
25·4	391·4	54·8	7·56
33·4	325·8	47·0	7·34
43·0	275·1	40·0	7·28

WHOLE SERUM (HORSE).

The sample of horse-serum selected for these experiments contained 7 % total protein. In order to obtain solutions of protein-content comparable to those employed above, some concentration of the serum proteins was necessary. By placing in shallow dishes for 48 hours in vacuo at room temperature material was readily prepared, which contained 18 % protein. The constituents of the serum did not appear to have been affected by the process, for, after diluting the concentrated material to obtain a solution whose protein-content was equal to that of the original serum, the viscosity was also found to be the same as that previously determined (see Table VI).

Influence of Concentration of Protein and of Temperature. The proportion of salt contained in serum was found to have an insignificant influence upon both the density and the viscosity of the system; hence the values of the coefficient of viscosity were calculated in relation to pure water as before. In Table VI are given the results of a series of experiments in which the protein-content varied from 1·8 % to 18 % by weight.

In obtaining values for the density of the various solutions, the same method was adopted as in the case of serum-albumin; after a direct determination had been made in case of one or two solutions, a curve was constructed from which intermediate values could be obtained. Such interpolated values are marked with an asterisk in Table VI.

TABLE VI.

Viscosity of horse-serum at concentrations above and below the normal; the normal serum contained 7·3 % protein.

Temperature 25° C. Time of flow in viscosimeter for water 55·5 seconds.

Concentration of protein, %	Time taken for flow in viscosimeter, secs.	Density of the solution (H_2O at 25° C. = 1)	Coefficient of viscosity ($H_2O = 1$)
1·836	62·4	1·0069*	1·13
3·665	70·3	1·0143	1·28
5·134	79·4	1·0193*	1·46
5·84	82·3	1·0220*	1·52
7·31[1]	92·8	1·0276	1·72
7·33[2]	92·5	1·0276	1·71
9·13	110·2	1·0344*	2·05
10·89	131·3	1·0405	2·46
12·67	156·6	1·0477*	2·96
14·71	206·2	1·0553*	3·92
16·27	253·6	1·0617	4·85
18·10	331·5	1·0679*	6·38

[1] Normal serum. [2] Prepared by dilution from concentrated serum.

* Interpolated and extrapolated values.

From Table VI, and more readily from curve (*c*) Fig. 1, it is seen that for low concentrations of total protein the viscosity of whole serum also remained low, varying from about 1·10 to 1·7 as the concentration of total protein was raised from 1·80 % to that of the normal serum (7·3 %). As the serum was concentrated, however, the viscosity increased rapidly with increasing concentration of total protein, and when it possessed a little more than twice the normal protein-content the viscosity relative to water reached the value of 5·0.

In comparison with serum- or egg-albumin the total proteins of serum yield much higher viscosity when in solution. For example, while 18% solutions of serum- and egg-albumin gave coefficients of viscosity equal to 4·9 and 2·8 respectively (see curves (*b*) and (*a*), Fig. 1), concentrated whole serum containing the same proportion of total protein had a viscosity of 6·38.

The influence of temperature upon viscosity was also greater with the proteins of whole serum. With the concentrated material, containing 18% protein, the coefficient of viscosity fell 34% (from 8·63 to 5·7) with increase in temperature of about 40° (see Table VII). A slightly greater rise of temperature in case of solutions containing 20% serum- and egg-albumin produced a decrease in viscosity coefficient of only about 29% and 19% respectively (see Tables V and II).

TABLE VII.

Influence of temperature upon the viscosity of whole serum (horse), (a) normal serum, (b) concentrated serum.

Exp.	Protein content, %	Density at 25° C. (H_2O at 25° C. = 1)	Temperature, °C.	Mean time of flow in viscosimeter, seconds: Serum	Mean time of flow in viscosimeter, seconds: Distilled water (from curve)	Coefficient of viscosity (H_2O at the same temperature = 1)
(*a*)	7·68	1·0290	0	218·2	111·6	2·01
	,,	,,	8·0	164·9	86·7	1·96
	,,	,,	17·0	125·5	67·1	1·92
	,,	,,	25·4	99·6	54·9	1·87
	,,	,,	33·0	83·6	47·3	1·82
	,,	,,	41·6	70·4	40·9	1·77
(*b*)	18·1	1·0679	0·2	897·2	111·0	8·63
	,,	,,	8·2	581·5	86·0	7·22
	,,	,,	14·3	462·3	71·9	6·86
	,,	,,	25·0	328·9	55·4	6·34
	,,	,,	32·2	270·3	48·0	6·01
	,,	,,	39·8	224·6	42·1	5·70

It is evident that one or more of the remaining proteins in horse serum must, when in solution, possess a viscosity much higher than that of the serum-albumin, and the results of some preliminary experiments suggest that the small proportion of "euglobulin" contained in the serum exercises a disproportionate influence upon the viscosity of the whole.

In a second communication it is proposed to publish the results of experiments, similar to the above, carried out with purified samples of

"euglobulin" and "pseudoglobulin," prepared from horse serum, and to include a general discussion of the theoretical bearing of the whole series of data.

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VIII. THE QUANTITATIVE ESTIMATION OF UREA, AND INDIRECTLY OF ALLANTOIN, IN URINE BY MEANS OF UREASE.

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For the rapid routine analysis of urea in urine Folin's magnesium chloride method [1901; 1902, 1; 1903; 1905] is probably the one most usually adopted, but it is not an easy method to acquire and it requires a considerable amount of attention. A good substitute might soon displace it. Folin himself has devised other methods for the estimation of urea in small quantities. We believe we have found a substitute for the first method by using the urease of the soy-bean [Takeuchi, 1909]. Marshall [1913, 1] has already published a method for the rapid clinical estimation of urea in urine by this means, but by his procedure the results are not sufficiently accurate, being only within about two per cent.; an extract of the soy-bean is required and results are only obtained after at least three hours. Marshall [1913, 2] has since modified his method by removing the ammonia after the conversion of the urea into ammonium carbonate by means of an air current.

Our procedure is similar to this latter one of Marshall, but it is much simpler; accurate results are obtained in a little over two hours, and it is not necessary to use an extract of soy-beans.

In its essential features the method is no more than Folin's method [1902, 2] of estimating ammonia in urine. By fitting together three or four cylinders and Allihn bottles in series with a sulphuric acid bottle at the end, duplicate estimations of ammonia and urea in urine can be carried out simultaneously. In the cylinders for the urea estimations are put 50 to 60 cc. of water, 1 g. of finely ground soy-bean and 5 (or 10) cc. of urine. These

cylinders are kept in a water bath at a temperature of 35–40° and an air current is drawn through the series. After about an hour the rubber connections between the cylinders and bottles are disjointed and 1 g. of anhydrous sodium carbonate is dropped into the cylinders; they are then connected together again and the air current drawn through for another hour. To prevent frothing liquid paraffin B.P. has been used; it is superior to petroleum or toluene as it does not evaporate and it obviates the necessity of using a tube containing cotton wool between the cylinder and Allihn bottle. It is not necessary to carry out a blank experiment with soy-bean alone, since no ammonia was evolved by two different samples of the bean which were tested several times. The Allihn bottles are charged with excess of 0·1 N sulphuric acid (25 or 50 cc.) which is titrated with 0·1 N alkali using alizarin red as indicator.

The ammonia estimations are carried out exactly as described by Folin, except that liquid paraffin is used to prevent frothing.

The following are some comparative data obtained with 5 cc. of human urine by the urease method and Folin's magnesium chloride method :—

Urease cc. 0·1 N acid		Folin cc. 0·1 N acid	
18·3 18·2	18·25	18·6 18·5	18·55
16·1 16·1	16·1	16·1 16·2	16·15
14·2 14·2	14·2	14·2 14·3	14·25
19·0 19·2	19·1	19·2 19·3	19·25

The urease method has been used daily for some weeks and the results are all of the same order, the two data always coming out together or differing at most by 0·4 cc.; in fact all our latest determinations of urea in urine have been made by the urease method as we have become convinced of its accuracy[1]. The values are very slightly lower than by the magnesium chloride method, in which a decomposition of other urinary constituents, e.g. allantoin, or possibly urochrome as suggested by Haskins [1906], may occur.

The method has been tested on solutions containing different amounts of pure urea and the results are correct against a nitrogen determination by Kjeldahl's method; thus

[1] It is advisable to cleanse the cylinders immediately after use and occasionally with formalin, as the residues of protein, if left, form a suitable medium for the growth of bacteria, which may produce ammonia from amino-acids and other compounds.

Urea solution taken			
2 cc.	19·5, 19·2	19·35 cc. 0·1 N acid = 9·68 for 1 cc.	N by Kjeldahl's method 19·5 cc. = 9·75 cc. for 1 cc. of solution.
4 cc.	38·5, 38·8	38·65 ,, ,, 9·66 ,,	
6 cc.	57·6, 57·9	57·75 ,, ,, 9·63 ,,	
8 cc.	77·2, 77·4	77·3 ,, ,, 9·66 ,,	

The urease of the soy-bean was shown to be specific by Takeuchi [1909] and its specificity has been more fully emphasised by Armstrong and Horton [1912], who also showed that its action is inhibited by ammonia; the removal of ammonia, as it is formed, by means of the air current will thus favour the completion of the reaction. Our method is in this way again more advantageous than Marshall's.

Not only urea, but also allantoin, is decomposed by the magnesium chloride method of Folin, as is stated by Cathcart [1906] and Haskins [1906]. Since urease has no action upon allantoin the two substances can therefore be readily estimated in urines which contain both compounds; the difference between the two data will give the amount of allantoin. The two compounds and mixtures of the two compounds have been tested with the following results:—

	5 cc. urea (from above)	5 cc. allantoin	10 cc. allantoin
Kjeldahl	48·75 cc. 0·1 N acid	—	9·8, 9·7, 9·5 — 9·7 cc. 0·1 N acid
Folin	—	4·5, 4·5, 4·6 — 4·5 cc. 0·1 N acid	9·7, 9·8 — 9·75 ,, ,,
Urease	48·5 cc. 0·1 N acid	0	0

Hence 5 cc. allantoin = 4·87 cc. 0·1 N acid.

	5 cc. urea + 5 cc. allantoin	5 cc. urea + 10 cc. allantoin
Folin	53·4, 53·6 — 53·5 cc. 0·1 N acid	57·5, 57·3 — 57·4 cc. 0·1 N acid
Urease	48·5, 48·5 — 48·5 ,, ,,	48·5, 48·5 — 48·5 ,, ,,
	∴ 5 cc. allantoin = 5·0 ,, ,,	4·45 ,, ,,

Several analyses of dog's urine (volume for 24 hours made up to 1000 cc.) have also been made, the figures being in all cases cc. of 0·1 N acid:—

Total N (5 cc.)	Ammonia (25 cc.)	Urea + allantoin (5 cc.) less ammonia	Urea (5 cc.) less ammonia	Allantoin in 5 cc.
19·5	8·3	16·7, 16·6 — 16·65	14·4, 15·1 — 14·8	1·85
15·5	6·1	13·0, 12·9 — 12·95	11·2, 11·6 — 11·4	1·55
7·4	2·0	6·6, 6·4 — 6·5	5·0, 5·4 — 5·2	1·3
21·3	3·0	19·1, 19·3 — 19·2	17·2, 17·4 — 17·3	1·9

The difference between the figures in columns 3 and 4 gives the amount of allantoin.

The estimation of allantoin in urine by Wiechowski's method does not lend itself to routine work and our procedure is undoubtedly more rapid than that adopted by Miss Lindsay [1909].

Summary.

The estimation of urea in urine is quickly and accurately made by decomposing it with urease (1 g. powdered soy-bean) at 35 to 40° for one hour. During this time the ammonia evolved is removed by an air current as in Folin's method for estimating ammonia. One gram of anhydrous sodium carbonate is then added and the air current is continued for another hour. Liquid paraffin B.P. is very convenient for lessening the frothing.

Since urease does not decompose allantoin and since both allantoin and urea are quantitatively decomposed by the magnesium chloride method of Folin, the amount of allantoin in those urines, which contain both compounds, is readily estimated by difference.

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IX. THE CHOLESTEROL OF THE BRAIN. II. THE PRESENCE OF "OXYCHOLESTEROL" AND ITS ESTERS.

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In a previous communication [1906] I was able to show, by the methods then available, that Baumstark's statement with regard to the occurrence of cholesterol esters in brain must be considered erroneous. My observations were in accordance with those of R. Bünz [1905], whose work was published a few months before mine. The methods used by Bünz and by myself were of an indirect nature, depending on variations in melting points etc., and my failure to isolate cholesterol esters from the oily mother liquors of the brain-cholesterol might be ascribed to the well-known difficulty inherent in this procedure. Since this work has been carried out, Windaus [1910] has described a quantitative method for the estimation of free and combined cholesterol, which depends on the insolubility of the digitonin-cholesterol compound. It seemed therefore of interest to reinvestigate the question of the presence of cholesterol esters in brain by the help of this new method. This was all the more desirable as indications of their presence may be taken from statements occurring in the recent literature on the subject. (Lapworth [1910], Lorrain Smith and Mair [1911], Lifschütz [1913, 1].)

Cholesterol esters are now usually estimated in an indirect way, i.e. by calculating as combined cholesterol the difference between the free and the total cholesterol content by Windaus' method, estimated in relatively small aliquot parts of suitably prepared extracts before and after saponification. As my previous work showed conclusively that cholesterol esters, if present at all in brain, would be found only in exceedingly small amounts, this indirect method of estimation does not offer much hope of success, as the limits of error of the method are greater than the possible differences to be looked for. It was therefore decided to work up the whole brain and to

remove the cholesterol completely by precipitation with digitonin, leaving the esters, if present, in the filtrate. One half of the filtrate was to be used for the actual preparation of the esters, whilst the other half was to be saponified, and the cholesterol, originally present as ester, precipitated with digitonin.

Although a whole adult human brain was examined in this way for cholesterol esters, no trace of them could be found. The same result was obtained with a child's brain. In both cases, however, digitonin produced a distinct weighable precipitate in the final solution. The examination of this precipitate brought out the fact that the substance in question certainly did not consist of cholesterol. The substance recovered from its digitonin compound, in contradistinction from cholesterol, gave with glacial acetic and sulphuric acid a violet colour, which on the addition of ferric chloride turned to a bright emerald green. This green solution, on spectroscopic examination, showed a very characteristic band in the red (between λ 630 and λ 650), which has been described by Lifschütz [1913, 1] as typical for the compound which he termed "oxycholesterol." According to him, "oxycholesterol" is present in blood and in most organs with the exception of the liver and bile[1] (see also Unna and Golodetz [1909], Lifschütz [1913, 2], Schreiber and Lénard [1913, 1 and 2]).

Lifschütz ascribes to this chemically ill-defined substance an important rôle in the metabolism of cholesterol, which seems to deserve more attention than it has hitherto received, the discussion of which, however, is outside the scope of this communication. The results of the present investigation in any case seem to justify the conclusion that in human brain a substance of this nature exists mainly in the form of esters.

Experimental.

For the first experiment the brain of a child of three months was used. Cause of death, pneumonia; weight of brain, 512 g. After removal of the membranes, the brain was finely minced. A water estimation was made in a sample of the mixed brain and showed 85·8 per cent. In order to obtain an idea as to the amount of digitonin necessary for the complete removal of cholesterol from the brain extracts, the percentage of cholesterol in the brain was estimated quantitatively. As the application of Windaus' method to the brain is complicated by the presence of the large amounts of lipoids, the

[1] In this connection it may be mentioned that, according to my observations, gallstones contain "oxycholesterol." If this result should be confirmed by a more extensive investigation, it might have a considerable bearing on the problem of their formation.

following method was used, which represents a combination of O. Rosenheim's method for the preparation of cholesterol [1906] with that of Windaus for its quantitative estimation. As the same method was used in the subsequent experiments it may be described here in detail.

Five grams of minced brain were mixed with 15 g. of plaster of Paris[1]. After the mass had set hard, it was broken up and the drying completed in a desiccator. The brain plaster mixture was then extracted with acetone in a Soxhlet apparatus for several days, the extraction flask being changed repeatedly. The extraction was considered complete when the last extract after evaporation of the solvent left only a trace of residue, which did not give any precipitate when digitonin was added to its alcoholic solution.

The combined acetone extracts were evaporated to dryness, the residue being taken up in 70 cc. of 95 per cent. alcohol, filtered and precipitated hot with a large excess (35 cc.) of a 1 % solution of digitonin (Merck) in 90 % alcohol. The contents of the flask were kept boiling for a few minutes and the precipitate allowed to settle at room temperature over-night. The digitonin-cholesterol was filtered on a weighed Gooch crucible, washed with alcohol and ether and dried to constant weight in a toluene oven at 105°. From 5 g. of fresh brain was obtained 0·1435 g. digitonin-cholesterol (mean of two analyses). According to Windaus the amount of cholesterol contained in digitonin-cholesterol is found by multiplication with the factor 0·2431; therefore the fresh brain contained 0·70 per cent. cholesterol.

The bulk of the minced brain was put into 1 litre of acetone, allowed to stand overnight and then filtered through a Buchner funnel under pressure. Five subsequent extracts each with 500 cc. cold acetone were made, and the extraction then continued three times with 800 cc. boiling acetone. Previous experience with other organs had shown me that by this procedure cholesterol and cholesterol esters were completely removed.

The dry residue of the combined extracts was treated repeatedly with boiling 95 % alcohol and filtered hot. An alcoholic extract, amounting to 1 litre, was thus obtained, which contained the whole of the cholesterol, free and combined, present in the brain.

The total quantity of free cholesterol contained in the whole brain, as calculated from the result of the quantitative estimation (see above), amounts to 3·5 g., which would require about 10·5 g. of digitonin. In order to ensure complete precipitation, 13 g. of digitonin dissolved in 1300 cc. of 90 % alcohol were added to the hot alcoholic brain extract. A copious precipitate formed and was allowed to settle over night. The complete removal of cholesterol was tested for by the formation of a precipitate on adding a few drops of an alcoholic solution of cholesterol to a sample (a few cc.) of the clear supernatant fluid. The precipitate was well washed with 90 % alcohol and with ether. A clear filtrate was thus obtained which should contain the whole of the cholesterol esters present in brain.

[1] It is essential that the best quality of plaster of Paris should be used. The "superfine" quality as sold by makers of plaster-casts was found to be suitable.

The filtrate was heated on the water-bath until most of the alcohol had evaporated and then transferred to an automatic extraction apparatus (Maassen's), in which it was extracted for many days with a mixture of ether and petroleum ether until the extraction was complete.

The ethereal extract was evaporated, the residue taken up with boiling absolute alcohol and saponified by boiling with sodium ethylate. Water was added and the saponified extract transferred again to the extraction apparatus. The final ethereal extract, which should contain the cholesterol of the whole of the cholesterol esters, was again evaporated, taken up in 70 cc. of hot 95 % alcohol and 70 cc. of 1 % digitonin solution were added. There was no immediate precipitate, but after standing for some time at room temperature a small flocculent precipitate settled, which appeared to be quite different from the crystallised digitonin-cholesterol as usually obtained. The precipitate was treated in the usual way and when dry weighed 0·2131 g.

It will be shown in connection with the next experiment that this precipitate did not consist of digitonin-cholesterol, but it is of interest to note that on the assumption that one quarter of it represents cholesterol, the percentage of combined cholesterol in the whole brain would only amount to 0·01 per cent.

In the absence of any evidence to the contrary, it may be argued that cholesterol esters, even if absent in a child's brain, may be present in the adult human brain.

In the next experiment, therefore, the whole of an adult human brain was treated in a similar manner. The brain of a man (cause of death, heart disease) weighing 1270 g. contained 78·9 per cent. of water and 1·95 per cent. of free cholesterol (mean of two determinations) estimated as in the previous experiment.

The extraction was carried out as in the first experiment, ten cold and three hot extractions with acetone having been made. In order to economise the valuable digitonin, of which at least 80 g. would have been necessary, a large bulk of the free cholesterol was allowed to crystallise out from the combined extracts. As the first crystallisation might possibly carry down any cholesterol ester present, the product was recrystallised twice from a mixture of alcohol and acetone, the mother liquors being added to the main extract. In this way, 21 g. of pure cholesterol were separated.

To the final extract, dissolved in 95 % alcohol, 850 cc. of 1 per cent. digitonin solution were added and the precipitated digitonin cholesterol removed as previously described. The whole of the free cholesterol was thus removed. The resulting filtrate was extracted with ether in the Maassen's

extraction apparatus as above described. One half (200 cc.) of the ether extract was reserved for the isolation of the esters as such, and the other half was saponified as before, and again extracted with ether. The final residue dissolved in 50 cc. of 95 % alcohol was precipitated with 70 cc. of 1 % digitonin solution. The formation and appearance of the precipitate resembled closely that observed in the previous case. The dry precipitate weighed 0·2635 g. The whole brain would therefore yield 0·5270 g. of this substance. Assuming again that one quarter of it consists of cholesterol, the percentage of combined cholesterol in the whole brain would amount to 0·01 g. per cent., i.e. exactly the same figure as calculated for the child's brain.

Examination of the digitonin compound.

Having obtained a weighable amount of a digitonin compound in the two cases, it became necessary to investigate it further and to establish its identity or otherwise with digitonin-cholesterol. Windaus has already shown that, by means of boiling xylene, cholesterol can be recovered from its combination with digitonin. Before subjecting the substances obtained from brain to this treatment, it was thought advisable to test the reliability of this method when dealing with such small quantities as are obtained from brain. For this purpose 0·26 g. of digitonin-cholesterol, as prepared by direct precipitation of a brain extract, was treated in a suitable extraction apparatus with boiling xylene for 7·5 hours. From the xylene extract, cholesterol was isolated in the typical crystals, without any difficulty.

The digitonin precipitates, obtained from brain after the removal of free cholesterol, were then treated separately with xylene in the extraction apparatus. In both cases I was unable to detect any cholesterol crystals. The xylene extract left on evaporation an oily residue (approximately 35 % of the digitonin compound), in which under the polarising microscope some anisotropic sphero-crystals could be detected. All attempts to bring this residue to crystallisation were unsuccessful.

The behaviour of the substance suggested a similarity to the product described by Lifschütz [1913, 1] as "oxycholesterol," which according to him also occurs as ester in ox brain. With this name Lifschütz designates for convenience sake the primary neutral oxidation products of cholesterol, which are as yet insufficiently defined chemically, but which under certain conditions give rise to a colour reaction possessing a highly characteristic absorption spectrum. By means of this spectroscopic behaviour "oxycholesterol" can not only be detected in the presence of cholesterol, but its quantity may be

estimated spectrometrically by comparison with a standard "oxycholesterol" solution.

In order to compare the substances obtained from brain with "oxycholesterol," I prepared some of this substance according to Lifschütz by oxidising pure cholesterol with benzoyl peroxide in glacial acetic acid solution. Following scrupulously the detailed directions of Lifschütz, there is no difficulty in obtaining the sticky amorphous product as described by him. This was boiled with alcoholic potash, extracted with ether and the residue from the ether extract purified by means of methyl alcohol. After careful evaporation of the methyl alcohol and drying *in vacuo*, the product obtained was a yellowish brittle resin, insoluble in water, but soluble in all the usual organic solvents.

This substance gave the typical reaction as described by Lifschütz. One milligram dissolved in chloroform gave with glacial acetic acid and sulphuric acid the first phase of the Lifschütz reaction (violet), which changed almost instantaneously on the addition of ferric chloride to a bright emerald green, showing a well defined band in the red between λ 630—650. A cholesterol solution remained perfectly colourless under the same conditions[1]. When these tests were carried out with the substances isolated as above described by means of their digitonin compound from brain, it was found that they gave the typical "oxycholesterol" reactions, even in minute quantities. One milligram of the substances gave a strong Lifschütz colour reaction, and the green solution showed the well marked typical absorption band in the red.

From some preliminary experiments, in which the coloured solutions were compared with a standard "oxycholesterol" solution in a Zeiss comparison spectroscope, it seems that their spectroscopic value is at least as high as that of the standard.

It is evident therefore that "oxycholesterol" exists in the brain in the form of esters and that free "oxycholesterol" is precipitated from its alcoholic solution by digitonin. This latter fact has also been demonstrated by Lifschütz [1913, 3].

[1] In conjunction with Dr O. Rosenheim I have found another characteristic reaction of "oxycholesterol" which may be carried out with the dry substance. If a minute quantity of dry "oxycholesterol" (or the residue of a drop of a solution to be tested) is put on a slide or watch-glass and a drop of methyl sulphate (techn.) is added, a brilliant purple colour is at once produced. Pure cholesterol remains unchanged under these conditions, but on warming or on prolonged standing it develops a bright cherry red colour. The reaction is not given with pure methyl sulphate. The reason for this as well as the characteristic behaviour of cholesterol, oxycholesterol and other substances towards methyl sulphate in solution and in the presence of ferric chloride etc. will be described in a subsequent communication.

With regard to the quantity of the esters present we may form an approximate estimate from the amount of the digitonin compound weighed as such. If we assume that in analogy to digitonin-cholesterol it contains a quarter of its weight of "oxycholesterol," the amount of the latter as ester would be 0·01 % in the fresh brain. Its amount, however, may be larger as we have at present no experimental data as to the completeness of its precipitation by digitonin.

An unsuccessful attempt was made to isolate the esters as such from the solution reserved for this purpose (see above, p. 78). Just as is the case with free "oxycholesterol," so also its esters are evidently not readily crystallisable [see also Lifschütz, 1913, 3]. Various solvents were tried, but the substance did not show any tendency to crystallise, although under the polarising microscope some anisotropic needles could be detected in it. It gave no reaction for free "oxycholesterol," but did so readily after saponification.

In this connection an experiment may be mentioned which I carried out in order to satisfy myself that "oxycholesterol" is not an artifact, i.e. a product formed from cholesterol esters during the lengthy process of saponification, etc. For this purpose 0·25 g. pure cholesterol palmitate was carried through the process in exactly the same way as the solution of esters described above. The product finally obtained did not give any trace of an oxycholesterol reaction.

The occurrence of free "oxycholesterol" in brain.

As the above results have clearly demonstrated the existence of "oxycholesterol" esters in brain, it seemed of interest to search for the presence of free "oxycholesterol." This substance forms, just like cholesterol, a compound with digitonin (Merck)[1], and it might therefore be expected that any free "oxycholesterol" present would be found in the primary digitonin precipitate. Its presence in this can be easily shown if a small quantity be dissolved in warm glacial acetic acid. After cooling, the solution gives

[1] This fact makes it unfortunately impossible to accept unconditionally the results of cholesterol determinations according to Windaus' method, unless the digitonin compound after weighing is tested qualitatively, and if necessary, quantitatively, for "oxycholesterol." The question becomes still more complicated since Windaus and Schneckenburger [1913] have recently discovered that "crystallised digitonin" contains, besides digitonin, in variable quantities, an amorphous glucoside called gitonin. It is possible that "oxycholesterol" is precipitated by "crystallised digitonin" only owing to the presence of gitonin. This would explain the contradictory statements of Lifschütz [1913, 3] and of Schreiber [1913], according to whom "oxycholesterol" is not precipitated by purified digitonin. In any case, the whole method of cholesterol estimation in tissues by means of digitonin seems to require further investigation.

Lifschütz's reaction. The dry digitonin-oxycholesterol compound also gives the methyl sulphate reaction mentioned in the foot-note on p. 79.

By means of these reactions I was able to show the presence of "oxycholesterol" in the adult brain referred to above, but not in the brain of the child.

This result seemed suggestive if we remember that in the brain of newborn children the cholesterol and galactoside percentage is known to be low, whilst the percentage of unsaturated phosphatides is high as compared with an adult's brain. One might assume that a similar relationship holds good for "oxycholesterol." In order to test this suggestion still further, I examined the extracts of another adult human brain, of a child's brain (5 days old) and that of a human foetus (36 weeks old). Here again the adult brain gave a strong "oxycholesterol" reaction, whilst that of the child gave a negative result. The brain of the foetus gave a positive reaction.

Before coming to a final conclusion with regard to this question, however, more experiments seem to be necessary.

Conclusions.

(1) The examination of the whole human brain by means of Windaus' method has shown the complete absence of cholesterol esters.

(2) Human brain contains "oxycholesterol" esters to the extent of at least 0·01 per cent.

(3) Free "oxycholesterol" seems to be present in adult human brain, but not in the brain of young children.

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X. THE CHOLESTEROL OF THE BRAIN. III. NOTE ON THE CHOLESTEROL CONTENTS OF HUMAN AND ANIMAL BRAIN.

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The data as to the percentage of cholesterol in brain available in the literature are still rather scanty, and most of the estimations carried out before the introduction of a trustworthy quantitative method can only be considered as approximate. Since Windaus' work has made it possible, by the use of digitonin, to obtain more accurate figures, some estimations of the cholesterol percentage in brain have been published. These were, however, mostly made incidentally with other work, and, moreover, they have been usually expressed in percentages of the moist organ or its chloroform extract. As the amount of water in brain is variable it is impossible, in the absence of any water estimations, to reduce the available figures accurately to a uniform standard.

The following estimations were made by a combination of O. Rosenheim's [1906] method for the preparation of cholesterol with Windaus' [1910] method for its quantitative estimation. The method, which obviates the difficulties due to contamination with other brain lipoids, has been published in full in the preceding paper [1914]. In some cases search was made for the presence of cholesterol esters with negative results.

Unfortunately this somewhat tedious work had been finished before the results of my previous communication [1914] on the occurrence of "oxycholesterol" in brain were obtained. In the light of the latter work, the figures must be taken to express the sum of the quantities of cholesterol and "oxycholesterol" present. Except in the case of human brain, "oxycholesterol" was not tested for.

Description of brain	Water percentage	Cholesterol percentage in moist brain	Cholesterol percentage in dry brain
Man (i)	78·86	1·93, 1·97 } 1·95	9·22
Man (ii) ...	78·90	1·91, 1·92 } 1·91	9·01
Child (aged 3 months)...	85·80	0·66, 0·73 } 0·69	4·89
Child (♂ aged 5 days) ...	89·99	0·53, 0·53 } 0·53	5·29
Foetus (♀ aged 36 weeks)	90·29	0·39, 0·40 } 0·39	4·07
Dog...	76·18	2·73, 2·79 } 2·76	11·59
Cat	76·53	2·29, 2·41 } 2·35	9·99
Ox (i)	78·83	2·39	11·28
Ox (ii)	78·32	2·57, 2·65 } 2·61	12·04
Sheep	79·50	2·13	10·37
Rabbit (i)	77·86	2·12	9·57
Rabbit (ii)	79·15	1·90	9·11
Fowl	80·34	1·43, 1·48 } 1·45	7·40
Codfish (i)	84·03	1·92	12·02
Codfish (ii)	84·94	1·79	11·89

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XI. ON THE RESISTANCE OF TRYPSIN SOLUTIONS TO HEAT.

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The action of heat on aqueous solutions of many enzymes has been studied more or less carefully for many years, and the general conclusion arrived at has been that all enzymes in aqueous solution are destroyed when heated for a short time to about 70° or 75°. So much is this the case that frequently the activity of a substance after its solution has been heated has been taken as proving that the substance in question is not an enzyme (e.g. secretin).

Since trypsin is practically without any digestive action in acid or neutral solution, the effect of heat on this enzyme was at first tested principally in alkaline solution, and it was found that such a solution rapidly became inactive at as low a temperature as 50° or sometimes even at 45° [Biernacki, 1891]. Vernon [1901] also found that fresh preparations of trypsin lost more than half their activity when kept in 0·4 % sodium carbonate at 37° for an hour. Similar results have been found by Vernon in later experiments, and by other observers.

On the other hand Vernon [1904] found that the presence of protein protected trypsin solutions from the effect of heat to a considerable extent, and the same protection was afforded by proteoses or peptone. Bayliss and Starling [1903] had previously noticed the protective effect of proteins or their hydrolytic products on solutions of trypsin.

The action of acids on trypsin, however, has not been so fully studied. Langley [1881] found that trypsin was considerably weakened by warming its solution for 2½ hours with 0·05 % hydrochloric acid. Wróblewski, Bednarski and Wojczynski [1901] found that trypsin when kept at 37° for a few hours in hydrochloric acid of over 0·14 % was considerably affected, and if 0·56 % acid were used the enzyme was sometimes completely destroyed.

The question of the effect of heat on trypsin was investigated later by

Schmidt [1910], who stated that trypsin in a slightly alkaline solution containing 5% of peptone could be boiled without being destroyed. The same protection against heat was afforded by a 2% solution of agar or a 10% solution of gelatin. Schmidt also stated that if trypsin powder were suspended in water-free glycerol it could be heated to 292° without being destroyed or much affected.

Schmidt's work was repeated by de Souza [1911], who however found that 5% peptone had hardly any effect in protecting trypsin solutions from destruction by heat. It appears from these experiments that an appreciable protection is afforded by 20% peptone under certain conditions, but if the heating is sufficient to cause complete destruction of the trypsin in pure aqueous solution, then the presence of peptone has only a slight protective effect. De Souza also tried the effect of heat on trypsin in presence of 20% peptone in acid, neutral and alkaline solutions. The solutions were heated to 80° for five minutes. No difference was observed between the acid and neutral solutions, the activity of these after heating being however slightly greater than that of the alkaline solution. Even in the case of the acid or neutral solution, however, over 85% of the trypsin was destroyed, and about 90% in the case of the alkaline solution.

Ohta [1912] also repeated the experiments of Schmidt, but failed to confirm his results.

In a paper just published, Mellanby and Woolley [1913] find that while trypsin is readily destroyed by heat in neutral or alkaline solution, if a solution of trypsin be made slightly acid, say with hydrochloric acid, it can be boiled for five minutes and yet retain considerable digestive power.

According to these observers, trypsin is destroyed in acid, more rapidly than in alkaline or neutral solution up to about 40°, but at higher temperatures the reverse is the case. At 40° there appears to be an optimum protective concentration of acid, above or below which the rate of trypsin destruction is accelerated.

Before the publication of the work of Mellanby and Woolley, and unaware of their experiments, I had tested the effect of heat on trypsin in connection with another research, and obtained results of a similar character. The method of testing the digestive power of the trypsin solutions was that of Hedin [1903]. The trypsin was allowed to act on a solution of caseinogen in presence of toluene and after a definite interval excess of tannic acid was added, to precipitate unaltered protein, meta-protein and proteoses. After standing 12 hours or more the precipitate was filtered off and the nitrogen

determined in a portion of the filtrate by Kjeldahl's method. Controls were also carried out to show the effect, if any, of the alkali used on caseinogen, and the amount of nitrogen not precipitated by tannic acid was also determined in each solution of trypsin used.

The following are the principal results obtained:—

1. Benger's Liquor Pancreaticus used as trypsin solution, 10 cc. of this requiring 1·6 cc. of N/10 sodium hydrate for neutralisation. 2 % caseinogen in normal sodium carbonate was the substrate. A portion of the trypsin was boiled for three minutes and cooled before adding the caseinogen. Digestion was continued at 37° for three hours.

	Digestion in cc. of N/10 nitrogen not ppted by tannic acid
(*a*) 1 cc. trypsin, 20 cc. water, 40 cc. caseinogen	49·8
(*b*) 1 ,, (boiled) ,, ,,	29·7

In this experiment the effect of boiling trypsin in slightly acid solution was to leave 60 % of the original digestive power unimpaired.

2. 10 cc. of the above trypsin solution were neutralised with sodium carbonate and made up to 25 cc. with water. Three portions (*a*, *b* and *c*) were boiled for three minutes in neutral, alkaline and acid solution respectively, cooled, and kept at 37° with 20 cc. of 2 % caseinogen in 2 N sodium carbonate for three hours.

	Digestion in cc. of N/10 nitrogen not ppted by tannic acid
(*a*) 2·5 cc. trypsin, 20 cc. water...	0·2
(*b*) 2·5 cc. trypsin, 19 cc. water, 1 cc. N Na_2CO_3 ...	0·1
(*c*) 2·5 cc. trypsin, 19 cc. water, 1 cc. N HCl ...	20·8
(*d*) 2·5 unboiled trypsin, 20 cc. water	20·9

It may here be mentioned that in all the experiments carried out, any differences in reaction due to the trypsin being boiled in acid etc. were adjusted before the caseinogen was added. Special care was taken also to ensure that none of the trypsin escaped being heated to 100°.

In the above experiment it will be seen that after being boiled in acid solution for three minutes, the trypsin still retained all its power of digesting caseinogen, while boiling in alkaline or neutral solution had completely destroyed the enzyme.

The digestive power of this trypsin before and after being boiled as above was also tested on boiled ox fibrin, the amount of nitrogen in the filtrate from the undissolved fibrin at the end of the digestion being taken as the measure of the action of the enzyme. It was found that on such fibrin trypsin acts

only slowly, producing much less effect in a given time than when acting on caseinogen. Nevertheless the trypsin boiled in acid dissolved as much fibrin as the unboiled trypsin, while that boiled in neutral or alkaline solution again had no digestive power.

3. Merck's trypsin used. A weak solution of this trypsin was dialysed against running water for 18 hours and filtered. The solution was neutral. The trypsin contained 0·02 % nitrogen. Three portions were boiled for three minutes (*a*, *b* and *c*) and then allowed to act on 20 cc. of 2 % caseinogen in 0·4 N Na_2CO_3 at 37° for three hours.

	Digestion in cc. of N/10 nitrogen not ppted by tannic acid
(*a*) 25 cc. trypsin, 1 cc. N Na_2CO_3	0
(*b*) 25 cc. trypsin, 1 cc. water	0·2
(*c*) 25 cc. trypsin, 1 cc. N HCl	21·4
(*d*) 25 cc. unboiled trypsin	28·9

In this experiment 75 % of the original digestive power remains after boiling the trypsin in acid solution, but the trypsin is destroyed in neutral or alkaline solution.

For the rest of the experiments the trypsin used was prepared in the method described by Hedin [1905]. An ox pancreas was minced and allowed to undergo autolysis at 37° in presence of water and toluene for a day and filtered. The filtrate was again kept at 37° for two days, dialysed against running water for two days, filtered, and kept with a little toluene.

This trypsin solution was neutral, contained less than 0·01 % nitrogen, and gave practically no biuret reaction.

4. Three portions of this trypsin were boiled for three minutes, and cooled before adding 20 cc. of caseinogen (same as in last experiment). Digestion lasted three hours.

	Digestion in cc. of N/10 nitrogen
(*a*) 25 cc. trypsin, 0·5 cc. N/10 Na_2CO_3	0·2
(*b*) 25 cc. trypsin, 0·5 cc. water	0·2
(*c*) 25 cc. trypsin, 0·5 cc. N HCl	6·5
(*d*) 25 cc. unboiled trypsin	10·5
(*e*) Control (water + caseinogen)	0

In this experiment we see that over 60 % of the original digestive power of the trypsin survives after the acid solution has been boiled, but none in the case of the neutral or alkaline solutions, the 0·2 cc. being within the limits of experimental error.

5. In order to see to what extent this trypsin would survive more prolonged heating, 25 cc. of the solution together with 5 cc. of N/10 HCl

were brought to boiling in a flask and then put in a steriliser for 20 minutes. During the whole of this time the temperature throughout the interior of the steriliser was 100°. The contents of the flask were then cooled and neutralised.

To a fresh portion of 25 cc. trypsin 5 cc. of N/10 HCl were added, and immediately neutralised. Then to both flasks were added 20 cc. of the usual caseinogen solution.

25 cc. of water were treated in exactly the same way as the fresh portion of trypsin, and the flasks were kept at 37° for 4·25 hours.

		Digestion in cc. of N/10 nitrogen
(*a*)	Boiled trypsin	4·2
(*b*)	Fresh trypsin	16·7
(*c*)	Control	0

The amount of nitrogen not precipitated by tannic acid, which was contained in the 25 cc. trypsin used, was also estimated, and allowed for in the above results. It corresponded only to 1 cc. of N/10 nitrogen.

From this experiment it appears that under suitable conditions a solution of trypsin can be heated to 100° for 20 minutes and yet retain 25 % of its original digestive power.

6. I have repeated one of the experiments described by Mellanby and Woolley to test the effect of varying concentrations of acid on trypsin. My experiment was carried out at 45°, at which temperature the acid solutions were kept for 15 minutes. 20 cc. of the usual caseinogen solution were added and digestion continued at 37° for three hours.

		Digestion in cc. of N/10 nitrogen
(*a*)	25 cc. trypsin, 0·2 cc. N HCl	8·0
(*b*)	25 cc. ,, 0·4 ,, ...	8·9
(*c*)	25 cc. ,, 0·6 ,, ...	8·9
(*d*)	25 cc. ,, 0·8 ,, ...	9·3
(*e*)	25 cc. ,, 1·0 ,, ...	8·9
(*f*)	25 cc. unboiled trypsin	9·8

In the above series the trypsin seems to be the least protected by the weakest acid (0·008 N), each of the other concentrations of acid having much the same effect. In all cases at least 80 % of the original digestive power remains after heating.

SUMMARY.

Solutions of trypsin when neutral or alkaline are rendered completely inactive by boiling.

Acid solutions of trypsin, on the other hand, after being boiled retain a considerable power to digest caseinogen. In some cases there is no destruction of this digestive power at all.

The power to digest caseinogen appears to be less affected by heat than the power to coagulate calcified milk, this being taken as the measure of the activity of trypsin by Mellanby and Woolley.

It may be that these two evidences of the action of trypsin are due to different sets of groupings of the trypsin molecule, and that the groupings to which the digestion of caseinogen are due are more thermostable than the others.

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XII. ON THE ACTION OF COAGULATING ENZYMES ON CASEINOGEN.

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Hammarsten [1872, 1874, 1877] first demonstrated that the rennin action on caseinogen was specific and independent of the action of the calcium salts. His explanation was that the caseinogen molecule was split up into a large molecule (Käse) and a smaller one (Molkeneiweiss). The "Käse" was rendered insoluble by the presence of soluble calcium salts and formed the clot. Since then little has been done to determine the chemistry of the clotting process, and our knowledge of this branch of the subject has up till recently been untouched by investigators.

The recent literature contains views which contradict the theory advanced by Hammarsten. Schryver [1913, 1 and 2] and Mellanby [1913] both consider that the rennin clot is probably a combination of enzyme and protein, and Schryver states definitely that rennin alone causes no proteoclastic change. Bosworth [1913] has found that the rennin does not split off any nitrogen from the caseinogen which remains in solution when the casein is precipitated by dilute acetic acid. The protein molecule has therefore undergone no cleavage into its components.

This, considered in connection with the results of his earlier work with van Slyke [1913], leads him to believe that the ferment breaks up the caseinogen molecule into two molecules of casein each half the size of the original molecule. In the case of basic calcium caseinogenate (containing 4 equivalents of calcium) the casein produced is soluble in water but is rendered insoluble by the presence of small quantities of calcium chloride. The caseinogenate containing two equivalents of calcium gives a casein insoluble in water[1].

[1] The English nomenclature—caseinogen for the unfermented protein and casein for the product of fermentation—is used throughout.

Enzymes employed.

The enzymes used were Witte's rennin powder 1:300,000, Grübler's trypsin, and a preparation made from the seeds of the Withania coagulans [Lea, 1884].

The rennin preparation was used in neutral aqueous suspension, was exceedingly active and fermented in a short time when the concentration was as low as 1:500,000. In the experimental work the concentration employed was 1 of rennin to 300,000 of caseinogen solution. The trypsin was used in aqueous suspension, in the proportion of 1 part of the solid preparation to 12,000 of caseinogen solution. The Withania coagulans enzyme was prepared by grinding up the seeds to a fine powder and extracting with 5 per cent. sodium chloride solution. Three volumes of absolute alcohol were added and the ferment precipitated. It was filtered, washed with absolute alcohol and dried at 37°. One gram of the powder thus obtained was made up to 100 cc. with 5 per cent. sodium chloride solution; 5 cc. of this solution were used to ferment 100 cc. of caseinogen solution. The trypsin and rennin contained no calcium or phosphorus estimable in three grams of the dried preparation, while the vegetable rennin contained a trace of phosphorus and no calcium.

Preparation and properties of the caseinogen.

At the commencement of the investigation it was found that the commercial preparations of caseinogen, even when obtained from reliable sources, did not give satisfactory results. In fact the rennin had scarcely any appreciable effect on them. A modification of Hammarsten's method was finally adopted which gave satisfactory caseinogen preparations, Schryver's plan of drying in different grades of alcohol being employed.

The method was as follows:—skimmed milk containing 0·1 per cent. of fat was taken, diluted with five times its volume of distilled water and 0·1 per cent. of acetic acid added slowly, the whole being constantly agitated until the protein separated out. The caseinogen was then allowed to settle and the supernatant fluid syphoned off. The mixture of caseinogen and diluted milk serum was poured on a cheese cloth filter and the remaining fluid drained off. The caseinogen was then washed by mixing it with a volume of distilled water equal to the quantity of milk from which it was obtained and shaking thoroughly. When settled the water was again syphoned off and the caseinogen washed a second and third time with the same volume of distilled water. It was next redissolved by shaking with 0·2 per cent. sodium carbonate

solution and the precipitation and washing carried out as before, the solution, precipitation and washing being repeated at least twice after the original precipitation from milk. Finally it was ground up successively with 33 per cent., 66 per cent. and absolute alcohol and given two half-hour extractions in an agitator with ether, filtered and dried for two hours at 37°.

These preparations upon analysis were found to be completely free from fat and had a phosphorus content of 0·87–0·90 per cent. The ash from 10 g. contained no estimable calcium. Solutions were prepared by grinding the dried powder with moist calcium carbonate in a mortar, centrifuging and filtering to remove the excess of calcium carbonate, the caseinogen when treated in this way having a solubility of 19–20 cc. (5 cc. gave by Kjeldahl's method enough ammonia to neutralise this volume of N/10 acid). The solutions were opaque and milky in appearance, neutral to litmus and contained the amount of calcium necessary to form the basic caseinogenate. After treatment by rennin these solutions readily gave precipitates when about 5 per cent. by volume of N calcium chloride was added. This amount of calcium chloride alone with the original caseinogen solutions produced no precipitate from 0°–40°, but when the temperature was raised to 50°–60° a precipitate was formed. The precipitates produced by the action of enzyme and calcium chloride rapidly contracted to a small fraction of the volume of the solution, while those produced by calcium chloride and heat settled very slowly and did not shrink in volume.

Composition of caseinogen and casein.

The experiments were carried out as follows: 200 cc. portions of caseinogen solution were put into beakers and the enzymes added in the proportions already mentioned. Control experiments were made, using equal amounts of caseinogen solution and enzyme solution inactivated by boiling, and submitting these to the same conditions as those containing the active enzyme. The mixtures were placed in an incubator at 37° for one hour, after which 10 cc. N calcium chloride (5·5 g. per 100 cc.) were added both to controls and fermented preparations. In the case of the fermented solutions the precipitate at once came down. Precipitates from the controls were obtained by heating them to 55°. When the precipitates had settled the supernatant liquid was decanted and the precipitates thoroughly broken up and shaken vigorously with 500 cc. of 10 per cent. alcohol in a tall cylinder. When they had again settled the clear fluid was syphoned off and this process repeated three times.

The last washing was found to be free from chlorides. To inactivate the enzyme the precipitates were mixed with water and boiled for five minutes, the controls being similarly treated. All the precipitates were finally ground up with absolute alcohol and ether and dried at 37°.

The nitrogen, calcium, phosphorus ratios were determined as an index of composition, nitrogen being taken as the fixed unit of comparison. Phosphorus was estimated by Neumann's method and nitrogen by Kjeldahl's. Calcium was estimated by ashing the dried caseinogen or casein, dissolving the ash in hydrochloric acid and neutralising with ammonia until the phosphates were precipitated. These were redissolved by adding strong acetic acid, and the calcium precipitated as the oxalate and weighed as the sulphate. No phosphorus was precipitated by this method.

The results are given in the following tables.

I. *Rennin action.*

Caseinogen (controls)				Casein			
	N	P	Ca		N	P	Ca
1.	100	5·8	10·4	3.	100	5·65	9·60
2.	100	5·8	10·5	4.	100	5·60	9·53
				5.	100	5·45	9·64
				6.	100	5·65	9·66

II. *Trypsin action.*

Caseinogen (controls)				Casein			
	N	P	Ca		N	P	Ca
7.	100	5·54	12·41	9.	100	4·85	10·42
8.	100	5·50	12·15	10.	100	4·32	10·17
				11.	100	4·71	10·47

III. *Withania coagulans.*

Caseinogen (controls)				Casein			
	N	P	Ca		N	P	Ca
12.	100	5·58	12·58	14.	100	5·48	12·40
13.	100	5·61	12·62	15.	100	5·45	12·35

These series of analytical results demonstrate clearly that the enzyme action results in some proteoclastic change in the caseinogen molecule. The first series on rennin action was carried out with a preparation of caseinogen which was different from that used in the other two series and had a somewhat higher phosphorus content. Two criticisms of these results may be advanced—that it may not be possible to remove all the absorbed calcium from the casein and that boiling the precipitates to inactivate the enzyme may split off phosphorus, a possibility which Schryver points out. With regard to the first point it has been found that the final washing is free from chlorides and that a uniformly lower calcium content appears in casein throughout the series. To settle the second point caseinogen was taken,

fermented by rennin and calcium chloride added. The precipitate was filtered off and the N : P ratio was determined in the filtrate and compared with that given by a caseinogen solution to which equal amounts of calcium chloride and inactive enzyme had been added under the same conditions. In no case was the temperature higher than 37°, so that any difference observed must be due to enzyme action.

	Caseinogen (controls)			Filtrate from casein	
	N	P		N	P
1.	100	5·76	3.	100	12·2
2.	100	5·80	4.	100	12·0
			5.	100	10·7
			6.	100	13·1

This clearly demonstrates that the phosphorus cleavage was due to the rennin action alone and not to the boiling with water.

These figures for caseinogen are in agreement both in phosphorus and calcium content with most of the preparations of the basic salt quoted in the literature published on this subject.

On the other hand the casein shows a slightly lower phosphorus or calcium content relative to the nitrogen. These figures are not comparable with the phosphorus or calcium content of the acid salts of casein described by other investigators. Schryver finds that no change in the N : P ratio takes place when caseinogen is converted into casein, and van Slyke and Bosworth [1913] have prepared a basic caseinogenate and a caseinate from milk of the same phosphorus and calcium content.

The cleavage is most marked in the case of trypsin where from 12–20 % of the phosphorus is split off, the action thus bearing a close resemblance to that exerted by this enzyme in alkaline solution [Plimmer and Bayliss, 1906]. The action is less marked in the case of the Withania enzyme.

Enzymic precipitation of caseinogen solutions without the addition of calcium salts.

Furthermore, caseinogen solutions after being subjected to ferment action give precipitates without addition of calcium chloride if the ferment be present in sufficient concentration. Caseinogen can thus be precipitated without the addition of calcium chloride in twenty-four hours by rennin when the latter is present in the proportion of 1 part of dried ferment to 1000 of solution. Trypsin in three to four hours also forms a precipitate in 1 : 5000 solution without the addition of calcium chloride. Withania coagulans also produced a small precipitate without addition of a soluble calcium salt in four

hours when one volume of ferment solution was added to five of caseinogen solution. Again, when the ferment was more dilute (rennin 1 : 100,000, trypsin 1 : 15,000, Withania 1 : 20) precipitates appeared when the temperature was raised from 45°–50° even when the ferments had acted only for twenty minutes and in the absence of added calcium salts. Controls containing the same concentrations of inactivated enzyme did not give these reactions.

Nature of the cleavage products.

The filtrates from casein preparations were taken and examined to ascertain the nature of the nitrogen and phosphorus split off. Cathcart's precipitating mixture as used by Plimmer and Bayliss [1906] was added to casein solutions to precipitate the protein and its higher cleavage products, and analyses were made on aliquot parts of the filtrate. In the case of the casein produced by rennin action no soluble phosphorus or nitrogen was found. No inorganic phosphorus was produced by any of the three ferments.

100 cc. of caseinogen solution were taken containing 0·521 g. N and 0·0295 g. P. N : P = 100 : 5·6.

		Produced from 100 cc. caseinogen solution and not precipitated by tannic acid		
		Soluble N	Soluble P	Ratio N : P
Trypsin action	1.	0·0203 g.	0·0033 g.	100 : 16·2
	2.	0·0084 g.	0·0015 g.	100 : 17·8
Withania action	3.	0·0028 g.	0·0006 g.	100 : 21·4
	4.	0·0022 g.	0·0009 g.	100 : 40·9

Trypsin as shown by these analyses gives the largest quantities of soluble nitrogen and phosphorus, and splits off some phosphorus group relatively rich in phosphorus, a N : P ratio considerably higher than the normal being obtained. In the case of the Withania enzyme the phosphorus is still higher in relation to the nitrogen but the total quantities of soluble nitrogen and phosphorus are very small. The amounts given above probably do not represent the total cleavage, some of the products in each case being presumably in a form precipitable by tannic acid.

As already mentioned the behaviour of trypsin in converting caseinogen into casein in neutral solution is somewhat like its proteoclastic action in alkaline solution, except that no inorganic phosphorus is produced as in alkaline solutions [Plimmer and Bayliss, 1906]. The effect of the trypsin used when allowed to act in alkaline solutions is shown by the following experiment.

Caseinogen solution and trypsin solution were taken in the same quantities as for the precipitation experiments, and enough alkali to make the concentration 0·2 per cent. NaOH added. This was incubated at 37° for three hours. Part was taken, the protein precipitated with dilute acetic acid and the inorganic phosphorus precipitated from the filtrate by magnesium citrate mixture, the phosphorus being estimated by Neumann's method in the phosphate precipitate. In another portion the soluble phosphorus was estimated as previously described.

100 cc. original solution.	Total P = 0·0268 g.
100 cc. solution after trypsin action.	Soluble P = 0·0150 g.
100 cc. solution after trypsin action.	Inorganic P = 0·0043 g.

The irreversibility of the change.

Caseinogen when precipitated from solution in the presence of calcium chloride at 55°, washed with 10 per cent. alcohol, redispersed in caustic soda, again precipitated with acetic acid, washed and dissolved by calcium carbonate gave solutions not nearly as concentrated as those obtainable from the original material. These latter had concentrations of 17–19·5 cc., but after the above process products were obtained, the solubilities of which were only from 7–13 cc. These could readily be acted on by any of the enzymes and a characteristic casein precipitate obtained.

Casein after the above treatment had very low solubilities, ranging from 0·5–4 cc. and the solutions were transparent, and when submitted to further enzyme action did not yield products more readily precipitated by calcium chloride than themselves.

This confirms the observations of Hammarsten and Schryver on this point.

Precipitation of caseinogen and casein by electrolytes.

The precipitating action of a number of salts was tested on caseinogen and casein solutions prepared by means of calcium carbonate as described. 5 cc. of solutions exposed to ferment action for one hour and the same amount from controls containing inactivated ferment were diluted to 100 cc., boiled, cooled and 1 cc. quantities put in test tubes in which 1 cc. of the various salt solutions had been previously placed. The concentrations indicated in the tables are those of the salts in the final mixture. The mixtures were allowed to stand at room temperature for one hour; + indicates a precipitate.

TABLE I.

1. *Rennin action.*

Concentration of salt in final mixture	$CaCl_2$		$BaCl_2$		$MgCl_2$		$SrCl_2$	
	Caseinogen	Casein	Caseinogen	Casein	Caseinogen	Casein	Caseinogen	Casein
2N	−	−	−	−	−	−	−	−
N	−	+	−	+	−	−	−	−
N/2	−	+	−	+	−	−	−	+
N/4	−	+	−	+	−	+	−	+
N/10	−	+	−	+	−	+	−	+
N/20	−	+	−	+	−	+	−	+
N/40	−	+	−	+	−	+	−	−
N/80	−	−	−	−	−	−	−	−

2. *Trypsin action.*

2N	−	−	−	−	−	−	−	−
N	−	+	−	+	−	−	−	−
N/2	−	+	−	+	−	+	−	−
N/4	−	+	−	+	−	+	−	+
N/10	−	+	−	+	−	−	−	+
N/20	−	+	−	+	−	−	−	−
N/40	−	−	−	−	−	−	−	−
N/80	−	−	−	−	−	−	−	−

3. *Withania coagulans action.*

2N	−	−	−	−	−	−	−	−
N	−	−	−	−	−	−	−	−
N/2	−	+	−	+	−	−	−	−
N/4	−	+	−	+	−	−	−	+
N/10	−	+	−	+	−	+	−	+
N/20	−	+	−	+	−	+	−	−
N/40	−	−	−	−	−	−	−	−
N/80	−	−	−	−	−	−	−	−

Calcium and barium salts in the case of all the enzymes are most effective as precipitating agents. The ranges of the action of strontium and magnesium salts are similar, but are narrower than in the case of calcium and barium salts. This holds for all the enzymes with slight individual variations. In no case was caseinogen precipitated within the range employed.

In the case of the lanthanum salt (Table II, p. 98) the requisite concentration is very low, being N/800 for caseinogen. This is in accord with the findings of other workers, the trivalent ion being an exceedingly powerful one as a precipitating agent. Again the variation for the different enzymes is seen, but to a greater extent, especially for casein obtained by trypsin action.

The variation in the different caseins is probably due to the influence of the cleavage products present in the mixture which, as shown earlier, are different for each ferment.

TABLE II.

Precipitating action of lanthanum nitrate.

Concentration of salt in final mixture	Rennin action		Trypsin action		Withania action	
	Caseinogen	Casein	Caseinogen	Casein	Caseinogen	Casein
N/50	–	–	–	+	–	–
N/100	–	–	–	+	–	–
N/200	–	–	–	+	–	–
N/400	–	+	–	+	–	–
N/800	+	+	+	++	+	+
N/1600	–	++	–	++	–	++
N/3200	–	–	–	–	–	–

It is remarkable that both caseinogen and casein are precipitated by the same concentration of lanthanum nitrate, whereas they require very different concentrations of calcium, barium, strontium and magnesium salts.

The investigation of the precipitating action of rennin on caseinogen in milk is an extremely difficult problem, since here the reaction of the liquid and the presence of salts, carbohydrates and fats all play a part which may obscure the essential factors of the process. For this reason solutions of pure caseinogen have been used throughout the foregoing experiments. Another point which is of the greatest importance is the method of preparation of the caseinogen. The different methods used for this purpose probably account for much of the diversity of opinion which exists at present on the nature of the enzyme action. In our preparations from milk the use of sodium hydrate was avoided so as to escape the possibility of hydrolysing the protein. Van Slyke and Bosworth have used ammonia in the final stages of their method, allowing it to remain in contact with the caseinogen overnight. Their preparations have a very low ash content and the phosphorus content is the lowest yet recorded. This is due possibly to removal of phosphorus by the action of the alkali.

The foregoing results indicate that the substance precipitated by calcium chloride from solutions of caseinogen, which have been submitted to the action of rennin, trypsin or Withania enzyme, differ not only in precipitability by salts and solubility but also in chemical composition.

Conclusions.

(1) The conversion of caseinogen into casein by enzyme action is accompanied by the cleavage of nitrogen, phosphorus and calcium.

(2) Rennin action produces no soluble nitrogen or phosphorus. Trypsin splits off both soluble nitrogen and phosphorus, while the Withania enzyme

also produces soluble nitrogen and phosphorus but in smaller absolute quantities.

(3) The cleavage products are specific for each enzyme and it is to this difference of enzyme action that the variation in behaviour of the resulting casein is to be ascribed.

(4) The precipitation of calcium caseinate by soluble calcium salts is not due to any chemical combination with these.

(5) The caseinogen once exposed to enzyme action and redispersed cannot be rendered more precipitable by renewed enzyme action.

(6) If the enzyme be sufficiently concentrated, precipitates are obtained without the addition of calcium salts and the same thing occurs with more dilute enzyme solutions when the temperature is raised above 45°.

In conclusion we acknowledge our indebtedness to the Director of the Imperial Institute for the supply of seeds of Withania coagulans from which the enzyme was prepared.

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XIII. THE ENZYMES OF WASHED ZYMIN AND DRIED YEAST (LEBEDEFF). II. REDUCTASE.

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The reducing powers of yeast have long been known, and it was shown by Hahn that yeast juice and zymin possessed similar properties.

Recently the relation of the reducing ferment of yeast to the enzymes concerned in alcoholic fermentation has become of some interest, owing on the one hand to the theories of Neŭberg, Kostytscheff and v. Lebedeff which postulate the reduction of acetaldehyde to ethyl alcohol as an essential step, and on the other hand to the experiments of Palladin [1908] who has found that during the fermentation of glucose by zymin the reducing action of the latter on sodium selenite and methylene blue is greatly diminished, and to those of Lvoff [1913, 1, 2, 3] who finds that during the reduction of methylene blue by dried yeast or maceration extract there is a correspondingly smaller fermentation of glucose, one molecule of methylene blue being equivalent to one molecule of glucose.

The reduction of selenite or methylene blue by living yeast is known [see Grüss, 1908] to be accelerated by the presence of glucose, and hence the conclusions of Palladin do not seem warranted without further examination.

In the following experiments the work of Palladin has been repeated and at the same time the effect of washing dried yeast and zymin upon the reducing power of the preparation has been ascertained.

The reducing powers of yeast, zymin and dried yeast towards sodium selenite.

Reduction of sodium selenite by living yeast. Influence of carbohydrates on the rate of reduction. The following mixtures were made up and incubated at 25°.

A.	2 g. yeast	+	30 cc. 0·5 per cent.	sodium	selenite	solution.	
B.	,,	,,	,,	,,	,,	,,	+1 g. glucose.
C.	,,	,,	,,	,,	,,	,,	+1 g. galactose.
D.	,,	,,	,,	,,	,,	,,	+1 g. lactose.
E.	,,	,,	,,	,,	,,	,,	+1 g. arabinose.

The red colour due to metallic selenium appeared first in B and was quite marked in one hour. At this time there was only a faint reduction in all the other flasks. Two hours later the flasks all showed reduction, but that in B was very much more marked than in the others, which were all equally reduced. Hence the rate of reduction is increased by the presence of a fermentable sugar as found by Grüss [1908]. The other sugars tried had no influence. A control experiment was carried out in which glucose was incubated with sodium selenite solution alone but no reduction took place. A second experiment in which other fermentable sugars, cane sugar and maltose, were employed, gave a similar result.

Reduction of sodium selenite by zymin. Similar experiments to those described above were next carried out, using zymin instead of living yeast. In no case was any acceleration produced by the presence of a fermentable sugar. Palladin [1908] states that the reduction in the case of zymin is greatly hindered and in some cases entirely inhibited under these circumstances. In the authors' experiments, however, such a retardation has only been observed when very high concentrations of sugar have been employed. With low concentrations (5 per cent.) of glucose little effect was produced.

These points are illustrated by the following experiments.

The following mixtures were made up and incubated at 25°.

								Concentration of glucose per 100 cc. in g.
A.	3 g. zymin	+	10 cc. 1 % selenite	+	10 cc. H_2O	...	...	0
B.	,,	,,	,,	,,	+9·4	,,	+ 1 g. glucose	5
C.	,,	,,	,,	,,	+8·8	,,	+ 2 g. ,,	10
D.	,,	,,	,,	,,	+7	,,	+ 5 g. ,,	25
E.	,,	,,	,,	,,	+4	,,	+10 g. ,,	50

The total volume was thus the same in each case, while the concentration of glucose varied from 5 to 50 g. per 100 cc. After incubation for 60 minutes the reduction in A, B, C and D was well marked and was approximately equal in extent. In E reduction had just started but was much less pronounced than in the other flasks. Hence glucose in concentrations up to 25 per cent. had little influence on the rate of reduction. Palladin's explanation of his results was that in the presence of glucose the reductase was directly concerned in the alcoholic fermentation and was therefore not free to reduce the selenite.

In view of the different results obtained by the authors, with lower concentrations of glucose, it seemed desirable to examine to what extent fermentation was influenced by the presence of selenite, for which purpose the following experiments were carried out:

A. *Living Yeast.*

(1) 2 g. yeast + 2 g. glucose + 20 cc. H_2O.
(2) ,, ,, ,, + 20 cc. 1 % sodium selenite.

The rate of fermentation of these mixtures at 25° was observed with the following results:

Duration of experiment, mins.	Total CO_2 evolved in cc. 1 Yeast + glucose	2 Yeast + glucose + selenite
5	9·1	10·4
10	17·8	20·6
15	28·0	31·2
20	36·6	40·9
30	54·6	60·8

In the above experiment therefore the mixture containing the selenite gave a slightly higher rate of fermentation than the control.

Reduction in (2) was well marked after incubation for 20 minutes. With zymin on the other hand a very different result was obtained.

B. *Zymin.*

(1) 2 g. zymin + 2 g. glucose + 20 cc. H_2O.
(2) ,, ,, ,, + 10 cc. 1 % selenite + 10 cc. H_2O.
(3) ,, ,, ,, + 20 cc. 1 % selenite.

Duration of experiment, mins.	Total CO_2 evolved in cc. 1 No selenite	2 0·5 % selenite	3 1 % selenite
50	11·0	2·4	2·1
65	25·5	2·7	2·3
80	33·8	3·1	2·4
95	40·3	3·4	2·4
155	64·2	4·4	3·1

With zymin therefore the presence of even 0·5 per cent. sodium selenite almost entirely inhibited the fermentation and this result was confirmed by further experiments. Both (2) and (3) showed signs of reduction after 50 mins. and the production of selenium increased steadily during the experiment. As Palladin [1908] used concentrations of selenite varying from 2 to 5 per cent. it is improbable that alcoholic fermentation was proceeding at all in any of his experiments.

C. *Dried Yeast.* (Lebedeff.)

(1) 2 g. dried yeast + 20 cc. H_2O + 2 g. glucose.
(2) ,, ,, + 20 cc. 1 % selenite + 2 g. glucose.
(3) ,, ,, + 10 cc. 1 % selenite + 10 cc. H_2O + 2 g. glucose.

Duration of experiment, mins.	Total CO_2 evolved in cc.		
	1 No selenite	2 1 % selenite	3 0·5 % selenite
35	18·8	0·6	0·9
95	36·6	1·0	1·5
155	55·6	1·5	1·9
380	98·6	3·8	3·8

Here, as with zymin, fermentation was almost entirely inhibited. Reduction was observed in (3) at the end of the experiment but not in (2).

The sample of dried yeast had a very low reducing power towards selenite as shown by the following experiment:

(1) 2 g. dried yeast + 20 cc. 1 % selenite.
(2) ,, ,, + 20 cc. 1 % selenite + 2 g. glucose.

These mixtures were incubated at 25°. After incubation for 4·5 hours no reduction could be detected in either. An hour later however reduction was visible in (2) and still later appeared in (1). These results with zymin and dried yeast cannot be explained on the ground of Lvoff's interpretation of his own experiments, according to which one molecule of glucose gives up two atoms of hydrogen to the reducible substance (in his case methylene blue), so that an amount of glucose equivalent to this escapes fermentation. In these experiments therefore in presence of 0·1 g. sodium selenite there should have been a deficit of about 29 cc. of CO_2, whereas as a matter of fact the deficit in the case of dried yeast was about 95 cc. and in the case of zymin 64 cc., without any sign of fermentation setting in.

It may here be remarked that the conclusions drawn by Lvoff [1913, 2 and 3] from his experiments cannot at present be accepted. In all the experiments in which the fermentation was continued beyond the stage at which the methylene blue was completely reduced, the deficit of carbon dioxide and alcohol increased considerably with the time. This indicates that the inhibition of fermentation even in the earlier stages cannot be solely attributed to the deviation of hydrogen, and indeed makes it doubtful whether any of it can be attributed to this cause. Experiments on the change in the amount of sugar during the process which are being carried out by Lvoff and, it may be suggested, an investigation on the degree of inhibition produced with different concentrations of dried yeast or maceration extract are required before any definite conclusion can be legitimately drawn. Experiments VI

and VIII [Lvoff, 1913, 3, pp. 304–5] suggest that the effect varies considerably with the amount of dried yeast employed.

Inactivation of dried yeast and zymin by washing.

It was observed that when zymin or dried yeast was washed several times with cold water and thus rendered incapable of fermenting sugar, it also lost its power of reducing methylene blue or sodium selenite.

It seemed therefore of interest to ascertain the cause of this loss of reducing power and also whether any substance capable of restoring it would at the same time restore the power of alcoholic fermentation. That the action is enzymic is shown by the fact that when dried yeast is boiled with water, the mixture does not reduce methylene blue. As a result it was found that the addition of certain aldehydes or of bouillon restored the reducing power but not the fermenting power, whilst the boiled washings restored both.

It seems probable therefore that washing removes some substance which acts as an acceptor for the oxygen activated during the reduction process and that the place of this can be taken by certain aldehydes or by some constituent of bouillon. The reducing enzyme of yeast therefore bears a close resemblance to that of potato juice recently investigated by Bach [1913, 1 and 2].

The zymin and dried yeast (Lebedeff) obtained from Schroder were washed in the manner previously described [Harden, 1913].

I. Washed zymin and sodium selenite.

5 g. zymin were washed and made to 60 cc.

1. 20 cc. zymin suspension + 20 cc. H_2O + 50 cc. 0·5 % sodium selenite.
2. ,, ,, ,, + 20 cc. boiled washings + 50 cc. 0·5 % sodium selenite.
3. ,, ,, ,, + 20 cc. H_2O + 50 cc. 0·5 % sodium selenite + 0·1 cc. formalin.
4. ,, boiled washings + 20 cc. H_2O + 50 cc. 0·5 % sodium selenite.

On incubation for 17 hours at 25° the only flask which showed reduction was (2), which contained washed zymin and boiled washings. The reducing power was not restored by the addition of formaldehyde (4).

II. Washed dried yeast and sodium selenite.

20 g. of dried yeast made to 100 cc.

1. 15 cc. yeast suspension + 10 cc. H_2O + 7 cc. 1 % selenite + 1 cc. toluene.
2. ,, ,, ,, + 10 cc. boiled washings + 7 cc. 1 % selenite + 1 cc. toluene.
3. 0 + 15 cc. H_2O + 10 cc. boiled washings + 7 cc. 1 % selenite + 1 cc. toluene.

Reduction was marked in (2) after 3 hours at 25° and did not occur in either (1) or (3) in 17 hours.

III. Washed dried yeast and (*a*) Methylene blue, (*b*) Schardinger's reagent.

10 g. dried yeast washed and made to 100 cc.

1. 20 cc. yeast suspension + 20 cc. H_2O + 1 cc. methylene blue + 1 cc. toluene.
2. ,, ,, ,, + 20 cc. boiled washings + 1 cc. methylene blue + 1 cc. toluene.
3. ,, H_2O + 20 cc. boiled washings + 1 cc. methylene blue + 1 cc. toluene.
4. ,, yeast suspension + 20 cc. H_2O + 1 cc. Schardinger + 1 cc. toluene.
5. ,, ,, ,, + 20 cc. boiled washings + 1 cc. Schardinger + 1 cc. toluene.
6. ,, H_2O + 20 cc. boiled washings + 1 cc. Schardinger + 1 cc. toluene.

The methylene blue was made by diluting 5 cc. of a saturated alcoholic solution to 200 cc.; Schardinger's reagent by mixing 5 cc. of saturated alcoholic methylene blue with 5 cc. formalin and diluting to 200 cc.

After 19 hours reduction had occurred in (2) and (5) but in none of the others.

Hence formaldehyde does not restore the reducing power.

IV. Reducing power of inactivated yeast in presence of various substances.

A number of qualitative experiments were made to ascertain the efficacy of various substances in producing reduction when added to washed dried yeast and methylene blue.

The results may be tabulated as follows:

Reducing power restored	No change
Salicylaldehyde*	Quinol*
Benzaldehyde	*p*-Phenylene diamine
Anisaldehyde	Pyrogallol
Isovaleraldehyde	Pyruvic acid
Bouillon*	Citral
	Acetaldehyde

The substances marked with an asterisk were tested as to their capacity to restore the power of alcoholic fermentation to washed dried yeast in presence of a small concentration of phosphate, in all cases with negative results.

Methylene blue and sodium selenite were also found to be inactive in this respect.

Summary.

1. The presence of a fermentable sugar favours the reduction of selenite by living yeast but has little influence on the reducing power of zymin unless the sugar is present in high concentration, when inhibition occurs.

2. Sodium selenite in concentration of 0·5 g. per 100 cc. almost totally inhibits the fermentation of glucose by zymin and dried yeast (10 g. per 100 cc. of 10 per cent. glucose solution).

3. When dried yeast or zymin is washed with cold water it loses its power of reducing methylene blue and sodium selenite.

4. Such washed preparations reduce methylene blue in presence of many aldehydes and of bouillon, but these do not restore to it the power of producing alcoholic fermentation.

5. Addition of the boiled washings to these washed preparations restores both the power of reducing methylene blue and of producing alcoholic fermentation.

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XIV. THE FORMATION OF A PEPTONE FROM CASEINOGEN BY THE PROLONGED ACTION OF DILUTE HYDROCHLORIC ACID IN THE COLD.

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The partial hydrolysis of proteins by strong acids in the cold was worked out very extensively by Emil Fischer, Abderhalden and their co-workers. As the result of the hydrolysis a number of well defined polypeptides were isolated. Methods were also devised for their separation.

We are not aware however that hydrolysis has been attempted by the prolonged action of weak acids, such for instance as hydrochloric acid in double the concentration in which it occurs in the gastric juice of the dog. The question arose whether hydrolysis took place at all in these conditions and if it did whether the products differed from those obtained by hydrolysis with strong acids.

For the purpose of testing these possibilities commercial caseinogen was left for three months in contact with 1 % HCl-solution. With the aid of the usual methods a product was separated, which, although not quite pure, proved to be a peptone. Although the amount of the substance available for the investigation was small it was possible to determine some of the amino-acids present in the preparation by using the excellent methods brought forward by van Slyke and Folin. As a result of this investigation it was found that the peptone obtained contained only a trace of tyrosine, whereas the peptones separated in this way are usually very rich in this amino-acid. This perhaps suggests that weak acids act on proteins differently from strong acids. A careful control of the commercial caseinogen was made to ascertain whether the product isolated was not present preformed in the commercial preparation, with entirely negative result. This control was performed because it seemed surprising that such weak acids as we had used were able to disintegrate the protein molecule to such an extent.

During this enquiry we had the opportunity to investigate the action of weak acids on the reaction of peptones with triketohydrindene hydrate and we have found that a drop of a decinormal solution of a mineral acid inhibits the reaction completely; the action of organic acids in a ten times higher concentration is the same.

Experimental.

1 kg. of commercial caseinogen was left in contact with 5 litres of a 1 % HCl-solution for three months. The suspension of caseinogen which had not been attacked was filtered off. The filtrate which had a deep brownish colour was acid and still contained free hydrochloric acid. The liquid which showed no sign of putrefaction gave a strong biuret reaction, but no Millon reaction. The solution was precipitated in the usual way with a 50 % solution of phosphotungstic acid. After 24 hours the precipitate formed was filtered off. The filtrate was still found to give a strong biuret reaction but was not investigated any farther.

The phosphotungstate obtained was decomposed in the usual way with baryta, and the filtrate evaporated *in vacuo* to a syrup which was dissolved in methyl alcohol and filtered into a large volume of absolute alcohol. The product separated was filtered off and washed with alcohol and ether. It was a white powder, fairly soluble in cold water and non-hygroscopic. Yield 14 grm.

The product contained a trace of ash, a trace of sulphur and no phosphorus. It contained 13·41 % N by Kjeldahl's method and 2·33 % of amino-nitrogen before hydrolysis.

The nature of the product was found to be that of a peptone as shown by the reactions described below:

I.	Millon	Trace. Traces of tyrosine.
II.	Diazo-reaction	+
III.	Triketohydrindene hydrate (Ninhydrin) ...	+
IV.	Folin's phenol-reagent	Trace.
V.	Xanthoproteic reaction	+
VI.	Biuret	Pink-violet.
VII.	Bromine	−
VIII.	Glyoxylic acid	+[1]
IX.	Diazo-reaction after benzoylation of the product	−. No histidine.

[1] The glyoxylic acid reagent was prepared following Benedict [1909] by the action of magnesium on oxalic acid.

For the control 100 grm. of commercial caseinogen were extracted for one hour on the shaking machine with HCl of the same concentration as that mentioned above. In contrast to the previous one the suspension

filtered with great difficulty. The filtrate was colourless but slightly opalescent. The solution gave a precipitate with phosphotungstic acid which yielded on decomposition under the same conditions as above 0·4 g. of a white substance which proved to be mostly inorganic; it consisted of ammonia salts chiefly and gave no trace of a biuret reaction. One can conclude therefore that the peptone obtained was formed by the action of the 1 % HCl on caseinogen.

With a view to determining some of the amino-acids present in this peptone a complete hydrolysis was performed according to van Slyke's method. An estimation of tyrosine was done separately according to the method of Folin and Denis [1912], but only a trace of this amino-acid was found, due very likely to an impurity. An attempt was made to estimate tryptophane by the recently described colorimetric method of Herzfeld [1913], but the method proved in our case to be unsatisfactory as a decolorisation took place[1].

The result of total hydrolysis of the peptone.

Total nitrogen	13·35 %	Histidine-N.	0
Melanine-N.	0·15 %	Lysine-N.	(?) 5·23 %
Ammoniacal-N.	0·90 %	Cystine-N.	0·30 %
Total amino-N.	5·78 %	Total N. of the phosphotungstic acid filtrate	6·16 %
N. of the phosphotungstic acid ppt.	5·53 %	Amino-N. of the phosphotungstic acid filtrate	1·92 %
Amino-N. of the phosphotungstic acid ppt.	2·78 %	Tyrosine-N.	0·09 %
Arginine-N.	0		

The results of the hydrolysis seem to suggest that the only hexone base in the product is lysine, the nature of the monoamino-acids being still unknown.

The effect of acids on the reaction of the peptones with ninhydrin.

One drop of a decinormal solution of hydrochloric or sulphuric acid added to 10 cc. of the peptone solution completely inhibits the reaction, both with the peptone described above and also with Witte peptone. The same effect was obtained with one or two drops of a normal solution of acetic or lactic acid.

[1] The same decolorisation took place when we tried to estimate tryptophane in maize by this method.

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XV. THE GALACTOSIDES OF THE BRAIN. II. THE PREPARATION OF PHRENOSIN AND KERASIN BY THE PYRIDINE METHOD.

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The methods hitherto used for the preparation of the galactosides from brain may be divided into two groups. In one group of methods, which may be traced back to Couerbe's work, a separation of the galactosides from the phosphatides is effected by purely physical means, mainly by the help of such solvents as alcohol (Thudichum), glacial acetic acid (Koch), methyl alcohol-chloroform (Wörner and Thierfelder), etc. In the second group of methods the relative resistance of the galactosides to hydrolysis by baryta is used to separate them from the easily saponifiable phosphatides. The latter method which dates back to the work of Müller has been applied directly to the brain (Parkus) or to the mixture of galactosides and phosphatides etc., which is obtained by cooling an alcoholic extract of brain (Kossel and Freytag, Thierfelder, Fränkel, Lapworth).

The product obtained by both methods consists of a mixture of at least two galactosides, a fact which was first recognised by Thudichum. To the latter investigator belongs also the credit of having introduced the only available method for their separation, which may be termed a temperature fractionation method.

Both groups of methods possess one inherent disadvantage, which has considerably retarded the study of brain lipoids in general. Whilst allowing the separation of the galactosides, they make it most difficult or even impossible to isolate the saturated phosphatides which are present in brain. Indeed since Thudichum, all the subsequent investigators, until quite recently, limited their work to the preparation of the galactosides, neglecting altogether the phosphatide moiety of the original product[1].

[1] Thudichum isolated one of these phosphatides, called by him sphingomyelin, by means of its cadmium salt, and it has since been prepared by other methods by Rosenheim and Tebb. According to unpublished investigations of Rosenheim and Tebb, the cadmium method is altogether unsatisfactory.

Further, none of the authors who used the baryta method seem to have taken into consideration the fact that the galactosides are more or less hydrolysed by prolonged boiling with baryta in aqueous or alcoholic solution. Only quite recently it has been shown by Thierfelder and Loening [1912] that relatively short boiling with aqueous baryta does not split off galactose from these substances. It remains, however, a significant fact that only those observers who used the alcoholic baryta method found a high nitrogen percentage in their products. This fact suggests that their preparations contained certain amounts of the nitrogenous base sphingosine, set free by partial hydrolysis from the galactosides or from the phosphatide sphingomyelin. This possibility must be borne in mind, especially as the alcoholic baryta method has recently again come to the fore [Fränkel, 1911; Lapworth, 1913], without apparently any previous investigation of the action of alcoholic baryta on the native galactosides.

Principle of new method.

The difficulties and failures of the older methods used for the study of the brain lipoids may be traced back to the general use of alcohol as the primary solvent. The solubilities in alcohol of the various substances composing the complex lipoid mixture, as it occurs in brain, are such that their separation has baffled investigators ever since Vauquelin first applied this solvent to brain over 100 years ago. The problem becomes much simplified by discarding altogether the use of alcohol as a primary solvent, and by applying to the brain the principle of selective extraction as first suggested by Rosenheim [1906], and subsequently worked out by Rosenheim and Tebb [1909, 1910]. The method consists shortly in extracting brain, suitably dehydrated, at various temperatures with solvents which as far as possible dissolve out only one group of lipoids at a time. The method has so far led to the separation of the following lipoids:

(1) Cholesterol (by extraction with cold acetone[1]).

(2) Lecithin and kephalin (by extraction with ether or petrol ether).

(3) Galactosides (by extraction with cold pyridine).

(4) Sphingomyelin and sulphatide (by extraction with warm pyridine).

The method has already been described in detail in a previous communication in this journal [1913], as far as the preparation of the crude galactosides is concerned.

[1] Acetone has subsequently also been adopted by S. Fränkel as the primary solvent in his method for the preparation of brain lipoids. After the removal of the unsaturated phosphatides, lecithin and kephalin, this investigator again reverts to the use of alcohol and proposes to separate the mixture of galactosides, saturated phosphatides and sulphatides by the use of the old methyl alcoholic baryta method [1911].

Nomenclature.

In his "Proposals for the nomenclature of the lipoids" the author has already stated in this journal [1909] the reasons for discarding the names cerebrin, pseudo-cerebrin, cerebron and homo-cerebrin. It was proposed, following the suggestion of Posner and Gies [1906], to retain the original names phrenosin and kerasin, introduced by Thudichum, for the two principal galactosides isolated by him from brain.

This nomenclature is now generally accepted by most writers on the subject, and it seems only logical to extend it to the cleavage products of these substances. The author has therefore followed Posner and Gies [1906] in adopting the name "phrenosinic acid" for the fatty acid obtained on hydrolysis of phrenosin. The term "kerasinic acid" proposed by Thierfelder for the corresponding acid from kerasin seems however to be unnecessary since this acid has been identified by Rosenheim and by Levene with the longer known natural lignoceric acid.

The separation of phrenosin and kerasin.

The difficulty of separating the galactoside mixture into its constituents is probably due to several causes, the most important of which are the following: (1) the close resemblance in their chemical constitution (see later); (2) the close resemblance of their physical properties, such as solubility etc.; (3) their chemical inertness, which prevents the formation of any characteristic derivative suitable for their purification[1]; (4) the remarkable property of these lipoids to exist in the liquid crystalline condition (see next communication) must also be held responsible to a large degree for the difficulties of their separation, especially since O. Lehmann, the discoverer of this interesting state of matter, has clearly demonstrated the easy miscibility of liquid crystals; (5) the absence of any criterion by means of which the completeness of the separation can be judged. Melting point estimation and elementary analysis are practically useless and may even be misleading in this case as has been clearly demonstrated by the experience of Thierfelder and of Levene and Jacobs[2].

[1] I have, however, found that these galactosides, when treated in pyridine solution with benzoyl chloride, easily give benzoyl products. A method for the separation of phrenosin and kerasin might be based on the different solubilities of their benzoyl derivatives.

[2] In spite of the close agreement of many analyses of "cerebron," Thierfelder and Loening were able to show later [1910] that it contained an admixture of another galactoside. Levene and Jacobs [1912] were so much struck by the agreement of their analyses of "cerebrine" and kerasin, that they considered them for this, amongst other reasons, to be stereo-isomeric substances.

The following observations, made by Rosenheim and Tebb six years ago in connection with some other work on brain lipoids [1908], are therefore of considerable value for the separation of phrenosin and kerasin.

(1) Phrenosin (in pyridine solution) was found to be dextrorotatory, whilst kerasin is laevorotatory in the same solvent.

(2) The behaviour of the two substances under the polarising microscope is characteristically different. It allows the detection of phrenosin in presence of kerasin, and *vice versa*, by a simple test which will be described later as the selenite-plate test.

By the help of these two tests, the progress of the separation of the galactoside mixture may be controlled. For the actual separation the principle of temperature fractionation, introduced by Thudichum, was made use of. As, however, the previous experience of Rosenheim and Tebb [1908] had shown that by the use of alcohol, which was the only solvent employed by Thudichum, a complete removal of the phosphatides is practically impossible, I have successfully used acetone containing 10 % of water for the primary fractionation.

The mixture is thus separated into: (*a*) *the phrenosin fraction*, deposited on cooling the acetone solution, from 50° to 36°, and (*b*) *the kerasin fraction*, deposited from the clear decanted mother liquor of (*a*) on cooling from 28° to about 0°.

It can easily be demonstrated by the use of the selenite-plate test that the phrenosin fraction still contains kerasin, and that phrenosin is present in the kerasin fraction. Their complete separation cannot be effected by the repetition of the temperature fractionation process with any single solvent. A series of fractionation experiments was therefore carried out with various mixtures of solvents and the products tested by the selenite-plate test as well as by the polarimeter. The following procedure was finally adopted.

Phrenosin is obtained free from kerasin by recrystallisation of the phrenosin fraction from a glacial acetic acid-chloroform mixture (3 : 2) at 37°. After the third recrystallisation from this solvent, it is usually found that the filtrate from the deposit formed at 37° no longer deposits kerasin when cooled to room temperature. The last traces of phosphorus are also removed by this solvent.

The selenite-plate test, however, still reveals at this stage the presence of small quantities of kerasin, which are removed by repeated temperature fractionation from a mixture of acetone-chloroform (3 : 2). The last filtrate from the deposit at 37° must remain perfectly clear on cooling to room temperature.

The product is finally once more recrystallised from acetone containing 10 per cent. pyridine.

Kerasin is obtained free from phrenosin in a similar way from the kerasin fraction by using the same solvents. In this case, however, the superficial criterion of the removal of phrenosin is given by the fact that the solution remains perfectly clear at 37° and only begins to deposit when cooled to 28°. The final product, recrystallised again from acetone-pyridine, should be laevorotatory, and when examined under the polarising microscope, show the complete absence of phrenosin.

A comparison of the substances thus obtained with phrenosin and kerasin prepared previously by Rosenheim and Tebb [1908] according to Thudichum's original method, showed a close agreement in elementary composition, melting point, optical activity, appearance under the polarising microscope and in their hydrolytic cleavage products. As far as our present knowledge admits, the identity of phrenosin and kerasin prepared by Thudichum's method and by the new pyridine method may be considered as proved.

Experimental.

The crude galactoside mixture, the preparation of which has already been described in detail in Part I of this series of communications [1913], was used for the isolation of the substances to be described. After two recrystallisations from an alcohol-chloroform mixture, it still contained a small percentage (0·08 %) of phosphorus. As the selenite-plate test was found to be most useful for controlling the progress of the separation of this mixture it may be described next.

The selenite-plate test. Both phrenosin and kerasin separate from a warm 10 % solution in pyridine on gradual cooling in the form of spherocrystals, possessing approximately the same refractive index as the solvent. They are therefore scarcely visible under the microscope in ordinary light. In polarised light, with crossed nicols, they stand out bright on the black background and show well-defined crosses. If a selenite-plate (Red I) is placed below the stage and immediately above the polariser in such a way that its axis lies diagonally to the planes of polarisation of the crossed nicols, a characteristic difference between phrenosin and kerasin will be noticed at once. On the red background the spherocrystals appear to be divided into quadrants, of which two opposite ones show the addition colour, blue, whilst the two others show the subtraction colour, yellow. But, while the spherocrystals of phrenosin, under the above conditions, show the blue colour in

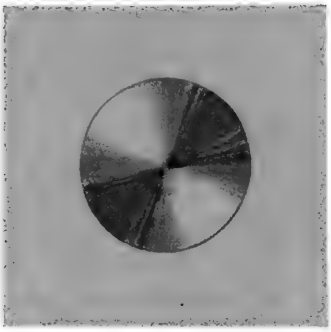

Fig. 1
Spherocrystal of phrenosin

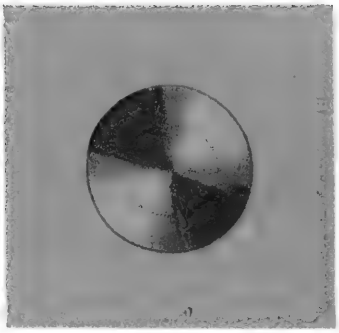

Fig. 2
Spherocrystal of kerasin

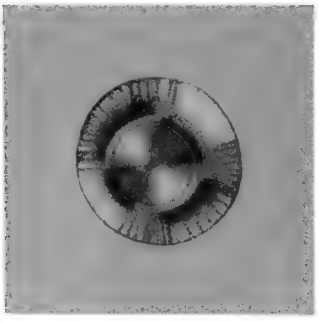

Fig. 3
Spherocrystal of a phrenosin-kerasin mixture

Reproduced from colour photographs (Lumière's process), taken in polarised light with a Selenite plate (Red I). The arrow indicates the direction of the axis of the Selenite plate.

the upper right and lower left quadrants (Fig. 1), the reverse is the case when the spherocrystals of kerasin are observed under exactly the same conditions (Fig. 2). Or, in other words, to use the nomenclature proposed by Göthlin [1913], the paragonal quadrants of the phrenosin spherocrystals show the addition colour, and the epigonal quadrants the subtraction colour, whilst the kerasin spherocrystals show the reverse behaviour. If we accept the current views according to which a spherocrystal is composed of radially arranged prisms, we may say that a spherocrystal of phrenosin is positively anisotropic, i.e. it is composed of optically positive prisms which are radially arranged. Kerasin on the other hand yields negatively anisotropic spherocrystals under the same conditions.

By means of this simple physical test, it is possible to establish in a very small sample the complete removal of kerasin from a phrenosin preparation, and *vice versa.*

It was found that from a galactoside mixture (i.e. either from any impure phrenosin or kerasin fraction, or from an artificial mixture), spherocrystals of both kinds are formed. They usually appear at first as separate crystals, varying in size from 0·05 to 0·5 mm. If the preparation is left for some hours, however, a kerasin crystal may deposit round a spherocrystal of phrenosin, or *vice versa.* A typical crystal of this kind, obtained from a phrenosin fraction, is reproduced from a colour photograph in Fig. 3. The limit of the sensitiveness of this test has not yet been established quantitatively. That it is, however, sufficiently sensitive for practical purposes, follows from the fact that a laevorotatory kerasin fraction, which was found to be free from phrenosin by the selenite-plate test, was also found on hydrolysis to be free from phrenosinic acid, the typical fatty acid of phrenosin.

In order to economise the valuable material, the test was carried out by dissolving a very small quantity (8–10 mg.) of the substance in a small test tube (0·5 × 4 cm.) by adding two drops of pyridine and warming to about 37°. A drop of the solution was transferred by means of a warm capillary pipette to a warmed slide, covered and allowed to cool gradually. A layer of spherocrystals forms at first round the edges of the coverslip, thus preventing further evaporation of the solvent, a fact which makes it possible to preserve the preparation for reference.

Fractionation of the galactoside mixture from acetone.

Previous work of Rosenheim and Tebb [1907] in connection with brain lipoids had demonstrated the advantage of acetone as a solvent for galactosides[1]. I have since found by a series of systematic experiments (omitted to save space), that the solvent power of acetone for galactosides is greatly increased if it contains 10 % of water.

The finely powdered galactoside mixture was therefore treated in portions of 50 g. with 3500 cc. of 90 % acetone in a water-bath kept at 56°. Only one extract was made and the insoluble part (about 15 %) has not yet been further examined. The clear acetone solution was allowed to cool in an incubator at 37°. After 16–20 hours a crystalline deposit, partially adhering to the sides of the glass vessel (phrenosin fraction), had formed, from which the supernatant fluid was easily decanted through a filter warmed to 37°. The filtrate was allowed to stand for 24 hours or longer in an ice chest, and the bulky gelatinous precipitate considered as the kerasin fraction.

The precipitates were filtered under pressure, washed with acetone and dried *in vacuo*. The kerasin fraction amounted to about 50 % of the phrenosin fraction, but the actual amount of kerasin contained in it is naturally much smaller, as only a rough separation is effected by this treatment.

Phrenosin.

32·5 g. of the phrenosin fraction were recrystallised from ten volumes of glacial acetic acid-chloroform (3 : 2). The finely powdered substance was first dissolved in 120 cc. of chloroform at about 60°, and to the solution was added 180 cc. of acetic acid previously warmed to the same temperature.

The clear solution was kept in an incubator at 37° over night, the deposit filtered and washed at 37° with the acetic acid-chloroform mixture. The moist precipitate was again dissolved in 200 cc. of the solvent and the solution treated in the same way. (The mother liquors of these two recrystallisations deposited on cooling to room temperature a considerable amount (6·64 g.) of a gelatinous precipitate, which was worked up with the kerasin fraction.)

The product deposited at 37° was granular and easy to filter. When recrystallised a third time in the same way, the mother liquor no longer

[1] This solvent has since been used for the same purpose by L. Smith and Mair [1910], and by Thierfelder and Loening [1912].

deposited on cooling to room temperature. After drying *in vacuo* the product weighed 20·1 g.

The selenite-plate test at this stage reveals only a small quantity of kerasin. A phosphorus estimation (Neumann's method) was intended, but 0·5532 g. of the substance carried through the process gave no trace of precipitate with the molybdic acid reagent, and the substance was therefore to be considered as phosphorus-free.

As the acetic acid-chloroform mixture did not seem to effect any further separation of kerasin, the product was now recrystallised from 100 volumes of acetone-chloroform (3 : 2). 19 g. of the product were first dissolved in 760 cc. chloroform and 1140 cc. warm acetone were added. The granular deposit, filtered at 37°, was twice more recrystallised in the same way. It then appeared to be perfectly uniform, as tested by the selenite plate.

In order to test its homogeneity, however, the product was separated into two fractions by using acetone-pyridine (equal parts) as a solvent. 13 g. were dissolved at 45° in 130 cc. pyridine and 130 cc. warm acetone were added. After keeping the solution for two hours at 35°, the deposit formed at this temperature was filtered off, and the solution cooled to room temperature. A further precipitate formed. The two fractions will be designated as phrenosin I a (6·5 g.) and phrenosin I b (5·2 g.).

From 30 g. phrenosin fraction of a different preparation there were obtained: 5·3 g. phrenosin II a and 4·7 g. phrenosin II b.

All the preparations, before being subjected to detailed investigation, were finally recrystallised from a large volume of acetone containing 10 per cent. of water.

All the preparations gave identical results on elementary analysis. They showed the same behaviour on melting and possessed the same optical activity. Further they gave on hydrolysis qualitatively and quantitatively the same products. These facts furnish strong evidence that a uniform substance had been isolated.

The product was further compared with phrenosin prepared according to Thudichum's original method. Thudichum fractionated his "white matter" from absolute alcohol at 37° and at room temperature. The fraction obtained at 37° serves for the preparation of phrenosin. Before or after the fractionation process, the product is treated with an ammoniacal alcoholic lead acetate solution in order to remove substances of the "cerebrin acid" type, which form insoluble lead salts. Thudichum's directions were closely followed, but as he had experienced considerable difficulty in obtaining his substance completely free from phosphorus, it was found advisable, in the light of later

experience, to use other solvents (glacial acetic acid, chloroform) as well as alcohol after the primary fractionation process. The lead treatment was carried out at the end of the fractionation process. Only traces of the "cerebrin acids" were found to be present. 90 g. "white matter," free from cholesterol, yielded finally 10 g. phrenosin. The product was free from phosphorus and sulphur and agreed in every detail with phrenosin prepared by the new pyridine method.

Kerasin.

From the kerasin fraction (see above), kerasin was separated in the following way: portions of 10 g. were dissolved in 40 cc. chloroform at 50° and to the solution were added 60 cc. glacial acetic acid previously warmed to about 60°. The solution remained clear until the temperature had fallen to 40° and was then kept in an incubator at 37°. A granular white layer, mainly phrenosin, collected on the surface and was filtered off at 37°. The filtrate began to deposit on cooling to 26° and solidified finally to a gelatinous mass. After filtering without pressure, and washing with the chloroform acetic acid mixture, the product was suspended in acetone and could be filtered under pressure. The product, when dry, weighed only 5 g. and was again recrystallised from 50 cc. of the chloroform acetic acid mixture as above described. Only 0·5 g. came down at 37°, the main quantity depositing at room temperature. The process was repeated twice more, when it was found that the solution no longer deposited at 37°, even when kept at that temperature for many hours. At this stage the selenite-plate test showed that the substance consisted practically only of kerasin, only a few isolated spherocrystals of phrenosin appearing after some hours.

Further purification was obtained by continuing the recrystallisation from 20 volumes of an acetone-pyridine mixture (equal parts). Three grams were dissolved in 30 cc. pyridine and 30 cc. acetone warmed to 45° were added. Only a faint cloud appeared on cooling to 37° in the incubator. This small deposit of phrenosin weighed 0·01 g. when filtered and dried *in vacuo*. The main filtrate began to deposit at 28° and was allowed to cool to room temperature before filtration. This process was repeated and the product finally recrystallised from a large volume of 90 % acetone containing 2 % pyridine.

The kerasin preparations were considered pure when only kerasin spherocrystals were found by the selenite-plate test and when their optical activity was not less than $[\alpha]_D = -2°$.

The separation by these purely physical means necessarily entails large losses, but leads finally to a pure product, a fact which was confirmed by the investigation of the hydrolytic cleavage products. From 10 g. of the original kerasin fraction, there were obtained on the average 1·36 g. kerasin (from 10–15 %).

The physical appearance of kerasin when depositing from its solutions is characteristically different from that of phrenosin. Whilst the latter usually deposits in loose granular masses, kerasin forms a coherent jelly which contracts on shaking. When filtered and dried *in vacuo*, it can easily be powdered, but when allowed to dry in air it forms a translucent white wax-like substance, thus fully justifying the name given to it by Thudichum.

Kerasin was also prepared according to Thudichum's method from the kerasin fraction of "white matter" (see above). It was subjected to the lead and cadmium treatment as described by Thudichum. It was found that only minute traces of substances precipitable by these reagents were present, no doubt owing to the fact that mixtures of solvents (see above) were used for recrystallisation instead of alcohol alone. The observation of Thudichum with regard to the change of solubility in alcohol as purification proceeds was fully confirmed. A product which contains only traces of phrenosin begins to deposit kerasin even at 37°, while originally, in presence of a large proportion of phrenosin, it only deposits below 28°.

From 90 g. cholesterol-free "white matter," there were obtained finally 5 g. kerasin.

The product was free from phosphorus and sulphur and agreed in its composition and all its properties with kerasin prepared by the pyridine method.

The results of the elementary analysis, the optical activity and hydrolytic cleavage products of phrenosin and kerasin will be described in a subsequent publication.

The expenses of this research have been defrayed from a grant from the Government Grant Committee of the Royal Society.

Summary.

1. The galactoside mixture obtained by extraction of brain with cold pyridine can be separated into a phrenosin and a kerasin fraction by temperature fractionation from acetone.

2. Phrenosin is obtained by recrystallisation of the phrenosin fraction

at 37° from chloroform-glacial acetic acid mixture, followed by recrystallisation from a chloroform-acetone mixture.

3. Kerasin is obtained from the kerasin fraction by recrystallisation at room temperature from chloroform-glacial acetic acid mixture, followed by a pyridine-acetone mixture.

4. A test (the selenite-plate test) is described, by means of which the progress of purification is controlled.

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XVI. THE GALACTOSIDES OF THE BRAIN. III. LIQUID CRYSTALS AND THE MELTING POINT OF PHRENOSIN.

By OTTO ROSENHEIM.

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(*Received February 24th, 1914.*)

The remarkable ease with which the brain galactosides are transformed into the liquid-crystalline state, was first observed by Rosenheim and Tebb [1908] in connection with some other work on the brain lipoids and has since been confirmed by Prof. O. Lehmann, who kindly examined phrenosin and kerasin (as well as sphingomyelin) prepared by the author. This phenomenon offers an explanation for the discrepant statements with regard to the melting points of these substances recorded in the literature. It further led to the discovery of the conditions under which phrenosin can be obtained in true crystalline form, which in its turn satisfactorily explains a hitherto unexplained property of phrenosin, for which the provisional term "Umlagerung" was used by Thierfelder and Wörner [1900]. A short description of my observations in this direction seems therefore justified.

(1) Liquid crystals, true crystals and myelin forms of phrenosin.

Phrenosin as usually obtained on cooling its solutions, consists of a white "crystalline" powder, which, however, does not possess any definite crystalline form in the usual sense. Under the microscope it is seen to consist of separate ball-shaped masses of approximately uniform size, which are only slightly anisotropic.

The formation of liquid crystals is best observed when a small quantity of the dry powder is carefully heated on a slide on Lehmann's polarisation microscope[1] [1910] until it is completely fused. In this condition it will be found to be completely isotropic, i.e. invisible in the dark field between crossed nicols. On allowing the slide to cool slightly a shower of separate anisotropic

[1] A very convenient electrical hot stage has been devised by Lorrain Smith and W. Mair [1910].

needle-shaped liquid crystals shoots out on the dark background (see Fig. 1). They may be best observed in the thinnest layers of the preparation and when pressure is exerted on the cover glass.

I made the further observation that phrenosin not only exists in the intermediary liquid-crystalline state when heated below its "clearing point," but also when it is allowed to settle out from its hot solutions in various solvents. This fact may also easily be demonstrated by means of Lehmann's microscope possessing a heating arrangement, using an alcohol solution of phrenosin. The liquid-crystalline globules which appear at first solidify on complete cooling to nodular masses, radially striated, thus forming the "crystalline" white powder as described above. The liquid-crystalline modification may even be observed macroscopically, if the dry substance is warmed with insufficient solvent. It then forms transparent globules which collect at the bottom of the flask and which were thought to be due to "decomposition" by the early investigators of brain lipoids.

It occurred to me that it might be possible to prevent the primary formation of the liquid crystals by some means and thus favour the formation of true crystals. After many unsuccessful attempts with various solvents under many conditions, it was found that true crystals were formed when a hot 2 % solution of phrenosin in 85 % alcohol was allowed to cool very gradually and without shaking, in an unsilvered Dewar flask. The flask was firmly plugged with cotton wool and kept in a water-bath at 75°. The temperature of the water-bath was allowed to fall and when the solution had reached a temperature of 65°, the first crystals appeared. Their quantity increased slowly and at 61° the whole solution was filled with a mass of glittering crystals, which were seen under the microscope to consist of thin, transparent, well defined plates, closely resembling cholesterol crystals (see Fig. 2). Highly purified phrenosin may thus be obtained completely crystallised. After filtration and drying it appears as silky flakes, its macroscopical appearance also resembling that of cholesterol. The substance may be recrystallised in the same way, but on allowing its solution to cool gradually in the ordinary way, the usual micro-crystalline powder (solidified liquid crystals) is obtained.

The air-dry crystals, when obtained from dilute alcohol, contain one molecule of water[1]. The water of crystallisation is not given up on drying *in vacuo* over concentrated H_2SO_4, but escapes on drying the substance at 105°.

[1] It is possible that the crystals obtained from absolute alcoholic or methyl alcoholic solution contain alcohol of crystallisation, but this question has not yet been experimentally investigated.

Fig. 1. Liquid Crystals of Phrenosin. ×90.

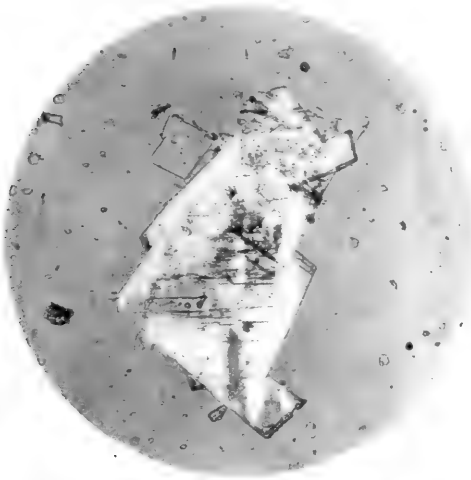

Fig. 2. Crystallised Phrenosin. ×50.

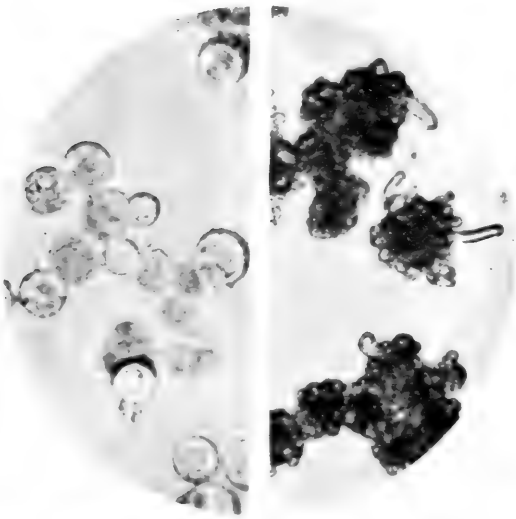

Fig. 3. *Left:* Phrenosin swelling up with water. *Right:* Early stage of Myelin forms. ×250.

0·4253 g. crystallised phrenosin, on drying in a toluene bath, lost 0·0100 g. = 2·35 % H_2O. Calc. for $C_{48}H_{93}O_9N + H_2O$: 2·13 % H_2O.

On combustion of the substance, dried at 105°, the following results were obtained:

0·1300 g.; 0·3305 g. CO_2; 0·1291 g. H_2O. 0·0960 g.; 1·7 cc. N at 17·5° and 722·5 mm.

		Calc. for $C_{48}H_{93}O_9N$
C	69·34	69·65
H	11·11	11·24
N	1·91	1·70

The fact that phrenosin may, under certain conditions, be obtained in the true crystalline condition offers an explanation for an interesting observation made by Thierfelder and Wörner [1900] many years ago. They found that when "cerebron" (= phrenosin) was kept at 50° suspended in 85 % alcohol or at 60° in methyl alcohol-chloroform [Thierfelder and Loening 1910], the previously "crystalline" product was gradually transformed into true crystals. This process was assumed to be due to an intra-molecular change (Umlagerung) but Thierfelder was at a loss to explain this "Umlagerung," which he showed was not due to the entrance of alkyl-groups into the molecule. I have repeated this experiment successfully with all my samples of pure phrenosin in the solidified liquid-crystalline condition and would suggest, in the light of the above observations, as an explanation of Thierfelder's "Umlagerung," that it consists simply in the gradual change of the solidified liquid-crystalline modification into the true crystalline condition at the temperature which is favourable for its formation[1].

Another characteristic property of phrenosin, which is related to the liquid-crystalline condition, consists in its tendency to give rise to myelin forms in the presence of water. If a fine suspension of phrenosin in water is gently warmed on a slide under the microscope, the small ball-shaped nodules swell up and are seen to be suddenly covered with spicules, which gradually develop into myelin forms (see Fig. 3). I may add the further observation that under the polarising microscope and a selenite plate, these peculiar forms behave exactly like the myelin forms obtainable from ammonium oleate or lecithin. In the paragonal position they show the subtraction colour (yellow) and in the epigonal position the addition colour (purple to blue).

[1] Phrenosin can only be obtained in the true crystalline state if certain conditions are observed, a fact which brings out the analogy of the brain galactosides to the vegetable glucosides, especially to those containing galactose like the digitalis glucosides. The latter have also so far only been separated by physical means, and digitonin, for instance, can only be obtained crystallised under certain conditions. Even then it may still contain the amorphous galactoside, gitonin, as has recently been shown by Windaus and Schneckenburger [1913].

The myelin forms appear on slight warming far below the boiling point of water, and indications of them may be seen even at 37°[1]. If a slide is kept at this temperature and then allowed to cool it will be seen that water has been taken up by the phrenosin powder, transforming it into transparent globules. Under the polarising microscope they are now strongly anisotropic, show the black cross of spherocrystals and behave with a selenite plate exactly like the spherocrystals of phrenosin as obtained from pyridine [see Rosenheim, 1914].

(2) The melting point (clearing point) of phrenosin.

The melting points recorded in the literature for phrenosin (pseudo-cerebrin, cerebrin, cerebron) show remarkable variations. Thudichum gave it at 176° and the subsequent statements vary from 170° (Parkus) to 212° (Thierfelder and Wörner). The last investigators state that the substance becomes "moist" at 130° and gradually covered with fine droplets, at 200° it becomes slightly yellowish, and fuses at 209° on slow heating, at 212° on rapid heating, to a clear yellowish fluid.

In my first observations on phrenosin (prepared according to Thudichum as well as by the pyridine method), I noticed a similar behaviour. On slow heating in a Thiele apparatus the samples soften between 130°–140°, shrink together and partially fuse between 170°–190°, and melt to a clear fluid at 205°–206°. On rapid heating the same substances melt, after the preliminary softening at 130°–140°, fairly sharply at 212°–215°. According to the rate of heating and the width of the tube, it is possible to obtain what appears as a melting point at any temperature between 170°–215°. Crystallised phrenosin behaves exactly like ordinary "crystalline" phrenosin.

An explanation for this peculiar behaviour was found in the above described discovery that this substance exists in the liquid-crystalline condition between the temperatures of 95° and about 210°. This fact may also be very conveniently demonstrated, without a polarising microscope, by the help of the arrangement recommended by Stoltzenberg [1911]. A thin glass rod is introduced into the melting point tube and the liquid-crystalline condition is recognised by pressure exerted on the glass rod. Under these conditions it was found that phrenosin enters into the liquid-crystalline

[1] G. F. Göthlin [1913] was unable to observe this fact, because he evidently made his observations in the absence of any heating arrangement. The myelin forms of pure phrenosin were first observed on my preparations in Prof. Aschoff's laboratory [see R. Kawamura 1911]. L. Smith and W. Mair [1910] have also made similar observations on the "cerebrosides" prepared by their method.

condition below 100° (approximately at 95°) and forms on pressure a perfectly clear viscous fluid at 130°, showing a final clearing point at 212°–215°. The liquid-crystalline phase evidently possesses a greater viscosity than the liquid-isotropic phase and the change from one to the other may be readily observed.

The discrepant statements about the melting point of phrenosin, which were quoted as evidence against the identity of the various preparations, are satisfactorily explained by the existence of the liquid-crystalline modification. Some observers evidently considered the substance as fused during the liquid-crystalline phase, whilst others took the temperature of formation of the isotropic-liquid phase as the melting point.

In view of the above observations there is no justification to speak of a "melting point" of phrenosin, the proper expression being the "clearing point," i.e. the temperature at which it changes from the liquid-crystalline anisotropic condition into the isotropic liquid-amorphous condition. [See also Lapworth, 1913.]

It may be mentioned here that kerasin behaves in every respect similarly to phrenosin. It gives rise to liquid crystals and to myelin forms, although the latter are not so readily produced as with phrenosin. Its clearing point is, however, much lower, namely 180°. Further I have so far been unable to obtain it in the truly crystallised condition like phrenosin. This fact may be connected with the constitution of the fatty acid (lignoceric acid) contained in kerasin, which, in distinction from phrenosinic acid, does not contain the hydroxyl group in its molecule. It may further be assumed that, in analogy to the derivatives of cholesterol, the ability of the galactosides to exist in the liquid-crystalline condition is mainly dependent on their basic constituent sphingosine, which resembles cholesterol in so far as it has the constitution of an unsaturated alcohol[1].

Prof. O. Lehmann, to whom I submitted samples of phrenosin and kerasin for more detailed investigation of their liquid-crystalline modification, has kindly given me permission to communicate the following abstract of his observations which he intends to publish in full elsewhere.

"The first of the two preparations of phrenosin received (labelled "crystallised") consists of thin transparent plates, approximately rectangular, and

[1] The property to form liquid crystals is not limited to the brain lipoids. I have found that "carnaubon" which was first obtained by Dunham and Jacobson [1910] from ox kidneys also possesses a liquid-crystalline phase. According to some preliminary experiments of the author the main fatty acid of carnaubon seems to be identical with lignoceric acid and carnaubon itself appears to be a mixture of a galactoside of the kerasin type with a phosphatide of the sphingomyelin type. The fact that these lipoids form mixed liquid crystals explains the difficulty of their separation.

slightly anisotropic; the second of small globules of fairly uniform size which show between crossed nicols the black cross of spherocrystals and which had probably been formed by solidification of globular myelin forms. The behaviour of both preparations is identical. On warming the dry substance it is suddenly transformed into the anisotropic liquid-crystalline modification and on further heating into the isotropic melt. The liquid crystals depositing from the latter do not show any tendency to run together, but they are deformed by pressure on the coverslip just like other liquid crystals. If the solid crystals are brought into water and gradually warmed, the change into the liquid-crystalline modification takes place below the boiling point of water. The liquid crystals assume the shape and structure of myelin forms. The ball-shaped solid structures are suddenly covered with radiary processes which, however, are not pointed but of uniform thickness and with rounded ends. On cooling, all the spicules suddenly contract to about one half of their size and their contents are transformed into an aggregate of fine plates of the solid modification. By repeated warming and cooling the extension and contraction of the rod-like myelin forms (liquid crystals) may be produced over and over again. We have to deal here with changes of form of a similar kind to those observed previously by Lehmann with protocatechuic acid [see Lehmann, 1911, p. 333]. Phrenosin forms with lecithin and ammonium oleate mixed liquid crystals in all proportions. Even with a high percentage of phrenosin the liquid crystals which separate out of dilute alcohol show the phenomenon of flowing together just as those of pure ammonium oleate (or lecithin).

By means of some aniline dyes the myelin forms of phrenosin may be artificially coloured, similarly to those of ammonium oleate. The phenomena are most striking, if the water in which the phrenosin granules are allowed to swell up contains just sufficient methylene blue to colour it faintly. The myelin forms, appearing on warming, will be seen to be stained intensely blue. On contraction by cooling, the blue colour changes into a reddish violet. If the processes are extended again to their former length by warming, the reddish violet colour just as suddenly changes into blue.

Kerasin behaves similarly to phrenosin, when heated in the dry state. At a certain temperature it suddenly passes into the liquid-crystalline modification, which changes on further heating into the isotropic melt. Warmed with water it swells up enormously, forming myelin forms which are only slightly more refractive than water. In consequence of their high water content they are of a slimy fluid consistency. With methylene blue they only stain very slightly, their size being reduced by the admixture of

the dye. On heating a few grains of the original substance with water to a higher temperature, it is transformed into the liquid-crystalline modification and then myelin forms appear which are distinctly anisotropic and easily stained. On cooling, the two kinds of myelin forms solidify, suddenly undergoing a contraction just like those of phrenosin. The solidified myelin forms are not brittle, but still soft and plastic. Kerasin forms with lecithin and ammonium oleate mixed liquid crystals in all proportions just like phrenosin."

The expenses of this research have been defrayed from a grant from the Government Grant Committee of the Royal Society.

Summary.

1. The galactosides phrenosin and kerasin exist in the liquid-crystalline condition at temperatures from below 100° up to 180° (kerasin) and 215° (phrenosin).

2. This fact explains the divergent statements with regard to the melting point of these substances. Instead of a melting point they possess a "clearing" point, i.e. a point at which the anisotropic liquid-crystalline phase changes into the isotropic liquid-amorphous phase.

3. They give rise to myelin forms on warming with water which possess the same optical properties as the myelin forms of lecithin, etc.

4. The conditions are described under which true crystals of phrenosin may be obtained.

5. An explanation is thereby afforded for Thierfelder's "Umlagerung" of phrenosin.

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XVII. REMARKS ON Dr SYMONS' "NOTE ON A MODIFICATION OF TEICHMANN'S TEST FOR BLOOD."

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and GOPAL CHANDRA MITRA.

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(*Received February 25th, 1914.*)

On p. 596 of Vol. VII there appear some strictures on the spectrum test for blood, which we consider to be not so well-grounded as the writer may think they are.

We may at the outset mention that we base our conclusions on the results obtained by us from the examination of articles in 749 cases, mostly of murder, that have come to our laboratory. In all 1819 articles, which were, or were supposed to be, bloodstained, have been examined by us in these cases.

The technique adopted by us is that devised by Hankin, whose long experience of medico-legal work led him to believe that it is the simplest and at the same time the most reliable method of obtaining the characteristic spectrum of *cyanhæmochromogen*, even when one has but a spot of blood no larger than a pin-point before one, a belief that our results have fully confirmed.

Briefly stated, the spectrum is obtained as follows:

If the stain be on a hard surface such as glass, steel, wood or cement, a fragment of it is removed by means of a sharp knife, conveyed to a microscope slide, and treated with a drop of 10 % solution of cyanide of potassium. Any superfluous fluid is removed by means of bibulous paper, and the preparation is then treated with a drop or two of solution of sulphide of ammonium, covered and examined *at once* under the $\frac{1}{2}''$ objective.

If it be a soft fabric that is stained, a minute portion of the stain is removed, plunged into boiling water for a couple of seconds, and then treated as above.

If blood be present, somewhere in the preparation there will be seen a spot of colour, the tint of which varies from the faintest pink to cherry-red, according to the amount of pigment present. If this spot be small it is brought into focus under the $\frac{1}{6}''$ objective; if it be of fair size the low power will suffice. The eye-piece of the microscope is removed and the long arm of a Browning's hand spectroscope, modified by Zeiss, is inserted into the tube. Under the short arm of the spectroscope is held a piece of paper, to reflect sufficient light for the reading of the wave-length scale, and the absorption-bands of *cyanhæmochromogen* are then identified. In rare cases only one band may be seen, but in all cases where blood is present this method of proving the existence of its pigment in the material examined will be found to be successful: for even stains that have been more than half washed-out give the characteristic spectrum when so treated.

In the wet season it often happens that all that can be seen on a blood-stained fabric is a spot or two of mould, but so far we have had no difficulty either in identifying blood in such a case, or in determining its origin by means of the precipitin reaction.

An *old* stain may require to lie soaking in the cyanide solution for about a minute, before the ammonium sulphide is added to it. The advantage of using a 10 % solution of potassium cyanide instead of a weaker solution is that the weaker solution may cause difficulty by more rapidly giving rise to diffusion of the pigment throughout the preparation: naturally the more concentrated the pigment at one spot the easier the recognition of its spectrum. Hence Hankin's use of boiling water to fix the pigment and prevent its diffusion along the fibrils of the piece of stained fabric, when the potassium cyanide solution is applied to it.

The ammonium sulphide solution should be prepared thus: strong solution of ammonia is diluted with four times its volume of water, and then saturated with hydrogen sulphide gas. Of the resulting preparation two parts are added to one part of ordinary dilute ammonia solution.

For routine practice we find it most convenient to use a small test-tube filled with this solution for the day's work, as the solution kept ready rapidly decomposes.

By so treating our stains we have no need of using Teichmann's test: for no substance save blood when treated as detailed above gives the colour reaction *plus* the spectrum of *cyanhæmochromogen*.

As to the "corpuscle test"—which Dr Symons states "has been found of very doubtful value, and very often impracticable in the case of old stains"—we would remark that we have nearly always been able to find enough

erythrocytes in a material—even earth—in which the presence of blood has been proved by the spectrum, to be able to say whether that blood was mammalian or non-mammalian. *A fortiori* we have been able to say that the cells before us were erythrocytes. By "nearly always" we mean in 97 % of cases. Only four out of 125 bloodstained specimens of earth gave rise to difficulty in determining the nature of the erythrocytes present.

For the determination of erythrocytes the fragment of stained material is allowed to soak in Vibert's fluid for half an hour, then teased out and examined under the oil-immersion lens. It will be remembered that Vibert's fluid is composed of common salt 2 grams, mercuric chloride half a gram, and distilled water 100 cubic centimetres. Of all the fluids devised by microscopists for the treatment of bloodstains we find it to be on the whole the most satisfactory, being thus able to confirm Masson's high praise of its merits.

XVIII. AN IMPROVED HYDROGEN ELECTRODE.

By GEORGE STANLEY WALPOLE.

From the Wellcome Physiological Research Laboratories, Herne Hill, S.E.

(*Received March 3rd, 1914.*)

Further use of hydrogen electrode vessels of the type described [Walpole, 1913] has resulted in some modifications justified by the more accurate results obtained.

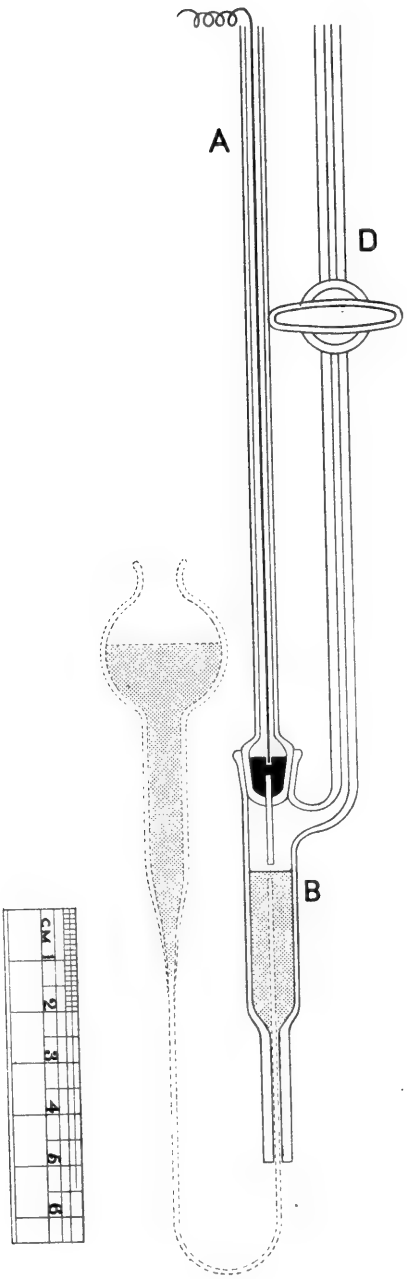
In the actual construction of the vessel the rubber stopper sometimes employed [1913, p. 411] was finally discarded, and a ground glass joint used instead where the glass tube *A*, carrying the electrode, fits into the outer tube *B*.

Both the electrode vessel, and the pipette necessary for refilling it when the fluid under investigation contains carbon dioxide, are made of hardest Jena glass, e.g. *Borosilicate glass, Jena* 59. 111. The slow rise of E.M.F. due to liberation of alkali from the glass is thereby avoided. The general dimensions are those given [1913, p. 415]. The description of a smaller electrode for electrometric titration [1913, p. 418] contains an error. Its capacity was 0·3 cc. not 3 cc. as stated.

In describing a determination it is assumed that the vessel is wet with the last fluid examined. Starting with a clean dry vessel the volume of fluid required is, naturally, smaller. The hydrogen supply is connected to *D*, and the air displaced. The tap on the T-piece [1913, p. 419] is shut, and, by means of the filling syringe, some of the liquid examined is drawn up into *B* and used to rinse it out. To do this *D* is shut and the electrode vessel tilted about the horizontal position.

For very careful work it is well to undo the ground glass joint and wet the surfaces with the experimental fluid before starting.

After emptying out the rinsing fluid by opening *D* and pushing home the piston of the filling apparatus, hydrogen is passed again for a second or two and the lower end of *B* dipped below the experimental fluid. The actual sample employed for the determination is then drawn up until the meniscus is just touching the blacked platinum point and the tap *D* is


A
D
B
CM 1
2
3
4
5
6

closed. The rubber tube from the filling apparatus may now be detached. If the fluid contains no carbon dioxide the electrode vessel is placed at once in the trough containing saturated potassium chloride at 18°.

If carbon dioxide is present the hydrogen bubble must be passed backwards and forwards (five minutes is sufficient) till equilibrium is established, and the liquid then replaced by a fresh sample of experimental fluid from the pipette. To do this, the pipette, filled with the solution, is brought into the position shown in the diagram and in a short while the contents of *B* will be replaced. The hydrogen bubble is again passed to and fro for five minutes and the fluid replaced again. Three replacements are sufficient.

The size of the bubble will be found now to have increased. Connection to the filling apparatus and gentle suction with the lower end of the electrode vessel immersed in experimental fluid enables the adjustment of the meniscus to the platinum point to be made accurately. The electrode vessel is now placed in the potassium chloride trough without delay.

The pipette has a capacity of about 4 cc. and is half filled for each replacement. There is no need for a stopper of any kind at the bottom of the electrode vessel as capillary forces are quite sufficient to prevent egress of fluid or intake of air during manipulation. In those cases where blood-protein and similar solutions are examined the lowered surface tension produces difficulties which may be overcome by dipping the lower end of the filled electrode vessel in distilled water for a moment.

A P_H^+ determination of a carbon dioxide-sodium carbonate mixture containing 0·02 N NaCl, made by mixing 20 cc. 0·1 N HCl and 25 cc. 0·1 N Na_2CO_3 and diluting to 100 cc., with water gave the following results:

Time	E.M.F. reading against sat. KCl calomel electrode	
12.4	(Vessel introduced into KCl)	
12.5	0·6100	
12.10	0·6100	
12.30	0·6100	
2.10	0·6100	$P_H^+ = 6·22$

The experiment was commenced with the apparatus wet from a previous determination on another fluid; volume of fluid taken for examination 10 cc.

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XIX. THE ESTIMATION OF LACTOSE AND GLUCOSE BY THE COPPER-IODIDE METHOD.

By SYDNEY WILLIAM COLE.

From the Physiological Laboratory, Cambridge.

(*Received March 3rd, 1914.*)

Experience of the various methods that have been described for the estimation of the sugars by volumetric analysis has convinced me that the most exact one we have at present is that of Amos W. Peters [1912, 2]. Unfortunately he only gives the copper values for glucose. Being anxious to utilise the method for a research involving the estimation of lactose, I determined the copper values for this sugar and publish them now in the hope that they may be of some value to those engaged in milk analysis. I also give a description of the method, with some slight modifications that I have made, as I found the original papers rather difficult to follow.

Principle of the method.

A large volume of copper sulphate is prepared and its copper content accurately determined as described below. A given volume of the copper sulphate is treated with an alkaline tartrate solution and a known volume of the sugar and the mixture is heated under definite standardised conditions for a certain time. The mixture is filtered through asbestos and the copper in the filtrate determined by treatment with potassium iodide and titration of the iodine liberated by means of a solution of sodium thiosulphate. The liberation of the iodine is effected under standard conditions of temperature, dilution and acidity, and the thiosulphate is previously standardised against the copper sulphate under the same conditions.

Solutions required.

A. Copper sulphate. 69·278 g. of the purest crystalline salt, $CuSO_4$, $5H_2O$, are dissolved in distilled water and the volume made up to 1 litre. It is advisable to prepare a large volume of this stock solution.

B. Alkaline tartrate. 346 g. of Rochelle salt and 250 g. of pure potassium hydroxide are dissolved in water and the volume made up to 1 litre.

C. Sodium thiosulphate, N/5. 99·2 g. of the purest thiosulphate are dissolved in boiled out water and the volume made up to 1 litre with boiled out distilled water. A considerable volume can be prepared and allowed to stand about a week before use. It then acquires a value which is almost constant for some months. It should be stored in the dark.

D. Potassium iodide. Saturated solution; 100 g. of the solid treated with 70 cc. of hot distilled water and allowed to cool.

Standardisation of the thiosulphate and determination of the copper value of the stock solution.

Of the three methods given by Peters [1912, 1] the simplest is that of calculating the copper from the weight of $CuSO_4, 5H_2O$ taken. I find that if Kahlbaum's purest copper sulphate "with certificate of analysis" be employed and allowance be made for the very small amount of impurity present as shown on the certificate, the results agree exactly with those obtained by the electrolytic method and by the "nitric acid-talcum" process of treating pure metallic copper. Consequently I shall only describe that method.

20 cc. of the copper sulphate solution are measured by means of a standard pipette into a 200 cc. Erlenmeyer flask, 40 cc. of distilled water and 20 cc. of strong acetic acid (33 %) are added, a thermometer is inserted and the mixture cooled or warmed to 20°. About 6·5 cc. of the saturated potassium iodide are run in, the thermometer being withdrawn and its stem washed with this solution. The iodine liberated is titrated at once with the thiosulphate. When approaching the end point 2 cc. of a 1 % solution of soluble starch are added and the titration completed. The colour changes to a chocolate-brown when very near the end point. This is best determined by the "spot test" method. Allow a drop of the thiosulphate to fall on the quiet surface of the liquid. If the end point has not been reached, a very perceptible white area is seen round the drop. This is very readily distinguished from the diminution of the slightly yellowish colour of the suspended cuprous iodide. The volume of the drop delivered by the burette must be deducted from the total volume added. After very little experience I could always obtain duplicates within 0·04 cc., the volume of the drop delivered by my burette.

The copper value of the thiosulphate is calculated as shown in the following example:

20 cc. of the copper sulphate required 27·60, 27·58 and 27·59 cc. (average, 27·59 cc.) of thiosulphate.

20 cc. of the copper sulphate = $69{\cdot}278 \times \frac{63{\cdot}57}{249{\cdot}57} \times 20 = 352{\cdot}93$ mg. Cu.

1 cc. of thiosulphate corresponds with 12·792 mg. Cu.

This result agrees well with that obtained by the electrolytic method (average, 12·789) and that of the "nitric-talcum" process (average, 12·786). It must be noted that N/5 thiosulphate theoretically corresponds to 12·71 mg. Cu per cc. The difference is undoubtedly outside the range of experimental error. The discrepancy cannot be explained on the assumption that the thiosulphate is impure, as in several cases I found that the normality of my thiosulphate agreed very closely with theory when titrated against standard iodine prepared from potassium iodide and iodate by the addition of standard acid.

The standard heating power.

The Erlenmeyer flask in which the mixture is heated is placed on asbestos gauze over a Bunsen or Meker burner. The height of the flame and of the gauze must be such that 60 cc. of distilled water in the flask are raised from 35° to 95° in 120 ± 2 secs. Owing to variations in the gas pressure I found it necessary repeatedly to check the heating power. I tried several methods of obtaining a constant pressure, and finally adopted the manometer shown in Fig. 1. Once the apparatus is assembled an estimation can be performed without the delay of having to determine the heating power.

On an adjustable ring stand about 4 cm. above the top of the burner place an unperforated sheet of asbestos gauze. Turn on the tap *B* to its full extent. Tighten the screw *A* till the pressure is reduced about one-third. Allow the gauze to get thoroughly heated before making a test. In a 200 cc. Erlenmeyer flask of Jena glass and of about 6 cm. basal diameter place 60 cc. of distilled water. The flask is fitted with a 2-hole rubber stopper carrying a thermometer so graduated that the stem above 34° is visible above the upper edge of the stopper. The lower end of the thermometer should be about 2 mm. from the bottom of the flask. By means of a stop watch note the time for the temperature to rise from 35° to 95°. If the time is less than 120 secs. tighten the screw *A* and repeat the experiment until the desired heating power is obtained. Note the reading of the manometer. This must be observed from time to time and the screw adjusted to maintain it at the correct level as the pressure of the gas supply varies. The height of the ring and the thickness of the asbestos should be such that the pressure

is well under the minimum supplied to the laboratory and yet sufficient to prevent any risk of the flame striking back. On making a fresh set of observations within a week it is only necessary fully to open the tap *B* and to adjust the screw *A* till the previously recorded pressure is obtained. With longer intervals it is desirable to make a fresh observation of the heating power owing to the possibility of the evaporation of some of the fluid in the manometer tube.

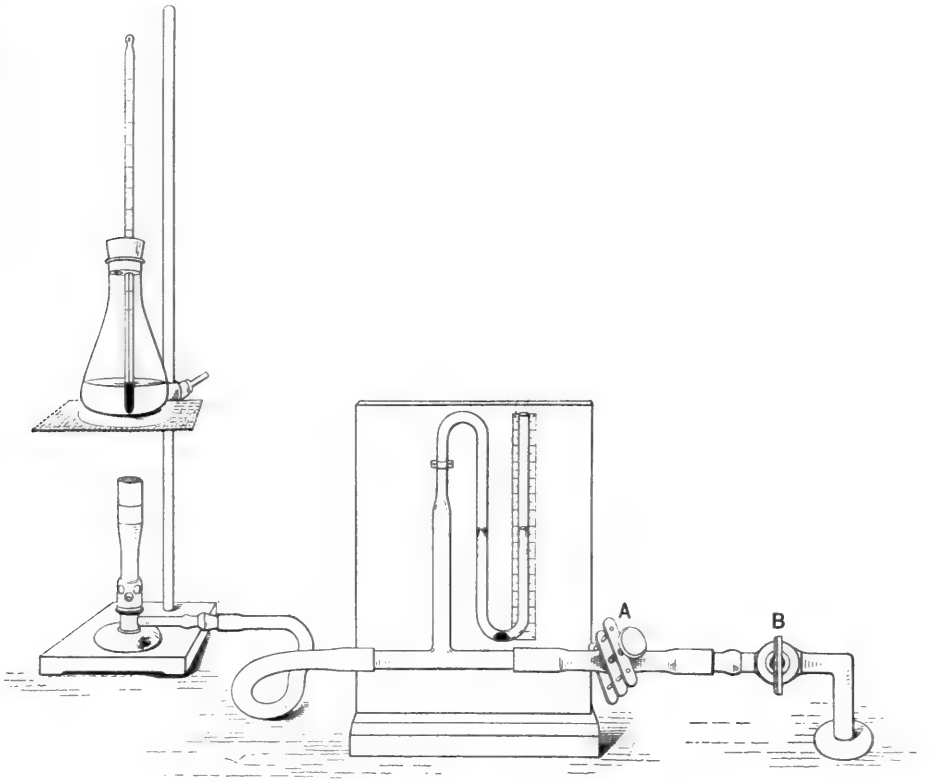

Fig. 1. Apparatus for maintaining a standard heating power. The manometer tube contains a dilute solution of eosin. It also contains a globule of mercury which nearly fills the bottom of the tube. This prevents the rapid oscillations of pressure due apparently to the explosions of local gas engines.

Filtering apparatus.

I have found it most convenient to use the apparatus shown in Fig. 2.

A is a Jena flask of 200 cc. capacity. If one is chosen with a perfectly flat bottom no trouble with fractures under pressure need be feared. The tube *B* is an ordinary calcium chloride tube. The lower end should project for

at least 3 cm. below the lower edge of the stopper to prevent loss by splashing during filtration. The filtering mat is made of glass wool, asbestos, powdered pumice and asbestos added in that order. After a test the cuprous oxide on the mat is dissolved in nitric acid diluted with an equal volume of water, and then thoroughly washed. I have used one tube for more than 100 estimations without having to add any more asbestos. The thickness of the mat should be such that the filtrate comes through under pressure in a steady stream.

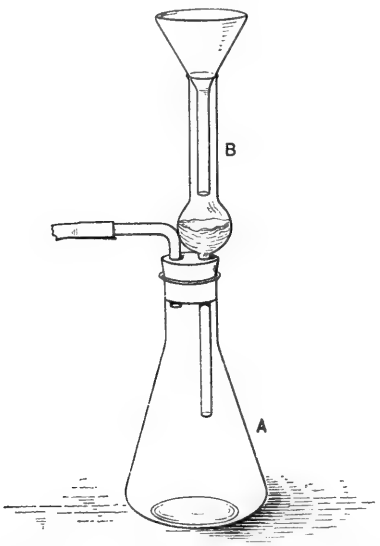

Fig. 2. Apparatus for the filtration of the reduced copper.

Method of analysis.

Into a 200 cc. Erlenmeyer flask measure 20 cc. of the standardised copper sulphate solution, 20 cc. of the alkaline tartrate and 20 cc. of the sugar solution (which must contain between 5 and 250 mg. of anhydrous lactose). Fit the two-holed rubber stopper firmly into the neck of the flask, adjust the thermometer so that its lower end is 2 mm. from the bottom of the flask and place on the heated gauze, note the time when the mercury indicates a temperature of 95°. Allow the heating to continue for exactly 20 secs. beyond this. Remove the flask by gripping the rubber stopper and swill it for a second or two under the tap or in a bowl of water. The lowering of the temperature practically stops the reduction. Filter the hot fluid at once using the stem of the thermometer as a stirring rod. Wash the flask twice with about 7 cc. of distilled water. Cool the filtrate by

holding the flask under the tap. Add exactly 4 cc. of strong sulphuric acid, insert a thermometer and cool to 20°. Add 6·5 to 7 cc. of the saturated solution of potassium iodide, washing the stem of the thermometer with this solution. Titrate at once with the standardised solution of sodium thiosulphate as described above, using soluble starch as an indicator when near the end point.

The reducing power of anhydrous lactose.

I give in table I the mean values I have obtained of the amounts of copper reduced by varying amounts of lactose. Duplicates agree to about 0·4 mg. Cu.

The stock solutions of lactose were prepared from the hydrate several times recrystallised. The values agreed exactly with those determined polarimetrically at the Cambridge University Chemical Laboratory by the kindness of Prof. Pope.

TABLE I.

Anhydrous lactose, mg.	Copper, mg.	Lactose / Copper	Anhydrous lactose, mg.	Copper, mg.	Lactose / Copper
3·9	3·3	1·182	70	88·2	0·793
5	4·4	1·136	75	94	0·798
8	7·4	1·081	80	100·9	0·793
10	9·8	1·020	85	107·3	0·792
12	13·3	0·902	90	113·6	0·792
15	16·6	0·903	95	120·1	0·791
20	24·1	0·830	100	127	0·788
25	30·5	0·820	120	152·8	0·786
30	36·9	0·813	126	160·6	0·785
35	43·2	0·810	130	166·1	0·783
40	49·9	0·802	140	179·4	0·780
45	56·1	0·802	150	191·6	0·783
50	62·5	0·800	175	223	0·785
55	69	0·805	200	255·5	0·782
57	71·3	0·800	240	307·3	0·781
60	75·2	0·798	250	320·7	0·780
65	81·3	0·799			

I have plotted these values and find that they are practically linear, except from 1 to about 20 mg. lactose. I have therefore constructed the curve shown in Fig. 3, by means of which the weight of anhydrous lactose present can be obtained from the amount of copper that it has reduced.

Example.

20 cc. of a solution of lactose were treated with 20 cc. of copper sulphate containing 348·8 mg. Cu.

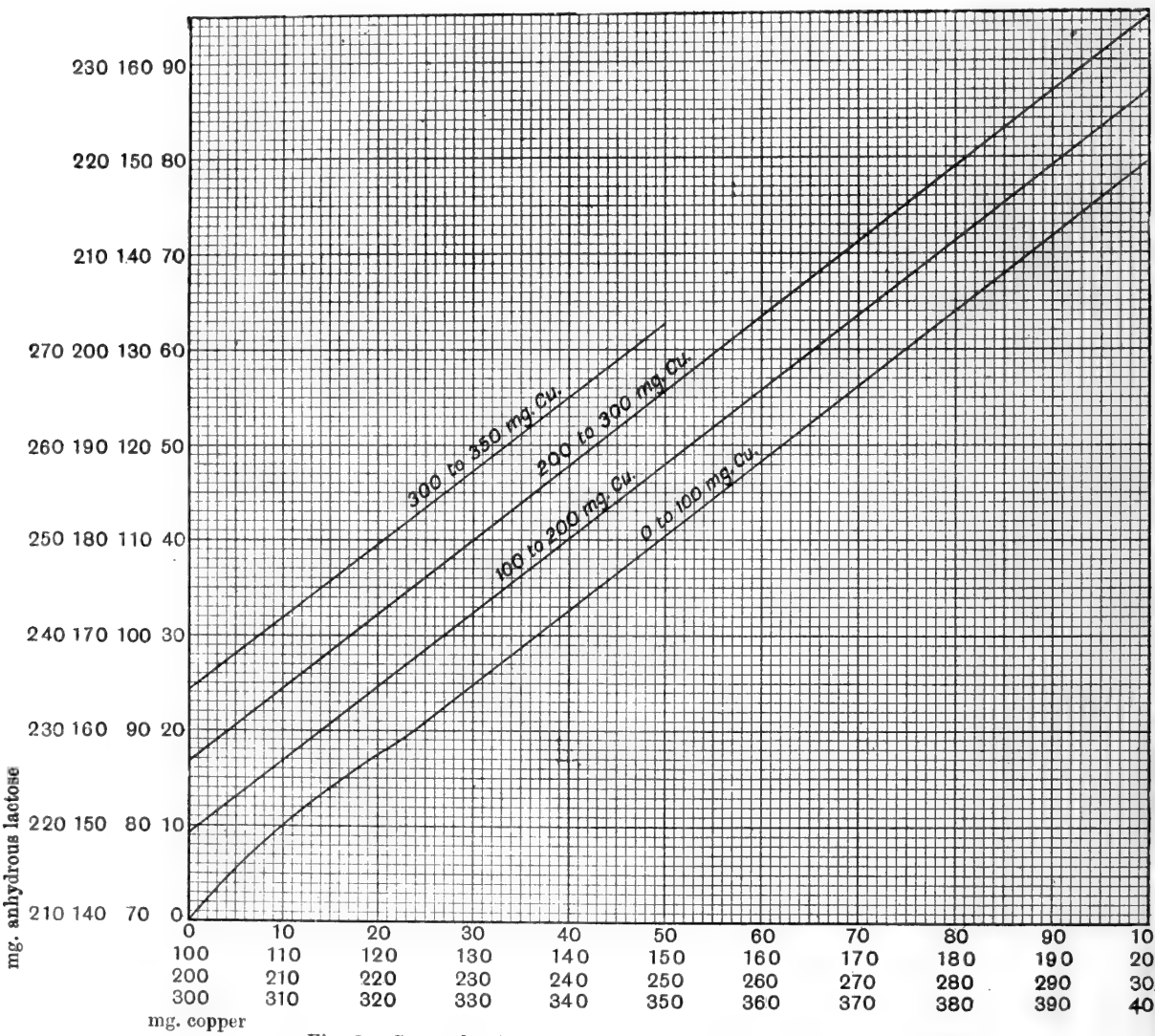

Fig. 3. Curve showing amount of copper reduced by lactose anhydride.

The copper in the filtrate liberated iodine which required **22·41** cc. of a thiosulphate, 1 cc. of which = 12·784 mg. Cu.

Cu in filtrate is $22{\cdot}41 \times 12{\cdot}784 = 286{\cdot}4$ mg.

Cu reduced is $348{\cdot}8 - 286{\cdot}4 = 62{\cdot}4$ mg.

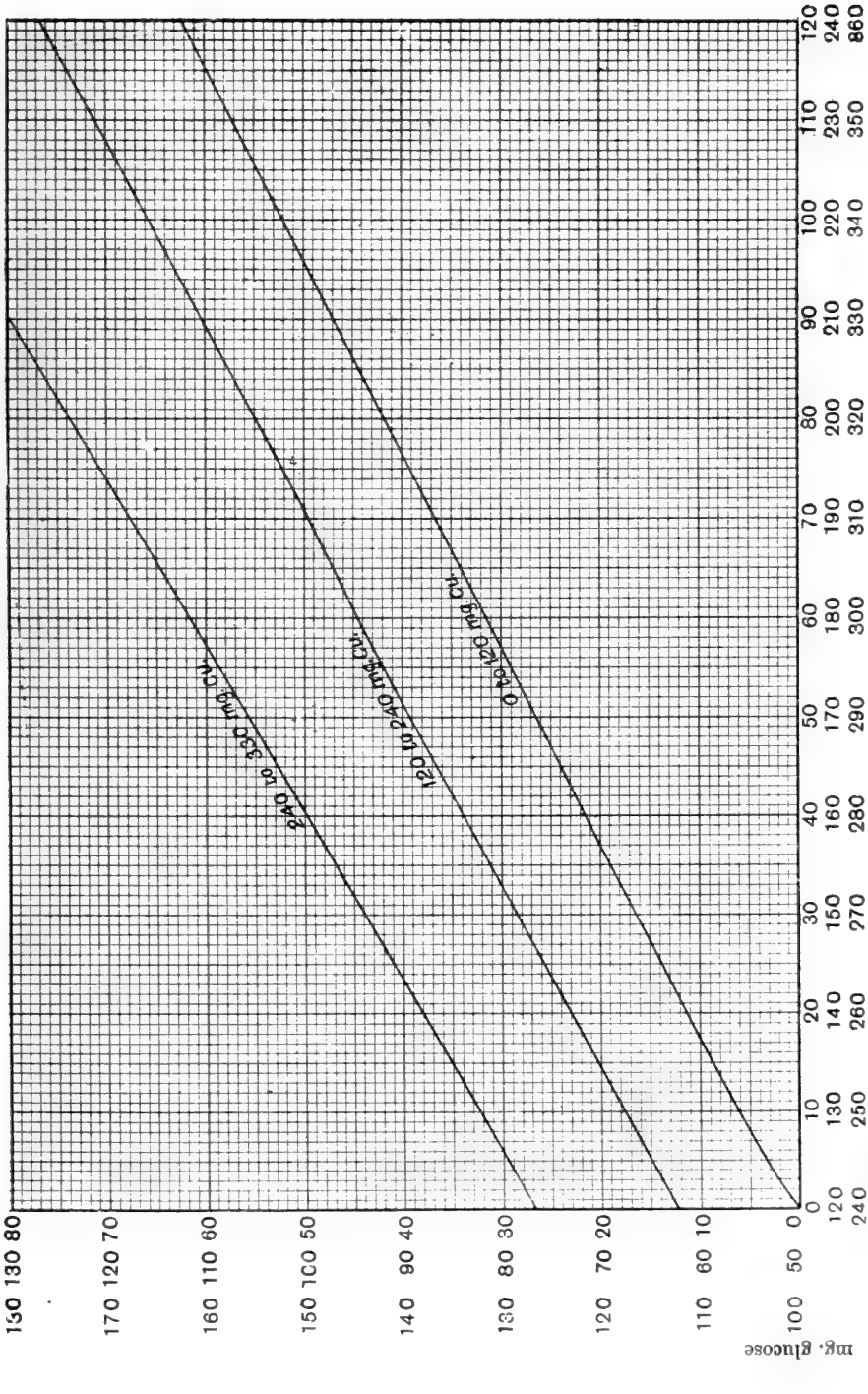

Fig. 4. Curve showing amount of copper reduced by glucose.

On reference to the curve in Fig. 3 this is seen to correspond to 50 mg. of anhydrous lactose.

The copper values above 25 mg. Cu can be converted to anhydrous lactose by the use of the following empirical formula, deduced from the curve.

mg. anhydrous lactose = $1{\cdot}25 + 0{\cdot}778 \times$ mg. Cu.

Thus in the above example

$$\text{Lactose} = 1{\cdot}25 + 0{\cdot}778 \times 62{\cdot}4 = 49{\cdot}71 \text{ mg.}$$

This agrees fairly well with the value deduced from the curve, i.e. 50 mg.

In Fig. 4 I give the corresponding values for glucose, taking the values obtained by Peters, except that I have not given the values above 180 mg. glucose. Peters states that 200 mg. glucose reduce 349·6 mg. copper. I find that even 192 mg. glucose completely reduce the whole of the copper present in the strongest of my solutions (352·9 mg.). In the range of the curve that I give I have obtained results in very close agreement with his.

It is of some interest to note that in the case of lactose the sugar/copper ratio decreases as the amount of sugar increases. The ratio lies between 0·81 and 0·78 between 30 and 250 mg. of lactose.

In the case of glucose the ratio falls to 0·522 at 25 mg. and keeps fairly constant to about 110 mg. It then rises again.

This is not in agreement with the usual text-book statements that "the greater the excess of the copper, the greater is the amount of copper reduced by a certain amount of sugar."

The steady fall in the case of lactose seems to indicate that there is a slight hydrolysis of the sugar by the alkali, so that the amount of copper reduced by the disaccharide is relatively greater when it is present in higher concentrations. I have previously had indications of the same phenomenon when attempting to estimate lactose by the application of Benedict's method.

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Peters (1912, 1), *J. Amer. Chem. Soc.* **34**, 422.
—— (1912, 2), *J. Amer. Chem. Soc.* **34**, 928.

XX. A VOLUMETRIC METHOD FOR THE ESTIMATION OF ETHEREAL AND INORGANIC SULPHATES IN URINE.

By OTTO ROSENHEIM AND JACK CECIL DRUMMOND.

From the Physiological Laboratory, King's College, London.

(*Received March 5th, 1914.*)

Ever since the discovery by Baumann of the existence in urine of ethereal sulphates in addition to inorganic sulphates, their estimation has been carried out by weighing them as $BaSO_4$ on the principle of Baumann's original method, as modified in some more or less important details by subsequent investigators[1].

Until quite recently it has been assumed that the estimation of sulphuric acid as barium sulphate is one of the simplest and most trustworthy methods of analytical chemistry. The large amount of research which has been devoted by analytical chemists to this question [Hulett and Duschak, 1904; van 't Kruys, 1910; Allen and Johnston, 1910; Järvinen, 1913, etc.] has, however, abundantly proved that this assumption is erroneous. It has been conclusively shown that even in comparatively simple solutions the method is liable to yield inaccurate results. Without going into detail it may be stated that correct results can only be obtained by a careful adjustment of the conditions, and Allen and Johnston, indeed, attribute the many good results, which are obtainable by this method, to the happy neutralisation of inaccuracies.

If we consider that all these difficulties are met with when dealing with simple salt solutions, we are led to assume that they are still greater when we are dealing with sulphate estimations in such a complex organic fluid as urine. This was indeed recognised by O. Folin [1906] when he said: "The investigations described in this paper are the outcome of a conviction that the published records of sulphate and sulphur determinations in urine, including many of my own, are intolerably unreliable."

[1] The methods proposed by R. v. Lengyel (estimation as strontium sulphate) and by Freund (titration with barium acetate) do not seem to have acquired more than academical interest.

Folin has overcome by his careful work the difficulties inherent in the method, but there remains always the fact that, even at its best, the method is a very tedious one, requiring a great deal of time and experience if accurate results are desired.

We have worked out a volumetric method for the estimation of ethereal and inorganic sulphates in urine, which is based on the well-known insolubility of the sulphate of the base benzidine (*p*-diamino-diphenyl $NH_2 . C_6H_4 . C_6H_4 . NH_2$). The method seems to be free from the objections of the barium method and has the advantage of being very rapid, whilst the results are at least as trustworthy as those obtained by the Baumann-Folin method, which we have adopted as a standard.

The principle of the method depends on the fact that insoluble benzidine sulphate is precipitated from solutions of sulphates on the addition of a soluble benzidine salt. As benzidine is a weak base, its salts with strong acids are readily dissociated and the sulphuric acid contained in benzidine sulphate may be quantitatively titrated with standard alkali solutions, using phenolphthalein as an indicator. The method was introduced in this form into inorganic analysis by Raschig [1903] and its reliability was confirmed by v. Knorre [1905, 1910] and by Friedheim and Nydegger [1907]. Järvinen [1913] came to the conclusion that the benzidine method gives the most trustworthy results and is preferable to the old barium method in inorganic analysis.

Before the benzidine method can be applied to urine it is necessary to investigate if it fulfils the essential conditions which are demanded. Any reagent which is intended to replace barium chloride for the estimation of ethereal and inorganic sulphates in urine must fulfil three conditions. (1) It must precipitate in the cold only the inorganic sulphates, free from ethereal sulphates. (2) It must not precipitate any other urinary constituents. (3) The precipitation of the total sulphates after hydrolysis (= inorganic + ethereal sulphates) must be complete.

With regard to the first condition we have tested the behaviour of benzidine solutions towards several ethereal sulphates which we prepared synthetically according to Baumann [1878]. It was found that aqueous solutions of the potassium salts of phenylsulphuric acid, *p*-cresylsulphuric acid and resorcinolsulphuric acid behave towards benzidine hydrochloride exactly in the same way as towards barium chloride, i.e. the organically bound sulphuric acid is not precipitated in the cold, but readily after boiling for a short time with dilute hydrochloric acid. We have further tested the behaviour of the ethereal sulphates contained in normal urine towards

benzidine solutions in the following way. 200 cc. of urine were freed from inorganic sulphates by precipitation with barium chloride in the usual way. The filtrate which contained only the ethereal sulphates remained perfectly clear after the addition of a solution of benzidine hydrochloride. We must therefore conclude that under the ordinary conditions of urinary analysis the ethereal sulphates are not precipitated by benzidine.

With regard to the second condition we have found that the addition of a benzidine solution to normal urine results at once in the production of a bulky cream coloured precipitate, which on examination is seen to be partially crystalline and partially amorphous. It can easily be shown that this precipitate contains benzidine phosphate besides benzidine sulphate. This fact alone would seem *a priori* to condemn the method. (See also K. Spiro [Neubauer-Huppert, 1910], which is the only reference to the subject we came across in the literature.) We found, however, that urine which is faintly acidified with dilute hydrochloric acid, gives a crystalline precipitate which rapidly settles. This precipitate consists entirely of crystals of benzidine sulphate. It was tested qualitatively with negative results for phosphoric and uric acids, and the absence of other acids than sulphuric acid was proved by the close agreement of control analyses in which sulphuric acid determinations were carried out in the same sample by the barium method and by direct titration. The experiment described above, in which it was shown that the addition of benzidine solution to urine freed from inorganic sulphates produces no further precipitate, may also be quoted as evidence that sulphates are the only urinary constituents which are precipitated from acid urine by benzidine.

With regard to the completeness of the precipitation of sulphuric acid by benzidine in the presence of the other constituents of urine, we consider this proved by experiments in which added sulphuric acid was quantitatively recovered, and further by the fact that after the removal of the benzidine precipitate no further precipitate was produced by the addition of barium chloride.

Benzidine seems therefore to fulfil the conditions laid down above and we felt justified in proceeding to the actual quantitative estimations. It now became necessary to carry out a series of experiments in order to investigate (1) the influence of increasing quantities of hydrochloric acid on the accuracy of the result, (2) the minimum time necessary for complete precipitation, and (3) the quantity of benzidine solution required for complete precipitation.

The results of these experiments showed that excess of hydrochloric acid prevents the precipitation of benzidine sulphate and that a quantity sufficient

to produce an acid reaction to Congo red paper gives accurate results. Further it was found that the precipitation is complete after five minutes and that one hour's standing does not influence the result. Even a large excess of benzidine solution does not affect the accuracy of the result.

The ethereal and inorganic sulphates in urine may obviously be estimated in several ways by means of benzidine. (1) Inorganic and total sulphates may be estimated directly and the amount of ethereal sulphates be calculated by the difference between the two. (2) Total and ethereal sulphates may be estimated directly and inorganic sulphates calculated by difference. (3) Inorganic and ethereal sulphates may be estimated separately.

As the direct estimation of ethereal sulphates, owing to the small amount present, requires a relatively large volume of urine, which is not always available, we have estimated them indirectly by the difference between the total and inorganic sulphates, a procedure which is analogous to that employed by Folin in the barium chloride method.

In our experience the practical application of the benzidine method to urine does not meet with any difficulties if the precautions are observed, which have already been described by Raschig and by Järvinen in the case of inorganic analysis. They refer to care in filtration and washing of the precipitate. It is also important that an excess of free hydrochloric acid be avoided. Partly for this reason and partly in order to avoid the excessive formation of dark pigments which might interfere with the end point of titration, we have found it advisable to reduce the amount of hydrochloric acid in the hydrolysis of the ethereal sulphates to the minimum necessary for complete hydrolysis.

It is evident that the benzidine method can also be employed for the estimation of the neutral sulphur, by subtracting the quantity of total sulphates from that of the total sulphur. The latter may be estimated by applying the benzidine method to the final solution of mineral salts, as obtained by Wolf and Oesterberg's modification [1910] of Benedict's method [1909]. The presence of copper in this solution does not in any way interfere with the accuracy of the results.

By working with 0·01 N KOH solutions and methyl-red as an indicator it seems also possible to use much smaller quantities of urine and to convert the method into a micro-analytical method. Such a method would be of use in metabolic experiments on small animals and we are still engaged with experiments in this direction.

Experimental.

The preparation of the benzidine solution. 4 g. benzidine (Kahlbaum) are rubbed into a fine paste with about 10 cc. of water and transferred with about 500 cc. of water into a 2 litre flask. 5 cc. of concentrated HCl (spec. grav. 1·19) are added and the solution made up to 2 litres with distilled water. 150 cc. of this solution, which keeps indefinitely, are sufficient to precipitate 0·1 g. H_2SO_4.

Influence of acidity, time and volume. The following series of experiments was carried out on a large sample of mixed urine. 25 cc. of urine were taken in each case. An estimation of inorganic sulphates by the barium method gave 0·104 % SO_3.

Influence of increasing quantities of HCl (1 : 4).

HCl, cc.	Benzidine solution, cc.	Time of standing	0·1 N KOH, cc.	SO_3 %
1	100	10 mins.	6·45	0·103
2	,,	,,	6·50	0·104
3	,,	,,	6·30	0·101
4	,,	,,	6·00	0·096
5	,,	,,	5·60	0·089
7	,,	,,	4·60	0·074
10	,,	,,	2·20	0·035
15	,,	,,	none	—

This table shows clearly that an excess of hydrochloric acid prevents the formation of the benzidine sulphate precipitate. The quantity of hydrochloric acid necessary to produce an acid reaction with Congo red paper was 0·8 cc. In all the subsequent experiments we used therefore 1–2 cc. HCl (1 : 4).

Influence of time of standing.

HCl, cc.	Benzidine solution, cc.	Time of standing	0·1 N KOH, cc.	SO_3 %
2	100	5 mins.	6·40	0·102
,,	,,	10 ,,	6·45	0·103
,,	,,	15 ,,	6·40	0·102
,,	,,	20 ,,	6·60	0·107
,,	,,	30 ,,	6·45	0·103
,,	,,	45 ,,	6·45	0·103
,,	,,	60 ,,	6·50	0·104

These results prove that precipitation is complete after five minutes and that the result is not affected by one hour's standing. The short time

required for precipitation is a distinct advantage as against the lengthy period usually required before barium sulphate precipitates can be filtered.

Influence of volume of benzidine solution.

HCl, cc.	Benzidine solution, cc.	Time of standing	0·1 N KOH, cc.	SO_3 %
2	70	5 mins.	6·50	0·104
,,	100	,,	6·40	0·102
,,	150	,,	6·50	0·104
,,	180	,,	6·50	0·104
,,	200	,,	6·50	0·104

The final result is evidently not influenced by an excessive quantity of the reagent and 100 cc. of the standard benzidine solution are sufficient to precipitate the whole of the sulphates contained in 25 cc. of normal urine.

An experiment may be mentioned here in which a known quantity of sulphate was purposely added to the urine. 2 cc. of a dilute sulphuric acid required 10·9 cc. 0·1 N KOH for neutralisation. This solution of sulphate, containing 53·4 mg. H_2SO_4, was added to 25 cc. urine which was previously found to contain 31·9 mg. H_2SO_4. In two experiments the final benzidine precipitates required 17·4 and 17·5 cc. 0·1 N KOH = 85·3 mg. and 85·4 mg. H_2SO_4.

	H_2SO_4 added	H_2SO_4 found
(1)	53·4 mg.	53·4 mg.
(2)	53·4 mg.	53·5 mg.

This experiment not only proves that the organic constituents of the urine do not interfere with the precipitation of sulphates by benzidine, but also that the precipitation is quantitative.

As an outcome of these preliminary experiments we have finally adopted the following procedure:

1. *The estimation of inorganic sulphates.*

25 cc. of urine are measured into a 250 cc. Erlenmeyer flask and acidified with dilute hydrochloric acid (1 : 4) until the reaction is distinctly acid to Congo red paper. Usually 1–2 cc. of dilute acid were added. 100 cc. of the benzidine solution (see above) are then run in and the precipitate, which forms in a few seconds, allowed to settle for ten minutes. The precipitate is filtered under pressure and washed with 10–20 cc. of water, saturated with benzidine sulphate. The precipitate and filter paper are transferred into the original precipitation flask with about 50 cc. water and titrated hot with

0·1 N KOH, after the addition of a few drops of a saturated alcoholic solution of phenolphthalein. 1 cc. 0·1 N KOH corresponds to 49 mg. H_2SO_4.

2. *The estimation of total sulphates (inorganic and ethereal).*

The hydrolysis of the ethereal sulphates may be carried out in the usual way by gently boiling 25 cc. of urine with 20 cc. dilute HCl (1 : 4) for fifteen to twenty minutes. In this case it is necessary carefully to neutralise the solution after boiling and to add again dilute HCl until the reaction is acid to Congo red. We obtained, however, equally good results by reducing the quantity of acid used for hydrolysis to 2–2·5 cc. HCl (1 : 4), adding also 20 cc. of water to prevent undue concentration. The hydrolysis is complete after 15–20 minutes' gentle boiling and the precipitation of the cold solution may be proceeded with at once without previous neutralisation. It seems also that the smaller quantity of acid produces only very little pigment, which interferes to a slight extent with the end point, if the larger amount of acid is used.

The filtration and titration of the precipitate is carried out exactly as under 1.

In order to obtain accurate results, it is most important that the precipitate should be finely suspended in water before titration, and this again entails certain precautions during filtration, so as to prevent the caking together of the precipitate. We used a funnel of 6 cm. diameter and a perforated porcelain filter plate (5–7 mm.), covered either with paper pulp or with a well-fitting filter paper. Filtration and washing are carried out under pressure with the precaution of not allowing the precipitate at any time to be sucked dry on the filter. When the filtrate is no longer acid to Congo red and the precipitate together with the filter paper have been transferred to the original flask, it is necessary to shake it thoroughly before titration. Raschig recommended closing the flask with a rubber stopper for this purpose, a precaution which we found not essential. It is necessary to employ a stronger phenolphthalein solution than usual, as it is adsorbed to a considerable extent by the filter paper.

We have tested the accuracy of the method by comparing the results of the benzidine method with those obtained by Folin's gravimetric barium method. As many as seventy-five separate analyses were carried out. Most of the results agreed absolutely, the largest difference amounted to − 0·0025 % in favour of the barium method and to + 0·001 % in favour of the benzidine method (calculated as SO_3 % of urine).

Some of the results, taken at random, may be quoted in full. 25 cc. of urine were used in each case.

	Gravimetric		Volumetric	
	Mg. $BaSO_4$	% SO_3	Cc. 0·1 N KOH	% SO_3
(*a*) *Inorganic sulphates.*				
Urine 1	87·1	0·120	7·54	0·120
	86·7	0·119	(6 identical	
		Mean 0·1195	estimations)	
Urine 2	57·6	0·079	4·90	0·078
	58·7	0·081	5·00	0·080
		0·080		0·079
Urine 3	85·7	0·117	7·28	0·116
			7·36	0·118
			7·32	0·117
			7·32	0·117
				0·117
(*b*) *Total sulphates.*				
Urine 3	93·1	0·128	7·99	0·128
			8·08	0·129
			8·08	0·129
				0·129

It will be seen that the results of the benzidine method agree not only *inter se* but also with those of the barium method as closely as can be expected[1].

Summary.

A rapid and accurate method for the estimation of sulphates in urine consists in their precipitation with benzidine solution and the subsequent titration of the insoluble benzidine sulphate by means of 0·1 N potassium hydrate.

[1] A full account of this method was given at the November meeting of the Biochemical Society at King's College, London. Since this paper was ready for the press, our attention has been drawn to a publication on the same subject by R. Gauvin and V. Skarzynski in the December number of the *Bull. Soc. Chim.* p. 1121. It is satisfactory to note that these authors arrived at practically the identical method and results, although they do not seem to have systematically investigated the requisite conditions.

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XXI. A NOTE ON THE PRODUCTION OF CASEIN FROM CASEINOGEN.

By SAMUEL BARNETT SCHRYVER.

(*Received March 7th, 1914.*)

In the last number of this Journal a paper by A. Harden and A. B. Macallum [1914] appeared, in which certain experiments on the production of casein from caseinogen are described. They draw the conclusion that this process is accompanied by a cleavage of nitrogen, phosphorus and calcium from the latter substance. These results are not in accord with my own [1913, 1, 2] and those independently obtained by Bosworth [1913]. I should like to draw attention to the reason of this apparent discrepancy. My own results were obtained with a solution of caseinogen in half-saturated lime-water, which had been obtained by rotating the lime-water with excess of caseinogen for several hours, and was consequently saturated with the acid and had a strongly acid reaction. Harden and Macallum, on the other hand, ground up their caseinogen preparation with excess of calcium carbonate and obtained thereby a solution of the basic caseinogenate. Now the acid solutions obtained by my method give clots with extraordinary readiness without addition of a soluble calcium salt when treated with rennin, even at ordinary room temperature. They also give clots with calcium chloride alone within certain limits of concentration when very gently warmed (to 25°). A condition for this very rapid clot formation is that the solution should be saturated with caseinogen. The rapidity of clot formation diminishes as soon as the acidity of the solutions is neutralised with calcium hydroxide. Under the conditions under which I worked, clots were obtained, the free acids from which had precisely the same empirical composition (N and P content and acidity) as caseinogen. The products were, however, only half as soluble in lime-water as caseinogen, and the solutions thus obtained could no longer be got to clot on rennin addition. A large number of attempts to produce a clottable solution from casein have been made, but without success. On the

question of the irreversibility of the casein formation, I am in accord with Harden and Macallum. I still think it quite likely that the casein is a compound of caseinogen with the ferment, although this suggestion has not yet been definitely proved.

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XXII. HERZIG AND MEYER'S REACTION APPLIED TO PROTEINS AND AMINO-ACIDS.

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From the Physiological Laboratory, Cambridge and the Wellcome Physiological Research Laboratories, Herne Hill.

(*Received March 9th, 1914.*)

This reaction [Herzig and Meyer, 1894] has been known for a considerable time as a means of detecting the presence of, and estimating, methyl groups attached to nitrogen in organic compounds. It consists in heating the substance to a high temperature in the presence of excess of hydriodic acid, so that the methyl groups leave the nitrogen to form methyl iodide, and the hydrogen of the hydriodic acid takes the place of the methyl group. The methyl iodide is then carried over into alcoholic silver nitrate, where silver iodide is formed. The precipitate of silver iodide can be weighed and from its amount the percentage of methyl attached to nitrogen can be calculated.

Certain precautions have to be taken in the estimation of methylated nitrogen in this way. Thus substances containing methoxy groups give a silver iodide precipitate, according to the well known Zeisel reaction. The precipitate from methoxy groups, however, forms when the reaction mixture is heated to 130°, whereas that from methylamino groups requires a temperature of 230°. In connexion with methoxy derivatives it must also be remembered that glycerol and fats (on account of their glycerol content) give rise to isopropyl iodide and to a silver iodide precipitate. Trier has published a criticism [Trier, 1913] of the method stating that amino-ethyl alcohol gives a precipitate under these conditions.

In the course of ordinary estimations by this method, it was found that commercial gelatin gave a positive result. At the end of the experiment the precipitate formed in the flask containing silver nitrate was black. This was due to the sulphur in the protein, for on boiling with dilute nitric acid, the black colour disappeared, leaving a clean precipitate of silver iodide. It was similarly found that Hammarsten's "Casein" gave the reaction, after

having been first carefully extracted with ether to make sure of the absence of fat. These results seemed to point to the possibility of the occurrence in protein of methylated nitrogen. The reaction could not be due to methoxy derivatives, as the precipitate of silver iodide was only formed when the reaction mixture was heated to about 230°.

To investigate this further, a dried pancreatic digest of protein, also carefully extracted with ether, was examined. This too gave a positive result, as did also a phosphotungstic precipitate from the digest, and the filtrate from the precipitation. It seemed desirable at this stage to make sure of the soundness of the reaction when applied to individual known amino-acids. Instead of the negative results expected, it was found that a mixture of glycine, tryptophane and a leucine-valine fraction gave a silver iodide precipitate. Of these separately, glycine and the leucine-valine fraction were positive, whilst tryptophane was negative.

On the basis that 100 grams of silver iodide correspond to 6·38 grams of methyl, the following percentages of methyl (CH_3) present in combination with nitrogen were found:

Glycine	0·34 %
	0·46
Alanine	0·43
	0·63
Leucine-valine	0·68
	0·95
Histidine	0·03
Tyrosine	negative
Tryptophane	negative
Commercial gelatin ...	0·77 %
Hammarsten's casein ...	0·97

In the case of the amino-acids the explanation seems to be that after the hydriodic acid has been distilled away, during the dry heating at 250°–270°, simple amino-acids break up, giving off carbon dioxide and leaving an amine, which should quite properly give the reaction. Thus glycine would decompose as follows:

$$NH_2 . CH_2 . COOH = NH_2 . CH_3 + CO_2.$$

Such an explanation would seem to apply even to leucine. If it broke up in this way, isoamylamine would be formed; and in fact when isoamylamine was tested by the reaction, a silver iodide precipitate was formed in amount corresponding to the above percentages.

It will be noticed that the values for protein are slightly greater than those for the individual amino-acids, and yet it is apparently only the amino-acids of simple constitution which do give a positive result. It may therefore

be argued that as these can only form a small fraction of the protein, the high values with protein are only partly explained. It is indeed possible that methylamino groupings do exist in protein, but this reaction does not afford a sufficiently reliable test for their presence. Quantitatively the limits of error are as wide as $+3\%$ and -15%. Thus sarcosine, which on the above considerations might be expected to give a higher value than the theoretical 16·8 % for methyl attached to nitrogen, actually gave 14·4 %. When it is seen that simple amino-acid groups are broken up at the high temperature of 250° and in the presence of hydriodic acid, the likelihood that there are other groupings in protein itself (not methylamino) which will also break up becomes extremely great.

The work out of which this note has arisen was begun during the tenure of the Michael Foster Studentship in Cambridge University. My thanks are due to Dr F. G. Hopkins for suggesting the work and for supervising its earlier stages.

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XXIII. STUDIES IN PROTEIN HYDROLYSIS.

By WILLIAM WYNN PRATT PITTOM (*St John's College, Government Research Scholar*).

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(*Received March 11th, 1914.*)

It is well known that proteins are built up of various proportions of different amino-acids, and that on hydrolysis they are broken down, giving proteoses, peptones, polypeptides, and finally their constituent amino-acids. The study of the ultimate products of hydrolysis has been extensively pursued, but, as yet, little light has been thrown upon the initial stages of the hydrolytic decomposition, and it was with the view of finding out something about the earlier steps in the degradation of the protein molecule, and incidentally to compare the behaviour of two proteins under the same conditions, that the following work was carried out.

The two proteins used were caseinogen and egg albumin; they were hydrolysed by strong hydrochloric acid for short intervals of time, and the various amounts of ammonia, amino-nitrogen, and nitrogen in bodies precipitated from the resulting liquid by phosphotungstic acid, were determined after each hydrolysis, the values so obtained being plotted on a curve.

The caseinogen used was "Merck's Pure" which had been extracted for several days with boiling alcohol in order to remove all traces of substances soluble in alcohol. It contained 14·92 per cent. of nitrogen and had an ash content of 4·27 per cent. so that the percentage of nitrogen in the ash free substance was 15·59.

The egg albumin used was prepared in the pure crystalline form from the whites of fresh eggs, Hopkins' modification of Hofmeister's method being used for the preparation. The crystalline product was twice purified by solution and precipitation, coagulated by absolute alcohol, washed free from ammonium sulphate, and carefully dried at 110° in an air oven. It contained 15·36 per cent. of nitrogen as determined by Dumas' method (Kjeldahl's method gave 14·15 per cent. nitrogen) and was practically ash free, containing 0·005 per cent. ash.

Method of experiment.

About one gram of protein was carefully weighed out into a hard glass, round-bottomed flask, fitted with a cork and glass tube to act as an air condenser, 20 cc. of boiling strong hydrochloric acid were added and the flask placed on a heated sand bath. When the time of hydrolysis (taken from the moment of adding the boiling hydrochloric acid) was completed, the flask was removed from the sand bath and at once cooled by the addition of cold water.

Estimation of ammonia (amide nitrogen).

The liquid was then introduced into a steam distillation flask, containing excess of a paste of magnesium oxide, the ammonia distilled off in steam into excess of standard acid, and the excess of acid titrated with standard alkali, using methyl orange as indicator.

This method of estimating the amide nitrogen of proteins, first used by Hausmann [1899 and 1900] was employed because, although it is well known that cystine is somewhat easily decomposed at 100°, yet it has been shown by Osborne that caseinogen and egg albumin, proteins which contain only minute quantities of cystine, if any, give the same results for amide nitrogen, whether the ammonia is distilled off at 100° by magnesia or *in vacuo* at low temperature by lime.

Humin nitrogen.

The liquid remaining in the flask was then filtered off from the magnesia, which was carefully washed with hot water until the washings were free from chloride. The filtrate and washings were then evaporated down and made up to 250 cc.

The magnesia precipitate was then dried, carefully detached from the filter paper and the nitrogen in it determined by Kjeldahl's method.

Amino-nitrogen (NH_2 *groups*).

This was estimated by Sörensen's [1908] formaldehyde method. To determine the amount of amino-nitrogen, a definite quantity (25 cc.) of the filtrate from the magnesia was made just faintly alkaline to phenolphthalein by adding dilute N/20 hydrochloric acid to the alkaline liquid. Then to this faintly alkaline solution were added 5 cc. of formalin (proved to be in

excess of what was actually required) made alkaline to the same faint pink colour by adding N/20 soda. The pink colour disappeared at once on mixing the solutions and the acidity produced was exactly measured by titrating with N/50 soda, the end point being taken when the solution reached the same tint as had been used previously.

The solutions were only slightly yellow in colour (due to humin substances in solution) and in no case was the colour sufficiently deep to necessitate clarifying the liquid, before titrating to the pink colour.

The titrations agreed very well among themselves, despite the very dilute alkali used, which tended to make the end point of the titration somewhat indefinite.

Diamino and polypeptide nitrogen, i.e. nitrogen precipitated by phosphotungstic acid in sulphuric acid solution.

This was estimated by precipitating in acid solution with phosphotungstic acid dissolved in dilute sulphuric acid, and then carrying out a Kjeldahl estimation on the carefully washed precipitate. 75 cc. of the liquid were used, made acid with dilute sulphuric acid, and excess of a 25 per cent. solution of phosphotungstic acid in 5 per cent. sulphuric acid added. The beakers containing the mixture were allowed to stand for 24 hours, during which time the precipitate had settled to the bottom leaving a clear liquid above. This was decanted and the precipitate washed with 5 per cent. phosphotungstic acid solution in 5 per cent. sulphuric acid, allowed to stand till the precipitate had again settled to the bottom of the beaker, and the clear liquid again decanted. Finally the precipitate was washed on to a filter, dried, and the nitrogen in it estimated by Kjeldahl's method, deduction for the nitrogen contained in the filter paper being made in each case.

It was found that the estimation of the nitrogen precipitated by phosphotungstic acid was a very difficult matter and for some time the duplicates did not agree. However, when the procedure above was adhered to, the results obtained agreed moderately well. The two chief difficulties to be overcome were the matter of washing all the precipitates in exactly the same way and for the same length of time, and then getting them filtered properly. The filtering was a very tedious business for, if great care was not exercised, the precipitate came through a Buchner filter, while it filtered extremely slowly through an ordinary filter paper. Finally, filtering through a Buchner funnel was adopted, great care being exercised not to get too big a pressure on the filter pump.

Experimental results.

I. *Estimation of Ammonia.* A. *Caseinogen.*

Time of hydrolysis in hours	Weight of caseinogen, g.	Number of cc. 0·1N HCl	Equivalent nitrogen, g.	Percentage nitrogen	Mean of duplicates
0	0·8376	0·20	0·00028	0·033	0·042
	0·9678	0·35	0·00049	0·051	
0·25	0·9994	9·55	0·01337	1·340	1·340
	1·0613	10·20	0·01428	1·340	
0·50	1·1179	10·90	0·01526	1·360	1·360
	0·9648	9·35	0·01309	1·360	
1	1·0627	10·60	0·01484	1·390	1·385
	1·0769	10·60	0·01484	1·380	
2	0·9842	9·90	0·01386	1·410	1·405
	1·1830	11·80	0·01652	1·400	
3	0·9467	9·65	0·01351	1·430	1·440
	0·9553	9·90	0·01386	1·450	
4	0·9026	9·40	0·01316	1·460	1·460
	0·9896	10·35	0·01449	1·460	
5	0·9373	10·10	0·01414	1·510	1·505
	1·0038	10·85	0·01519	1·500	
6	1·2733	13·50	0·01890	1·490	1·495
	1·0307	11·10	0·01554	1·500	

I. *Estimation of Ammonia.* B. *Egg albumin.*

Time of hydrolysis in hours	Weight of egg albumin, g.	Number of cc. 0·1N HCl used	Equivalent nitrogen, g.	Percentage nitrogen	Mean of duplicates
0	0·8824	0·40	0·00056	0·063	0·063
0·25	0·9942	6·70	0·00938	0·940	0·940
	1·0085	6·80	0·00952	0·940	
0·50	0·8607	6·10	0·00854	0·990	0·975
	0·9895	6·80	0·00952	0·960	
1	0·8164	6·20	0·00868	1·060	1·050
	1·0275	7·65	0·01710	1·040	
2	0·8806	6·90	0·00966	1·100	1·100
	0·7636	6·05	0·00847	1·100	
3	1·0956	9·10	0·01274	1·160	1·150
	0·9860	8·00	0·01120	1·140	
4	0·7748	6·70	0·00938	1·210	1·200
	1·0865	9·20	0·01288	1·190	
5	0·9522	8·45	0·01183	1·250	1·245
	1·0181	9·00	0·01260	1·240	
6	1·0429	9·20	0·01288	1·240	1·260
	1·0098	9·10	0·01274	1·280	
7	0·9884	8·90	0·01246	1·260	1·260
	0·8228	7·40	0·01036	1·260	

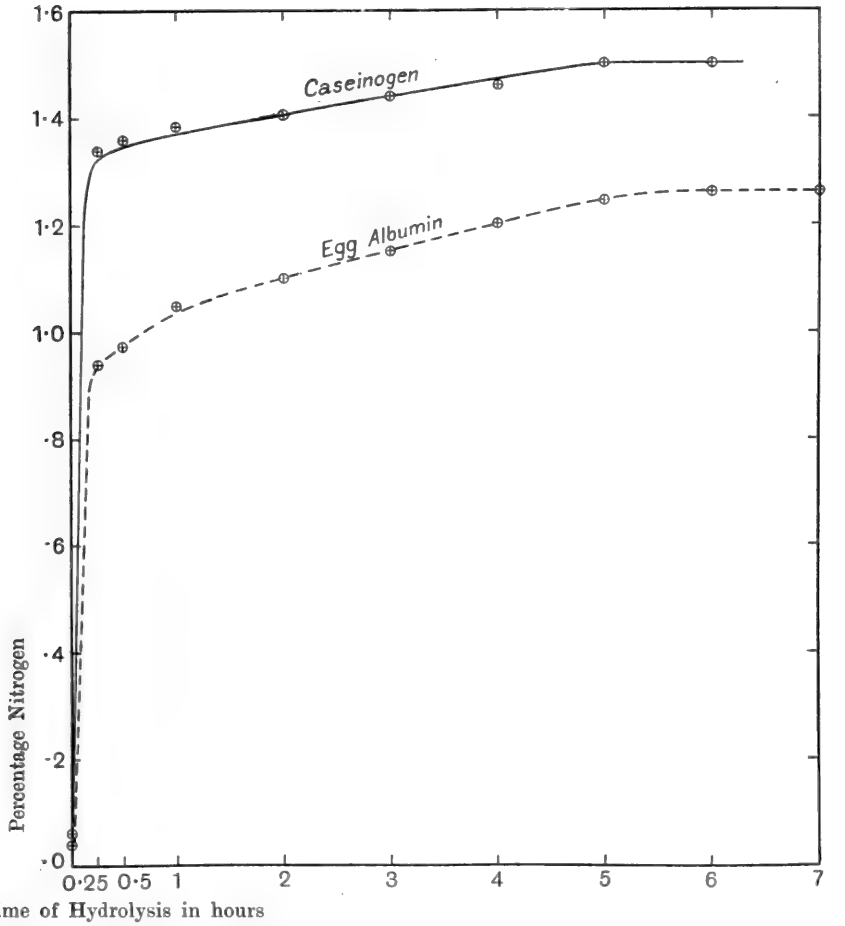

Fig. 1. Curves showing the rate of liberation of Ammonia. (Amide Nitrogen.)

II. *Estimation of Humin Nitrogen.* A. *Caseinogen.*

Time of hydrolysis in hours	Weight of caseinogen, g.	Number of cc. 0·1 N HCl used	Equivalent nitrogen, g.	Percentage nitrogen	Mean of duplicates
0·25	0·9994	4·75	0·00665	0·67	0·625
	1·0613	4·40	0·00616	0·58	
0·50	1·1179	2·60	0·00364	0·33	0·320
	0·9648	2·10	0·00294	0·31	
1	1·0627	2·50	0·00350	0·33	0·340
	1·0769	2·70	0·00378	0·35	
2	0·9842	1·60	0·00224	0·23	0·235
	1·1830	2·00	0·00280	0·24	
3	0·9467	1·90	0·00266	0·28	0·290
	0·9553	2·00	0·00280	0·30	
4	0·9026	1·80	0·00252	0·28	0·280
	0·9896	2·00	0·00280	0·28	
5	0·9373	1·50	0·00210	0·22	0·240
	1·0038	1·90	0·00264	0·26	
6	1·2733	2·90	0·00406	0·32	0·310
	1·0307	2·20	0·00308	0·30	

Mean of values (excluding 0·25 hour) = 0·29 per cent.

II. *Estimation of Humin Nitrogen.* B. *Egg albumin.*

Time of hydrolysis in hours	Weight of egg albumin, g.	Number of cc. 0·1 N HCl used	Equivalent nitrogen, g.	Percentage nitrogen	Mean of duplicates
0·25	0·9942	4·30	0·00602	0·61	
	1·0085	11·90	0·01666	1·65	
0·50	0·8607	2·00	0·00280	0·32	0·31
	0·9895	2·10	0·00294	0·30	
1	0·8164	2·10	0·00294	0·36	0·32
	1·0576	2·10	0·00294	0·28	
2	0·8806	1·90	0·00266	0·30	0·30
	0·7636	1·60	0·00224	0·30	
3	1·0956	2·15	0·00301	0·28	0·28
	0·9860	2·00	0·00280	0·28	
4	0·7748	1·90	0·00266	0·34	0·33
	1·0865	2·50	0·00350	0·32	
5	1·0181	3·00	0·00420	0·41	0·36
	0·9522	2·15	0·00301	0·31	
6	1·0098	1·95	0·00273	0·27	0·27
	1·0429	2·00	0·00280	0·27	
7	0·9884	2·00	0·00280	0·28	0·32
	0·8228	2·10	0·00294	0·36	

Mean of values (excluding 0·25 hour) = 0·31 per cent. nitrogen.

III. *Estimation of Amino-nitrogen.* A. *Caseinogen.*

25 cc. of the standard volume of liquid were titrated in each case with N/50 soda.

Time of hydrolysis in hours	Weight of caseinogen, g.	Number of cc. N/50 soda used	Equivalent nitrogen, g.	Percentage nitrogen	Mean of duplicates
0·25	0·9994	$\left.\begin{matrix}11\cdot30\\11\cdot10\end{matrix}\right\} \times 10 = 112\cdot00$	0·03136	3·14	3·190
	1·0613	$\left.\begin{matrix}12\cdot30\\12\cdot25\end{matrix}\right\} \times 10 = 122\cdot75$	0·03447	3·24	
0·50	1·1179	$\left.\begin{matrix}14\cdot65\\14\cdot75\end{matrix}\right\} \times 10 = 147\cdot00$	0·04116	3·68	3·705
	0·9648	$\left.\begin{matrix}12\cdot85\\12\cdot85\end{matrix}\right\} \times 10 = 128\cdot50$	0·03598	3·73	
1	1·0627	$\left.\begin{matrix}18\cdot45\\18\cdot45\end{matrix}\right\} \times 10 = 184\cdot50$	0·05166	4·86	4·885
	1·0769	$\left.\begin{matrix}18\cdot95\\18\cdot85\end{matrix}\right\} \times 10 = 189\cdot00$	0·05292	4·91	
2	0·9842	$\left.\begin{matrix}19\cdot30\\19\cdot40\end{matrix}\right\} \times 10 = 193\cdot50$	0·05418	5·50	5·525
	1·1830	$\left.\begin{matrix}23\cdot35\\23\cdot55\end{matrix}\right\} \times 10 = 234\cdot50$	0·06566	5·55	
3	0·9467	$\left.\begin{matrix}20\cdot20\\20\cdot30\end{matrix}\right\} \times 10 = 202\cdot50$	0·05670	6·00	6·020
	0·9553	$\left.\begin{matrix}20\cdot60\\20\cdot60\end{matrix}\right\} \times 10 = 206\cdot00$	0·05768	6·04	
4	0·9026	$\left.\begin{matrix}20\cdot50\\20\cdot80\end{matrix}\right\} \times 10 = 206\cdot50$	0·05782	6·40	6·350
	0·9896	$\left.\begin{matrix}22\cdot25\\22\cdot25\end{matrix}\right\} \times 10 = 222\cdot50$	0·06230	6·30	
5	0·9373	$\left.\begin{matrix}21\cdot75\\21\cdot85\end{matrix}\right\} \times 10 = 218\cdot00$	0·06104	6·51	6·565
	1·0038	$\left.\begin{matrix}23\cdot45\\23\cdot30\end{matrix}\right\} \times 10 = 233\cdot75$	0·06650	6·62	
6	1·2733	$\left.\begin{matrix}24\cdot90\\24\cdot70\end{matrix}\right\} \times 10 = 248\cdot00$	0·08694	6·74	6·785
	1·0307	$\left.\begin{matrix}31\cdot10\\31\cdot00\end{matrix}\right\} \times 10 = 310\cdot50$	0·06944	6·83	

III. *Estimation of Amino-nitrogen.* B. *Egg albumin.*

25 cc. of the standard volume (250 cc.) of liquid were titrated in each case with N/50 soda.

Time of hydrolysis in hours	Weight of egg albumin used, g.	Number of cc. of N/50 soda used	Equivalent nitrogen, g.	Percentage nitrogen	Mean of duplicates
0·25	0·9942	$\left.\begin{matrix}5{\cdot}00\\4{\cdot}80\end{matrix}\right\} \times 10 = 49{\cdot}00$	0·01372	1·38	1·400
	1·0085	$\left.\begin{matrix}5{\cdot}15\\5{\cdot}05\end{matrix}\right\} \times 10 = 51{\cdot}00$	0·01428	1·42	
0·50	0·8607	$\left.\begin{matrix}9{\cdot}60\\9{\cdot}50\end{matrix}\right\} \times 10 = 95{\cdot}50$	0·02674	3·09	3·045
	0·9895	$\left.\begin{matrix}10{\cdot}30\\10{\cdot}50\end{matrix}\right\} \times 10 = 104{\cdot}00$	0·02912	3·00	
1	0·8164	$\left.\begin{matrix}14{\cdot}40\\14{\cdot}30\end{matrix}\right\} \times 10 = 143{\cdot}50$	0·04018	4·93	4·930
	1·0576	$\left.\begin{matrix}18{\cdot}20\\18{\cdot}30\end{matrix}\right\} \times 10 = 182{\cdot}50$	0·05210	4·93	
2	0·8806	$\left.\begin{matrix}17{\cdot}20\\17{\cdot}30\end{matrix}\right\} \times 10 = 172{\cdot}50$	0·04816	5·47	5·540
	0·7636	$\left.\begin{matrix}15{\cdot}40\\15{\cdot}20\end{matrix}\right\} \times 10 = 153{\cdot}00$	0·04284	5·61	
3	1·0956	$\left.\begin{matrix}22{\cdot}90\\23{\cdot}10\end{matrix}\right\} \times 10 = 230{\cdot}00$	0·06440	5·88	5·900
	0·9860	$\left.\begin{matrix}20{\cdot}90\\20{\cdot}80\end{matrix}\right\} \times 10 = 208{\cdot}50$	0·05838	5·92	
4	0·7748	$\left.\begin{matrix}16{\cdot}40\\16{\cdot}60\end{matrix}\right\} \times 10 = 165{\cdot}00$	0·04620	6·00	6·100
	1·0865	$\left.\begin{matrix}24{\cdot}10\\24{\cdot}05\end{matrix}\right\} \times 10 = 240{\cdot}75$	0·06741	6·20	
5	1·0181	$\left.\begin{matrix}22{\cdot}90\\23{\cdot}30\end{matrix}\right\} \times 10 = 231{\cdot}00$	0·06496	6·38	6·430
	0·9522	$\left.\begin{matrix}22{\cdot}00\\22{\cdot}10\end{matrix}\right\} \times 10 = 220{\cdot}50$	0·06174	6·48	
6	1·0098	$\left.\begin{matrix}23{\cdot}80\\23{\cdot}85\end{matrix}\right\} \times 10 = 238{\cdot}25$	0·06671	6·66	6·605
	1·0429	$\left.\begin{matrix}24{\cdot}30\\24{\cdot}50\end{matrix}\right\} \times 10 = 244{\cdot}00$	0·06832	6·55	
7	0·9884	$\left.\begin{matrix}23{\cdot}70\\23{\cdot}80\end{matrix}\right\} \times 10 = 237{\cdot}50$	0·06650	6·74	6·765
	0·8228	$\left.\begin{matrix}20{\cdot}00\\19{\cdot}80\end{matrix}\right\} \times 10 = 199{\cdot}00$	0·05572	6·77	

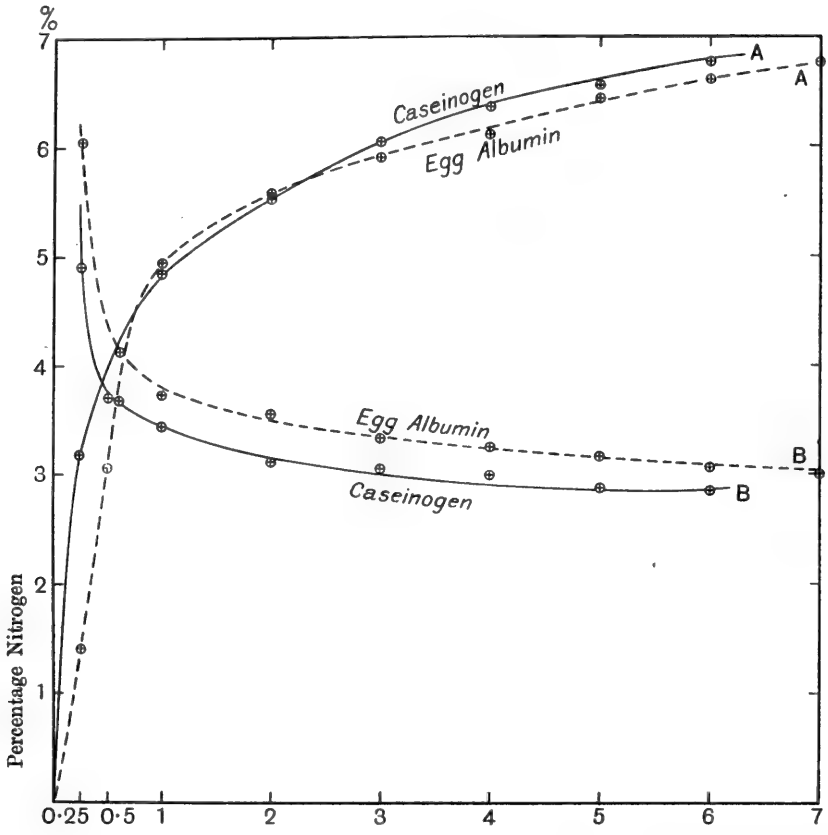

Fig. 2.

Curves showing: A. Rate of Liberation of Amino-Nitrogen.
B. Rate of Liberation of Nitrogen precipitated by Phosphotungstic acid.

IV. *Estimation of Nitrogen precipitated by Phosphotungstic Acid.*

A. *Caseinogen.*

75 cc. of the liquid were used.

Time of hydrolysis in hours	Weight of caseinogen, g.	Number of cc. 0·1 N HCl used (actual titration × 3·33)	Nitrogen equivalent, g.	Percentage nitrogen	Mean of duplicates
0·25	0·9994	35·34	0·04947	4·96	4·900
	1·0613	36·67	0·05131	4·84	
0·50	1·1179	29·17	0·04083	3·66	3·695
	0·9648	25·67	0·03594	3·73	
1	1·0627	26·16	0·03662	3·40	3·440
	1·0769	26·34	0·03687	3·48	
2	0·9842	21·50	0·03010	3·06	3·100
	1·1830	26·50	0·03710	3·14	
3	0·9467	20·83	0·02912	3·08	3·040
	0·9553	20·50	0·02870	3·00	
4	0·9026	19·00	0·02660	2·95	3·010
	0·9896	21·67	0·03034	3·07	
5	0·9373	19·00	0·02660	2·88	2·860
	1·0038	20·67	0·02894	2·84	
6	1·2733	25·67	0·03594	2·84	2·830
	1·0307	20·83	0·02916	2·82	

IV. *Estimation of Nitrogen precipitated by Phosphotungstic Acid.*

B. *Egg albumin.*

Time of hydrolysis in hours	Weight of egg albumin, g.	Number of cc. 0·1 N HCl used (actual titration × 3·33)	Nitrogen equivalent, g.	Percentage nitrogen	Mean of duplicates
0·25	0·9942	42·34	0·05922	5·96	6·055
	1·0085	44·34	0·06202	6·15	
0·50	0·8607	25·67	0·04018	4·17	4·110
	0·9895	28·67	0·03594	4·05	
1	0·8164	21·67	0·03034	3·72	3·735
	1·0576	28·34	0·03966	3·75	
2	0·8806	22·50	0·03150	3·58	3·550
	0·7636	19·16	0·02684	3·52	
3	1·0956	25·67	0·03594	3·28	3·320
	0·9860	23·67	0·03314	3·36	
4	0·7748	18·17	0·02541	3·28	3·260
	1·0865	25·00	0·03500	3·24	
5	1·0181	22·67	0·03174	3·14	3·185
	0·9522	22·00	0·03080	3·23	
6	1·0098	21·34	0·02988	2·96	3·025
	1·0429	23·00	0·03220	3·09	
7	0·9884	21·34	0·02988	3·02	2·990
	0·8228	17·34	0·02428	2·96	

Discussion of results.

I. *Ammonia.* In the very early stages of the hydrolysis the rate of liberation of ammonia is very rapid, and consequently the curves (Fig. 1) rise very steeply. This early period is then followed by one in which the ammonia comes off at a steady slow rate, so that the curves during this second period rise slowly. Finally the amount of ammonia liberated reaches a limit and the curve becomes a straight line parallel to the time axis.

The ammonia liberated on protein hydrolysis is always considered as being combined in the protein in the form of an amide, and Osborne and his fellow workers have shown that the amount of ammonia liberated on complete acid hydrolysis of a protein roughly corresponds to what would be obtained from the half amides of the two dibasic acids, glutamic and aspartic, contained in the protein. The results here obtained are in accord with such an explanation.

The experimental error in the estimation of ammonia is probably rather high, chiefly owing to the smallness of the quantities measured, but still there is no doubt that the curves indicate the rate at which the ammonia is set free.

II. *Humin Nitrogen.* From the mean of all the values obtained it appears that 0·31 per cent. nitrogen in the case of egg albumin and 0·29 per cent. nitrogen in the case of caseinogen is removed by the magnesia.

The values for 0·25 hour's hydrolysis are not included here as evidently in these cases the magnesium oxide carries down with it bodies other than humin substances.

III. *Amino-nitrogen.* The two curves (Fig. 2 *A*) obtained showing the rate of liberation of amino-nitrogen are very similar, although of course by no means identical. Moreover, they resemble in a striking manner the curves obtained for the rate of liberation of amino-nitrogen when the hydrolysis of the protein has been accomplished by means of an enzyme, such as trypsin. Hence the process of hydrolysis is the same whether the hydrolysing agent be a vigorous one, such as hydrochloric acid, or a slow one, such as trypsin. These curves for the amino-nitrogen illustrate very clearly the difference in composition and behaviour of the primary products of hydrolysis of the two proteins. The curves twice intersect, indicating that the rate of liberation of amino-nitrogen from the primary products of hydrolysis of the two proteins, twice changes.

In the first phase of the hydrolysis the amount of amino-nitrogen set free is greater in the case of caseinogen, indicating that more amino-acids and polypeptides are formed during this period in the hydrolysis of caseinogen than in that of egg albumin. In the second phase of the hydrolysis this state of affairs is reversed, showing that during this period the complex polypeptides derived from egg albumin are broken down more readily than those formed in the primary decomposition of caseinogen.

Finally after the second intersection of the curves the rate of liberation of amino-nitrogen is greater again in the case of caseinogen, proving that the simpler polypeptides derived from caseinogen are more easily broken down to amino-acids than those produced from egg albumin. This of course is what one expects when it is remembered that the complete hydrolysis of egg albumin takes longer to accomplish than that of caseinogen.

IV. *Nitrogen precipitated by phosphotungstic acid.*

Here again the two curves (Fig. 2 *B*) are very similar in form, but throughout the whole period investigated the caseinogen curve lies below that of the egg albumin.

In the early part of the hydrolysis there is a large amount of nitrogen in the phosphotungstic acid precipitate in both cases, showing that there

is a good deal of polypeptide nitrogen in the precipitate. Afterwards, however, the phosphotungstic acid contains very little polypeptide nitrogen, for from three hours onwards the curve runs nearly parallel to the time axis, though it does fall just a little.

The big difference between the values of the phosphotungstic acid nitrogen after 0·25 and after 0·5 hour's hydrolysis, is undoubtedly due to the decomposition of complex polypeptides into simpler ones, which are not precipitated by phosphotungstic acid. This point is brought out more clearly by drawing the curve total nitrogen accounted for, against time.

In the figures which follow no account is taken of the fact that the diamino-acids, which are precipitated by phosphotungstic acid, contain a certain amount of amino-nitrogen and hence some of the nitrogen is accounted for twice. This of course has no bearing on the point at issue.

Total Nitrogen accounted for in the above Estimations.

Time of hydrolysis in hours	Percentage ammonia (amide nitrogen)	Percentage amino-nitrogen	Percentage phosphotungstic nitrogen	Percentage humin nitrogen	Total percentage
A. *Caseinogen.*					
0·25	1·340	3·190	4·900	0·63	10·06
0·50	1·360	3·705	3·695	0·29	9·05
1	1·385	4·885	3·440	0·29	10·00
2	1·405	5·525	3·100	0·29	10·33
3	1·440	6·020	3·040	0·29	10·77
4	1·460	6·350	3·010	0·29	11·11
5	1·505	6·565	2·860	0·29	11·22
6	1·495	6·785	2·830	0·29	11·40
B. *Egg albumin.*					
0·25	0·940	1·400	6·050	1·13	9·52
0·50	0·975	3·045	4·110	0·31	8·44
1	1·050	4·930	3·735	0·31	10·02
2	1·100	5·540	3·550	0·31	10·50
3	1·150	5·900	3·320	0·31	10·68
4	1·200	6·100	3·260	0·31	10·87
5	1·245	6·430	3·180	0·31	11·16
6	1·260	6·605	3·025	0·31	11·20
7	1·260	6·765	2·990	0·31	11·32

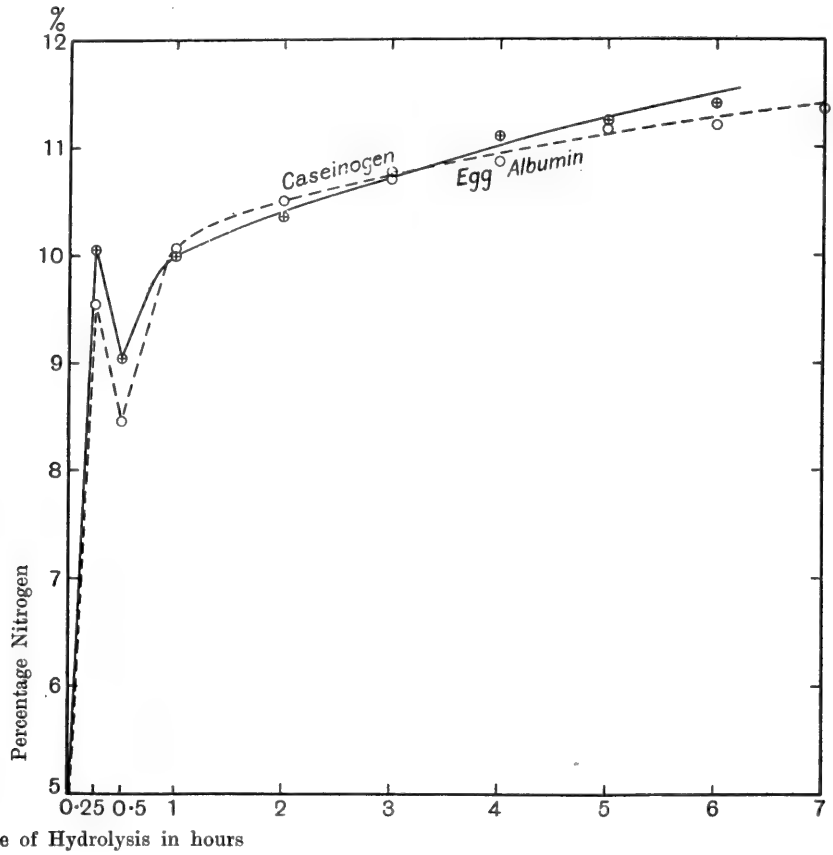

Fig. 3. Curves showing total nitrogen accounted for.

These figures and the curves (Fig. 3) plotted from them show that the total amount of nitrogen accounted for rises steadily with the time except in the case of 0·5 hour, where the total nitrogen accounted for is considerably less than that at 0·25 hour.

This proves definitely that the huge decrease in the amount of nitrogen precipitated by phosphotungstic acid at these two times of hydrolysis, is not nearly accounted for by the increase in the amount of amino-nitrogen found, or in other words it is clear proof that between 0·25 and 0·5 hour's hydrolysis some complex polypeptides which were precipitated by phosphotungstic acid after 0·25 hour's hydrolysis, have been broken down into simpler products, which are not precipitated by this reagent.

The fact that at no time is the whole of the nitrogen of the proteins accounted for shows that there are many polypeptides which are not precipitated by phosphotungstic acid, a point which is further emphasised by

the fact that the phosphotungstic nitrogen curves run nearly parallel to the time axis after a few hours' hydrolysis, i.e. long before the hydrolysis of the protein is nearly complete.

Another point which the phosphotungstic nitrogen curves indicate is that the diamino-acid content of caseinogen is less than that of egg albumin, a result which is in direct opposition to the published figures, viz.:

		Hart [1901] Caseinogen	Hugonenq et Galimard [1906] Egg albumin
Lysine	...	5·80 %	2·15 %
Arginine	...	4·84	2·14
Histidine	...	2·59	0·00

from which figures it appears that caseinogen should have 3·36 per cent. of diamino-nitrogen and egg albumin 1·10 per cent.

Although in the above experiments the hydrolysis was not complete, the curves show that it is impossible to obtain, on complete hydrolysis, results compatible with the published figures, and the indicated differences seem large enough to warrant a re-determination of the diamino acids in both cases.

Summary.

These results prove definitely that many of the simpler polypeptides are not precipitated by phosphotungstic acid, and moreover indicate that a definite point exists at which the more complex peptides are broken down into simpler ones, most of which are not precipitated by phosphotungstic acid, at which therefore it is advantageous to stop the hydrolysis so that the complex polypeptides may be isolated. The isolation of such bodies, and the study of their properties, it is my intention to essay in the near future.

Moreover the experiments here described emphasise the difference in the constitution of caseinogen and egg albumin and the consequent difference in composition of their primary products of hydrolysis.

Finally, I wish to thank Professor T. B. Wood for suggesting this work, and to say how much I appreciate the interest he has shown in its progress.

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XXIV. DIAGRAMMATIC CO-ORDINATION OF PHENOMENA RELATING TO AGGREGATION OF SOLS.

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The published investigations of the following phenomena embrace a considerable portion of the facts so far accumulated on the causes inhibiting or actuating the aggregation of sols, especially when they are mixed with proteins and electrolytes:

1. *The action of electrolytes on sols*—causing the running together of sol particles when added to the sol in sufficient concentration.

2. "*Protection*"—the property of proteins and certain other substances in preventing the aggregation of sols by electrolytes.

3. *Change of sign of the electrostatic charge on the protected sol particle.*

4. *Non-coincidence of point of maximum flocculation and point where sign of observed particle changes.*

5. *Change of sign of charge on the protein at its isoelectric point.*

6. *Mutual precipitation of dissimilarly charged colloids* in solutions free from electrolytes, including the particular case where one of the colloids is amphoteric and has the properties of "protection," e.g. is of a protein nature; also the solubility of the precipitate formed in excess of either constituent.

7. "*Irregular series*" (Bechold) and "*Pre-zone phenomenon*" (Buxton).

8. "*Reversible and irreversible aggregation.*"

Up to the present these phenomena have been generally investigated one at a time. Each worker has naturally chosen experimental conditions particularly suitable for the study of one phenomenon only. Little or no co-ordinated work has been done, linking up on a quantitative basis the known facts, and demonstrating them as parts of a well-ordered scheme of things, easy to remember, and, on the surface, easy to understand.

The plotting of results, obtained in this class of work, in chart form, undertaken primarily for my own use, has proved so helpful to me in

following out these relationships that I feel in a position to recommend them for general use in expressing the results of experiments of this type.

In working with a very finely divided oil emulsion, the particles of which were so small that, without misapplying the term, it could be called an "oil sol," I found, in common with previous experience, that the addition of hydrochloric acid in sufficient strength caused the particles to run together and the oil to separate. The presence of a sufficient quantity of gelatin, however, prevented this from happening; the sol was "protected" in this case. When the gelatin was in certain concentrations, not too small and not too great, the oil particles aggregated immediately if just the right amount of acid was added, but not if the acid was too strong or too weak.

This seemed to point to some new and peculiar phenomenon where the gelatin acted as an "activator" to the acid, for in this case the acid was far too weak to aggregate the sol alone. It was not till later that it was seen that this fitted into the general scheme of things and was capable of expression in terms of things known, and that the invention of a new "phenomenon" and a corresponding vocabulary was unnecessary. In other cases where gelatin of a certain strength caused immediate aggregation of the oil in the presence of a small quantity of acid incapable of doing this alone, it was found that all strengths of acid greater than this brought about the same result.

To simplify the matter of making and studying these mixtures it was decided that each should contain 2 cc. of oil sol, 2 cc. of gelatin solution and 2 cc. of hydrochloric acid solution. Rows of these mixtures were put up in test tubes. In any one row the 2 cc. of acid placed in each tube was of the same strength throughout; the concentration of the 2 cc. of gelatin solution added varied from tube to tube. The strength of acid used was changed from row to row.

As very dilute solutions were sometimes used it was found necessary from the outset to express their concentrations as powers of ten, either of actual concentration or normality. For instance the strength of a gelatin solution was expressed in terms of its concentration. A 1 in 10 solution was expressed as "gelatin 10^{-1}," for example, and a 1 in 15,000 solution as $10^{-4\cdot2}$. Similarly 0·001 N hydrochloric acid was written 10^{-3} N HCl.

After the mixtures had been made two hours they were examined carefully, and notes made of those which showed to the eye no change of state of the sol, those in which partial aggregation had occurred, and those in which the separation of the oil was complete.

Observations in the electric field using the microscope method showed

that the oil particles in some of these mixtures were positively and in others negatively charged. The sign of the charge did not depend upon the state of aggregation—either charge was observed in tubes containing mixtures which did not aggregate just as in those that did.

When the results of these two sets of observations were expressed in tabular form little that was intelligible could be made of them; in the form of a chart they are all summarised in Fig. 1.

Aggregation Diagram No. 1.

It will be remembered that each mixture consisted of 2 cc. of oil sol, 2 cc. of gelatin solution and 2 cc. of hydrochloric acid. As ordinates are expressed negative exponents of normality of the hydrochloric acid put into the tube; as abscissae the negative exponent of the concentration of gelatin. The tubes were considered one at a time and a mark plotted on the chart at the point corresponding with the concentration of acid and gelatin in the tube indicating (1) that no change had taken place in the distribution of

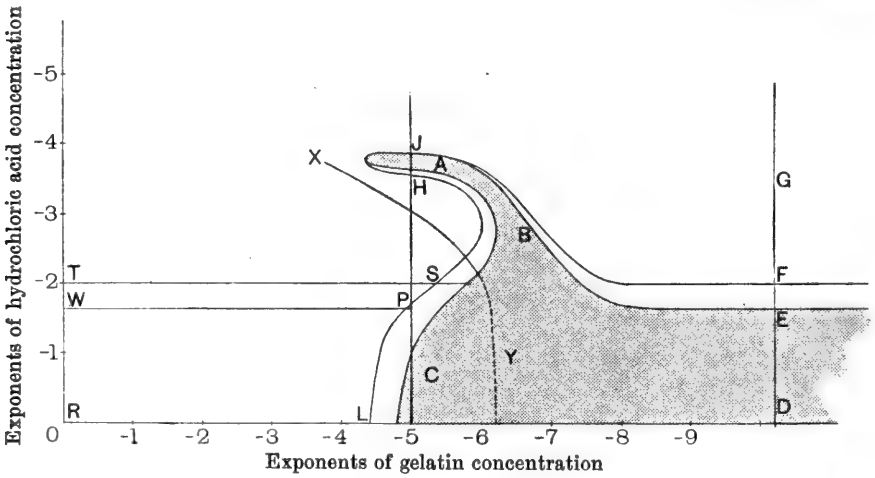

Fig. 1. Aggregation diagram No. 1. Oil sol-gelatin-hydrochloric acid.

the oil particles, (2) that complete aggregation and separation of the oil had occurred, or (3) that aggregation was only partial.

The sign of the oil particles, whether aggregated or not, was indicated by a small + or −. Lines were now drawn on the diagram by the help of these distinguishing marks. The first, *LHJF*, enclosed all points where aggregation, whether partial or complete, was observed. Another, *CABE*, enclosed all points where separation of the oil was complete.

The line *XY* was drawn through all points where the oil particles appeared to have no electric charge; that is, they did not wander in the electric field. In all mixtures represented by points to the right of this line, the particles were negatively charged. All points to the left of this line correspond to mixtures in which particles were positively charged.

The chart—called for convenience an "aggregation diagram"—represents the state of aggregation and sign of the dispersed particle, two hours after mixing, of every possible mixture of equal volumes of oil sol, gelatin solution and hydrochloric acid. Mixtures stronger in HCl than 0·33 normal were not plotted in the present instance simply to avoid unnecessary complication of the diagram.

At this stage it will be well to examine this chart with reference to the list of phenomena given at the beginning of this paper.

1. *The action of electrolytes on sols.* The action of electrolytes in the absence of protein is seen in the extreme right of the diagram. In the particular case shown in Fig. 1 the protein in a dilution of one in one hundred million or more has no effect. The lines bounding the aggregation area become parallel to the abscissae, and may be produced to infinity to the point where the protein is infinitely dilute. The ordinate *DEFG* represents, therefore, the behaviour of mixtures of 2 cc. sol, 2 cc. water and 2 cc. of every strength of electrolyte solution, in this case HCl. No aggregation occurs with extremely dilute acid, but when the strength of acid used is 10^{-2} N a slight separation of the sol occurs. As the strength of acid increases the amount of sol separation increases until at $10^{-1\cdot6}$ N and all concentrations above this it is described as "complete."

In the case of an electronegative sol and an electrolyte with a polyvalent cation the ordinate *DEF* would be much shorter—a result obtained equally if the sol were electropositive and the electrolyte contained a polyvalent anion.

At present the actual mechanism of electrolyte precipitation of sols is not very well understood. It is hoped that in the course of this work some phenomena may be observed which can be co-ordinated with the facts already known about the conditions determining the stability or otherwise of sols.

2. "*Protection.*" Any point in the area *WPLR* corresponds to a mixture in which the gelatin has prevented the acid from aggregating the oil sol. Enough acid is present to bring this about completely if the gelatin were not present. It may therefore be referred to as the "area of complete protection against aggregation." In the same way the area *WPST* is "the area of complete protection against partial aggregation," for in the mixtures

the behaviour of which is represented by points in this area the acid alone would only partially aggregate the sol in two hours.

Areas of "partial protection against partial aggregation" and "partial protection against complete aggregation" are to be found in the diagram and may some day become matters of study.

3. *The change of sign of the "protected" sol particle.* It is interesting to note that the "area of protection" lies entirely inside the area where the sign of the sol particles, or rather the sol-gelatin complexes, is positive. Also, and this is shown better in Fig. 2 where mastic sol was used, it is possible to have a sol protected by gelatin in a mixture containing as much as one-third of its volume of N HCl, while a mixture exactly similar except that the acid is 6000 times more dilute, flocculates immediately. A result of this kind was observed by Mines [1912] using a gold sol.

4. *Non-coincidence of point of maximum flocculation and point where the sign of the observed particle changes.* The type of flocculation which may be observed in extremely dilute acid concentration if the concentration of the protein be adjusted with great care, is remarkable in that no change of sign of the electronegative sol particle necessarily takes place.

In Fig. 1 the ordinate *CPHJ* passes through points representing a series of mixtures in which are placed say 2 cc. of oil sol, 2 cc. gelatin 10^{-5}, and 2 cc. of hydrochloric acid decreasing in concentration from tube to tube. From normal acid to decinormal acid (10^{-1}) complete flocculation is observed, from there to $10^{-1\cdot7}$ the flocculation is incomplete, and as far as can be made out in solutions containing so much electrolyte the sign of the visible particles is +. With acid strengths from $10^{-1\cdot7}$ to $10^{-3\cdot5}$ there is no change whatever visible in the state of aggregation of the sol, but at HCl 10^{-3} N no charge can be detected on the particles, while at $10^{-3\cdot1}$ and all tubes following, which contain weaker acid, the sign of the particles is −. Following on the series; partial separation is observed with acid $10^{-3\cdot6}$; at $10^{-3\cdot7}$ and $10^{-3\cdot8}$ the separation is complete; at $10^{-3\cdot9}$, 10^{-4} and onwards no change in sol aggregation can be seen.

The change of sign of the sol particles with constant gelatin concentration and diminishing acid takes place outside either of the ranges where aggregation occurs; and this assumes a particular interest when it is remembered that globulin suspensions are negatively charged at the point of maximum flocculation, and on increasing the acid concentration they are well dispersed before the visible particles are electrically neutral. This observation recorded by Chick [1913] I have confirmed. The figures given in the paper referred to are $H^{\cdot}$ concentration $32{\cdot}10^{-7}$ for the point of maximum flocculation [cf.

Michaelis and Rona, 1910]. At H· concentration $748 \cdot 10^{-7}$ the sign is still −, changing to + at $1140 \cdot 10^{-7}$.

5. *Change of sign of the protein at its isoelectric point.* A line may be drawn through all points having H· ion concentration equal to the isoelectric point of the protein. This will divide the diagram into two parts representing mixtures containing + charged and − charged protein respectively.

6. *Mutual precipitation of dissimilarly charged colloids.* Biltz [1904] in his classical confirmation of earlier work by Picton and Linder [1897], and Lottermoser [1901] expressly state that *in the absence of electrolytes* oppositely charged colloidal solutions precipitate one another from solution when mixed in certain definite proportions, and that the precipitate is soluble in excess of either constituent. Whether this limiting condition is ever fulfilled in practice is questionable; but still the mixtures containing only hydrochloric acid $1/3 \cdot 10^{-3}$ N HCl may be considered in the class of mixtures of colloidal solutions to which Biltz referred. Following the abscissa representing a row of mixtures made by mixing say 2 cc. acid 10^{-3} N, 2 cc. oil sol and 2 cc. gelatin solution, decreasing in concentration from tube to tube, it is seen that mutual precipitation only occurs within a certain range of gelatin-hydrosol ratios. The large number of the variables from tube to tube, however, all of which affect aggregation, makes the analysis of this phenomenon in the present state of our knowledge very difficult, and postpones the complete understanding of the curious shape of the aggregation area.

7. "*Irregular Series,*" and "*Pre-zone phenomenon.*" The first of these terms was used by Bechold [1904] to describe the results obtained by aggregating various sols and bacterial suspensions with solutions of ferric and aluminium chlorides. Buxton and Rahe [1910] saw in these experiments the demonstration of the action of the acid solution when concentrated, and the precipitating action of a colloidal solution in greater dilutions. In order to avoid confusion they referred to their numerous flocculation experiments in which irregular series occurred from other causes as demonstrations of the "pre-zone phenomenon."

8. *Reversible and Irreversible Aggregation.* Sols such as gold, oil and mastic are referred to as irreversible hydrosols because when flocculated by electrolytes they do not disperse again when the electrolytes are removed by dialysis, and, when flocculated by traces of acid, the reversal of this effect is not obtained by addition of corresponding traces of alkali.

In the aggregation area of Fig. 1 there may be recognised two types of flocculation. To the extreme right, where separation of the sol has occurred

in the absence of protein, the aggregation is not reversible by adding alkali. At *JH* where the protein plays an all-important *rôle* in the flocculation, the addition of a trace of alkali results at once in the almost complete reversal of the phenomenon. In other parts of the aggregation area it would appear as if both the reversible and irreversible type of aggregation were found varying in relative amount with the position on the diagram. When the sol is of oil the former type shows a grape bunch appearance under the ultramicroscope, while the latter aggregates are single drops of oil formed by the coalescence of the minute particles. With gold sol-gelatin-HCl mixtures both types are also readily observed.

With mastic-gelatin-HCl mixtures these phenomena may be observed with even greater readiness. If care be taken to adjust the proportions so that aggregation occurs with a maximal quantity of gelatin and traces only of hydrochloric acid, the electronegative aggregates are, as would be expected from the diagram, dispersed by traces of alkali or acid. The reversibility of the flocculation diminishes on standing just as globulin suspensions after a time become insoluble. When in addition it is found that the flocculae are dispersed and not, as would be expected, aggregated further by the addition of a small quantity of sodium chloride, the analogy to euglobulin appears so striking that the possibility of its being a complex physically comparable with the mastic-gelatin or oil-gelatin complexes here dealt with cannot be overlooked[1].

Aggregation Diagrams Nos. 2, 3 and 4 a.

After this preliminary description of the method of investigation pursued in coordinating various phenomena connected with the aggregation of sol-protein complexes, three particular experiments may be considered. These refer to the behaviour of mixtures of equal volumes of a particular *mastic* sol, gelatin and an electrolyte solution, and may be called for convenience experiments 2, 3 and 4. This particular sol I found more easy to work with than an oil sol, for the various stages of aggregation from a slight clouding of the blue opalescence in twenty-four hours to an immediate and complete flocculation of the whole of the material could be picked out more or less accurately and recorded.

The difference between the three experiments lay in the nature of the

[1] The flocculated complex made from pseudoglobulin (horse) and a sol of the lipoid from euglobulin (horse) resembles euglobulin still more closely. Even after several days' standing it is instantly and completely dispersed by traces of alkali or acid.

electrolyte constituents of the mixtures. In all three experiments the electrolyte varying in amount from row to row was hydrochloric acid. In experiment 3, however, every mixture was in addition 0·011 N with respect to sodium chloride: in experiment 4, every mixture was in addition 0·011 N with respect to sodium acetate. When the mixtures had been prepared and

Mastic-gelatin-electrolyte Aggregation Diagrams.

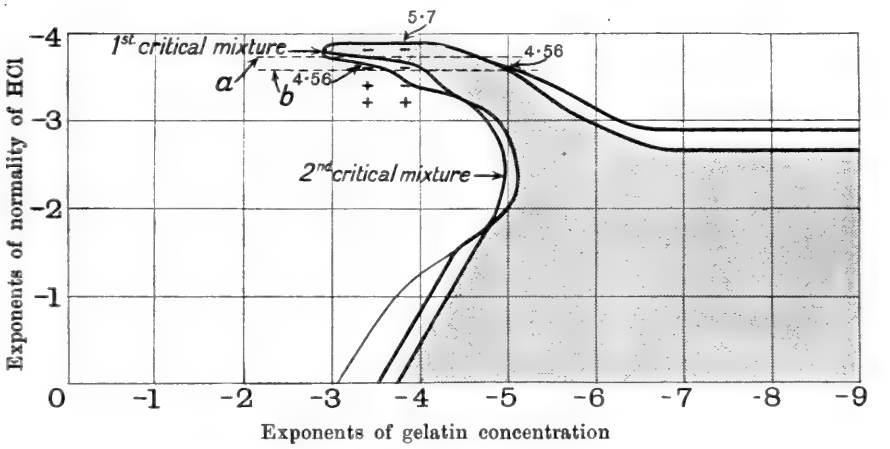

Fig. 2. Aggregation diagram No. 2. Electrolyte HCl in varying amounts.

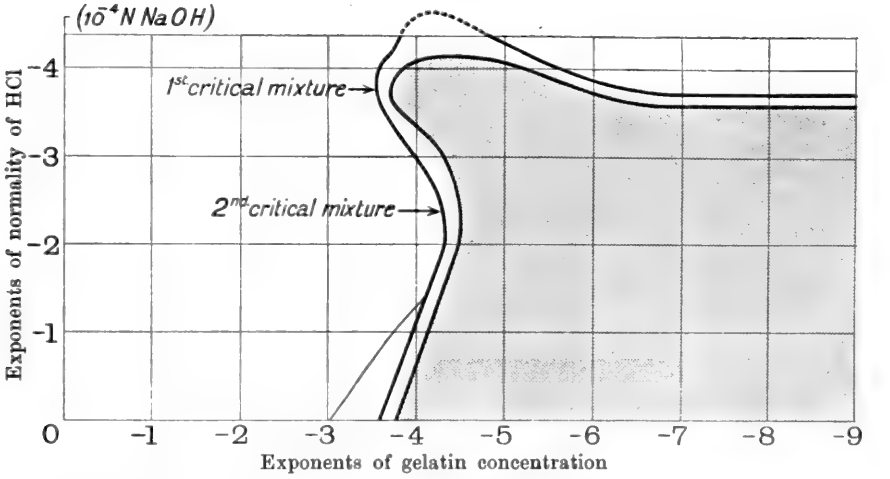

Fig. 3. Aggregation diagram No. 3. Electrolyte HCl in varying amounts + 0·033 N sodium chloride.

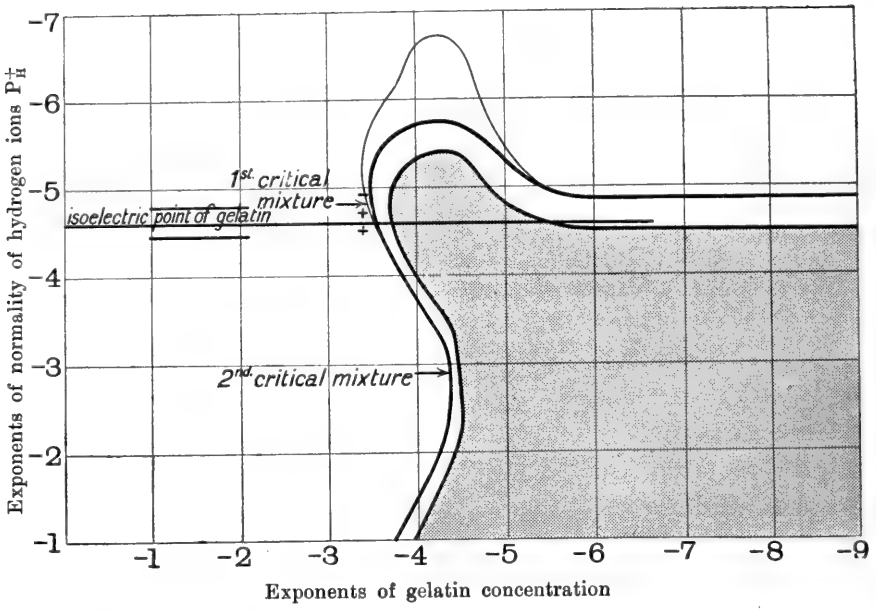

Fig. 4. Aggregation diagram No. 4A. Electrolyte HCl in varying amounts +0·033 N sodium acetate.

NOTE.—Each mixture contains equal volumes of the three constituents. The concentrations of gelatin and HCl whose exponents are plotted in these diagrams are the concentrations used to make the mixtures. To find the actual concentration in a mixture add log 3 = ·477 to the negative exponent. In Fig. 4 the H· ion concentrations, plotted as ordinates, refer to the H· ion concentrations of the mixtures.

allowed to stand 24 hours, one aggregation diagram for each experiment was plotted in which negative exponents of acid concentration were ordinates and the negative exponents of the gelatin concentration abscissae. The sign of the electrostatic charge on the observable particles was also recorded where expedient. The diagrams from experiments 2 and 3 are reproduced in this paper. In each the stippled area represents complete flocculation: the space between the two thick lines just outside this area includes all points representing mixtures which show considerable aggregation of the particles in twenty-four hours but do not leave perfectly clear supernatant fluid above the flocculae. The areas bounded by a thin line represent slight aggregation only. The diagram from experiment 4 which is not necessary for discussion of these results has not been reproduced.

The next step was to determine the hydrogen ion concentration of every mixture in the three experiments.

In experiments 2 and 3, the rows containing acid less than $10^{-3\cdot4}$ N

presented very considerable difficulties. The reaction inertia and the hydrogen ion concentration were low, and in those tubes to which no sodium chloride was added the conductivity was also low. By working with sufficient care accurate results were obtained in tubes where no flocculation had occurred but in the remaining tubes the results were of such a nature that they are best considered separately and recorded with certain reservations.

In experiment 4 the mixtures all lent themselves readily to accurate P_H^+ determinations and so a second aggregation diagram could be prepared in which the ordinates were the negative exponents of hydrogen ion concentration. This diagram 4A has been reproduced (Fig. 4). A comparative study of this diagram and the two preceding is instructive.

It is possible to regard diagram 3 as being derived from diagram 4A by submitting the ordinates to a process similar to that of opening out a telescope, the telescope having a large number of joints which do not slide out necessarily at the same rate. This suggests that if the influence of changing hydrogen ion concentration be ignored, sodium chloride and sodium acetate in strength 0·011 N behave very similarly in relation to the flocculation of mastic-gelatin mixtures. A similar relationship between diagrams 2 and 4A does not hold.

Hydrogen ion determinations for certain important mixtures in experiments 2 and 3 were made quite satisfactorily and a few are recorded on the diagram 2, but in view of the labour involved, corresponding diagrams, 2A and 3A, in which the ordinates would have been hydrogen ion concentrations, could not be prepared complete for the present paper (see p. 182).

In experiment 4 it was found that throughout each row of mixtures the $H^{\cdot}$ ion concentration did not vary appreciably, that is to say, the mixtures along the row were *isoxyntic* (i.e. of equal $H^{\cdot}$ ion concentration) in spite of a variation in gelatin content from $10^{-3\cdot48}$ to infinite dilution. At this stage certain relationships can be established by comparing diagrams 2, 3 and 4A.

(1) *Relationships between composition and possibility of flocculation.*

(*a*) In each of the three experiments there is one mixture which is just on the verge of flocculation and in which flocculation would not have occurred at all if the gelatin concentration had been a little greater. Neither would it have flocculated if a little more acid or a little less acid had entered into its composition. Its reaction is invariably in the region of the isoelectric point of gelatin. It may be conveniently called the "1st critical mixture" of the system mastic-gelatin-electrolyte. In experiment 2 the "1st critical mixture" contains five times as much gelatin as in experiments 3 and 4.

(*b*) It is seen that at the upper limits of HCl concentration flocculation is not appreciably influenced by the presence of 0·011 N NaCl. The effect of this concentration of sodium chloride is shown markedly, however, when the minimal concentrations of hydrochloric acid at which complete flocculation of mastic alone can be observed are examined.

In experiment 2 if the mixture made from HCl $10^{-2\cdot4}$ N, gelatin $10^{-5\cdot0}$ and mastic be considered it is seen that partial flocculation has occurred. If stronger acid with the same gelatin solution, or weaker acid with the same gelatin solution, or weaker gelatin with the same acid strength, were used to make the mixture flocculation would be complete.

Similarly in experiment 3 if the mixture made from HCl $10^{-2\cdot4}$ N, gelatin $10^{-4\cdot4}$ and mastic be considered the same fact is evident. More acid, or less acid, or less gelatin, other constituents of the mixture being left unaltered, will give mixtures flocculating to the full extent. And, again, in experiment 4 there is one mixture made from gelatin $10^{-4\cdot4}$ to which the same considerations apply.

The three mixtures, one in each experiment, which have this curious relationship to those contiguous to them may also be called "critical mixtures." To distinguish them from those of the type described in (*a*) they may be called "2nd critical mixtures."

Critical mixtures whether "1st" or "2nd" have this in common—a slight increase in gelatin concentration would inhibit flocculation completely. They differ in that a "1st critical mixture" is not flocculated by more acid or less acid, while a "2nd critical mixture" is.

The three "2nd critical mixtures" considered—one from each of the experiments 2, 3 and 4—were found experimentally to have the same hydrogen ion concentration $P_H^+ = 2\cdot9$.

They are made from gelatin $10^{-5\cdot0}$ when electrolyte is not added and $10^{-4\cdot4}$ when sodium chloride or acetate is present, i.e. those which are 0·011 N with respect to sodium salt contain four times as much gelatin as the other. It is suggested that the increased thickness of coating of gelatin (at $P_H^+ = 2\cdot9$ in all three cases) resulting from the inclusion of four times as much gelatin per unit volume in the mixture, balances the flocculation effect of 0·011 N sodium ions. On p. 186 an explanation of the existence of the "2nd critical mixture" has been attempted.

In Figures 2 and 3 it is seen that as the acid concentrations increase towards the foot of the diagram, the effect of the added sodium chloride in experiment 3 becomes less and less noticeable. The apparent discrepancy of diagram 4A in this respect disappears when the nature of the ordinates

is recognised. Diagrams 2A, 3A (not reproduced) and 4A are identical towards the base line—$P_H^+ = 10^{-0}$ (normal $H^{\cdot}$ ion concentration).

Relationships between flocculation area and (a) the isoxyntic line corresponding to the isoelectric point of gelatin; (b) the line passing through all points where the observed particles are electrically neutral.

Experiment 4.

In experiment 4 the following determinations were made with a transport apparatus similar in design to that used by Michaelis [1909]. It was made of glass of one cm. bore all in one piece. Rubber was dispensed with entirely. Non-polarisable electrodes, Cu in $CuCl_2$, and Ag in N NaCl, were employed, and many test experiments were made which left no doubt as to the accuracy of the results.

Fluid in side tubes—32 cc. water + 30 cc. acetate mixture + 30 cc. 5 per cent. alcohol.
Fluid in middle tube—30 cc. gelatin $10^{-3.6}$ + 30 cc. acetate mixture + 30 cc. mastic sol.

Composition of acetate mixture	P_H^+ of middle tube	Direction of migration	
3·3 cc. N sod. acetate + 2·2 cc. N HCl + water to 100 cc.	4·28	+	Isoelectric
,, ,, 2·0 ,, ,, ,,	4·47	+	point of
,, ,, 2·0 ,, ,, ,,	4·52	+	gelatin
,, ,, 1·5 ,, ,, ,,	4·75	+	4·6
,, ,, 1·2 ,, ,, ,,	4·92	−	(Michaelis)
,, ,, 1·2 ,, ,, ,,	4·92	−	
,, ,, 1·0 ,, ,, ,,	5·05	−	

The meniscus of the moving mastic suspension remained sharp and could be observed with great accuracy. After about an hour the direction could be ascertained definitely even in the solutions $P_H^+ = 4{\cdot}75$ and $P_H^+ = 4{\cdot}92$. Gelatin concentration $10^{-3.6}$ was used throughout these determinations because it was the weakest solution that could be used without involving flocculation.

On the diagram + and − signs represent the results of some of the determinations just described. It is remarkable to note that the observed particles in the 1st critical mixture are almost without any electric charge. The isoelectric point of gelatin is possibly slightly more acid than this, indicating that the uncharged particles result from a combination of neutral or possibly slightly negative gelatin and the electronegative nucleus.

Experiment 2.

In the 1st critical mixture of experiment 2, in which electrolytes were excluded as far as possible, the effect of the electronegative nucleus on the charge of the mastic-gelatin complex will be seen to be much more marked.

As previously stated a considerable time was spent in determining under the best experimental conditions the hydrogen ion concentrations of certain mixtures in experiment 2 which had not flocculated. In every case a series of mixtures was made simultaneously in which the position of the flocculation area was verified and then the non-flocculated mixtures of this same series examined with respect to their hydrogen ion concentration and the sign of the electrostatic charge of the observable particles by the microscope method. The mixtures were prepared and the observations repeated many times with concordant results.

Flocculated mixtures were also examined in the same way. The particles were invariably electronegative but the hydrogen ion concentrations did not furnish results sufficiently trustworthy to be considered in this connection.

The results of the observations are given below. Some are recorded on the diagram.

Mixtures of equal volumes of mastic sol, gelatin solution and HCl taken from Exp. 2.

Exponent of gelatin concentration used	Exponent of normality of HCl used	E.M.F.	P_H^+	Sign of observed particle
−3·6	−3·0	0·455	3·53	+
,,	−3·4	0·490 : 0·488	4·12	±
,,	−3·6	0·515 : 0·515	4·56	−
,,	−3·9	0·580	5·70	−
−4·0	−3·0	0·455	3·54	+
,,	−3·4	0·491 : 0·485	4·10	−
,,	−3·6	0·502 : 0·507	4·40	−
,,	−4·0	0·534 : 0·544	5·00	−
−4·4	−3·0	0·455	3·54	+
,,	−3·4	0·492	4·18	−
−5·0	−3·5	0·513	4·54	−
,,	−3·6	0·515	4·56	−

From the evidence of these figures the following statements may be made:

(*a*) In the "horn" like part of the flocculation area the observed particles are negatively charged. In mixtures, just below this, which have not flocculated, the particles are also negatively charged. Comparison with aggregation diagram No. 1 where an oil sol was used shows that the

line XY passing through all mixtures where the observed particles of the diagram are electrically neutral does not pass so close to the "horn" like part of the flocculation area as in diagram 2. The other striking difference between the two diagrams is the relative stability of the two sols to $H^{\cdot}$ ion concentration. It is suggested that these two phenomena have a common factor.

(*b*) *Passing upwards through the "horn"* along a series of mixtures made from gelatin solution of concentration $10^{-3\cdot6}$ *the* $H^{\cdot}$ *ion concentration is changing very rapidly,* viz. from $P_H^+ = 4\cdot56$ at acid $10^{-3\cdot6}$ N to $P_H^+ = 5\cdot70$ at acid $10^{-3\cdot9}$.

It is therefore established that the isoxyntic lines P_H^+ 4·45 and P_H^+ 4·80 representing the upper and lower limits of the isoelectric range of gelatin as measured by Michaelis, either both pass below the "horn" or that the upper P_H^+ line 4·8 passes through the lower part of the "horn."

The precise positions of these lines cannot be determined with the same accuracy as in experiment 4, so that they are represented by dotted lines in the diagram labelled *a* and *b*.

(*c*) Mixtures which, if the available data with regard to the isoelectric point of gelatin be correct, would show migration of gelatin to the cathode nevertheless contain observable particles carrying a definite negative charge.

These results from experiments 4 and 2 may be summarised as follows:

It is established, within the limits of experimental error, that, in the presence of 0·011 N sodium acetate, the 1st critical mixture contains electrically neutral particles, and has the hydrogen ion concentration at which no wandering of gelatin can be detected in transport experiments. The sign of the particle in this and contiguous mixtures is, therefore, that of its coating: the electronegative character of the mastic nucleus does not manifest itself at all.

In the absence of electrolytes except minimal traces of HCl, and gelatin of such concentration that when the HCl content is increased the line representing the series of mixtures passes downwards through the middle of the "horn" like projection of the aggregation area, the above coincidences do not occur. Instead the following phenomena occur in the following order:

(1) No aggregation; gelatin negative; all observable particles negative.

(2) Aggregation; gelatin negative; all observable particles negative.

(3) Aggregation; gelatin sign not discoverable by transport experiments; all observable particles negative.

(4) No aggregation; gelatin positive; all observable particles negative.

(5) No aggregation; gelatin positive; all observable particles positive.

The isoelectric range of gelatin was determined in the presence of sodium acetate and hence the above results, in so far as they depend upon this determination, are open to discussion. It is extremely probable, however, that, in the absence of added electrolyte, the electrically neutral particle is made up of an electropositive gelatin coating and an electronegative mastic nucleus. On the other hand it may be stated definitely that the particles in the 1st critical mixture and all other mixtures represented by points in the "horn" like part of the aggregation area are electronegative. All mixtures containing electrically neutral particles are relatively more acid, and show no sign of aggregation.

I can offer no explanation of the results. Every endeavour has been made to discover faults in the observations.

Liberation of alkali from the glass of the microscopic cell was at first considered a possible source of error, but a considerable number of experiments showed that this did not account for the effects observed.

Theoretical discussion of the observed results.

The existence of the "horn" in Figs. 1 and 2, or rather its suppression in Figs. 3 and 4, due to the presence of 0·011 N sodium chloride or sodium acetate, is the manifestation of a phenomenon the explanation of which may for the present be referred to the conjectures concerning the salt dispersion of globulin [Hardy, 1905; Mellanby, 1905; Chick, 1913], denaturated serum protein [Chick and Martin, 1912] and methylimino compounds [Schryver, 1910].

Since, in experiments 3 and 4, 0·011 N sodium salt was present, while it was not present in experiment 2, the series containing gelatin $10^{-5\cdot0}$ in experiment 2 is taken as being comparable with those containing $10^{-4\cdot4}$ in experiments 3 and 4. The reasons for this are stated on p. 180. These series are now to be considered first of all independently, and then with regard to their mutual relationship.

Experiment 2. *Series of mixtures made from gelatin* 10^{-5} *and hydrochloric acid of increasing concentration.*

The facts observed are that with acid from infinite dilution to $10^{-3\cdot7}$ no flocculation occurs; with acid $10^{-3\cdot6}$ there is a partial flocculation; with all stronger acids flocculation occurs except with one strength of acid, namely,

$10^{-2\cdot4}$ N, which is on the verge of flocculation, and is the "2nd critical mixture."

Also, the P_H^+ of the mixture which is on the verge of flocculation made from $10^{-3\cdot6}$ acid is 4·56, and the observed particles of this mixture and all the neighbouring mixtures are negatively charged. It will be remembered that the isoelectric range of gelatin is $10^{-4\cdot45}$ to $10^{-4\cdot8}$.

Among the phenomena known to be concerned in this flocculation or non-flocculation are the surface tension effects at the surfaces of the particles. These are partly of an electrical nature with purely surface effects as concomitant factors. Practically the whole of the gelatin present is in this series on the surfaces of the mastic particles—a point probably capable of demonstration by differential filtration experiments. The series may be divided into two parts. The first consisting of mixtures containing acid from infinite dilution to say $10^{-2\cdot9}$ N HCl and the latter from this strength to normal acid.

In the first part of the series it is known that hydrogen ions are not present in sufficient quantity to flocculate mastic at all even when not coated with gelatin.

This factor can therefore be put aside for the moment and purely surface effects—electrical and otherwise—considered. Gelatin is present in the solution in three forms—just as is any other electrolyte. In accordance with the mathematical investigations of Michaelis and others, the proportion of it which is un-ionised is a maximum at its isoelectric point, and the proportions which are negatively charged and positively charged respectively are equal in amount. If the solution be made more acid the concentration of positively charged gelatin increases. On the alkaline side of the isoelectric point electropositive gelatin ions also exist in concentrations, compared with the total amount of the gelatin present, diminishing with diminishing acidity.

Their existence is necessitated on theoretical grounds but they cannot be detected by the direct transport method. Is it not possible that the flocculation observed on the alkaline side of the isoelectric range established by transport observations is due to traces of electropositive gelatin—and, in fact, that the flocculation of mastic is a very sensitive indicator of gelatin in this condition? The particles are observed to be electronegative—the effect of the electronegative mastic nucleus is not abolished—but it is suggested that the tendency to minimise the surface energy of the system through flocculation becomes operative in spite of electrostatic repulsion when this repulsion is diminished through the acquisition by the particle

of a film of gelatin originally electropositive. The behaviour of the series of mixtures all made from gelatin 10^{-5} and HCl acid from infinite dilution to $10^{-2\cdot9}$ N is seen to be in accordance with the above hypothesis. Continuing the same series from acid $10^{-2\cdot9}$ N to normal acid the phenomenon of the "2nd critical mixture" is observed.

Throughout these mixtures purely surface effects may be taken as being overwhelmed by phenomena of an electrical nature. There are two factors involved. The *first*, which did not come into consideration over the first half of the series, is the flocculating effect of the hydrogen ions on the gelatin-coated mastic particles. The *second* is a factor having the opposite effect—the increasing electropositiveness of the coat, a quantity which may be referred directly to gelatin cation concentration. Adopting the nomenclature used by Sörensen [1912] and one of the equations deduced from fundamental principles quoted by him—

$$\rho = \frac{1}{1 + \frac{k_a}{H^{\cdot}} + \frac{k_b H^{\cdot}}{k_w}},$$

an expression may be obtained giving the rate of change of gelatin cation concentration at any $H^{\cdot}$ ion concentration,

$$A^{\cdot} = \frac{k_b x}{OH'} = \frac{k_b A \rho}{OH'} = \frac{k_b A \rho H^{\cdot}}{k_w}$$

$$= \frac{k_b A H^{\cdot}}{k_w \left(1 + \frac{k_a}{H^{\cdot}} + \frac{k_b H^{\cdot}}{k_w}\right)}.$$

Differentiating with respect to $H^{\cdot}$,

$$\frac{dA^{\cdot}}{dH^{\cdot}} = \frac{k_w k_b A H^{\cdot} (H^{\cdot} + 2k_a)}{(k_w k_a + k_w H^{\cdot} + k_b H^{\cdot 2})^2}.$$

Putting the dissociation constants and A, the total gelatin concentration, equal to unity and substituting for $H^{\cdot}$ 0·01, 0·10, 0·20, 0·25, 0·33, 0·50, 1·00, 1·50, 2, 10, the values for the expression are 0·02, 0·19, 0·28, 0·32, 0·37, 0·41, 0·33, 0·19, 0·16, 0·01.

From these figures it is seen that the rate of change of gelatin cation concentration increases and then diminishes again with increasing $H^{\cdot}$ ion concentration from its isoelectric point. On the other hand, the rate of change of the tendency to flocculate, that is the factor above, probably increases steadily when referred to the same variable.

The actual result is the algebraic sum of these two factors and it follows from the above relationship that there is a certain $H^{\cdot}$ ion concentration where this algebraic sum is a minimum. This I conceive to be the case

in the mixture which contains acid $10^{-2\cdot4}$ N, and which I have called the "2nd critical mixture."

Experiments 3 *and* 4. *Series of mixtures made from gelatin* $10^{-4\cdot4}$ *with addition of sodium chloride* + HCl, *and sodium acetate* + HCl *respectively. Hydrogen ion concentration increases from each mixture to the next.*

Throughout the series in experiment 4 the same general phenomena are observed with one important difference. The range of hydrogen ion concentration over which flocculation is observed extends to $P_H^+ = 6\cdot56$. In Fig. 2 flocculation ceases before the value of hydrogen ion concentration falls to $P_H^+ = 4\cdot56$ at the gelatin concentration 10^{-5} which is taken as comparable; and at $P_H^+ = 5\cdot7$ under the peculiar circumstances of the series with gelatin $10^{-3\cdot6}$. The addition of 0·011 N sodium acetate makes all this difference. In similar fashion in Fig. 3 complete flocculation was observed in tubes to which no acid at all was added, and almost complete flocculation in tubes containing 10^{-4} NaOH.

If this result of added electrolyte is taken as indicating a widening of the isoelectric range it is comparable with Michaelis and Davidsohn's observations on the isoelectric point of oxyhaemoglobin. In transport experiments they showed [1912] that though the position of the P_H^+ range over which no definite inference could be drawn as to the direction of wandering remained unchanged, its upper and lower limits widened with increasing electrolyte addition.

Proceeding along these two series "2nd critical mixtures" are again observed at $P_H^+ = 2\cdot9$. The explanation is probably the same in all three cases even if that put forward is not correct.

Experimental details.

Materials used. The electronegative sols used were of oil, mastic and gold. The *oil sol* found most convenient was the result of many trials and was prepared in the following manner. To 94 cc. of Kahlbaum's acetone, 6 cc. of water are added and 20 cc. of olive oil "sublime." After thorough shaking and settling 40 cc. of the acetone layer are blown in from a pipette as quickly as possible into 1000 cc. of "conductivity" water. The rapidity of mixing of the acetone solution of oil and the water is a contributing factor to the success in preparing a finely divided sol. It should show to the naked eye only traces of oil on the surface, even after standing many months, and appear transparent with a blue fluorescence. A "white"

appearance indicates that the oil particles are much larger than when the solutions are transparent and fluorescent. The material extracted by wet acetone from the olive oil is probably richer in free fatty acid than the original oil, and to this I have ascribed the fine emulsions obtained by this method. Adding the same oil drop by drop to 100 cc. acetone + 6 cc. water until no more dissolves even on prolonged shaking, I obtained from the solution coarse "white" emulsions on blowing 4 cc. of it into 100 cc. of water [Donnan, 1910; Dubrisay, 1913].

A freshly prepared oil sol does not differ in appearance from one many months old. It is found, however, that when an endeavour is made to prepare a flocculation diagram with some protein and an electrolyte solution the sol does not seem to be so sensitive to minute differences of protein or electrolyte concentration after long standing. And the sharp well defined arcs on the diagram give way to regions similar in shape but of blurred outline.

The *mastic sol* used in all these experiments was prepared from commercial gum mastic in the following manner. Excess of the gum was boiled under a reflux condenser with 99 per cent. alcohol for several hours, the vessel cooled and then allowed to stand for several days. The clear solution resulting contained 1·7 per cent. of total solids. Five cc. blown forcibly from a pipette into 95 cc. of conductivity water furnished an excellent sol, pale straw colour by transmitted light, and with a marked blue fluorescence. If the alcoholic solution is run out from the pipette into the water in the normal way a milky fluid results, the particles of the disperse phase of which are comparatively large. The difference between the solution resulting is in the two cases very striking; much more so than with oil or any other suspension I have observed.

Unlike the particles of the oil sol the mastic particles increased in size hourly in a very marked manner, though even after months of standing no flocculation occurred. Fresh suspensions were therefore prepared for each experiment. As their magnitude increased the particles seemed to change in size at a progressively lower rate.

Contrary to expectation it was found quite possible to measure hydrogen ion concentrations with accuracy in solutions containing mastic or oil in the colloidal condition.

The mastic and oil sols used contained small quantities of acetone and alcohol respectively. It was thought that possibly 1·7 per cent. of alcohol or acetone might affect the charge on the protein particles in the mixtures made, but no evidence of this could be observed. Samples of sols were

distilled until quite free from alcohol or acetone under diminished pressure, and then half made up to the original volume with the correct proportion of alcohol, and half made up with water. No difference in the behaviour of these two sols on flocculating with traces of gelatin and acid could be observed either in the rapidity or the limits of flocculation. Scarcely any difference was observable between the original sol and the same sol after distillation and making up to the original volume with the correct proportion of alcohol. In transport experiments the addition of 1·7 per cent. of alcohol to both middle and outer limbs made no difference to the direction of migration of gelatin in the neighbourhood of the isoelectric point.

The *gold sols* (Au_F) were prepared by Zsigmondy's [1901] formalin method followed by dialysis. The removal of the alkali rendered these sols less stable, and after several days' dialysis an increase in size of the particles was invariably observed. The finer sols (Au_P) obtained by the ethereal phosphorus method were also prepared.

Gelatin. Coignet Fils et Cie's Gold Label Gelatin was soaked in frequent changes of conductivity water in a hard glass vessel for several days till twelve hours' contact at 0° did not change 3 gemmho water to more than 5 gemmhos. No signs of mould or bacterial growth were observed. A solution, approximately 1 per cent., was prepared by dissolving in boiling distilled water and the exact strength determined by refractive index at 17·5°. Suitable dilution gave a concentration 10^{-3} from which other solutions could be made. A typical 10^{-3} gelatin solution had conductivity 9·6 gemmhos.

The effect of the dialysis could be demonstrated by comparing different concentrations of dialysed gelatin with corresponding concentrations of undialysed gelatin in their effect on mastic sol and N/10 sodium acetate. Equal volumes of gelatin solution, mastic sol and N/10 sodium acetate solution are taken in each test tube. Flocculation is more rapid and complete in the latter case and extends over a wider range of gelatin concentration—a result attributable to the additional electrolytes present.

Apparatus. Determinations of H· ion concentration were in all cases made electrometrically, using a gas electrode of convenient type in conjunction with Michaelis' saturated KCl calomel electrode. The connecting fluid was saturated KCl and no correction for contact potential of the fluid investigated and saturated KCl was made. For nearly all of the determinations an electrode vessel of borosilicate glass with the electrode mounted in the same material and a ground joint [Walpole, 1914] was used.

Conductivity determinations were made by the Kohlrausch method, at

18°, in stoppered vessels of the Henry-Zörkendörfer pattern, using well-blacked electrodes.

As far as possible all solutions were stored in special bottles of hard glass. The test tubes employed were remarkable for their insolubility in water. As the interactions observed were many of them almost instantaneous, the slow absorption of carbon dioxide from the atmosphere by the mixture could be ignored.

The *ultramicroscopic* apparatus was Leitz' 1913 pattern illuminated by 5 amp. arc light. Observations on direction of migration in the electric field could be made in solutions containing only small quantities of electrolytes by using a cell 0·3 mm. deep provided with platinum electrodes [Chick and Martin, 1912].

Migrations in the electric field were also observed in an apparatus similar to that employed by Michaelis. Every due precaution was taken to avoid the fallacies contingent to the method.

In connection with the experimental part of this work I wish to express my indebtedness to my assistants Messrs R. Defries and S. Scott.

Summary.

A method is given by which the relationship of the phenomena of the inhibition or actuation of the aggregation of mixtures of sols and electrolytes, especially in the presence of proteins, may be coordinated diagrammatically on a quantitative basis.

This method has been applied to oil and mastic sols to which gelatin and hydrochloric acid have been added and the effect of the addition of sodium chloride and sodium acetate to the mastic-gelatin-hydrochloric acid mixtures has been studied in a similar manner.

The chief phenomena observed may be summarised here:

1. In the presence of not too much gelatin flocculation of mastic or oil sols may occur by the addition of a trace of acid quite unable to flocculate the sol alone. The same quantity of gelatin "protects" the sol against acid 6000 times more concentrated.

2. This aggregation may be observed equally well with gold sols and in all cases it is reversible.

3. In the absence of electrolyte except HCl, two critical mixtures are observed which are on the verge of flocculation. These have been worked out in the case of one particular mastic sol and called the "1st" and "2nd" critical mixtures.

1st critical mixture. The hydrogen ion concentration of this mixture is in the neighbourhood of the isoelectric point of gelatin but is probably on the alkaline side of this point. The particles are negatively charged in this mixture, and in contiguous mixtures whether flocculated or non-flocculated. Mixtures differing from this by having more gelatin, more acid or less acid do not flocculate.

2nd critical mixture. The hydrogen ion concentration of this mixture is $P_H^+ = 2{\cdot}9$. Mixtures having more acid or less acid flocculate, but here again more gelatin inhibits flocculation. The particles are positively charged in this mixture and in contiguous mixtures whether flocculated or non-flocculated.

4. In the presence of hundredth normal sodium chloride or sodium acetate there are also two critical mixtures showing precisely similar flocculation changes with changing acid or gelatin concentration. The following differences are noticeable:

(*a*) The two critical mixtures approximate much more closely than before in gelatin concentration.

(*b*) The *1st critical mixture* is either at or only just on the alkaline side of the isoelectric point of gelatin. The gelatin coated mastic particles are almost exactly electrically neutral. The *2nd critical mixture* in the presence of added electrolyte resembles the *2nd critical mixture* in the absence of added electrolyte in all respects including $H^{\cdot}$ ion concentration except that four times as much gelatin is present in the former case. Apparently the additional thickness of gelatin coat corresponding to the greater gelatin concentration counterbalances the flocculating effect of the added electrolyte.

5. Whether sodium chloride or acetate be present or no, the *1st critical mixture* contains more gelatin than the *2nd critical mixture.*

6. In any series of oil-gelatin or mastic-gelatin mixtures containing minimal traces of electrolyte, and differing only in $H^{\cdot}$ ion concentration, those which are flocculated are not those which contain electrically neutral particles. This lack of coincidence of the "point of maximum flocculation" and the point where the particles have no "electric charge" was pointed out in an earlier paper: an endeavour has been made here to analyse this phenomenon further.

7. Miss H. Chick's observation that "electrolyte-free" globulin at the reaction of maximum flocculation is not electrically neutral has been confirmed.

8. The wide difference of gelatin concentration (100 times) between the *1st critical mixtures* with and without salt addition is related to an area

of aggregation over which this phenomenon is more or less reversible experimentally by acid, alkali, or sodium chloride. The reversal by alkali is much more readily observed than that by acid.

9. A possible physical similarity between the particles of a mastic gelatin mixture and those of a euglobulin suspension is suggested. Preliminary attempts to make a suspension strictly comparable with euglobulin from pseudoglobulin and a sol from the lipoid of euglobulin have been made, and work on this subject is being continued.

These simple systems were originally examined in the hope that the results would assist in the elucidation of the phenomena met with in the investigation of the acid agglutination of bacteria. The system in the case of a bacterial suspension is, however, more complicated and this paper can only be regarded as a contribution to the general subject of flocculation.

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XXV. ACTION OF PEPSIN AND TRYPSIN ON ONE ANOTHER.

(Preliminary Paper.)

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Hitherto only a small amount of work has been done in investigating the action of enzymes on one another, and many of the earlier results are inconclusive or contradictory. It was stated by Corvisart (quoted by Wróblewski) that pepsin and trypsin were mutually destructive. Kühne [1876], on the other hand, found that pepsin digested trypsin, but that the reverse did not hold good. A few years later Engesser [1880] however found that pepsin had practically no action on trypsin, digestion being continued for two hours. Further uncertainty was introduced by the work of later authors.

Langley [1881] tested the action of certain enzymes on one another, taking the precaution to determine the action of acid or alkali of the appropriate strength on the enzymes in question. He found that pepsin was destroyed considerably more rapidly by trypsin in an alkaline solution than by alkali of the same strength acting on the pepsin alone. Langley also found that trypsin lost much of its digestive power when kept for some time in 0·05 % hydrochloric acid before being allowed to act on fibrin in alkaline solution, but he does not appear to have done any experiments himself to show the action of pepsin on trypsin. As the result of a considerable number of experiments on different enzymes, Wróblewski, Bednarski and Wojczynski [1901] conclude that not only does trypsin increase the destructive effect of alkali on pepsin, but also that pepsin increases the destructive effect of acid on trypsin. While different observers have found considerable variations in the range of hydrogen or hydroxyl ion concentration compatible with digestive power on the part of pepsin or trypsin respectively, there seems little doubt that the former enzyme has no appreciable action in an alkaline medium, trypsin on the other hand being active only in presence of alkali. Consequently previous investigations of the action of these enzymes

on one another appear to have been confined to discovering if pepsin would digest trypsin in acid solution, or if trypsin on the other hand would have a destructive action on pepsin in an alkaline medium.

In the light of the older view that enzymes were of a protein nature it was not unreasonable to expect that in such cases the pepsin for example would in an acid medium break up the trypsin into a number of less complex substances, and that thus the specific digestive properties of the trypsin would be lost.

Now that considerable doubt has been thrown on the protein nature of the enzyme molecule, however, such a breakdown of the molecule is by no means certain to take place in every case.

Within the last few years the mode of action of enzymes has been rendered clearer by experiments which appear to show that the enzyme acts on its appropriate substrate by means of different chemical groups or side chains.

Pollak [1905] found that on diluting a trypsin solution with a quantity of the same trypsin solution which had been previously heated to 70° much less digestion took place than in the case where the enzyme was diluted with a corresponding amount of water. Similar results were obtained by Schwartz [1905] in the case of pepsin.

Bayliss [1904] found that trypsin became altered in the above way on being heated, and suggested that the altered enzyme or "zymoid" still retained the power of combining with the substrate, but that the specific digestive power had been destroyed.

Bearn and Cramer [1907] also found that pepsin and rennin when heated to about 60° inhibited the action of the fresh enzyme when excess of the heated enzyme was added. Generally it was found that if the enzyme were heated to 100° this inhibitory power was lost, the reason probably being that the groups which combine with the substrate are themselves destroyed at 100°. Different enzyme preparations, however, varied considerably in their resistance to heat.

Although trypsin is a more energetic proteolytic enzyme than pepsin, nevertheless they are both capable of acting on a large number of proteins with the formation of similar hydrolytic products to a certain stage of digestion. This led to the idea that both enzymes might attack proteins by combining with the same side chains, the specific digestive action of the enzymes, however, being due to dissimilar side chains, the latter in the case of pepsin acting only in acid solution, and in the case of trypsin only in presence of alkali.

If this were the case we should expect that an excess of trypsin would inhibit the action of pepsin in acid solution, and similarly excess of pepsin would inhibit the action of trypsin in alkaline solution. The enzyme present in excess in such cases would thus act in a manner analogous to the zymoid of the other enzyme.

Experimental methods.

Various enzyme preparations were used in these experiments, such as Benger's Liquor Pepticus and Liquor Pancreaticus, and the pepsin and trypsin in scales or powder of Merck, Fairchild and Grübler.

The enzyme solutions were put in small flasks and a definite amount of dilute hydrochloric acid or sodium carbonate added. In each flask was then put an equal amount of the protein to be digested and the flasks were kept in a water bath at 37° for a certain time. Any slight accidental variations in temperature would affect all the flasks equally, and thus introduce no difficulty.

Two methods of estimating the amount of digestion were employed. The first, principally used for determining the amount of digestion by pepsin, was that of making the mixture of enzymes act on fibrin for a definite time. The undissolved fibrin was then filtered off and the nitrogen in a portion of the filtrate determined by Kjeldahl's method.

Since the object of these experiments was to determine the activity of one enzyme under different conditions, for example the relative digestive power of pepsin in dilute acid and of pepsin in the same strength of acid containing trypsin in addition, such a method of estimating the amount of digestion seems quite satisfactory, as the stages of digestion must presumably be the same in both cases, and where digestion has gone on for a definite time the amount of dissolved nitrogen must give a true indication of the digestive power of the enzyme under these conditions. The fibrin was always obtained from ox blood, and was minced, thoroughly washed till free from blood, suspended in water and heated to 70° in order to destroy any enzymes present. The water was then pressed out and the fibrin preserved in glycerol. When the fibrin was required for use, the glycerol was washed out with water, the excess of water pressed out and equal weighed amounts of the fibrin taken for experiment.

Control experiments were also carried out with the same amount of fibrin and acid or alkali, but no enzyme. No appreciable amount of fibrin was found to be dissolved in any of the controls.

The amount of nitrogen present in the solutions of enzymes used was also

determined in every case, and that amount deducted from the total nitrogen in solution at the end of the experiment. The difference represented the nitrogen of the protein dissolved by the enzyme.

The other method of determining digestive power employed in these experiments was that described by Hedin [1903]. The enzyme is allowed to act on a solution of caseinogen, and at the end of the period of digestion excess of tannic acid is added. The unchanged protein and proteoses are precipitated, and the nitrogen left in solution is estimated in the filtrate by Kjeldahl's method. This gives a measure of the amount of protein digested to the stage of peptone or amino-acids.

Control solutions of the enzymes and also of the caseinogen used were also treated in the same way with tannic acid, and the nitrogen in the filtrate allowed for in calculating the amount of protein digested. Toluene was added to each flask in order to prevent the action of bacteria. As a rule it was found that the fibrin method was the more suitable when digestion by pepsin was being studied, as pepsin digests caseinogen only very slowly to the stage at which it is not precipitated by tannic acid.

On the other hand, the caseinogen method was found more suitable for digestion by trypsin, the latter enzyme dissolving heated ox fibrin too slowly for convenience in experiment.

EFFECT OF EXCESS OF TRYPSIN ON THE DIGESTIVE POWER OF PEPSIN.

		Digestion in cc. of N/10 nitrogen
1.	1 cc. pepsin, 20 cc. trypsin, 30 cc. N/10 HCl... ...	11·4
	1 cc. pepsin, 20 cc. water, 30 cc. N/10 HCl	26·9
	1·4 g. fibrin added. Digestion 2·5 hours.	
2.	1 cc. pepsin, 20 cc. trypsin, 30 cc. N/10 HCl... ...	9·0
	1 cc. pepsin, 20 cc. water, 30 cc. N/10 HCl	26·3
	1·5 g. fibrin. Digestion 1·5 hours.	
3.	1 cc. pepsin, 20 cc. trypsin, 30 cc. N/10 HCl... ...	9·9
	1 cc. pepsin, 20 cc. water, 30 cc. N/10 HCl	28·8
	1·6 g. fibrin. Digestion 2·25 hours.	
4.	1 cc. pepsin, 10 cc. trypsin, 20 cc. water, 30 cc. N/10 HCl	37·1
	1 cc. pepsin, 20 cc. trypsin, 10 cc. water, 30 cc. N/10 HCl	14·2
	1 cc. pepsin, 30 cc. trypsin, 30 cc. N/10 HCl... ...	5·8
	1 cc. pepsin, 30 cc. water, 30 cc. N/10 HCl	47·6
	2 g. fibrin. Digestion 2·75 hours.	
5.	1 cc. pepsin, 20 cc. trypsin, 30 cc. N/10 HCl... ...	5·2
	1 cc. pepsin, 20 cc. water, 30 cc. N/10 HCl	18·9
	1·7 g. fibrin. Digestion 1·25 hours.	
6.	1 cc. pepsin, 20 cc. trypsin, 30 cc. N/10 HCl... ...	4·8
	1 cc. pepsin, 20 cc. water, 30 cc. N/10 HCl	13·3
	1 g. fibrin. Digestion 2 hours.	

		Digestion in cc. of N/10 nitrogen
7.	1 cc. pepsin, 20 cc. trypsin, 30 cc. N/10 HCl... ...	8·9
	1 cc. pepsin, 20 cc. water, 30 cc. N/10 HCl	22·7
	1·1 g. fibrin. Digestion 1·5 hours.	
8.	1 cc. pepsin, 10 cc. trypsin, 20 cc. water, 30 cc. N/10 HCl	7·2
	1 cc. pepsin, 20 cc. trypsin, 10 cc. water, 30 cc. N/10 HCl	5·1
	1 cc. pepsin, 30 cc. trypsin, 30 cc. N/10 HCl... ...	4·2
	1 cc. pepsin, 30 cc. water, 30 cc. N/10 HCl	18·5
	1·8 g. fibrin. Digestion 1·5 hours.	

Of these experiments Nos. 1 to 4 were carried out with Benger's preparations, the others being with Merck's pepsin and trypsin (1 % solutions). As Liquor Pancreaticus is slightly acid, it was first neutralised, and a corresponding small amount of sodium chloride added to the control flask though this was probably not at all necessary.

It may be mentioned that exactly similar results were obtained by using Grübler's pepsin and trypsin in 1 % solution, and that many other experiments carried out with Benger's and Merck's preparations confirmed in every respect those described above, but those quoted are sufficient to show very clearly the effect of an excess of trypsin in inhibiting the digestive action of pepsin solutions. Experiments 4 and 8 also show that this inhibition varies with the proportion of trypsin present. If the amount of the latter enzyme be very large, the action of the pepsin is almost stopped entirely. This is what was to be expected if we assume that trypsin can combine with protein in acid solution.

The objection might be raised that the action of the trypsin was really due to the presence of protein or the products of protein digestion in the trypsin solutions used. That such is not the case, however, is shown by the following experiments.

		Digestion in cc. of N/10 nitrogen
9.	1 cc. pepsin, 20 cc. 2·5 % albumin, 30 cc. N/10 HCl ...	26·3
	1 cc. pepsin, 20 cc. water, 30 cc. N/10 HCl	25·4
	1 g. fibrin. Digestion 1·5 hours.	
10.	1 cc. pepsin, 20 cc. 2 % caseinogen, 20 cc. water, 10 cc. N/10 HCl	29·7
	1 cc. pepsin, 20 cc. water, 30 cc. N/10 HCl	24·9
	1 g. fibrin. Digestion 2 hours.	
	(The caseinogen was dissolved in N/10 HCl.)	
11.	1 cc. pepsin, 20 cc. water, 30 cc. N/10 HCl	31·9
	1 cc. pepsin, 20 cc. water, 30 cc. caseinogen	30·7
	1 cc. pepsin, 20 cc. water, 30 cc. albumin	37·6
	(The caseinogen and albumin were 1·5 % in N/10 HCl.)	
	1·2 g. fibrin. Digestion 1 hour.	

In these three experiments Liquor Pepticus was used.

		Digestion in cc. of N/10 nitrogen
12.	1 cc. pepsin, 20 cc. water, 20 cc. N/10 HCl	25·8
	1 cc. pepsin, 20 cc. water, 20 cc. caseinogen	24·4
	1 cc. pepsin, 20 cc. water, 20 cc. albumin	26·1
	1·1 g. fibrin. Digestion 1·75 hours.	
	(The caseinogen and albumin were 1 % in N/10 HCl.)	
Merck's pepsin (0·1 %) was used in this experiment.		
13.	1 cc. pepsin, 20 cc. water, 20 cc. N/10 HCl	32·3
	1 cc. pepsin, 20 cc. water, 20 cc. caseinogen	32·6
	1 cc. pepsin, 20 cc. water, 20 cc. albumin	31·7
	1·2 g. fibrin. Digestion 0·75 hour.	
	(Caseinogen and albumin 1 % in N/10 HCl.)	
Grübler's pepsin (1 %) was used in this experiment.		

It is clear from these experiments that digestion of a solid protein such as fibrin is not less rapid when there is also present in solution a protein such as caseinogen or egg albumin. The action of the pepsin on the fibrin seems to begin immediately in such a case, there being no interval during which the protein in solution is first broken down into less complex substances.

The action of the trypsin is not an actual destruction of the pepsin. This is at once seen if the amounts of fibrin used vary considerably. It has been shown by Hedin [1905] that the amount of caseinogen digested in a given time by a certain amount of trypsin increases with the concentration of the substrate, until a maximum is reached when sufficient substrate is present to unite with the whole of the trypsin.

Similar results are obtained in the digestion of fibrin by pepsin.

		Digestion in cc. of N/10 nitrogen
14.	1 cc. pepsin, 20 cc. trypsin, 20 cc. N/10 HCl, 0·9 g. fibrin	9·9
	1 cc. pepsin, 20 cc. trypsin, 20 cc. N/10 HCl, 2·7 g. fibrin	28·8
	1 cc. pepsin, 20 cc. water, 20 cc. N/10 HCl, 0·9 g. fibrin...	20·7
	1 cc. pepsin, 20 cc. water, 20 cc. N/10 HCl, 2·7 g. fibrin...	65·6
	Digestion 1 hour.	
15.	1 cc. pepsin, 20 cc. trypsin, 30 cc. N/10 HCl, 0·9 g. fibrin	10·9
	1 cc. pepsin, 20 cc. trypsin, 30 cc. N/10 HCl, 3·4 g. fibrin	42·2
	1 cc. pepsin, 20 cc. water, 30 cc. N/10 HCl, 0·9 g. fibrin...	25·5
	1 cc. pepsin, 20 cc. water, 30 cc. N/10 HCl, 3·4 g. fibrin...	94·1
	Digestion 0·75 hour.	
In these two experiments Benger's preparations were used.		
16.	0·8 cc. pepsin, 25 cc. trypsin, 30 cc. N/10 HCl, 1 g. fibrin	7·9
	(*a*) 0·8 cc. pepsin, 25 cc. trypsin, 30 cc. N/10 HCl, 3·5 g. fibrin	17·1
	(*b*) 0·8 cc. pepsin, 25 cc. water, 30 cc. N/10 HCl, 1 g. fibrin	22·4
	0·8 cc. pepsin, 25 cc. water, 30 cc. N/10 HCl, 3·5 g. fibrin	84·5
	Digestion 0·75 hour.	
17.	0·8 cc. pepsin, 25 cc. trypsin, 30 cc. N/10 HCl, 0·7 g. fibrin	4·9
	(*a*) 0·8 cc. pepsin, 25 cc. trypsin, 30 cc. N/10 HCl, 5 g. fibrin	12·7
	(*b*) 0·8 cc. pepsin, 25 cc. water, 30 cc. N/10 HCl, 0·7 g. fibrin	18·5
	0·8 cc. pepsin, 25 cc. water, 30 cc. N/10 HCl, 5 g. fibrin...	121·9
	Digestion 0·75 hour.	

		Digestion in cc. of N/10 nitrogen
18.	0·8 cc. pepsin, 20 cc. trypsin, 30 cc. N/10 HCl, 0·5 g. fibrin	4·7
	0·8 cc. pepsin, 20 cc. trypsin, 30 cc. N/10 HCl, 1·5 g. fibrin	12·6
	0·8 cc. pepsin, 20 cc. trypsin, 30 cc. N/10 HCl, 7 g. fibrin	13·1
	0·8 cc. pepsin, 20 cc. water, 30 cc. N/10 HCl, 0·5 g. fibrin	10·5
	Digestion 0·5 hour.	

0·5 % pepsin and 1 % trypsin (Grübler) were used in these three experiments.

Those experiments show well the effect of varying the quantity of fibrin added to the enzyme solutions. It is clear that the effect of adding an excess of trypsin to a given amount of pepsin can be apparently counteracted by increasing the amount of fibrin. The reason evidently is that when the fibrin is increased in amount a larger number of pepsin molecules can carry on their digestive action, although in those experiments at least the amount of digestion by pepsin + trypsin relative to that by pepsin alone is no greater when much fibrin is present than when a smaller amount of the substrate only is used.

In experiments 16 and 17 it will be noticed that the amount of digestion in (*a*) is still less than that in (*b*) in spite of the fact that the amount of fibrin in the former is considerably greater than in the latter case. In these two experiments the proportion of trypsin to pepsin is probably higher than in experiments 14 and 15, consequently the proportion of fibrin should have been correspondingly higher in (*a*) than in (*b*) to produce the same effect. By using still smaller amounts of fibrin as in No. 18 this difficulty is got over, and in this last experiment we see again that by employing suitable proportions of fibrin it is possible to get a greater amount of digestion by the pepsin + trypsin than by the pepsin alone.

Since it has been clearly shown that the inhibition of pepsin by excess of trypsin is not due to the presence of soluble protein in the trypsin solution, also that it is not due to the pepsin being destroyed by the trypsin, it seems reasonable to suppose that this inhibition is due to the trypsin itself. If this were the case, we should expect that such an inhibition would no longer occur if the trypsin were destroyed.

The simplest way appeared to be by boiling the solution of trypsin before adding it to the pepsin. This, however, proved somewhat ineffective as the following show.

		Digestion in cc. of N/10 nitrogen
19.	1 cc. pepsin, 20 cc. trypsin, 30 cc. N/10 HCl	9·9
	1 cc. pepsin, 20 cc. boiled trypsin, 30 cc. N/10 HCl ...	16·8
	1 cc. pepsin, 20 cc. water, 30 cc. N/10 HCl	28·8
	1·6 g. fibrin. Digestion 2·25 hours.	
20.	1 cc. pepsin, 20 cc. trypsin, 30 cc. N/10 HCl	15·4
	1 cc. pepsin, 20 cc. boiled trypsin, 30 cc. N/10 HCl ...	20·7
	1 cc. pepsin, 20 cc. water, 30 cc. N/10 HCl	40·2
	1·7 g. fibrin. Digestion 1·5 hours.	

In these two experiments Liquor Pepticus and Liquor Pancreaticus were used. The latter was boiled under a reflux condenser for about 30 minutes and filtered. The precipitate was found to be entirely and readily soluble in the same concentration of pepsin as was used for the main experiment. As mentioned in a previous paper by me [1914] this trypsin preparation is slightly acid and retains a considerable proportion of its digestive power even after being boiled. In the last two experiments therefore, the digestive power of the trypsin was probably not entirely destroyed, or at least the power of the trypsin to unite with its substrate. In this case it is easy to understand why boiled trypsin still inhibits the action of pepsin on fibrin.

It may be mentioned here that it was through these unexpected results that I was led to investigate the effect of heat on trypsin more fully. As it has been shown in the paper referred to and by other workers that trypsin loses its digestive power readily when boiled in neutral or alkaline solution, I tried the effect of trypsin, which had been thus boiled, on pepsin, but found that it was still effective in inhibiting the digestion of fibrin by pepsin.

There is reason to believe, from preliminary experiments, that it is much harder completely to inactivate the trypsin molecule than the pepsin molecule, so that the zymoid modification of trypsin would still inhibit the action of pepsin even after prolonged heating of the trypsin solution. Different trypsin preparations also are found to behave differently in this respect. It was also found by Bearn and Cramer that different preparations of pepsin varied considerably in their behaviour to heat. The results with trypsin will be described in a later paper on this subject.

Trypsin was found to inhibit the action of pepsin on caseinogen solutions also, but the effect of pepsin on this substrate is slight, so far, at least, as rendering the nitrogen non-precipitable by tannic acid goes.

		Digestion in cc. of N/10 nitrogen not ppted. by tannic acid
21.	1 cc. pepsin, 20 cc. trypsin, 15 cc. N/10 HCl... ...	3·1
	1 cc. pepsin, 20 cc. water, 15 cc. N/10 HCl	6·2
	20 cc. of 1 % caseinogen in N/20 HCl added. Digestion 4 hours.	
22.	1 cc. pepsin, 20 cc. trypsin, 30 cc. 2 % caseinogen in N/10 HCl	9·8
	1 cc. pepsin, 20 cc. water, 30 cc. 2 % caseinogen in N/10 HCl	14·3

Liquor Pepticus and Liquor Pancreaticus used in these two experiments.

In No. 22, digestion lasted 18 hours, so that while these experiments show that trypsin again inhibits pepsin, they show still more clearly that this method is unsuitable for estimating the digestive activity of pepsin solutions.

EFFECT OF EXCESS OF PEPSIN ON THE DIGESTIVE ACTION OF TRYPSIN.

The experiments were carried out similarly to those already described. When Liquor Pepticus was used as the pepsin solution, a considerable allowance had to be made for the acidity of this solution, the control solution having sodium chloride added in amount corresponding to that in the neutralised pepsin solution.

		Digestion in cc. of N/10 nitrogen
23.	1 cc. trypsin, 20 cc. pepsin, 40 cc. 1 % Na_2CO_3 ...	6·7
	1 cc. trypsin, 20 cc. water, 40 cc. 1 % Na_2CO_3 ...	12·2
	1 g. fibrin. Digestion 2 hours.	
24.	1 cc. trypsin, 20 cc. pepsin, 40 cc. 1 % Na_2CO_3 ...	7·9
	1 cc. trypsin, 20 cc. water, 40 cc. 1 % Na_2CO_3 ...	18·6
	1·2 g. fibrin. Digestion 2 hours.	
25.	1 cc. trypsin, 10 cc. pepsin, 20 cc. water, 35 cc. 1 % Na_2CO_3	26·4
	1 cc. trypsin, 20 cc. pepsin, 10 cc. water, 35 cc. 1 % Na_2CO_3	21·1
	1 cc. trypsin, 30 cc. pepsin, 35 cc. 1 % Na_2CO_3 ...	12·8
	1 cc. trypsin, 30 cc. water, 35 cc. 1 % Na_2CO_3 ...	33·3
	2 g. fibrin. Digestion 1·5 hours.	

Liquor Pancreaticus and Liquor Pepticus were used in these experiments.

It will be seen from the above that an excess of pepsin inhibits the action of trypsin on fibrin, the amount of inhibition varying with the amount of pepsin added.

On the whole it was found that much more digestion was effected when caseinogen was used as the substrate, and the nitrogen estimated in the filtrate from the tannic acid precipitate.

		Digestion in cc. of N/10 nitrogen not ppted. by tannic acid
26.	1 cc. trypsin, 20 cc. pepsin, 40 cc. caseinogen ...	20·3
	1 cc. trypsin, 20 cc. water, 40 cc. caseinogen ...	46·9
	Caseinogen was 2 % in 1 % Na_2CO_3. Digestion 3 hours.	
27.	1 cc. trypsin, 20 cc. pepsin, 40 cc. caseinogen ...	28·2
	1 cc. trypsin, 20 cc. water, 40 cc. caseinogen ...	53·6
	Caseinogen as above. Digestion 4 hours.	

The effect of a soluble protein such as caseinogen on the digestion of fibrin by trypsin was tested by two experiments.

		Digestion in cc. of N/10 nitrogen
28.	1 cc. trypsin, 20 cc. caseinogen, 20 cc. N/10 Na_2CO_3 ...	19·3
	1 cc. trypsin, 40 cc. N/10 Na_2CO_3	16·3
	1 g. fibrin. Digestion 2 hours.	
	The caseinogen was 2 % in N/10 Na_2CO_3.	
29.	1 cc. trypsin, 20 cc. caseinogen, 20 cc. N/10 Na_2CO_3 ...	17·9
	1 cc. trypsin, 40 cc. N/10 Na_2CO_3	16·8
	0·8 fibrin. Digestion 2 hours.	
	The caseinogen was 2 % in N/10 Na_2CO_3.	

These two experiments show that the presence of excess of a soluble protein such as caseinogen does not delay the digestion of fibrin by trypsin. Egg albumin, as is well known, inhibits the action of trypsin, and I found that only a small amount of fibrin was digested if albumin was added in place of the caseinogen in these two experiments. The above results show, however, that the inhibition of trypsin by excess of pepsin is not due to the trypsin digesting the proteins in solution before attacking the fibrin.

Several experiments were carried out to see if pepsin solutions which had been boiled lost their power of inhibiting the action of trypsin. Here again, as in the corresponding experiments with boiled trypsin in excess acting on pepsin, the results varied considerably.

		Digestion in cc. of N/10 nitrogen not ppted. by tannic acid
30.	1 cc. trypsin, 20 cc. pepsin, 40 cc. caseinogen ...	14·9
	1 cc. trypsin, 20 cc. boiled pepsin, 40 cc. caseinogen ...	40·3
	1 cc. trypsin, 20 cc. water, 40 cc. caseinogen ...	52·8

The caseinogen was 2 % in N/10 Na_2CO_3. Digestion 3 hours.

Liquor Pepticus and Liquor Pancreaticus were used in this experiment.

This shows that pepsin by being boiled loses most of its power to inhibit trypsin. In this case the pepsin had been boiled for about 10 minutes. Probably it still retained some of its zymoid properties, which would have been destroyed by more prolonged heating.

Although I had previously noticed that boiled trypsin still inhibited the action of pepsin, it was in the course of this last experiment that I found, in one of the controls containing boiled trypsin and caseinogen, that the boiled enzyme had brought about a considerable amount of digestion of the protein. I was led from that to consider the question of the resistance of trypsin to heat, and since then have been unable to get clear evidence of the destruction of the inhibitory power of pepsin by heat. As was shown by Bearn and Cramer, different preparations of pepsin vary widely in their resistance to heat, and it may be that in my experiments the solutions have always retained their power of combining with the substrate. This question is being now investigated, and the results will be published in a later paper.

It may be mentioned that Bayliss found that he could not always produce the zymoid modification of trypsin, or at least demonstrate the action of the zymoid, and it is evident from the experiments described in this preliminary paper that a number of points have still to be settled in regard to the varying behaviour of different enzyme solutions after being heated.

SUMMARY.

Excess of trypsin inhibits the digestive action of pepsin in acid solution. Such inhibition is not due simply to the presence of protein associated with the trypsin. Nor is it due to the pepsin being destroyed by the trypsin.

The power of trypsin to inhibit pepsin is to some extent lost if the trypsin solution is boiled before being added to the pepsin.

It is suggested that trypsin can combine with protein in acid solution, so that although unable to digest such protein, an excess of trypsin would prevent pepsin from combining fully with the protein.

Similarly an excess of pepsin inhibits the digestive action of trypsin in alkaline solution. The amount of inhibition in this case is usually less than that caused by an excess of trypsin on the digestive power of pepsin in acid solution.

This inhibition again is not simply due to the presence of protein associated with the pepsin. Heating the pepsin solution destroys to a considerable extent its power to inhibit trypsin.

In this case it is suggested that the pepsin acts by combining with the protein in alkaline solution, thus preventing the digestion of the protein by the trypsin.

In both cases the amount of inhibition produced depends on the relative proportions of the enzymes present. Thus, the greater the amount of pepsin, the less digestion will be effected by a given amount of trypsin acting in alkaline solution.

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XXVI. THE FLOWER PIGMENTS OF *ANTIRRHINUM MAJUS*. III. THE RED AND MAGENTA PIGMENTS.

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An account of the Mendelian factors for flower colour in ***Antirrhinum***, of the preparation of the pigment and of the analyses of the yellow pigments has been given in previous communications [Wheldale, 1913; Wheldale and Bassett, 1913, 1914].

Of the yellow pigments in *Antirrhinum*, only two occur in quantity and are concerned in the inheritance of colour. One is present in the so-called ivory variety and has been identified with the flavone, apigenin, $C_{15}H_{10}O_5$; the other is present (in addition to apigenin) in the yellow variety and has been identified with the flavone, luteolin, $C_{15}H_{10}O_6$. The ivory variety is dominant to the yellow and contains a factor "I" which is absent from yellow and may be expressed as the power to inhibit the formation of luteolin. The true white or albino variety does not contain either apigenin or luteolin.

The remaining varieties contain anthocyanin in addition to flavones and may be enumerated as follows:

1. Yellow tinged with bronze.
2. Ivory tinged with rose doré.
3. Bronze.
4. Rose doré.
5. Yellow tinged with crimson.
6. Ivory tinged with magenta.
7. Crimson.
8. Magenta.

It has hitherto seemed probable that, in addition to flavones, one anthocyanin pigment only—red—is responsible for varieties 1–4, rose doré being coloured by red anthocyanin and apigenin, and bronze by red anthocyanin and luteolin. This assumption has now been confirmed and also the similar view that varieties 5–8 contain only magenta anthocyanin; crimson being coloured by magenta anthocyanin and luteolin, and magenta by the same anthocyanin and apigenin.

The purification of red and magenta anthocyanins was carried out as follows:—The crude pigment (obtained by hydrolysis of the glucosides from flowers containing, of course, flavone in addition to anthocyanin) is finely powdered and extracted with absolute alcohol and in this way is purified from a considerable amount of brownish insoluble substance which is probably a decomposition product produced by boiling with sulphuric acid during the hydrolysis of the glucoside. The alcohol extract is taken to dryness again, finely ground, and extracted in a Soxhlet thimble suspended immediately above boiling ether. After some months most of the flavone pigments are removed by the ether, the process being continued with fresh quantities of ether till the latter remains practically colourless after boiling for a few days. The residue consisting almost entirely of anthocyanin, is then taken up in the minimum amount of absolute alcohol and a large volume of ether added which precipitates the anthocyanin, any flavone present remaining in solution. The precipitated anthocyanin is filtered off, dried, and again extracted with ether to remove traces of flavone.

Red anthocyanin thus prepared is an Indian red powder. We have not yet been able to obtain anthocyanin as a satisfactory crystalline product. It does not melt even when heated to 350°. It is readily soluble in absolute alcohol and pyridine, slightly soluble in dilute acids, ethyl acetate and acetone, almost insoluble in water and insoluble in ether, chloroform and benzene. In concentrated sulphuric acid it forms a reddish solution with a slight green fluorescence. It is soluble in alkalies to a green solution. With ferric chloride solution it gives a green colouration changing to brown. Lead acetate forms a brownish yellow precipitate, but barium, silver, cadmium and mercury do not form precipitates.

Pure magenta anthocyanin is a magenta red powder with similar properties and solubilities to the red. In concentrated sulphuric acid it gives a red solution with a slight greenish fluorescence. It is soluble to a green solution in alkalies. With ferric chloride solution it gives a green colouration changing to blue. With lead acetate it forms a dull green precipitate of a lead salt. In alkaline solutions it is strongly fluorescent, green by transmitted, red by reflected light.

The combustion results for pure red anthocyanin derived from different varieties were:

		C	H	O (by difference)
From rose doré		51·93 %	5·02 %	43·05 %
From bronze		51·37	5·05	43·58
,, ,, ...		52·12	4·97	42·91
	Mean	51·81	5·01	43·18

Of magenta anthocyanin from different varieties:

From magenta	50·26 %	4·89 %	44·85 %
From ivory tinged with magenta	50·68	5·54	43·78
From crimson	50·56	4·90	44·54
Mean	50·50	5·11	44·39

The corresponding numbers for the yellow pigments are as follows:

Apigenin	66·66 %	3·70 %	29·64 %
Luteolin...	62·90	3·49	33·61

The anthocyanins were always obtained in amorphous form, but although we were unable to get a crystalline product, the combustion results, combined with the failure to effect any separation even though a large number of solvents were used, made it appear certain that the pigments were single substances and not constant mixtures, a view confirmed by the fact that the same substance could be obtained from different varieties.

This note is suggested by the following observations. In working with the yellow pigments we, for a long time, obtained a mixture of apigenin and luteolin of constant melting point and great difficulty was experienced in separating the constituents, but in this case the mixture gave very variable combustion results, quite in contrast with those of the anthocyanins quoted above.

Attempts were made to determine the molecular weight by depression of freezing point, using phenol as a solvent, but the results, though consistent in a series of experiments with anthocyanin from different varieties, were obviously far too low. In the case of magenta, the average value was 150, of red 380. Since the value for magenta is clearly too small, it is probable that the red is also below the correct value. The remarkable agreement between the results in the series of determinations carried out suggests however that the numbers obtained bear a simple relation to the molecular weights and that the low values are to be explained by some type of dissociation in the phenol.

Acetic acid and various other solvents did not dissolve sufficient substance to give readable depressions.

Attempts to determine the molecular weight by elevation of boiling point in absolute alcohol gave mean values of 572 for red and 717 for magenta. The elevation of the boiling point, for the concentrations it was possible to use, was so slight that the possible experimental error might be very considerable. Added to this, there appeared to be some change in the anthocyanin during the experiment and towards the end varying amounts were precipitated.

The combustion results give as simplest formulae, $C_8H_9O_5$ for the red and $C_{15}H_{18}O_{10}$ for the magenta. The boiling point determination of the molecular weight would appear to indicate that the molecule is $3(C_8H_9O_5)$, i.e. $C_{24}H_{27}O_{15}$ which has a molecular weight of 555 for the red, and $2(C_{15}H_{18}O_{10})$, i.e. $C_{30}H_{36}O_{20}$ which has a molecular weight of 716 for the magenta.

Lead salts of both the anthocyanins were prepared by mixing alcoholic solutions of lead acetate and anthocyanin. The precipitates were washed with hot alcohol, dried at 130° C. and the lead determined as $PbSO_4$.

In the case of red, 1 gram-atomic weight of lead was found to be equivalent to 224 g. of anthocyanin. Taking the molecular weight of the anthocyanin to be 555, this would indicate that 5 atoms of lead combine with 2 molecules of the pigment and the molecular weight of red anthocyanin calculated in this way from the lead equivalent would be 560.

In the case of magenta, 1 gram-atomic weight of lead was found to be equivalent to 204 g. of anthocyanin. Taking the molecular weight of magenta anthocyanin to be 716, this would indicate that 7 atoms of lead combine with 2 molecules of the pigment and the molecular weight of magenta anthocyanin calculated from the lead equivalent would be 714.

An attempt was made to estimate the number of hydroxyl groups present in the anthocyanin molecule by means of Zerewitinoff's [1907] modification of Hibbert and Sudborough's method. This consists in dissolving the substance in thoroughly dried pyridine, treating it in a suitable apparatus with a considerable excess of methyl magnesium iodide dissolved in dry amyl ether, and collecting the gas evolved. Each hydroxyl group causes the evolution of a molecule of methane. It should be noted that "hydroxyl groups" as determined by this method include those forming part of carboxyl groups, and also such ketone groups as can give rise to hydroxyl by a tautomeric change.

The values obtained indicate that the red anthocyanin, assuming the formula to be $C_{24}H_{27}O_{15}$, contains twelve hydroxyl groups as defined above, while the magenta, taking the formula as $C_{30}H_{26}O_{20}$ contains fifteen hydroxyl groups.

It will be seen that in the formation of the lead salts, a number of the hydroxyl groups do not react.

As far as our researches are concerned, it still remains to be shown that anthocyanin is a flavone derivative. The order of magnitude indicated for the molecular weights suggests that the anthocyanins of *Antirrhinum*, if flavone derivatives, are considerably more complex than flavones and may be formed by condensation of two molecules accompanied by oxidation or by condensation of a flavone with phenols or hydroxybenzoic acids.

In a recent paper, Willstätter [1913] gives some account of the extraction and analyses of pigments (and their derivatives) from the Corn-flower. Three pigments are identified; a purple pigment which he considers to be a flavone derivative and which is an acid; a blue pigment, the potassium salt of the purple, and a red pigment which is a salt, of the oxonium type, of the anthocyanin with an acid present in the plant.

The relation between the magenta and red pigments of *Antirrhinum* cannot be of the nature of that suggested by Willstätter for the pigments of the Corn-flower. It seems that the anthocyanins of the two genera differ considerably from each other. For instance Willstätter's anthocyanins form oxonium salts with mineral acids with great ease, whereas we have entirely failed to obtain such salts by any method. The very dissimilar range of colour varieties thrown off in the two cases also lends support to the view that the anthocyanins concerned are different in character.

From the standpoint of genetics, the chief items of interest contained in the present paper are the following:

1. There are only two kinds of anthocyanin in *Antirrhinum*, red and magenta. Mixed with ivory pigment (apigenin) the colours of red and magenta are unaffected, but a mixture with yellow pigment (luteolin) gives two other colours, bronze and crimson respectively. Both red and magenta anthocyanin may also occur in varying amounts giving rise to tinged, pale and deep varieties.

2. Both red and magenta anthocyanin contain a considerably higher percentage of oxygen than the flavones, and of the two, the percentage in magenta is the higher. Hence if the anthocyanins are derived from flavones, the process must be in part one of oxidation.

3. There is evidence also that the anthocyanin molecules are larger than the flavone molecule. Hence, again, if a flavone constitutes the chromogen, condensation must take place, either of two flavone molecules, or of a flavone with one or more molecules of an aromatic acid or phenol, etc.

We were unfortunately obliged to discontinue work at this point owing to our supply of pure material being exhausted, but we hope to carry out further investigations and to try to elucidate the constitution of anthocyanins.

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XXVII. THE CONSTITUTION OF PSEUDO-MUSCARINE ("SYNTHETIC MUSCARINE").

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(*Received March 12th, 1914.*)

Pseudo-muscarine or "synthetic muscarine" as the base has generally been termed was first obtained by Schmiedeberg and Harnack [1877]. Schmiedeberg and Koppe [1869] had obtained from the "Fly Agaric" (*Amanita muscaria*) a very powerfully active base "muscarine," which in its chemical behaviour closely resembled choline with which it was found associated in the fungus. Harnack [1876] showed that the muscarine obtained by Schmiedeberg and Koppe was still mixed with choline, and effected a purification of the base by repeated recrystallisation of the aurichloride. From the analyses of this salt he assigned to muscarine the formula $C_5H_{15}O_3N$, and on account of the close chemical relationship of the base to choline and its presence in the fungus with the latter base, suggested that its constitution could be expressed by the formula

$$(OH)\,N\,(CH_3)_3CH_2\,.\,CH\,(OH)_2,$$

muscarine thus being represented as a hydrated aldehyde derived from choline.

This hypothesis was apparently confirmed a little later when Schmiedeberg and Harnack [1877], by evaporating to dryness a solution of choline (or still better the platinichloride of the base) in concentrated nitric acid, obtained a base which they stated was chemically and physiologically indistinguishable from the natural muscarine obtained from *Amanita muscaria*. This base was, therefore, regarded as "synthetic" muscarine, and the supposition that muscarine was indeed an oxidation product of choline was apparently confirmed.

This straightforward solution of the problem of the constitution of muscarine was however shown to be incorrect by Boehm [1885] who found that "synthetic" muscarine, while closely resembling the natural base in physiological action, in some respects differed markedly, the most important

differences being that the synthetic base showed a powerful curare-like action which the natural base does not possess, and that the synthetic base did not constrict the mammalian pupil whereas natural muscarine does.

The problem was still further complicated by the fact that the aldehyde corresponding to choline, the so-called "betaine aldehyde" which was first synthesised by Berlinerblau [1884] and later by E. Fischer [1893] (who proved its constitution by oxidising it to betaine), was found to differ considerably in its action from both the natural and "synthetic" muscarines.

The problem was later re-investigated by Nothnagel [1894]. He obtained both natural and "synthetic" muscarine; the former from *Amanita muscaria*, the latter by Schmiedeberg and Harnack's method from choline. He too stated that so far as could be judged from the small amount of material available, the natural muscarine was chemically identical with the synthetic base, and confirmed Schmiedeberg and Harnack's formula for the latter. He also repeated and confirmed Berlinerblau's work. The physiological action of these bases was examined by Hans Meyer who confirmed Boehm's observations with regard to synthetic and natural muscarine and in addition pointed out further differences in their action.

Briefly then it appeared that there were two chemically indistinguishable bases which possessed different physiological actions and for this no satisfactory explanation has as yet been put forward.

Recently during an investigation into the nature of a muscarine-like base which is present in extracts of ergot, and was found to be acetyl-choline, as already described [Ewins, 1914], I had occasion to prepare some pseudo-muscarine for purposes of comparison and it occurred to me that, in view of the facts detailed above, confirmation of the constitution of this base was desirable, and that further examination might throw some light on the general problem. The preparation of the base gave no difficulty and a pure crystalline platinichloride was obtained, the base from which showed all the characteristic physiological effects[1] of synthetic muscarine as described by Boehm [1885], Hans Meyer [v. Nothnagel, 1894] and Honda [1911].

The formula assigned by Schmiedeberg and Harnack to their platinichloride and confirmed by Nothnagel was as follows:

$$[(1)\,N\,(CH_3)_3CH_2\,.\,CH\,(OH)_2]_2\,PtCl_4\,.\,2H_2O.$$

The salt thus contained two molecules of water of crystallisation. It is to be noted, however, that these were not completely removed, except by heating to a temperature of 130–135°.

[1] The physiological experiments involved in this investigation were in all cases carried out by Dr H. H. Dale.

As a preliminary step it was decided to attempt to confirm the above formula. The platinichloride was obtained "air dry" by standing in an ordinary desiccator over calcium chloride. The salt then remained unaltered in weight when placed *in vacuo* in a desiccator over sulphuric acid, and when heated under ordinary pressure in a steam bath (98°). Loss of weight occurred when the salt was heated to a temperature of about 115°, but it was found that by heating to 130–135° the loss of weight was considerably greater than that required for the loss of two molecules of water, and it was evident that the salt was undergoing decomposition. Finally it was found that the air dry platinichloride could be heated *in vacuo* at 100° for some hours without undergoing any loss of weight; a fact which afforded conclusive evidence of the absence of water of crystallisation.

An examination of the analytical data recorded by Schmiedeberg and Harnack, as well as by Nothnagel, further showed that there was no record of a determination of the nitrogen content of the molecule, although an otherwise complete analysis had been carried out. The platinichloride was accordingly analysed for nitrogen according to Dumas' method, when figures were obtained showing the presence of four nitrogen atoms in the molecule, i.e. two atoms of nitrogen in each molecule of free base. Accepting the remaining analytical data as more or less accurate for the supposedly hydrated platinichloride the actual formula for this salt was presumably the following:

$$(C_5H_{13}O_2N_2)_2\,PtCl_6.$$

Subsequently complete analysis proved this to be correct.

For the constitution of this base the two most probable formulae were the following:

$$\underset{\text{I}}{(OH)\,N\,(CH_3)_3\,CH_2\,.\,CH_2\,.\,NO_2}, \qquad \underset{\text{II}}{(OH)\,N\,(CH_3)_3\,CH_2\,.\,CH_2\,.\,O\,.\,NO}.$$

Formula (I) is that of an aliphatic nitro-derivative, the nitrogen being directly linked to a carbon atom while (II) represents the base as the choline ester of nitrous acid. That formula (II) correctly represents the constitution of the base is shown by the following facts:

In the first place the base gives a typical Liebermann's nitroso-reaction with phenol and sulphuric acid, which would not be the case if it were a nitro derivative. In the second place, on hydrolysis of the base by means of dilute acid or alkali, nitrous acid is very readily liberated, and choline may be recovered from the solution.

This base was actually obtained and recognised as such by Nothnagel

[1894] (who termed it "nitroso-choline") in the preparation of pseudo-muscarine by Schmiedeberg and Harnack's method. He considered this substance, however, to be a bye-product of the reaction, chiefly on account of a difference of crystalline form of the platinichlorides of the base and of pseudo-muscarine respectively. It was, however, remarked by Schmiedeberg and Harnack [1877] that the platinichloride of pseudo-muscarine (synthetic muscarine) which crystallises ordinarily in octahedra might also form "Federfahne ähnliche Aggregaten," a description which corresponds with that of "federbartartige Krystallen" recorded by Nothnagel for the platinichloride of "nitroso-choline."

That the supposed difference of constitution of these two bases in reality had very little foundation is supported by the analytical figures recorded by Nothnagel [1894] and shown in the following table:

Pseudo-muscarine platinichloride $[N(CH_3)_3CH_2.CH(OH)_2]_2PtCl_6 2H_2O$ Found	Nitroso-choline platinichloride $[N(CH_3)_3CH_2CH_2O.NO]_2PtCl_6 2H_2O$ Found	Choline nitrous ester platinichloride (anhydrous) Calculated
C = 17·66	17·69	17·8
H = 4·92	3·70	3·85
N —	8·31	8·31
Cl = 31·07	—	31·6
Pt = 28·54, 28·60, 28·44	28·73	28·93
Aurichloride	Aurichloride	Aurichloride
Au = 42·66	Au = 41·79	Au = 41·7

It will be observed that Nothnagel also formulates the "nitroso-choline" platinichloride as containing two molecules of water of crystallisation which, he states, were not removed by long heating at 100°. Nevertheless after drying the salt at 100° he obtained figures which, as is seen, agree well for the anhydrous salt. This fact was also pointed out by Schmidt and Wagner [1904], who obtained "nitroso-choline" by the prolonged action of concentrated nitric acid on choline at the ordinary temperature. The identity of this base with pseudo-(synthetic) muscarine is further supported by the similarity of the melting points of the aurichlorides ("pseudo-muscarine" aurichloride m.p. 234° after sintering from 174°; "nitroso-choline" aurichloride m.p. 240°) and by the fact stated by Nothnagel that the solubilities of the platinichloride are very similar.

In view, therefore, of the foregoing facts, it must be accepted that by the action of concentrated nitric acid upon choline as originally described by Schmiedeberg and Harnack, only one base yielding a platinichloride comparatively little soluble in cold water is produced. This base, which, as will

be shown in detail elsewhere by Dale, produces all the physiological effects described by various authors as brought about by pseudo-muscarine (synthetic muscarine), is, in fact, the choline ester of nitrous acid, and the constitution originally suggested for this base by Schmiedeberg and Harnack must be considered to be disproved.

If the formula ascribed by Harnack [1876] to the natural base be accepted as correct the difference in physiological action of the two bases is easily accounted for by their different chemical constitutions. It is, however, by no means certain that this formula can be accepted as correctly representing the constitution of the base. The facts that many choline-esters have a high degree of activity [Hunt and Taveau, 1911], which in the case of acetylcholine has been shown to approximate to the muscarine type of action [Dale, 1914], and that the so-called pseudo-muscarine is the choline-ester of nitrous acid, make it possible that muscarine itself may be a base of this class. In such a case recrystallisation of the aurichloride might well be accompanied by partial hydrolysis of the base.

With these possibilities in mind I prepared the choline-ester of nitric acid $(OH)\,N\,(CH_3)_3\,CH_2\,.\,CH_2\,.\,O\,.\,NO_2$ by the method described by Schmidt and Wagner [1904]. This base, again, is possessed of very considerable activity and in fact its action in some respects resembles that of natural muscarine much more closely than does that of pseudo-muscarine; nevertheless it still produces a marked curare effect upon frogs. How far these facts have any bearing upon the question of the constitution of natural muscarine must, however, for the present remain an open question.

Experimental.

Preparation of pseudo-muscarine. 3·8 grams of pure choline platinichloride were dissolved in about 5 cc. of concentrated nitric (D 1·4) on the water bath and then rapidly evaporated just to dryness on a sand bath. The reaction product was washed successively with absolute alcohol and once or twice with small quantities of cold water to remove the bulk of the unchanged choline platinichloride. The residue was crystallised from a little hot water. The platinichloride of pseudo-muscarine separated on standing as extremely well formed octahedra. The product was homogeneous and melted sharply with decomposition at 250–251° (bath at 200° and temperature slowly raised). Recrystallisation from water caused no alteration either of crystalline form or of melting point. The salt was anhydrous. Analysis of this salt gave the following results:

0·1043 g.; 0·0300 g. Pt. Pt = 28·76.
0·1071 g.; 0·0312 g. Pt. Pt = 29·13.
0·1384 g.; 0·0401 g. Pt. Pt = 28·97.

*0·1641 g.; 0·1114 g. CO_2; 0·0654 H_2O; C = 18·5; H = 4·4.
0·1320 g.; 0·0846 g. CO_2; 0·0475 H_2O; C = 17·5; H = 4·0.

*0·1346 g.; 9·0 cc. N_2 (moist) at 766 mm. and 11°. N = 8·0.
0·1184 g.; 8·5 ,, ,, 753 ,, 13°. N = 8·38.

Calculated for $[C_5H_{13}O_2N_2]_2PtCl_6$. Pt = 28·93; C = 17·80; H = 3·86; N = 8·31.

The analysis of this salt for C, H, and N gave some difficulty since NO was readily evolved. Unless special precautions were taken and doubly long reduced copper or silver spirals employed some of the oxides of nitrogen escaped reduction and results were obtained such as those instanced above and marked with an asterisk. In such cases nitrous acid was readily shown to be present in the potash bulbs or nitrometer.

The platinichloride was converted into the aurichloride by decomposing the former by means of excess of potassium chloride, evaporating the solution to dryness, extracting with alcohol, evaporating off the alcohol and precipitating the concentrated aqueous solution of the residue with an aqueous solution of gold chloride. The aurichloride thus formed was recrystallised from water, in which it is moderately soluble, and was obtained in the form of plates which melted with decomposition at 256° after sintering from about 200°.

Analysis:

0·1753 g.; 0·0727 g. Au; Au = 41·47.

Calculated for $[C_5H_{13}O_2N_2]AuCl_4$. Au = 41·73.

Hydrolysis of pseudo-muscarine. 0·4 gram of the platinichloride of pseudo-muscarine was boiled under reflux for 2 hours with 5 cc. of 10 % hydrochloric acid. The solution was then concentrated and on standing there was obtained 0·07 gram of a platinichloride crystallising in needles and hexagonal plates, melting at 237–238°.

Analysis gave the following:

0·0646 g.; 0·0199 g. Pt. Pt = 30·8.

Calculated for $[C_5H_{14}ON]_2PtCl_6$. Pt = 31·6 per cent.

The mother liquors from the platinichloride were decomposed by potassium chloride and the aurichloride of the base obtained as described above. This was moderately soluble in hot water and melted at 261–262°.

Analysis:

0·1387 g.; 0·0610 g. Au. Au = 44·0.

Calculated for $[C_5H_{14}ON)AuCl_4$. Au = 44·4 per cent.

The mother liquors from the aurichloride were decomposed by H_2S and on treatment with mercuric chloride gave a crystalline salt melting sharply at 249° which when mixed with choline mercuric chloride showed no alteration of melting point.

On hydrolysis therefore pseudo-muscarine yields choline. The formation of nitrous acid on hydrolysis was readily shown by boiling a solution of the chloride of the base for a few moments with dilute alkali, acidifying the solution, and adding to a solution of potassium iodide and starch. The dark blue colour of the starch-iodine complex at once appeared.

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XXVIII. THE ENZYMES OF WASHED ZYMIN AND DRIED YEAST (LEBEDEFF). III. PEROXYDASE, CATALASE, INVERTASE AND MALTASE.

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Peroxydase.

On the assumption that hydrogen peroxide and peroxydase might possibly be capable of oxidising some degradation product of sugar, Bach [1906] submitted sugar to the simultaneous action of hydrogen peroxide, peroxydase and an enzyme capable of decomposing sugar. He chose for his experiments the fermenting enzyme of yeast and employed zymin. Being however of the opinion that yeast contains no peroxydase he made use in his investigations of a peroxydase prepared from horse-radish. Although unable to observe any of the oxidation processes the probability of which he had anticipated, he found that when the peroxydase used was previously boiled the amount of carbon dioxide evolved was invariably more than when it was not boiled. This led him to conclude that the presence of active peroxydase had a deterrent effect on the fermenting power of zymin.

The authors find that fresh English brewery yeast contains active peroxydase. When however the yeast is dried for 17 hours at a temperature of 37° it no longer gives the reaction. On washing the dried yeast however the presence of peroxydase can again be detected.

Dried Munich yeast (Schroder) behaves like dried English yeast and does not react for peroxydase when unwashed, but regains that activity on being washed. Whether the peroxydase in yeast has a deterrent effect on the fermentation the authors are at present not prepared to say. It is to be observed that Bach in his experiments used a peroxydase from an outside source (horse-radish) and the presence of an agent in that preparation that would have a retarding effect on fermentation and which could be destroyed by boiling is not impossible.

Experimental.

Experiment 1. Ten g. of pressed English brewery yeast were washed four times to remove adhering wort, etc., and suspended in 300 cc. of distilled water. The washing in this and all the other experiments was done by stirring up the yeast in pots with 120 cc. of distilled water for the first time and 100 cc. for all subsequent times and centrifuging. Into two small Erlenmeyer flasks were introduced:

Flask 1. 10 cc. of yeast suspension (equivalent to 0·1 g. of dried yeast) + 1 cc. 1 per cent. p-phenylenediamine + 1 cc. H_2O_2 (10 per cent. dilution of 3 per cent. H_2O_2 neutralised to litmus).

Flask 2. 10 cc. of yeast suspension + 1 cc. 1 per cent. p-phenylenediamine.

Flask No. 1 showed the first sign of a colouration in one minute and assumed an intense violet colouration in three minutes.

Flask No. 2 remained unchanged.

It is evident from this experiment that English yeast contains an active peroxydase.

Experiment 2. A sample of the yeast used in the previous experiment was then dried for 17 hours at a temperature of 37° and one gram of the dried yeast suspended in 100 cc. of distilled water. Into two flasks were introduced:

Flask No. 1. 10 cc. of yeast suspension (0·1 g. of dried yeast) + 1 cc. 1 per cent. p-phenylenediamine + 1 cc. H_2O_2 (10 per cent. dilution of 3 per cent. H_2O_2).

Flask No. 2. 10 cc. of yeast suspension + 1 cc. 1 per cent. p-phenylenediamine.

Although flask No. 1 after one minute assumed a slightly darker colour than the control flask No. 2, it did not show any violet colouration and the reaction was decidedly negative. The repetition of the above experiments gave similar results and we may conclude that drying inhibits the activity of the peroxydase in the yeast.

Experiment 3. Some dried English yeast was washed four times; this process removes soluble phosphates, the coenzyme of zymase and, doubtless, other substances. Into two flasks were introduced:

Flask No. 1. 10 cc. of washed yeast suspension (0·1 g. of dried yeast) + 1 cc. 1 per cent. p-phenylenediamine + 1 cc. dilute H_2O_2.

Flask No. 2. 10 cc. of unwashed dried English yeast suspension (0·1 g. of dried yeast) + 1 cc. 1 per cent. p-phenylenediamine + 1 cc. dilute H_2O_2.

Flask No. 1 showed a colouration after 2 minutes. Flask No. 2 showed no colouration after 5 minutes. This experiment shows that the activity of the dried yeast can be restored by washing.

Experiment 4. 5 g. of dried English yeast were washed four times. The first washings amounted to 32 cc. Six Erlenmeyer flasks were taken and in each were placed 10 cc. of washed yeast suspension (0·1 g. of dried yeast) + 1 cc. 1 per cent. p-phenylenediamine + 1 cc. dilute H_2O_2.

To flask No. 1 nothing was added.
„ No. 2 0·6 cc. of the washings was added.
„ No. 3 1 cc. „ „ „
„ No. 4 1·5 cc. „ „ were added.
„ No. 5 5 cc. „ „ „
„ No. 6 5 cc. of the neutralised washings were added.

No. 1 showed a colouration after one minute, No. 2, No. 3 and No. 4 after 3, 4 and 4 minutes respectively. No. 5 and No. 6 showed no colouration after 15 minutes. This experiment shows that the addition of the washings inhibits the action of the peroxydase in yeast.

Experiments similar to Nos. 3 and 4 were carried out with dried Munich yeast (Schroder) and zymin, and the results obtained were identical with those obtained with English dried yeast. It is however to be observed that various samples of zymin gave reactions of different intensity.

It is evident from these results that after the yeast cell has been dried or treated with acetone washing removes some substance which has an inhibiting effect on the peroxydase of the yeast. The washings not only inhibit the action of the peroxydase in yeast but also exert a deterrent effect on that of milk as shown by Experiment 5.

Experiment 5. Into two flasks were introduced:

Flask No. 1. 1 cc. dilute H_2O_2 + 1 cc. 1 per cent. p-phenylenediamine + 1 cc. of milk + 5 cc. of water.

Flask No. 2. 1 cc. dilute H_2O_2 + 1 cc. 1 per cent. p-phenylenediamine + 1 cc. of milk + 3 cc. of water + 2 cc. of washings from dried Munich yeast.

Flask No. 1 showed a colouration immediately. Flask No. 2 showed no colouration after 5 minutes.

It was further observed that the action of the inhibiting agent seems to increase in a higher proportion than that of the peroxydase as the amount of yeast is increased.

The following experiment illustrates this point.

Experiment 6. Flask No. 1. 0·05 g. of dried English yeast suspended in 10 cc. of H_2O + 1 cc. 1 per cent. p-phenylenediamine + 1 cc. H_2O_2.

Flask No. 2. 0·2 g. of dried English yeast suspended in 10 cc. of water + 1 cc. 1 per cent. p-phenylenediamine + 1 cc. H_2O_2.

Flask No. 1 showed the first signs of a colouration only after 4 minutes and eventually assumed a violet colouration which, although not so intense as in the case of washed yeast, was well marked. Flask No. 2 showed no colouration after 15 minutes.

Several other substances have been found, the addition of which to washed dried Munich yeast (Schroder) inhibits the action of the peroxydase. These are beef broth (0·1 cc.), sodium lactate (0·5 cc. of 1 per cent.), peptone (1 cc. of 1 per cent.), alkalis and acids. Beef broth previously treated with H_2O_2 for 20 minutes still has an inhibiting effect. As to the acids and alkalis, small traces of either will inhibit the action of the peroxydase in yeast as shown by the following experiment.

Experiment 7. Flask No. 1. 10 cc. of washed dried Munich yeast (Schroder) suspension (0·1 g.) + 1 cc. 1 per cent. p-phenylenediamine + 1 cc. H_2O_2 + 0·5 cc. N/10 KOH.

Flask No. 2. 10 cc. of washed dried suspension (0·1 g.) + 1 cc. 1 per cent. p-phenylenediamine + 1 cc. H_2O_2 + 0·5 cc. N/10 H_2SO_4.

Flask No. 3 contained 10 cc. of washed dried yeast (Schroder) suspension (0·1 g.) + 1 cc. 1 per cent. p-phenylenediamine + 1 cc. H_2O_2.

No. 3 showed a colouration after two minutes. No. 1 and No. 2 showed no colouration after 5 minutes.

As the washings of dried yeast are slightly acid to litmus the possibility that the acidity of the washings acted as the inhibiting agent on the peroxydase suggested itself, but it was experimentally proved that this was not the case.

Experiment 8. In this experiment the washings were carefully neutralised to litmus.

Flask No. 1. 10 cc. of washed dried Munich yeast suspension + 1 cc. 1 per cent. p-phenylenediamine + 1 cc. H_2O_2 + 3 cc. H_2O.

Flask No. 2. 10 cc. of washed dried Munich yeast suspension + 1 cc. 1 per cent. p-phenylenediamine + 1 cc. H_2O_2 + 1·5 cc. of neutralised washings + 1·5 cc. H_2O.

Flask No. 3. 10 cc. of washed dried Munich yeast suspension + 1 cc. 1 per cent. p-phenylenediamine + 1 cc. H_2O_2 + 3 cc. neutralised washings.

No. 1 showed a colouration after 2 minutes while No. 2 and No. 3 showed no colouration after 5 minutes.

Precisely the same result was obtained when the washings were made neutral to phenolphthalein.

Washed and unwashed Munich dried yeast, washed and unwashed English dried yeast, washed and unwashed fresh English brewery yeast give no reaction with p-phenylenediamine and with benzidine in absence of H_2O_2, which proves the absence of an oxydase. Similarly the above-mentioned yeasts under the same conditions give no reaction for tyrosinase.

Catalase.

In the experiments of Bach mentioned earlier in this paper this experimenter also observed that the amount of oxygen evolved by the catalase of the zymin was almost equal to the theoretical amount capable of being liberated from the hydrogen peroxide. In order to ascertain with precision whether the washing of dried Munich yeast influenced the catalase, experiments were carried out as follows: 0·1 g. of dried Munich yeast (Schroder) was introduced into a small Erlenmeyer flask connected with an azotometer filled with mercury over which the liberated oxygen could be collected and measured. 25 cc. of hydrogen peroxide were run in from a burette passing through the stopper of the flask, and the volume of the oxygen liberated and the time taken for the liberation of the gas noted. Then a second experiment was carried out in which 25 cc. of the hydrogen peroxide were treated in the same apparatus with excess of acidified $KMnO_4$, and the volume of oxygen liberated again measured. The available oxygen of the hydrogen peroxide, i.e. half the amount evolved on treatment with $KMnO_4$, was thus ascertained and compared with the volume evolved by the action of the catalase of the yeast. The washed dried Munich yeast was treated in a similar way.

Experiment 1. 0·1 g. of unwashed dried Munich yeast liberated from 25 cc. of H_2O_2 17·1 cc. of oxygen in 5 minutes.

Acidified $KMnO_4$ and 25 cc. of the same H_2O_2 liberated 33·6 cc. of oxygen.

Experiment 2. 0·1 g. of washed dried Munich yeast liberated from 25 cc. of another sample of H_2O_2 11 cc. of oxygen in 5 minutes.

Acidified $KMnO_4$ and 25 cc. of the same H_2O_2 gave 21·9 cc. of oxygen.

It is evident from these results that the catalase in the dried Munich yeast is capable of liberating the entire available oxygen of the H_2O_2 and that the washing of the yeast makes no difference either to the volume of the oxygen liberated or to the rate of evolution.

Invertase and Maltase.

Experiments were next made to ascertain whether by repeated washing at ordinary room temperature it was possible to remove all traces of invertase and maltase from dried Munich yeast (Schroder) and zymin. The investigations were carried out by means of two different sets of experiments. In one set the yeast was washed six times. The first washings were boiled in order to destroy any invertase and maltase they might contain and added to the washed yeast, the mixture being then digested with cane sugar and maltose respectively for 17 hours at 25° and the CO_2 evolved, if any, noted. One flask containing glucose and another containing water in addition to the mixture of washed yeast and boiled washings were also digested at the same time for comparison. In the event of the invertase and maltase being entirely washed out no fermentation of cane sugar or maltose could be expected to take place, whereas if hydrolysis occurred the resulting hexoses would be fermented by the zymase in presence of the coenzyme added in the washings.

In the second set of experiments the cane sugar and the maltose were respectively digested with the washed yeast without the addition of the washings for 3 hours at a temperature of 25°. The suspensions were then filtered and the filtrate examined in order to ascertain whether any hydrolysis had occurred. In this case, no coenzyme being present, no fermentation could take place.

1. Invertase.

Experiment 1. The Munich dried yeast used in this experiment was washed six times.

No. 1. 25 cc. (5 g.) of washed Munich yeast + 20 cc. (1 g.) of cane sugar solution + 30 cc. of boiled washings + 0·5 cc. of toluene.

No. 2. 25 cc. (5 g.) of washed Munich yeast + 20 cc. (1 g.) of glucose + 30 cc. of boiled washings + 0·5 cc. of toluene.

No. 3. 25 cc. (5 g.) of washed Munich yeast + 20 cc. H_2O + 30 cc. boiled washings + 0·5 cc. toluene.

The digestion was continued for 17 hours at 25°.

The contents of all three flasks fermented and the following volumes of CO_2 were evolved :

Time	Flask No. 1	Flask No. 2	Flask No. 3
After 30 minutes	8·9 cc.	8·2 cc.	3·6 cc.
,, 60 ,,	14·8	14·1	5·5
,, 90 ,,	18·8	17·7	7·2
,, 17 hours	54·8	57·3	16·0

An experiment in which zymin was substituted for the dried Munich yeast washed six times was also carried out and the results obtained were similar. In 17 hours 25·3 cc. of CO_2 were evolved from the cane sugar, 25·2 cc. from the glucose, and 4·9 cc. from the autofermentation of the zymin.

These results show that the rate of evolution and the volume of the CO_2 produced from cane sugar are almost the same as from glucose, and it may therefore be concluded that the amount of invertase left in the dried Munich yeast and the zymin after washing is very appreciable.

The above results are confirmed by the following set of experiments.

Experiment 2. The dried Munich yeast used was washed six times.

Flask No. 1. 25 cc. (5 g.) of washed dried Munich yeast + 20 cc. (1 g.) of cane sugar solution + 0·5 cc. of toluene.

Flask No. 2. 25 cc. (5 g.) of washed dried Munich yeast + 20 cc. of water + 0·5 cc. of toluene. The contents of the two flasks were incubated for 3 hours at 25° and were then filtered, and the ratio

$$\frac{\text{Rotation in 400 mm. tube}}{\text{Reduction (g. glucose per 100 cc.)}}$$

of filtrate No. 1 was determined, the rotation of filtrate No. 2 being reckoned for in calculating the actual ratio.

$$\text{Ratio } \frac{\text{Rotation in 400 mm. tube}}{\text{Reduction (g. glucose per 100 cc.)}} \text{ of filtrate No. 1} = \frac{-0{\cdot}373}{0{\cdot}500} = -0{\cdot}746.$$

$$\text{Ratio } \frac{\text{Rotation in 400 mm. tube}}{\text{Reduction (g. glucose per 100 cc.)}} \text{ of invert sugar} = -0{\cdot}800.$$

Experiments similar to Experiment 2 were carried out with dried Munich yeast washed with a mixture of water and toluene and with zymin washed both with water only and with water and toluene. The following are the results obtained:

With zymin washed with water Ratio $= \frac{-0{\cdot}586}{0{\cdot}736} = -0{\cdot}796.$

„ „ „ and toluene Ratio $= \frac{-0{\cdot}629}{0{\cdot}760} = -0{\cdot}827.$

With dried Munich yeast washed with toluene and water

$$\text{Ratio} = \frac{-0{\cdot}614}{0{\cdot}730} = -0{\cdot}840.$$

All the above results show that the entire cane sugar was inverted during the three hours.

In view of these results it became of interest to ascertain whether there was any difference in the rate of inversion of cane sugar between washed and unwashed zymin. Zymin was chosen because small quantities of that preparation in a concentrated solution of sugar do not commence fermenting for

some time and consequently it is possible to ascertain the amount of sugar inverted during that time and compare it with the amount inverted in the same time by an equal weight of zymin after being washed.

Experiment 3. The mixtures were incubated for 20 minutes at a temperature of 25°.

Flask No. 1. 0·1 g. of unwashed zymin suspended in 20 cc. of water + 50 cc. of 10 per cent. cane sugar solution.

Flask No. 2. 0·1 g. of washed zymin suspended in 20 cc. of water + 50 cc. of 10 per cent. cane sugar solution.

No fermentation was observed during the twenty minutes. The flasks were then placed in boiling water for twenty minutes in order to stop any further action of the invertase. After cooling to the room temperature they were made up to 100 cc. and filtered and the reducing powers of the filtrates calculated in terms of glucose were determined.

Reduction of filtrate No. 1 = 1·04 g. of glucose per 100 cc.
„ „ No. 2 = 0·67 g. „ „ „

It is thus seen that about $\frac{1}{5}$ of the total cane sugar was inverted by the unwashed zymin during the twenty minutes and that 64·4 per cent. of the amount inverted by the unwashed zymin was inverted by the washed preparation. This would suggest that the amount of invertase left behind even after six washings is a very considerable fraction (approximately 65 per cent.) of that originally present.

2. Maltase.

A series of experiments similar to those carried out with cane sugar were made with maltose.

Washed dried Munich yeast (Schroder) and washed zymin readily fermented maltose on addition of the boiled washings. The volumes of CO_2 were as follows:

	Maltose	Glucose	Autofermentation
Munich dried yeast	58·0 cc.	57·3 cc.	16·0 cc.
Zymin	25·1	25·2	4·9

As from cane sugar the rate of evolution and volume of the CO_2 evolved from maltose are almost equal to those given by glucose, and consequently the amount of maltase left behind in the preparations after washing must also be very considerable.

The resulting ratios $\frac{\text{Rotation in 400 mm. tube}}{\text{Reduction (g. glucose per 100 cc.)}}$ of filtrates obtained after digesting maltose without the addition of the washings for 3 hours at 25° with dried Munich yeast (Schroder) and zymin respectively, each of these preparations washed with water alone and with a mixture of water and toluene, were :

Dried Munich yeast washed with water only $\frac{+1{\cdot}095}{0{\cdot}550} = +1{\cdot}99.$

„ „ „ „ and toluene $\frac{+1{\cdot}848}{0{\cdot}680} = +2{\cdot}7.$

Zymin washed with water only $\frac{+2{\cdot}360}{0{\cdot}574} = +4{\cdot}1.$

„ „ „ „ and toluene $\frac{+2{\cdot}886}{0{\cdot}490} = +5{\cdot}89.$

Ratio for glucose + 2·1. Ratio for maltose + 9·8.

It appears from these figures that the whole of the maltose was converted into glucose by the dried Munich yeast while only a part of the maltose was converted by the zymin. Washing with a mixture of water and toluene seems however to have made no great difference to either preparation.

The idea of the possibility of removing the maltase from the zymin by a more thorough process of washing suggested itself and an experiment was accordingly instituted in which the zymin was washed 8 times. Each time it was vigorously shaken up in the pot for several minutes and the last time it was placed in a shaker for an hour previous to being centrifuged. On addition of the boiled washings the mixture fermented maltose freely at a rate almost equal to that produced with glucose. It is therefore evident that the most thorough method of washing will not entirely remove the maltase from zymin at ordinary room temperature.

As in Experiment 3 with cane sugar, 50 cc. of 10 per cent. maltose were also digested with 0·1 g. of washed and unwashed zymin respectively for 20 minutes. The resulting filtrates obtained showed the following reductions per 100 cc. calculated in terms of glucose :

Washed zymin 2·2 per cent. Unwashed zymin 2·2 per cent.

In this case although only a part of the maltose was converted into glucose the amounts hydrolysed were equal in both cases, and therefore the quantity of maltase removed by the washing can only have been very small.

SUMMARY.

1. Fresh English yeast reacts with H_2O_2 and p-phenylenediamine for peroxydase.

2. Dried yeast (both English and Munich) does not react for peroxydase with p-phenylenediamine and H_2O_2 but on being washed it regains that activity.

3. The addition of the washings and some other reagents to washed dried yeast inhibits the action of peroxydase.

4. Washing does not affect the activity of the catalase of dried yeast (Schroder).

5. The power of hydrolysing cane sugar is partially but not entirely removed from zymin and dried Munich yeast (Schroder) by washing at ordinary room temperature whilst the power of hydrolysing maltose is not affected.

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XXIX. AN INVESTIGATION INTO THE PHYSICO-CHEMICAL MECHANISM OF HAEMOLYSIS BY SPECIFIC HAEMOLYSINS. No. II. THE ELECTRICAL CONDUCTIVITY OF SENSITISED CORPUSCLES AND THE ACTION OF INORGANIC FERMENTS OR METAL-SOLS UPON THEM.

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In my previous communication [1913] I pointed out that in the process of haemolysis by specific haemolysins, the amboceptor molecules are at first adsorbed into the pores in the walls of the erythrocytes and subsequently a true chemical combination takes place between these and the molecules of the corpuscular walls.

Observations have since been carried on to determine the electrical conductivity of the sensitised corpuscles as compared with the unsensitised ones. The following methods were adopted.

Sheep's corpuscles after being washed with normal saline were treated with six times the working dose of anti-sheep amboceptor and the mixture kept for a variable period, from two to twenty-four hours, in the ice-chamber. Subsequently the corpuscles were centrifuged and thoroughly washed with normal saline. In a second series of observations the sensitised as well as the unsensitised corpuscles were suspended in 5 % glucose solution and then separated by centrifugalisation.

The conductivity of the corpuscles was measured by Kohlrausch's method and the following results were obtained. In all cases the conductivity was calculated at 26° C.

TABLE I.

The electrical conductivity of the sediment of corpuscles from the emulsion in normal saline.

(*a*)	(1)	Unsensitised corpuscles	K (26° C.) $\times 10^5 = 360$
	(2)	Sensitised corpuscles	K (26° C.) $\times 10^5 = 300$
(*b*)	(1)	Unsensitised corpuscles	K (26° C.) $\times 10^5 = 300$
	(2)	Sensitised corpuscles	K (26° C.) $\times 10^5 = 120$
(*c*)	(1)	Unsensitised corpuscles	K (26° C.) $\times 10^5 = 190$
	(2)	Sensitised corpuscles	K (26° C.) $\times 10^5 = 160$

TABLE II.

The electrical conductivity of the sediment of corpuscles from the emulsion in 5 % glucose solution in distilled water.

(*a*)	(1)	Unsensitised corpuscles	K (26° C.) $\times 10^5 = 250$
	(2)	Sensitised corpuscles	K (26° C.) $\times 10^5 = 100$
(*b*)	(1)	Unsensitised corpuscles	K (26° C.) $\times 10^5 = 70$
	(2)	Sensitised corpuscles	K (26° C.) $\times 10^5 = 38$

It will be seen from the above tables that the adsorption of amboceptor by erythrocytes was followed by a diminution of the electrical conductivity of the corpuscles and this was specially marked when the sediment of corpuscles was obtained from an emulsion in isotonic glucose solution.

In the case of the sediment from the suspension in normal saline, the conductivity is mainly due to the ions still in contact with the corpuscular walls. Therefore the corpuscular walls of the sensitised corpuscles obstruct the passage of the ions more than the unsensitised ones. In the case of the sediment from glucose solution, the conductivity is mostly due to the envelopes. From these the conclusion follows that the walls of the sensitised corpuscles conduct electricity worse than those of the unsensitised ones.

From the above it may be assumed that the diminution of conductivity of the corpuscular walls in the case of sensitised corpuscles is due to the action of amboceptor on the walls and therefore that at least a portion of the amboceptor is in some sort of combination with the corpuscular walls in sensitised corpuscles.

Action of inorganic ferments or metal-sols upon sensitised corpuscles.

A series of experiments was undertaken to determine the action of catalysts, such as animal charcoal and platinum black, upon sensitised corpuscles. No haemolysis was brought about by their action. Colloidal solution of iodine (Collo-Iode of Dubois) also gave negative results. Various

metal-sols were mixed in varying concentration with the sensitised corpuscles. The following have, up to the present time, been used. Electargal and electro-selenium prepared by Clin and Co. (Paris), collo-sol silver and collo-sol mercury prepared by Oppenheimer and Sons.

Up to now, all such experiments with colloids have failed to bring about haemolysis of the sensitised corpuscles.

Conclusions.

(1) In the process of adsorption of amboceptor by the erythrocytes, it is in the corpuscular walls that the molecules of the amboceptor remain adsorbed.

(2) The electrical conductivity of the corpuscular walls of the amboceptor-loaded corpuscles is less than that of normal erythrocytes.

(3) Metal-sols, as well as other catalysts, such as animal charcoal, platinum black, colloidal iodine do not bring about any haemolysis of sensitised corpuscles.

I am deeply indebted to Col. Sutherland, I.M.S., and Dr G. C. Mitter of the Medical College, Calcutta, for providing me with the haemolytic antisera for my experiments, and to Professor Nilratan Dhar of the Presidency College, Calcutta, for assisting me in determining the electrical conductivities.

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XXX. ON THE ESTIMATION OF β-HYDROXY-BUTYRIC ACID.

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(Received April 29th, 1914.)

The experiments described in this paper were undertaken in order to test those methods for the estimation of β-hydroxybutyric acid which are applicable to mixtures of its two optical isomers. Two such methods are available, dependent upon the conversion of the acid into crotonic acid, and into acetone, respectively.

The production of crotonic acid has been applied as a method of estimation by Darmstädter [1903], Ryffel [1905], and Pribram [1912]. Darmstädter's method has been shown by Shindo [1907] to be very inaccurate. Pribram obtained 99 % of the theoretical amount of crotonic acid in as little as 350 cc. of distillate. In the present investigation a large number of estimations were made by Pribram's method, and the yield was not more than 96 % in from 700 to 1200 cc. of distillate. The form of the curve obtained is very similar to that given below for the formation of acetone from β-hydroxybutyric acid; in the later stages of the distillation the crotonic acid comes over more and more slowly. In Ryffel's method this difficulty was avoided by calculating the ultimate result of the distillation from the rate of evolution observed in fractions of the first 400 cc. of distillate.

The conversion of β-hydroxybutyric acid into acetone by oxidation with sulphuric acid and potassium bichromate was applied as a method of estimation by Shaffer [1908]; the acetone produced was estimated by means of iodine and thiosulphate in the ordinary way. It seems preferable to employ the method of Scott-Wilson [1911] for determining the amount of acetone, since the reagent introduced by him is less likely than is iodine to be affected by the presence of other substances. This combination of the methods of Shaffer and Scott-Wilson has been used in the investigation of a series of cases of acidosis [Kennaway, Pembrey, and Poulton, 1913].

Results obtained with the Scott-Wilson method.

The acetone is precipitated by a reagent containing mercuric cyanide, silver nitrate, and caustic soda; the amount of mercury in the precipitate is estimated by titration with potassium sulphocyanide. On the assumption that one molecule of acetone combines with five atoms of mercury, Scott-Wilson obtained results which were about 3 % too low. The following experiments were carried out with acetone from the bisulphite compound (Kahlbaum).

TABLE I.

			Mg. acetone in 10 cc.
I.	(1)	0·0990 gm. acetone weighed and diluted to 1 litre ...	0·990
	(2)	100 cc. of above solution estimated by iodine method ...	(1) 0·981
			(2) 0·981
	(3)	10 cc. estimated by Scott-Wilson method. Mean of ten estimations (max. 0·980, min. 0·960)	0·9718
	(4)	5 cc. estimated by Scott-Wilson method. Mean of ten estimations (max. 0·986, min. 0·963)...	0·9707
II.	(1)	0·0747 gm. acetone weighed and diluted to 1 litre ...	0·747
	(2)	100 cc. of above solution estimated by iodine method ...	(1) 0·722
			(2) 0·722
	(3)	10 cc. of above solution estimated by Scott-Wilson method ...	(1) 0·719
			(2) 0·713
III.	Acetone solution of unknown strength:		
	(1)	100 cc. estimated by iodine method	0·8180
	(2)	10 cc. estimated by Scott-Wilson method	0·8177

In Scott-Wilson's experiments, the precipitation of acetone by the mercuric cyanide reagent was found to be complete in 10 minutes if the reagent were added in the proportion of not less than 25 cc. for 1 mg. of acetone. These estimations were made with a $\frac{N}{1000}$ acetone solution (i.e. 0·58 mg. in 10 cc.). Since the concentration of acetone in distillates from urine is often much lower than this, the question of the relation between this concentration and the rate of precipitation requires some further testing.

TABLE II.

Precipitation of acetone in different concentrations with the same time of precipitation.

Portions of 10 cc. of the acetone solution given under (II) above were precipitated with Scott-Wilson reagent, the concentration being in some cases lowered by addition of water, as shown in the third column below. The precipitation was in all cases allowed to proceed overnight.

Acetone solution, cc.	Scott-Wilson reagent, cc.	Total volume, cc.	Result of estimation	
			Cc. KCNS used	Mg. acetone
10	25	35	12·40	0·719
,,	25	35	12·30	0·713
,,	25	35	12·20	0·707
,,	20	30	12·35	0·716
,,	20	30	12·30	0·713
,,	20	100	12·20	0·707
,,	20	100	12·05	0·699
,,	20	200	12·10	0·702
,,	20	200	11·90	0·690
,,	20	300	11·40	0·661
,,	20	300	10·90	0·632
,,	20	300	10·70	0·620

These results show that the precipitation can be rendered incomplete and irregular by sufficient dilution. The rate of precipitation was tested as follows:

TABLE III.

Precipitation of acetone at the same concentration with different times of precipitation.

Mixtures of Acetone solution ... 10 cc.
Scott-Wilson reagent ... 20 ,,
Water ... ... 70 ,,

Hours allowed for precipitation	Result of estimation	
	Cc. KCNS used	Mg. acetone
½	12·00 12·00	0·696
1	12·30 11·95	0·703
1½	11·90 11·90	0·690
2	12·00	0·696
2½	12·00	0·696
5	12·10 12·20	0·7047
18	12·30 12·20 12·05	0·7064

The differences observed in this series are almost within experimental error. A full investigation of the relations between different concentrations of acetone and of mercuric cyanide, and the rate of precipitation, would obviously be very laborious. In practice, this question does not give much trouble. With a little experience, it is easy to judge from the rate at which the precipitate develops whether an excessive amount of acetone has been added to the amount of reagent taken. In any case of doubt, one can (1) add more reagent to one of two duplicates; (2) keep one duplicate for a longer time before filtration; or (3) keep the filtrate to see whether any further precipitate develops in it. It is well to obtain distillates which contain more than 0·7 mg. acetone in 100 cc. (see Table II), to aim at using 30 cc. reagent for 1 mg. acetone, and to leave the mixtures when possible overnight before filtration.

Except where it is otherwise stated, all the acetone estimations given in this paper were made by the Scott-Wilson method.

Results obtained with Shaffer's method.

(*a*) *The estimation of β-hydroxybutyric acid.*

Shaffer [1908] obtained approximately correct yields (98·8 to 103·3 %), using a preparation of β-hydroxybutyric acid the purity of which was not tested in a satisfactory manner. Cooke and Gorslin [1911] showed that they could obtain concordant duplicates by this method, but did not attempt to establish its accuracy. Mondschein [1912] obtained an average yield of 97·8 %, but he gives no particulars of the preparation of the acid used in his experiments.

The preparation used in the estimations given below was made from the impure inactive acid (Kahlbaum). The liquid was diluted with water, filtered, and extracted with ether; the extract was distilled in vacuo, the residue neutralised with caustic soda, and the salt recrystallised five times from alcohol. It gave the following result on combustion[1]:

0·1600 g.; 0·0806 g. H_2O; 0·2240 g. CO_2.

	C %	H %
Found	38·20	5·60
Calculated for $C_4H_7O_3Na$	38·09	5·55

When weighed amounts of this salt were treated by Shaffer's method,

[1] I am indebted to Mr V. Steele for carrying out this analysis.

the yield of acetone obtained was from 90 to 96·5 % of the theoretical amount. The concentration of acetone in successive portions of distillate becomes less and less until the amount, if any be present, is too small for estimation. (Table IV and Fig. 1.)

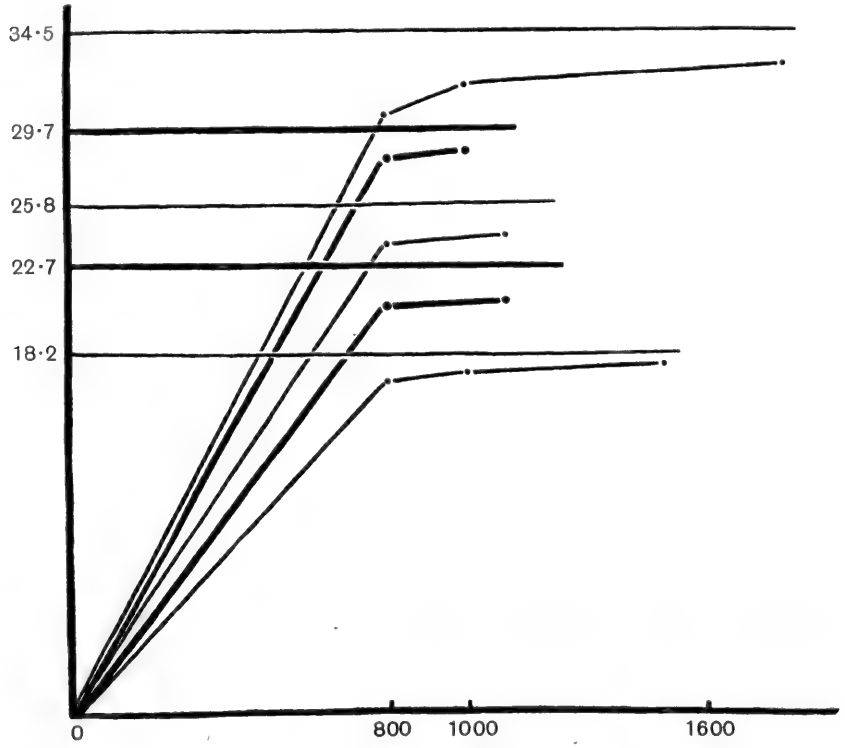

Fig. 1. Formation of acetone from β-hydroxybutyric acid.

Abscissae represent cc. distillate.
Ordinates represent mg. acetone.
Horizontal lines show the theoretical amounts of acetone.

Various attempts were made to increase the yield by altering the manner in which the oxidation was carried out. If the concentration of sulphuric acid be increased to six volumes per cent., which is twice that recommended by Shaffer, the reaction comes to an end sooner, but the total yield is not increased (see Expts. VII and VIII, Table IV). Slow addition of the bichromate produces a somewhat higher result than the more rapid addition of the same amount (Fig. 2). Theoretically, 1 part of acetone should be produced by 1·7 parts of bichromate. Large excess of bichromate certainly diminishes the yield, as Shaffer stated; on the other hand, the theoretical

amount of bichromate produces only about $\frac{2}{3}$ of the theoretical yield of acetone (see Expt. VI, Table IV; and Fig. 2). Between these two extremes of excess and defect, there is a wide range over which the amount of bichromate seems to have no regular influence on the result. This is shown in Table V, in which the yields are arranged in descending order.

TABLE IV.

Sodium β-hydroxybutyrate distilled with 3 volumes % sulphuric acid, and potassium bichromate.

Expt.	Distillate, cc.	Acetone found, mg.	Acetone calculated, mg.	Yield %	Potassium bichromate, mg. used	Potassium bichromate, mg. calculated
I	500	18·94				
	300	1·63				
	300	0·26				
	1100	20·83	22·69	91·9	250	40
II	800	81·78				
	500	trace				
	1300	81·78	91·2	90	700	155
III	800	28·10				
	200	0·17				
	1000	28·27	29·64	95·4	600	50
IV	800	29·69				
	200	1·95				
	800	1·22				
	1800	32·86	34·47	95·3	440	59
V	800	23·74				
	300	0·49				
	1100	24·23	25·77	94	400	44
VI	800	39·45			100	
[Effect of theo-	800	16·54			150	
retical amount	300	0·72				
of bichromate]	1900	56·71	60·39	93·9	250	103
VII	800	26·216				
[6 vols. % sul-	800	Nil				
phuric acid]	1600	26·216	27·93	93·9	300	48
VIII	800	12·934				
[6 vols. % sul-	800	Nil				
phuric acid]	1600	12·934	13·96	92·6	200	24

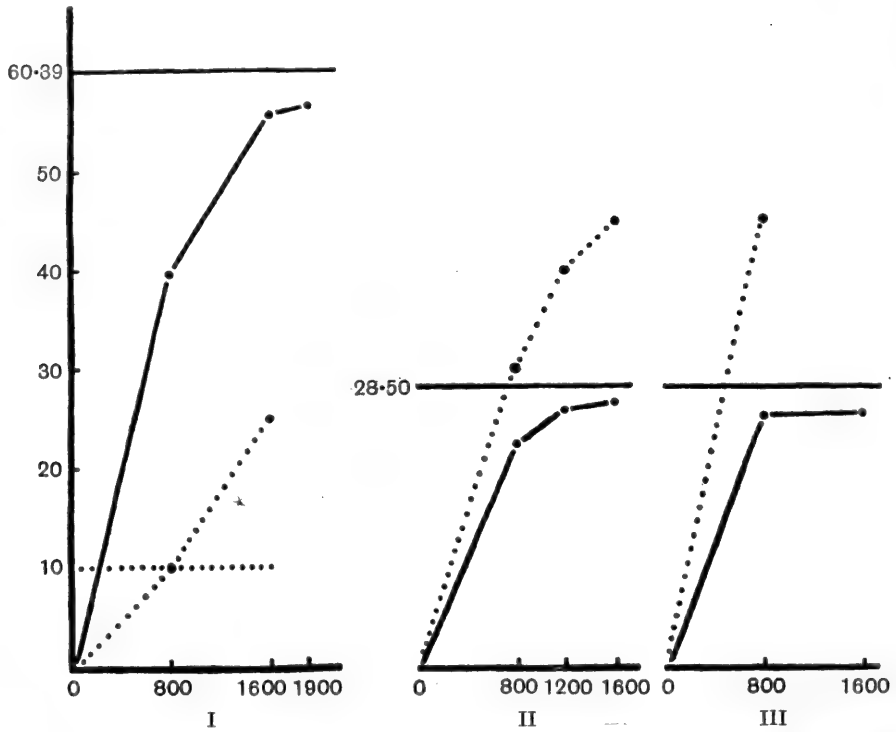

Fig. 2. Effect of amount of bichromate on the yield of acetone from β-hydroxybutyric acid.
Abscissae represent cc. distillate.
Ordinates represent mg. acetone or cg. bichromate.
Continuous lines represent acetone; the horizontal lines show the theoretical yield.
Dotted lines represent bichromate.
Curve I. Effect of theoretical amount of bichromate (horizontal dotted line).
Curves II and III. Duplicate determinations on urine with added sodium β-hydroxybutyrate. Slow (II) and rapid (III) addition of bichromate.

TABLE V.

Effect of amount of bichromate upon the yield of acetone from sodium β-hydroxybutyrate.

$\frac{\text{Bichromate used}}{\text{Bichromate calculated}}$ (Quotient)	Yield %
12	95·4
7	95·3
9	94·0
2·5	93·9
6	93·9
8	92·6
6·5	92·1
6·25	91·9
4·6	90·0
Mean 6·6	93·2

When the sodium salt is added to urine, and the urine then treated in the ordinary way by Shaffer's method, the yield is as a rule somewhat higher than that given by a pure solution (Table VI); probably the increase is due to concentration of the fluid brought about by the bulky precipitate of lead compounds[1].

TABLE VI.

Sodium β-hydroxybutyrate added to urine and treated by Shaffer's method.

Expt.	Distillate, cc.	Acetone found, mg.	Acetone calculated, mg.	Yield %
I	800	23·90		
	300	0·08		
	1100	23·98	25·69	93·4
II	800	28·72		
	300	0·44		
	1100	29·16	31·65	92·1
III	800	16·07		
	400	1·30		
	400	0·17		
	1600	17·54	18·52	94·6
IV	800	16·80		
	800	0·30		
	1600	17·10	18·19	94
V	800	16·88		
	200	0·44		
	500	0·23		
	1500	17·55	18·19	96·5
VI	800	15·72		
	700	1·71		
	600	trace		
	2100	17·43	18·19	95·8
VII	800	16·72		
	600	0·62		
	1400	17·34	18·19	95·3

It is then easy to show that Shaffer's method does not give a quantitative yield of acetone, but it is of course much more difficult to find out whether the acetone obtained from urine by oxidation is derived wholly from β-hydroxybutyric acid, and there seems to be no satisfactory way of doing this. However, the following experiment was made to investigate this question. A diabetic urine containing 3·4 % sugar, 0·228 % aceto-acetic acid, and

[1] In these and all other cases where substances were added to urine, the amount yielded by the urine alone was of course subtracted from the result.

0·925 % β-hydroxybutyric acid, was acidified and extracted with ether until the extract was optically inactive. The second distillate from the residue when treated by Shaffer's method gave with Scott-Wilson's reagent a barely perceptible trace of precipitate, such as might well have been due to incomplete extraction of the β-hydroxybutyric acid. This experiment of course shows merely that the ether-insoluble substances did not yield acetone.

After nearly all the estimations given in this paper had been made[1], Shaffer and Marriot [1913] published some very similar results on the formation of acetone from β-hydroxybutyric acid. They obtained from 90 to 94 % of the theoretical amount; by very slow oxidation this was increased to from 95 to 97 %.

(*b*) *The estimation of aceto-acetic acid.*

The accuracy of Shaffer's method as a means of estimating aceto-acetic acid, which is greatly preponderant over acetone in urine [Arnold, 1900], appears never to have been tested. The estimations made with this substance were not altogether satisfactory, as difficulty was experienced in establishing a constant boiling point for the ester employed. However, the following experiments were carried out.

3·2525 gram aceto-acetic ester was saponified in the cold with the calculated amount of N caustic soda (25 cc.), and diluted to 1 litre (Solution I).

	Acetone mg.		
	Found by Scott-Wilson estimation	Calculated from weight of ester	Yield %
10 cc. of above solution distilled by Shaffer's method	14·15	14·50	97·6
25 cc. ,, ,, ,, ,, ,,	35·96	36·25	99·0

This solution gave low results when added to urine. Probably some ester was still present, which was decomposed to acetic acid by the strong ammonia used in Shaffer's method. The solution was therefore shaken out five times with ether to remove ester; it had then taken up acetone from the ether, but the results obtained before and after addition to urine were compared. (Solution II.)

Solution II. 25 cc. distilled gave 54·37 mg. acetone.
25 cc. added to urine gave 53·12 mg. acetone = 97·7 %.

Another preparation (III) gave the following results:

50 cc. distilled gave 10·526 mg. acetone.
50 cc. added to urine gave 10·522 mg. acetone.

[1] A preliminary communication was given to the Physiological Society, Nov. 15th, 1913. [Kennaway, 1913.]

The recovery of acetone from urine was tested as follows:

10 cc. acetone solution precipitated with Scott-Wilson's reagent gave 0·597 mg.
100 cc. added to urine gave ... ... 6·037 mg. = 101 %.

Analyses of mixtures of sodium aceto-acetate and sodium β-hydroxybutyrate.

		Acetone, mg.		Yield
		Calculated	Found	%
1.	Aceto-acetic acid (I)	36·25	35·23	97·2
	β-hydroxybutyric acid	31·94	29·41	92·1
2.	Aceto-acetic acid (I)	36·25	35·23	97·2
	β-hydroxybutyric acid	25·76	24·23	94·0
3.	Added to urine			
	Aceto-acetic acid (III)	6·737	6·826	101·0
	β-hydroxybutyric acid	28·50	26·34	92·1

A combination of the Shaffer and Scott-Wilson methods.

This may be illustrated by stating in detail the quantities used in a particular estimation. The method is taken from the papers of Shaffer [1908], Cooke and Gorslin [1911], and Scott-Wilson [1911], with various modifications which are mentioned below.

25 cc. of diabetic urine giving a moderately strong ferric chloride reaction was placed in a 250 cc. flask, diluted with water, and mixed with 50 cc. basic lead acetate solution[1] and 10 cc. strong ammonia. A drop of dilute sulphuric acid in the supernatant fluid showed that excess of lead was present. The mixture was made up to 250 cc. 100 cc. of the filtrate was made faintly acid with 9 cc. 20 vol. % sulphuric acid, and made up to 250 cc. 100 cc. of this solution was placed in the distilling flask with 400 cc. water, 15 cc. concentrated sulphuric acid and talc, and 300 cc. distilled off at constant volume. The distillate was made up to 500 cc., and two portions of 50 cc. precipitated with 20 cc. Scott-Wilson reagent.

800 cc. was then distilled off at constant volume with addition of 400 cc. 0·2 % potassium bichromate and water; the distillate was made up to 1 litre, and two portions of 50 cc. precipitated with 20 cc. Scott-Wilson reagent.

300 cc. was then distilled off at constant volume with addition of water,

[1] The U.S.P. solution used by Shaffer is prepared as follows: 180 g. lead acetate is dissolved in 700 cc. water; the solution is boiled for half an hour, while 110 g. of litharge is added in small portions. The solution is made up to 1 litre. The amount required is in most cases from one-half to twice the volume of urine.

and made up to 500 cc.; 100 cc. of this with 15 cc. reagent gave no precipitate on standing overnight.

The Scott-Wilson titrations gave the following results:

(1) Aceto-acetic acid + acetone.

$$\text{KCNS}\ \begin{matrix}9{\cdot}45\text{ cc.}\\9{\cdot}45\text{ cc.}\end{matrix}\Big\} = 0{\cdot}548\text{ mg. acetone in } \tfrac{1}{10}\times\tfrac{10}{25}\times\tfrac{10}{25}\times 25 = 0{\cdot}4\text{ cc. urine.}$$

(2) β-hydroxybutyric acid.

$$\text{KCNS}\ \begin{matrix}10{\cdot}70\text{ cc.}\\10{\cdot}75\text{ cc.}\end{matrix}\Big\} = 0{\cdot}622\text{ mg. acetone in } \tfrac{1}{20}\times\tfrac{10}{25}\times\tfrac{10}{25}\times 25 = 0{\cdot}2\text{ cc. urine.}$$

The estimation of such small quantities does not involve any considerable error. If the last two titration figures given above had been 10·5 cc. and 10·9 cc., and the day's volume of urine had been 7000 cc., the total amount of β-hydroxybutyric acid reckoned separately from the two titrations would have been 38·3 or 39·8 grams. The titrations as a rule do not differ by more than 0·2 cc.

The reasons for taking as much as 25 cc. of urine when the ultimate estimation is carried out upon the equivalent of 0·2 or 0·4 cc. are (1) the errors of the series of measurements are lessened by working with large volumes; (2) it is important that the concentration of acetone in the distillate should not be too low (see Table II)[1]; (3) with some urines, but not with all, the conversion of a larger quantity of β-hydroxybutyric acid into acetone takes place more rapidly and completely than that of a smaller quantity (Fig. 3).

It is most convenient to have from 0·5 to 1 mg. of acetone in 25 to 50 cc. of distillate. 1 mg. of acetone requires 17·3 cc. N/100 KCNS. The amount of acetone bodies in a urine can be judged roughly by colour tests. If Rothera's reaction gives a strong colour within a few seconds, probably more than 0·25 % aceto-acetic acid is present; the reaction is still given by a concentration of 0·0005 % or less [Hurtley, 1913]. The ferric chloride reaction is not obvious unless more than 0·07 % aceto-acetic acid is present. In order to form some idea of the amount of β-hydroxybutyric acid, one may remember that in well-marked cases of acetonuria this acid forms from 60 to 80 % of the total acetone bodies [Neubauer, 1910]; if this percentage were 66, the concentrations of acetone in the two distillates obtained by the method given above would be equal.

The urine is measured into the flask first, and diluted before adding the basic lead acetate; there is then less frothing. The froth can be

[1] Direct distillation into the reagent, as practised by Scott-Wilson with normal urine, would not be practicable in pathological cases.

dispersed, when brought up into the neck of the flask, by rubbing with a glass rod and piece of filter-paper[1].

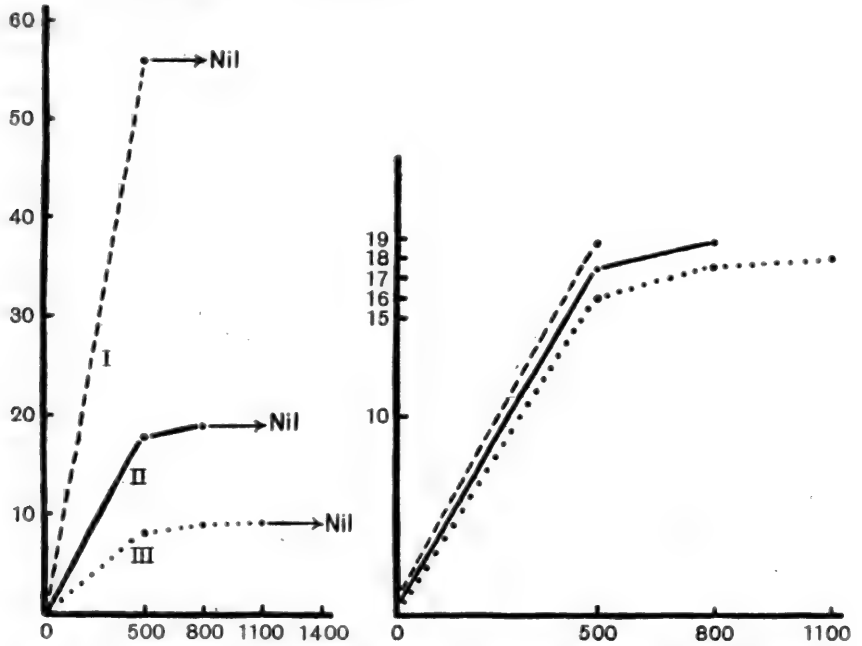

Fig. 3. Conversion of different amounts of β-hydroxybutyric acid into acetone.
Abscissae represent cc. distillate.
Ordinates represent mg. acetone.
I. Estimation on equivalent of 12 cc. urine.
II. ,, ,, ,, 4 ,,
III. ,, ,, ,, 2 ,,
In the right-hand figure, the results given in curves I and III are calculated for 4 cc. urine, to facilitate comparison.

Estimation on equivalent of—	12 cc. urine	12 cc.	4 cc.	2 cc.
1st distillation, 500 cc.	55·98	55·97	17·57	8·062
2nd ,, 300 ,,	Nil	Nil	1·14	0·823
3rd ,, 300 ,,			Nil	0·14
4th ,, 300 ,,				Nil
Mg. acetone, total	55·98	55·97	18·71	9·025
	÷3=18·66			×2=18·05

The apparatus which has been used in these estimations is shown in Fig. 4. The distillate from the first flask is passed through a second flask, as in Scott-Wilson's method, containing about 30 cc. 40 % caustic soda and an equal volume of water, with a little talc; this is kept at more or less constant volume by a small flame, and towards the close of the distillation

[1] A drop of ether is of course highly effectual, but must on no account be used, as ordinary ether, and even that sold as "distilled over sodium," contains quite enough acetone to affect a Scott-Wilson estimation.

is boiled down to a small volume. This flask must rest on a sand-bath, as it is apt to crack on wire gauze. The distillate is received into a 500 cc. or 1000 cc. measuring flask; the condenser and delivery-tube should be in one piece, as no rubber junction at this point is satisfactory. The measuring flask is connected with a water trap in a 50 cc. cylinder; it is well to let the water suck back here soon after the start of the distillation, to make sure that the apparatus is tight. In carrying out the oxidation, it is generally convenient to keep the funnel filled with 0·2 % bichromate, and if necessary to add water or a few cc. of 5 % bichromate.

In the estimation of β-hydroxybutyric acid, a second distillation is necessary to show whether the formation of acetone is completed. The result is in many cases nil, or an amount too small to estimate; in other cases it may be as much as 10 % of the whole. The obvious method of avoiding this lengthy distillation and the large volume of distillate is to carry out the oxidation with a reflux condenser and bulbs, and then distil off the acetone formed. The results obtained thus were always too low, this being due probably to loss of uncondensed acetone when changing to the distillation apparatus. In estimations on urine, the formation of acetone is as a rule completed more rapidly than when the pure acid is used.

The second distillation with hydrogen peroxide described by Shaffer was not found to make any considerable difference in those cases where it was tested (Table VII). When a somewhat lower result is obtained from the

TABLE VII.

Comparison of results with Iodine and Scott-Wilson methods.

Milligrams acetone.

	From aceto-acetic acid + acetone		From β-hydroxybutyric acid			
			Before H_2O_2		After H_2O_2	
	Iodine method	Scott-Wilson method	Iodine method	Scott-Wilson method	Iodine method	Scott-Wilson method
Urine 1	15·46	15·48	41·82	41·90	42·30	41·83
,, 2	16·19	15·98	39·40	38·64	38·68	38·78
,, 3	14·75	14·93	52·22	51·11	50·76	50·60
,, 4	15·59	15·83	50·76	50·60	49·05	49·45
,, 5	5·560	5·568	17·88	17·57	17·52	17·40
,, 6	43·51	41·76	111·19	106·15		
,, 7	6·16	6·32	21·75	21·97		
,, 8	9·91	9·12	49·31	46·40		
,, 9	4·83	5·18	24·65	24·48		
Blood 1	11·12	11·34	24·17	20·88		
,, 2	12·33	12·29	28·04	24·52		

second distillation, the diminution may be due to loss of acetone rather than to removal of other substances; possibly these are eliminated by the method of distillation through caustic soda adopted in these experiments (Fig. 4). When blood is treated in the same way as urine, besides acetone, other substances which combine with iodine are undoubtedly formed (Table VII).

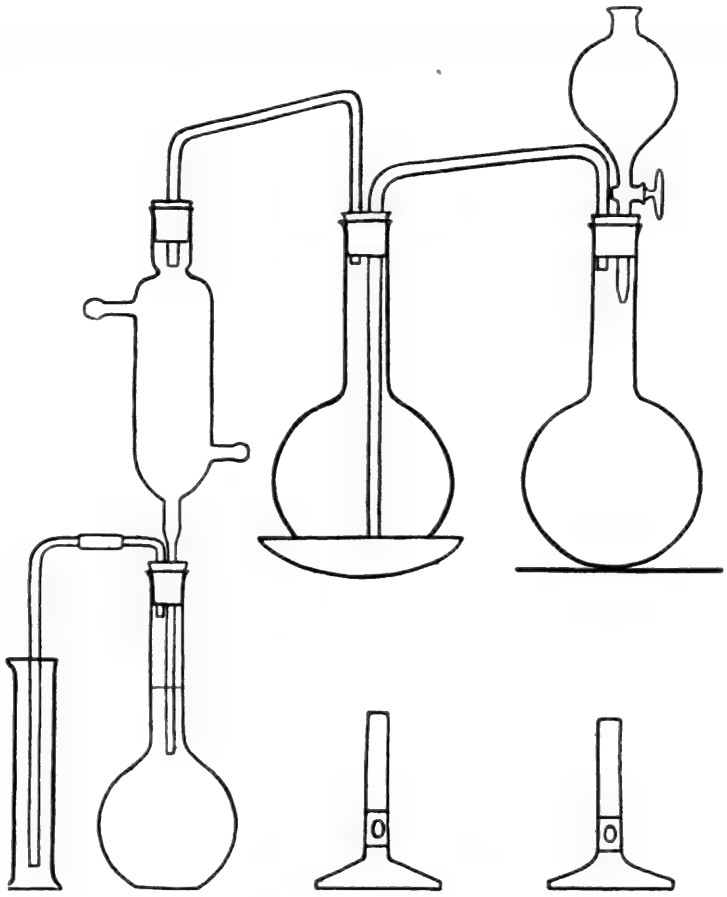

Fig. 4.

Scott-Wilson showed that urine when decomposed by bacterial action yielded more acetone, or some substance reacting like acetone with his reagent. This change occurs slowly even when the urine is preserved with thymol in a cold room, and remains acid and apparently fresh (Table VIII); the β-hydroxybutyric acid is as a rule unaffected. Thymol when oxidised with bichromate and sulphuric acid yields some substance which is precipitated by Scott-Wilson's reagent, but comparative experiments show that the amounts

of thymol present in the filtered and diluted urine are not sufficient to affect the result. Chloroform cannot be used, as it causes reduction of the reagent, and the results are too high.

TABLE VIII.

	In 100 cc. urine	
	Aceto-acetic acid + acetone as acetone, grams	β-hydroxybutyric acid as acetone, grams
Urine I. Estimated fresh.	0·187	0·812
Kept with thymol at room temperature 6 months later (acid)	0·229	0·819
Urine II. Estimated fresh.	0·062	0·1911
Kept with thymol in cold room 5 months later (acid)	0·068	0·1907
Urine III. Estimated fresh.	0·1305	0·300
Kept with thymol in cold room 10 days later	0·1298	0·2994
10 weeks later	0·138	0·311
12 weeks later (acid)	0·137	0·311

Some notes on the Scott-Wilson method.

Scott-Wilson [1911] says that the mercuric cyanide reagent remains perfectly clear for months; in my experience it invariably develops a finely divided precipitate soon after preparation. This substance no doubt contains mercury or silver, or both, and must be removed. It is best to make up the reagent at least a week before it is required, and to filter through glass wool before use.

The constituents of the acid mixture are given by Scott-Wilson in "parts," but it is not stated whether these are by weight or by volume. The following mixture has been used in the present investigation: water 550 cc., concentrated sulphuric acid 27 cc., concentrated nitric acid 280 cc.

The precipitation can be carried out in beakers, as the precipitate is removed from these much more easily than from Erlenmeyer flasks. The precipitate after standing adheres very tenaciously to the vessel; it is best removed by rubbing with a glass rod and piece of filter paper; the paper is transferred on to the filter, and is used again in the same way to wipe out the funnel before the titration. If rubber tubing on a glass rod is used, the very fine precipitate gets inside the rubber however closely this be attached.

A Gooch crucible does not seem very suitable for the filtration. If an ordinary glass funnel be used with a porcelain disc, the final removal of the

precipitate is much easier, and the white particles are more visible on glass than on porcelain. The filter paper on the disc is covered with a suspension of talc in water, the talc being well sucked down before beginning the filtration. Such filters will stand the full force of the pump, in spite of the destructive action of the reagent upon filter paper. The precipitate and beaker should be washed five times after wiping the precipitate out of the beaker.

After washing, the material is lifted into an Erlenmeyer flask with a glass rod, and the funnel wiped with filter paper. Not more than 25 cc. of acid mixture should be used in washing down, as much dilution renders the end-point very vague. About 2 cc. of N/5 permanganate are added, and the mixture boiled till it is colourless; the sides of the flask are then washed down with a jet of acid mixture, about 1 cc. of permanganate is added, and the boiling repeated. No concordant results were obtained till this method of decomposing the precipitate was adopted.

The titration is carried out with N/100 KCNS, which can be standardised against silver nitrate. The first faint brown colour is the end-point; three more drops of sulphocyanide produce an obvious reddish-brown tint, and it is well always to obtain this second colour in order to be sure of the first.

Summary.

The oxidation of β-hydroxybutyric acid with sulphuric acid and potassium bichromate in Shaffer's method of estimation gives from 90 to 96% of the theoretical amount of acetone.

A combination of Shaffer's method with Scott-Wilson's method for the estimation of acetone is described.

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XXXI. COUNTER DIFFUSION IN AQUEOUS SOLUTION.

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(*Received May 4th, 1914.*)

Although we cannot put forward a satisfactory theory for the results of our experiments yet we think that they are of sufficient interest to merit publication—they may at least direct attention to a peculiar feature of diffusion so far undescribed. The following experiments are based upon the discovery of one of us (W. A. O.) that if a strong concentration of solute is allowed to diffuse into some solvent, and if both solution and solvent contain, in addition, another substance in weak concentration, in this case sodium chloride, there is an increase in concentration of the latter dilute substance in the path of the advancing diffusion. If, for instance, there are two solutions, one vertically placed above the other and in contact over a relatively small surface, and if both contain decinormal sodium chloride and one of them, necessarily the lower, contains ammonium sulphate of three molecular strength, then after some days' diffusion it is found that the sodium chloride concentration in the upper fluid is greater than decinormality whilst that in the lower fluid is correspondingly reduced.

Methods.

Vertical glass cylinders were employed each of 23 millimetres internal diameter and with a tap near the middle which had a bore exactly the same diameter as that of the cylinder. The volume from the bottom of the cylinder to the top of the bore of the tap was estimated and a corresponding volume measured off above the closed tap. In the earlier experiments the volume of fluid employed was 100 cm.^{3} below and 100 cm.^{3} above, but we found later that quicker results could be obtained by taking 30 cm.^{3} instead of 100. The lower diffusion fluid was poured into the cylinder until it

rose above the tap. The tap was closed and the upper part of the cylinder cleaned out with distilled water and dried. The upper diffusion fluid was then placed in the cylinder above the tap and a glass stopper inserted. The cylinder, still kept vertical, was then set up in a sheltered part of the laboratory, the tap was turned on, and diffusion allowed to take place at room temperature. When a sufficient interval of time had elapsed the tap was turned, the upper fluid well mixed and poured out. The upper part of the cylinder was then washed with distilled water and dried. The tap was then opened and the lower portion of fluid poured into a separate beaker and well mixed. Sodium chloride, or rather the chloride present, was estimated by the Volhardt procedure. Sulphate was determined gravimetrically as barium sulphate in the usual way and the concentration of ammonium sulphate calculated from the results. Glucose was estimated polarimetrically care being taken as regards mutarotation. The hypobromite method was considered sufficiently accurate for urea. By percentages are meant grams of solute in 100 cm.^{3} of solution.

The following experiments are typical of many others which we performed showing results of strong ammonium sulphate solution in affecting the increase of concentration of chloride.

Experiment I.

Before diffusion—
Above tap, 30 cm.^{3} NaCl 0·58 %.
Below tap, 30 cm.^{3} NaCl 0·57 %, $(NH_4)_2SO_4$ 39·6 %.
After diffusion for seven days—
Above tap, 30 cm.^{3} NaCl 0·63 %, $(NH_4)_2SO_4$ 5·6 %.
Below tap, 30 cm.^{3} NaCl 0·53 %, $(NH_4)_2SO_4$ (by difference) 34·0 %.

Experiment II.

Before diffusion—
Above tap, 30 cm.^{3} NaCl 0·58 %.
Below tap, 30 cm.^{3} NaCl 0·57 %, $(NH_4)_2SO_4$ 39·6 %.
After diffusion for 21 days—
Above tap, 30 cm.^{3} NaCl 0·66 %, $(NH_4)_2SO_4$ 6·0 %.
Below tap, 30 cm.^{3} NaCl 0·46 %, $(NH_4)_2SO_4$ (by difference) 33·6 %.

The differences in the chloride concentration are certainly striking and well outside the limits of error. We next tried the effect of two ammonium sulphate solutions of different concentrations in separate cylinders placed together under similar conditions of temperature and for the same time.

Experiment III.

A. Before diffusion—
Above tap, 30 cm.^{3} NaCl 0·56 %.
Below tap, 30 cm.^{3} NaCl 0·56 %, $(NH_4)_2SO_4$ 26·4 %.
After diffusion for 7 days—
Above tap, 30 cm.^{3} NaCl 0·62 %, $(NH_4)_2SO_4$ 4·1 %.
Below tap, 30 cm.^{3} NaCl 0·49 %, $(NH_4)_2SO_4$ (by difference) 22·3 %.

B. Before diffusion—
Above tap, 30 cm.^{3} NaCl 0·56 %.
Below tap, 30 cm.^{3} NaCl 0·56 %, $(NH_4)_2SO_4$ 52·8 %.
After diffusion for one week.
Above tap, 30 cm.^{3} NaCl 0·64 %, $(NH_4)_2SO_4$ 7·3 %.
Below tap, 30 cm.^{3} NaCl 0·46 %, $(NH_4)_2SO_4$ (by difference) 45·5 %.

The stronger solution of ammonium sulphate effected therefore a greater heaping up of the chloride. In order to avoid the difficulties associated with an ionisable salt we tried diffusion with cane sugar and also with glucose.

Experiment IV.

Before diffusion—
Above tap, 100 cm.^{3} NaCl 0·58 %.
Below tap, 100 cm.^{3} NaCl 0·58 %, Cane sugar 34·2 %.
After diffusion for seven days—
Above tap, 100 cm.^{3} 0·60 % NaCl.
Below tap, 100 cm.^{3} 0·56 % NaCl.

Experiment V.

Before diffusion—
Above tap, 30 cm.^{3} NaCl 0·58%.
Below tap, 30 cm.^{3} NaCl 0·57 %, Glucose 36 %.
After diffusion for seven days—
Above tap, 30 cm.^{3} NaCl 0·60 %, Glucose 5·6 %.
Below tap, 30 cm.^{3} NaCl 0·54 %, Glucose (by difference) 30·4 % .

Organic non-electrolytes have therefore the same property. On the assumption of a simple kinetic explanation based upon molecular volumes we tried equimolecular mixtures of glucose, $C_6H_{12}O_6$, and urea, CON_2H_4.

Experiment VI.

Before diffusion—
Above tap, 100 cm.^{3} NaCl 0·56 %, Urea 12 %.
Below tap, 100 cm.^{3} NaCl 0·54 %, Glucose 36 %.
After diffusion for seven days—
Above tap, 100 cm.^{3} NaCl 0·56 %.
Below tap, 100 cm.^{3} NaCl 0·54 %.

Experiment VII.

Before diffusion—
Above tap, 100 cm.^{3} NaCl 0·57 %, Urea 12 %.
Below tap, 100 cm.^{3} NaCl 0·58 %, Glucose 36 %.

After diffusion for 14 days—
Above tap, 100 cm.^{3} NaCl 0·58 %.
Below tap, 100 cm.^{3} NaCl 0·56 %.

Experiment VIII.

Before diffusion—
Above tap, 100 cm.^{3} NaCl 1·16 %, Urea 12 %.
Below tap, 100 cm.^{3} NaCl 1·16 %, Glucose 37·2 %.

After diffusion for 14 days—
Above tap, 100 cm.^{3} NaCl 1·17 %, Glucose 2·8 %.
Below tap, 100 cm.^{3} NaCl 1·15 %, Glucose (by difference) 13·4 %.

Experiment IX.

Before diffusion—
Above tap, 30 cm.^{3} NaCl 0·58 %, Urea 12 %.
Below tap, 30 cm.^{3} NaCl 0·55 %, Glucose 35 %.

After diffusion for seven days—
Above tap, 30 cm.^{3} NaCl 0·59 %, Urea (by difference) 10·9 %, Glucose 3·3 %.
Below tap, 30 cm.^{3} NaCl 0·55 %, Urea 1·1 %, Glucose (by difference) 31·7 %.

The results of this last group are not quite so definite. We tried the same solution as in Experiment IX allowing diffusion to continue for seven weeks but got no greater effect. It will be seen, however, that the differences obtained, though they may extend but little beyond the limits of error, all point in the same direction. We leave the investigation at this stage trusting that some light may be thrown on the subject from the theoretical side.

XXXII. THE CURATIVE ACTION OF AUTOLYSED YEAST AGAINST AVIAN POLYNEURITIS.

By EVELYN ASHLEY COOPER, *Beit Memorial Research Fellow, Lister Institute.*

(*Received May 5th, 1914.*)

In a previous communication [Cooper, 1913] a method was described for the preparation from voluntary muscle of a substance, small amounts of which readily cured polyneuritis in pigeons. The method employed however was not only expensive, but the yield of active substance was very small, so that an attempt has since been made to discover a less costly process of preparing a non-toxic strongly curative substance suitable for use in the treatment of human beri-beri.

Yeast has for some time been known to be very effective in curing polyneuritis in birds, its curative action having been first observed by Schaumann [1910], and Cooper and Funk [1911] subsequently showed that the yeast still retained its curative properties after hydrolysis with 20 % sulphuric acid for 24 hours.

As yeast contains a powerful proteolytic ferment and is easily obtainable in the East, it was thought that possibly a suitable curative solution could be prepared from it by autolysis.

325 gms. of pressed brewers' yeast was accordingly placed in a flask in the hot room (35° C.) for 36 hours, and the yeast was rapidly converted into a brown fluid. This was filtered, and the residual cell-debris washed with a little water. The total volume of the combined filtrate was 270 cc.; 3 cc. of the liquid readily cured pigeons (300 gms.) affected with polyneuritis in 3 to 12 hours, and even 1—2 cc. improved the condition of the birds. As from 3 to 6 gms. of pressed yeast was necessary to bring about complete recovery, it is seen that the solution resulting from autolysis was at least as strongly curative as the original yeast.

A solution of similar potency could be obtained by the addition of 95 per cent. alcohol to the autolysed yeast, but, apart from the fact that the alcohol coagulated the cell-debris and so facilitated filtering, this method had no advantage over the one described above.

By treating the alcohol-free filtrate with basic lead acetate, a copious precipitate was obtained, and the active substance passed almost entirely into the filtrate. In this way much of the colouring matter could be removed, and the highly curative filtrate, although not superior to the original solution therapeutically, can be so readily obtained in large amount that it should be suitable raw material for fractionation with a view to the isolation of the anti-neuritic substance.

Five kilos. of pressed yeast were next autolysed as before for 36 hours, and the filtered solution measured 4500 cc. Three cc. of this readily cured pigeons affected with polyneuritis in a few hours, and the solution stored in the cold room retained its curative power for at least 8 weeks. Birds were then given orally large volumes of this active solution to ascertain if it exerted a toxic action, and the following results were obtained:—

Bird	Weight	Dose	Effect
Pigeon	300 gms.	30 cc. (15 cc. twice in 8 hrs.)	Still healthy 1 week afterwards.
,,	300 ,,	30 ,, ,, ,,	
Chicken	1800 ,,	100 cc. (50 cc. ,, ,,	
,,	500 ,,	36 cc. (18 cc. ,, ,,	

The results show that as much as 10 times the curing dose (3 cc.) given to pigeons had no injurious effect, and even 100 cc. given to the chickens did no harm.

Some experiments were also carried out to ascertain whether a strongly curative solution could be as easily prepared when the yeast was first air-dried at 20° C. To 75 gms. of the dry preparation, which had been stored for six weeks in a dry tin, 250 cc. of water was added, and the yeast allowed to autolyse at 35° C. for 48 hours. The mixture was then filtered, and the insoluble matter washed with water, the combined filtrates measuring 250 cc. Three cc. of the solution again rapidly cured neuritic pigeons. As 1–2 gms. of the dried yeast were required to effect complete recovery, it is seen that the solution was quite as strongly curative as the material from which it was derived. 0·5 cc. of the solution injected subcutaneously rapidly cured a pigeon, and 1–2 cc. had no toxic action.

Air-dried yeast retained its marked anti-neuritic properties even after storage in a dry tin for two years, and after four months' storage still readily autolysed when mixed with water and kept at 35° C.

By autolysing yeast it is thus possible to prepare a highly active solution which being non-toxic and inexpensive should be suitable for the treatment of human beri-beri on a large scale. Owing to the stability of the anti-neuritic substance and proteolytic ferment contained in yeast, as shown above, the yeast can be air-dried, stored under dry conditions, and autolysed as required.

Summary.

1. By the autolysis of brewers' yeast a solution can be obtained which possesses as marked curative properties towards avian polyneuritis as the original yeast.

2. The solution retains its curative power for at least eight weeks and when given orally to birds in doses 10 times as great as the minimum curing dose has no toxic action.

3. Air-dried yeast retains its curative power after storage for two years in a dry tin, and still autolyses after storage for four months.

Conclusion.

The autolysis of brewers' yeast should afford a simple inexpensive method of preparing a non-toxic solution suitable for the oral treatment of human beri-beri.

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XXXIII. A NOTE ON THE BASES OF GASWORKS COAL-TAR WHICH ARE BELIEVED TO BE THE PREDISPOSING CAUSE OF PITCH CANCER, WITH SPECIAL REFERENCE TO THEIR ACTION ON LYMPHOCYTES, TOGETHER WITH A METHOD FOR THEIR INACTIVATION.

PART I. AUXETIC ACTION.

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(*Received May 12th, 1914.*)

It has long been known that gasworks coal-tar, the pitch derived from it and soot (which contains tar), cause chronic dermatitis, warts and ulcers leading to epithelioma, in the skins of the workmen employed at the briquette (patent fuel) works and tar distilleries. This specific predisposition to cancer brought about by these commodities has recently formed the subject of Home Office enquiries. The clinical evidence shows that it is only what is commonly known as coal-tar and the pitch derived from it by distillation that have this action; tar and pitch obtained from the blast-furnaces are harmless. The mechanical irritation caused by both varieties is identical, so that it is evident that some chemical factor is responsible for the predisposition to cancer above referred to, a fact borne out by the incidence of pitch cancer and warts caused by liquid petroleum [Legge, 1911; Lush, 1911; Ross, H. C., 1913, 1].

Although it is well known that gasworks tar differs widely in its chemical composition from blast-furnace tar, the latter being manufactured at a much lower temperature, it must also be remembered that the tar made at the gasworks and coke-ovens which is dangerous, is derived mostly from bituminous coal; the harmless blast-furnace tar, on the other hand, being made from the harder Scotch coal. [Ross, 1913, 2.]

H. C. Ross and J. W. Cropper [1910] put forward a working hypothesis connecting the onset of carcinoma and the predisposition to it with chemical substances called auxetics and kinetics. Auxetics are substances (most of them are amino-bodies) which induce cell-division in white blood cells, such as lymphocytes and other cells; kinetics are another group of substances (including most of the alkaloids) which excite amoeboid movements in cells.

The method of testing for both auxetics and kinetics is to mix the suspected solution with agar jelly, and to spread some blood cells on the surface of films prepared from this jelly. If auxetics are present in sufficient strength the lymphocytes after 10 minutes incubation at 37° will be seen to exhibit characteristic division figures; if kinetics are present, the cells (being examined without incubation) show exaggerated amoeboid movements. It is this test that has been employed in the following chemical experiments; the full details of the method have been described by Ross and Cropper [1911, 1]. In every case the test was repeated several times and throughout the experiments gave consistent results.

The present paper is concerned only with auxetic action, and the clinical bearing of this is not discussed; the question of the isolation of kinetics from tar will be described at a later date. In investigating the question of pitch cancer, Ross and Cropper [1911, 2] have detected auxetics and kinetics in gasworks tar and pitch, in soot and in liquid petroleum they were however unable to detect any kinetic whatever, and only a trace of auxetic in the harmless blast-furnace tar and pitch. A long series of experiments was made by these investigators and the subject is also dealt with in the Report of the second Home Office enquiry on pitch cancer [Lush, 1913].

From observations made by Ross and Cropper it was found that on fractionally distilling tar which had been found capable of yielding an aqueous extract active in inducing cell-division, the fraction distilling between 260°–320° comprised the whole of the active portion, that is to say the activity was associated with the anthracene fraction. Various pure substances known to occur in this fraction were tested by the above observers but found to be without action on lymphocytes. The following experiments were therefore undertaken to see whether the active agent or agents could be isolated.

Experimental.

Isolation of active basic fractions.

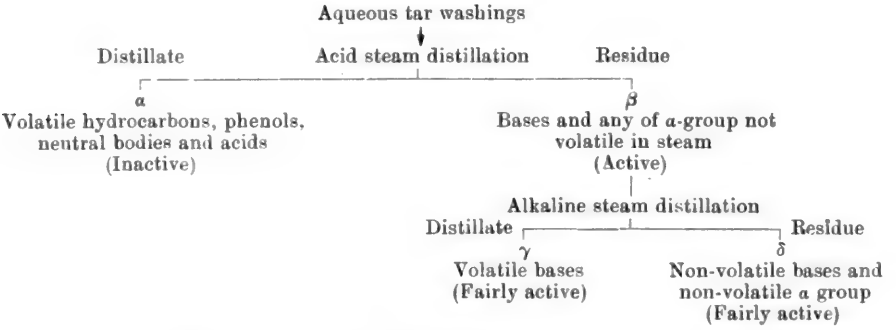
The tar used in the following experiments was a sample of the common variety which causes disease at the tar distilleries and briquette works and was kindly placed at our disposal by Mr Atkinson Butterfield.

Aqueous extracts were faintly acid in reaction.

Preliminary tests were made to ascertain the chemical nature of the substance or substances responsible for this auxetic action and in the first place an aqueous extract of the tar was used.

This was prepared by taking two volumes of water at 60° to 1 volume of tar, shaking for 1 hour, allowing to stand overnight and then centrifugalising or decanting off the aqueous layer. The washings prepared in this way were always faintly acid in reaction.

It was then found that on steam distilling with this original acid reaction, the substances capable of action on lymphocytes remained in the residue, but the kinetic action had apparently been destroyed or inhibited in some way as it was neither in the distillate nor residue. On steam distillation with a faintly alkaline reaction auxetic could be detected in both distillate and residue, that is to say the auxetic is slightly volatile in steam. The two steam distillations were then carried out consecutively on the same sample of washings and the result shown diagrammatically below, pointed to the active constituent being basic in character.



The active substance being therefore apparently of basic nature, acid extracts of the tar were made and it was found that this treatment yielded solutions which had a much stronger action on lymphocytes than had the aqueous ones. Acid extracts were accordingly used in all the following experiments, the acid employed consisting of two volumes of 5 per cent. hydrochloric acid to 1 of tar, and the extraction was carried out at 60° as in the previous case with water.

An attempt was then made actually to isolate the bases from the portion γ in the scheme above by means of chloroplatinic acid in the usual way, but after decomposing the platinum salt by means of sulphuretted hydrogen and then removing the excess of the latter by a current of air, no auxetic action could be detected. One explanation of this may be the adsorption of the active substance by the platinum sulphide which at first was formed in colloidal solution. A further explanation of this loss of activity was afterwards discovered and will be referred to later. The filtrate from the platinum salt was also found to be inactive after the removal of the platinum as above.

A further attempt was made using basic lead acetate as precipitant, decomposing the lead salt with sulphuric acid and then removing excess of acid by means of baryta. Here again no trace of auxetic action remained.

It then seemed necessary to obtain a larger and more concentrated supply of the active substance and methods with this end in view were next tried. It was found that on the addition of 10 per cent. caustic soda to an aqueous extract a precipitate was obtained which redissolved on continued addition of alkali and that this precipitate when filtered off and dissolved up in a little dilute acid (HCl) was a strong auxetic but did not keep well, rapidly losing its activity. In the same way the aqueous and acid extracts of tar likewise lose their activity on long standing in the air. With this method it was extremely troublesome to remove the excess of caustic soda, and if this is not done properly it is impossible to carry out the auxetic test with any hope of success as the alkali kills the cells, and an excess of salts even if neutral must be avoided[1].

The following method was then tried and found to be quite reliable. The tar was extracted as before with twice its volume of 5 per cent. hydrochloric acid, being shaken for one hour, and allowed to stand overnight and then centrifugalised. The supernatant liquor was precipitated with strong ammonia, care being taken not to add excess. A precipitate was obtained which on testing was found to be highly active while the supernatant liquor was inactive. The precipitate was collected, dried in a desiccator and again tested when dry and was still found to be highly active.

It is interesting to note that samples of alkaline condenser liquors and hydraulic main liquors from gasworks, which were kindly given by Dr Colman, were found after removal of sulphur to give the auxetic test. It is not certain whether these materials would be good sources of auxetics, as the latter deteriorate rapidly when exposed to the air in alkaline solution.

[1] The solutions must always be neutralised before testing on lymphocytes. Salts delay the diffusion of substances into cells so that they must not be in excess.

The precipitate from the ammonia may also be taken up in benzene and dried over anhydrous sodium sulphate, but before testing, the benzene must be very thoroughly blown off under a fan or distilled off under reduced pressure; the previous treatment is however to be preferred. In this way 85 g. of bases were extracted from about 1 cwt. of tar. These were distilled under reduced pressure and separated into five fractions.

Fraction	B.-P. at 16 mm.	Weight of distillate
I	0—100°	3·4 g.
II	100—150°	19·2
III	150—200°	18·2
IV	200—220°	8·5
V (Residue)	—	33

Fractions II and III were found to be highly active, I was extremely poor and IV and V were quite inactive.

II and III were then refractionated, but no great difference in activity could be detected in the fractions thus obtained. Both II and III oxidised rapidly in the air and in so doing lost their auxetic power, and this fact presumably accounts for the loss of action previously referred to in the weak aqueous and acid extracts of tar.

Further treatment of basic fractions.

Experiments were next made on the isolation of these active constituents and qualitative experiments showed the presence of nitrogen and possibly of sulphur.

An estimation kindly made by Dr Hartley of this Institute with van Slyke's apparatus showed that the nitrogen was not contained in any side chain but was bound up in the ring.

In this connection it was found that Kahlbaum's pseudocumidine, which is closely allied to the cumidine occurring in coal tar, was very feebly active. The test in this case requires great care as the conditions must be exactly right for any activity to be shown at all.

ψ-cumidine is easily benzoylated in the usual way yielding a white crystalline compound, Mp. 174–5° which has no longer any detectable action on lymphocytes. Attempts were then made to isolate individual bases from the mixture obtained by precipitation with ammonia, by means of benzoyl chloride, but little evidence of benzoylation could be obtained and no crystalline benzoyl compounds could be isolated. Had this been successful it would have been possible to separate the aniline bases from the pyridine bases and to ascertain whether auxetic action was an inherent property of either or both.

The next method tried was fractional crystallisation of the picrates. A saturated solution of picric acid was made, preferably in alcohol, and the basic fraction added drop by drop. A yellow solid at once separated out which crystallised readily from acetic acid. On fractional crystallisation two main fractions were obtained. The first more insoluble portion A, having a Mp. 199°–201°, the second B (after several recrystallisations) Mp. 161°–2°.

Analysis of A gave the following figures.

0·0895 g.; 0·1709 g. CO_2; 0·0219 g. H_2O.
C = 52·11 %, H = 2·71 %.
0·0999 g.; 0·1924 g. CO_2; 0·0252 g. H_2O.
C = 52·52 %, H = 2·80 %.
0·1188 g.; 16·8 cc. N_2 at 16° and 761·75 mm.
N = 16·52 %.
0·1319 g.; 19·1 cc. N_2 at 16° and 760·5 mm.
N = 16·80 %.

The figures obtained for picrate A correspond with the following empirical formula, $C_{22}H_{14}O_9N_6$ which requires

$$C = 52{\cdot}17\ \%\ H = 2{\cdot}76\ \%\ N = 16{\cdot}60\ \%,$$

but nothing further is at present known with regard to the constitutional formula.

The quantity of picrate B, Mp. 161°–2°, obtained was insufficient for analysis.

Experiments on the hydrolysis of picrate A were then undertaken with the object of isolating again and if possible identifying the active base from it, but the results so far have been somewhat unsatisfactory. In the first place the picrate was only hydrolysed with difficulty, and secondly it was found almost impossible to separate the very small amount of free base produced from the metallic picrates formed in the hydrolysis. Only when this was carried out with litharge was any free base obtained and the amount of this was much too small for identification to be possible.

The solution of this free base did however produce cell-division showing that it was one of the active constituents.

The hydrolyses were all carried out in air and possibly better results might be obtained by using an atmosphere of nitrogen, the active bases being very readily oxidised to inactive compounds.

Experiments on the inactivation of the auxetics of coal-tar.

In view of the theory that the susceptibility of pitch workers is due to the presence in the pitch used of the auxetics above described it seemed highly desirable to devise a method whereby these substances might be removed from the tar and pitch or at any rate inactivated. As has been

described above the auxetics can be almost completely removed from the tar by thorough washing with water or dilute acid. These methods however leave the tar in a state which renders its further distillation extremely troublesome and hence have practical objections.

A method has been recently patented by H. W. Robinson [1913] for the treatment of the tar or pitch by formaldehyde whereby the auxetics are inactivated, and an important test on a practical scale is now being made at the briquette works at Cardiff to see if this inactivation of auxetics prevents the disease occurring among the workmen.

The experiments described above, however, have suggested a simpler method of inactivation. It was noticed that aqueous extracts of tar rapidly lost their auxetic power if left exposed to the air for any considerable time. This was especially noticeable if the extracts had been made alkaline and hence the free bases unless dried or kept in an evacuated desiccator rapidly become inactive. The above facts suggested that the loss of activity was due simply to the oxidation of the auxetics and hence experiments were at once commenced with a view to rendering the tar innocuous by means of oxidation methods.

The following experiments illustrate the method finally adopted. 100 g. of tar were heated in a flask in an oil bath to 160° and a blast of air blown through. The tar was then extracted as before with acid, and the extract after neutralisation tested for auxetic action and found to be inactive in every case. A similar experiment was carried out with ozonised air and again the extract of the residue was inactive. The oxidation was quicker in this case than in the experiment where ordinary air was used. The auxetics present can therefore readily be rendered harmless by oxidation according to the method described and it is hoped shortly to try the process on a large scale. The experiments just described refer only to the auxetics, the inactivation of the kinetics being at present under investigation.

Summary.

1. Methods are discussed for the separation of basic constituents of gas-works coal-tar which excite cell division.

2. The bases capable of exerting this action are found to occur in the anthracene fraction of the tar.

3. Two of these bases have been isolated as picrates but have not as yet been satisfactorily identified.

4. A method for the inactivation of these bases in tar is described.

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XXXIV. THE VISCOSITY OF PROTEIN SOLUTIONS. II. PSEUDOGLOBULIN AND EUGLOBULIN (HORSE).

By HARRIETTE CHICK.

From the Lister Institute.

(*Received May 18th, 1914.*)

The following experiments form a continuation of those published recently by Lubrzynska and the author [1914], on the influence of concentration of protein upon the viscosity of horse serum and solutions of crystallised egg and serum albumin (horse). In the present case the investigation has been extended to the pseudoglobulin and euglobulin of horse serum. The significance of both sets of results is discussed at the end of the present paper.

Preparation of the Material.

By pseudoglobulin is signified the protein, soluble in distilled water at its iso-electric point, which is separated from serum by one-half saturation with ammonium sulphate[1] (addition of an equal volume of saturated ammonium sulphate solution to the diluted serum). The term euglobulin is used to indicate the protein insoluble in distilled water at its iso-electric point and therefore precipitated by dilution of the serum and acidification until the reaction is adjusted to the iso-electric point of the protein.

The protein precipitated by one-third saturation with ammonium sulphate (addition of one volume of saturated ammonium sulphate to two volumes diluted serum) is frequently considered to be euglobulin, and this method is a standard one for separating this protein from pseudoglobulin. The precipitate obtained, however, is a mixture of the two, and the total amount is considerably more than is obtained by dilution and acidification. Further, it does not include all the euglobulin, for the precipitate of pseudoglobulin, obtained when the concentration of ammonium sulphate in the filtrate is

[1] There is no reason to suppose that this method sharply separates pseudoglobulin from albumin [see Chick and Martin, 1913].

raised to one-half saturation, is found to contain more protein insoluble in water (euglobulin), than if the latter had previously been removed by dilution and acidification.

Euglobulin. In the present case the euglobulin was always prepared by the last named method. Serum was diluted ten times and the reaction adjusted to the iso-electric point of the euglobulin by means of the addition of a small amount of acetic acid, about 2–3 cc. normal acetic acid being, as a rule, found necessary for each litre of diluted serum. The optimum amount was determined by making preliminary trials with small samples, and was easily recognised by the ease and rapidity with which the precipitate settled. After one to two days' standing the precipitate was separated by centrifuging the deposit, after decanting the top liquor. It was purified three or four times by redissolving (dispersing) in a minimum of caustic soda and precipitating with an exact equivalent of hydrochloric acid. The stronger acid is here employed in place of acetic, because a smaller quantity is found to be necessary and a lower concentration of salt results on neutralising. The final precipitate was washed once with slightly acidified water. For this purpose water, saturated with carbon dioxide and diluted about 30 times with ordinary distilled water, has been found to yield the convenient, slight, acidity.

Pseudoglobulin. The pseudoglobulin, used for the experiment given in Table V, was prepared from diluted horse serum by one-half saturation with ammonium sulphate, after removal of the precipitate given by one-third saturation. The crude precipitate was dissolved in water and twice re-precipitated, the final material being dialysed for two days against running water (tap) and for sixteen days against distilled water, changed daily, in presence of toluene.

The method of precipitation by one-third saturated ammonium sulphate, as stated above, does not remove all the euglobulin. The small proportion remaining can be detected even before the dialysis is commenced, by diluting the material and acidifying with minute amounts of dilute acetic acid, a method by which euglobulin can readily be detected in presence of a small concentration of salt [see Chick, 1913]. After dialysis for nine days, as much as one-tenth of the total protein was found to be insoluble in distilled water. At the completion of the process the material contained about 14 % protein, of which about one-eighth was found to be in this condition. This insoluble material, the presence of which must, I think, be attributed to a gradual degradation of the pseudoglobulin to a water-insoluble condition, shows very remarkable analogies with euglobulin and has been made the

subject of a separate investigation, the result of which will be published later. When the dialysis was complete[1], the insoluble material could be completely separated by diluting and centrifuging. The top liquor was found to be free from insoluble protein and was concentrated over sulphuric acid in a vacuum in order to obtain material of the right strength for the viscosity measurements.

In order to investigate material which had suffered no degradation, dialysis was dispensed with in preparing that used for the experiments in Table VI. The euglobulin was removed in the ordinary way from the serum by dilution (1 in 10) and acidification and a further precipitate separated by addition of ammonium sulphate to one-third saturation (19·8 g. $(NH_4)_2SO_4$ to 100 cc.); ammonium sulphate was then added to the filtrate until one-half saturation was reached (11·3 g. $(NH_4)_2SO_4$ to 100 cc., final density 1·138). The pseudoglobulin was purified as follows. The precipitate was pressed between filter paper to free it from mother liquor, redissolved in water and an equal volume of saturated ammonium sulphate was added. The resulting mixture contained an excess of ammonium sulphate, over one-half saturation, owing to the salt contained in the precipitate. This extra amount was ascertained by boiling a small sample of the mixture, filtering the protein and determining the density of the filtrate. The requisite amount of water could be then calculated and added to the mixture. The precipitate was filtered, freed from mother liquor as far as possible by pressing between filter paper and finally dissolved in a little water. The solution was found to have the following composition: protein 13·75 %, ammonium sulphate 10·46 %. It contained only the faintest trace of insoluble protein (tested by dilution and acidification).

Euglobulin.

Influence of concentration of protein.

The experiment, of which the results are detailed in Table I, was so arranged that the conditions of solution (dispersion) should approximate to those obtaining in normal serum. The euglobulin was dispersed with a small amount of alkali, the concentration of hydrogen ions, 10^{-8} N, approximated to that of serum, and in addition a small amount of sodium chloride was present, about 1 gram per 6 grams protein. The results are shown graphically

[1] During the dialysis of proteins, the reaction gradually approximates to the slight acidity characteristic of the iso-electric point, and as a consequence any euglobulin or "denaturated" protein is gradually precipitated.

in curve *c*, Figure 1, where the results of similar experiments with pseudo-globulin are also plotted, together with those for serum albumin and whole serum [see Chick and Lubrzynska, 1914, p. 61] for purposes of comparison. The much greater viscosity of euglobulin is at once apparent. A solution containing 6·6 % protein has a viscosity of 3·49, i.e. more than twice that shown by solutions of serum albumin or by whole serum of equal protein-content. With higher concentration of protein (12·95 %), the viscosity of euglobulin reaches the high figure of 21·7, a value not approached by the strongest solutions obtained of serum albumin (20·6 % protein, coefficient of viscosity 7·54) or of the proteins of whole serum (18·1 % protein, coefficient of viscosity 6·38).

TABLE I.

Influence of concentration of the protein upon the viscosity of solutions (dispersions) of euglobulin (horse) in dilute sodium chloride solution and alkali.

* Concentration of sodium chloride 2·2 % to 0·5 %.
* ,, ,, hydrogen ions $10^{-8.0}$ to $10^{-8.2}$ normal.
Temperature 25°. Time of flow in viscosimeter for water = 18·5 seconds.

Concentration of protein, %	Mean time of flow in viscosimeter, secs.	Density of solution (H_2O at 25° = 1)	Coefficient of viscosity, $H_2O = 1$
12·95	379·3	1·0582	21·69
10·81	191·7	1·0479†	10·86
9·84	144·9	1·0435	8·17
6·60	62·7	1·0290	3·49
3·27	31·2	1·0141	1·71

* Concentration of salt and alkali was adjusted to give results comparable with those obtained for whole horse serum [see Chick and Lubrzynska, 1914, Table VI, p. 67].
† Interpolated value.

Influence of hydrogen ion concentration and of salt content.

Hardy [1905] found that the viscosity of euglobulin, when dissolved by salt, was much lower than when dispersed by alkali. When small amounts of alkali are used, this is undoubtedly the case, the opalescent material obtained being much more viscous than the clear solution formed when salt is used to dissolve the protein. If, however, the concentration of alkali is increased, the viscosity falls rapidly. This is seen in Table II, where, in Experiment I, the result is given of three determinations of viscosity in solutions containing 5·6 % protein, but with concentration of hydrogen-ions falling from $10^{-7.46}$ N to $10^{-10.02}$ N. Corresponding to this change in reaction, there is a fall in the coefficient of viscosity from 12·17 to 3·62. The last

result is not greatly in excess of that obtained in Experiments II and III which deal with solutions of salt-globulin of the same protein concentration. The reaction in these two instances is not far removed from that of the iso-electric point which was determined by Michaelis and Rona [1910] to be at a concentration of hydrogen-ions equal to about 10^{-6} N. The direct influence of salt in lowering viscosity[1] is seen by a comparison of Experiment IV with Experiment I. In the former case, a small concentration of salt, 1 %, reduced the coefficient of viscosity from about 12·2 to 2·4 in presence of, roughly, the same concentration of hydrogen-ions. The figures for Experiments II and IV are interpolated values obtained from curves *b* and *c* respectively, in Fig. 1.

TABLE II.

Influence of concentration of hydrogen-ions upon the viscosity of euglobulin (horse) (a) dispersed by alkali, Exp. I; (b) by NaCl, Exps. II and III, and (c) by alkali + NaCl, Exp. IV.

Concentration of protein 5·68 %.
Temperature 25°.

Exp.	Concentration of NaCl, %	Concentration of alkali, NaOH, in terms of normality	Concentration of hydrogen-ions, in terms of normality	Density of the solution (H_2O at 25° = 1)	Coefficient of viscosity (H_2O, or salt solution, = 1)
I	—	—	$10^{-7.46}$	1·0164	12·17
	—	0·01	$10^{-9.34}$	,,	6·84
	—	0·02	$10^{-10.02}$	,,	3·62
II	3·6	—	$10^{-5.7}$	—	2·65*
III	3·5	—	$10^{-6.2}$	—	2·77†
IV	1·0	—	$10^{-8.1}$	—	2·39*

* Interpolated values from curves *b* and *c*, Fig. 1, see also Table I and Table III (Exp. II).
† See Exp. I, Table III.

In Table III are given the results of two experiments showing the influence of protein concentration when the euglobulin, at or about its iso-electric point, is dispersed by means of salt alone. It is seen that, when the concentration of protein is sufficiently high, very high values are obtained for the coefficient of viscosity. For example, in Experiment II, when the protein concentration was 13·2 %, the coefficient of viscosity was 29·56.

From a comparison of Tables II and III with Table I, which deals with an experiment in which the conditions obtaining in serum were closely imitated, it is evident that the euglobulin in serum must be regarded as salt-globulin.

[1] This influence would appear even more marked if, in calculating the concentration of protein in the solutions of salt-globulin, any allowance were made for the water appropriated by the salt.

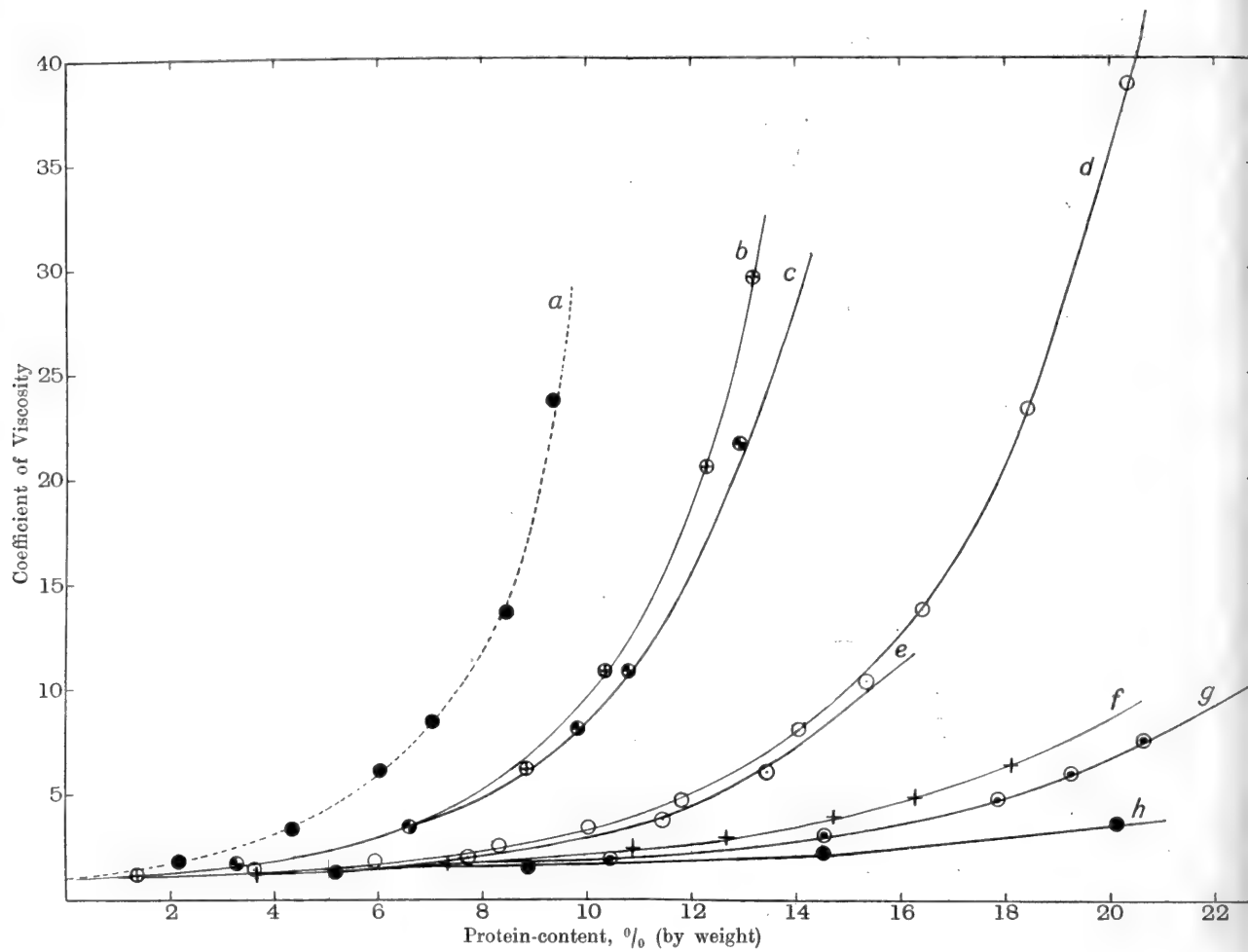

Fig. 1. Influence of protein concentration upon the viscosity of various protein solutions.

Curve *a* ----●---- , sodium caseinogenate [Chick and Martin, 1913].
,, *b* —⊕— , euglobulin (salt). (See Exp. II, Table III.)
,, *c* —◕— , euglobulin (salt + alkali). (See Table I.)
,, *d* —○— , pseudoglobulin. (See Table V.)
,, *e* —⊙— , pseudoglobulin (salt). (See Table VI.)
,, *f* —+— , whole serum (horse). [Chick and Lubrzynska, 1914, Table VI.]
,, *g* —⊙— , serum albumin. [,, ,, ,, ,, ,, IV.]
,, *h* —●— , egg albumin. [,, ,, ,, ,, ,, I.]

TABLE III.

Influence of concentration of protein upon the viscosity of euglobulin (horse) dissolved (dispersed) in sodium chloride solutions.

Exp. I. Concentration of hydrogen-ions $= 10^{-6.2}$ normal.
Exp. II. ,, ,, ,, $= 10^{-5.5}$ to $10^{-6.0}$ normal.
Temperature $= 25°$.
Mean time of flow for water $= 55{\cdot}4$ seconds.

Exp.	Concentration of NaCl, %	Concentration of protein, %	Mean time of flow in viscosimeter, secs.		Density (H_2O at $25° = 1$)		Coefficient of viscosity, H_2O containing 3 % NaCl = 1
			Euglobulin solution	Salt solution (3·0 % NaCl)	Euglobulin solution	Salt solution (3·0 % NaCl)	
I	3·0	8·02	241·1	57·6	1·045	1·0211	4·28
	3·7	5·82	142·3	,,	1·038*	,,	2·48
	4·3	3·62	95·2	,,	1·032	,,	1·62
	3·5	1·44	68·6	,,	1·025	,,	1·18
				(3·6 % NaCl)		(3·6 % NaCl)	H_2O containing 3·6 % NaCl = 1
II	3·6	13·20	1654·1	58·1	1·0650	1·0257	29·56
	,,	12·32	1153·3	,,	1·0626*	,,	20·56
	,,	10·57	612·3	,,	1·0574*	,,	10·87
	,,	8·86	352·8	,,	1·0522*	,,	6·23
	,,	6·58	189·4	,,	1·0451	,,	3·32
	,,	1·36	69·1	,,	1·0298*	,,	1·19

* Interpolated values.

Influence of Temperature.

The solution used to investigate the influence of temperature contained 10·81 % protein and 1·82 % sodium chloride, and had a hydrogen-ion concentration equal to 10^{-8} N, the conditions being so arranged as to approximate to those obtaining in normal serum. The viscosity at various temperatures is expressed in relation to that of a 1·82 % solution of sodium chloride, estimations of the latter being made in the same viscosimeter at points over the same range of temperature. The results were expressed in a smoothed curve, from which the values in the 3rd column of Table IV were obtained. With a rise of temperature from 2·1° to 41·3°, the coefficient of viscosity was decreased to less than one quarter (see Table IV), an effect very much greater than that obtained with the other serum proteins investigated. A solution of serum albumin containing 20·65 % protein had its viscosity reduced by only about 30 % for a similar change of temperature, and while, for the whole proteins of serum, the effect of temperature was somewhat greater, it was insignificant compared to the result obtained for euglobulin.

TABLE IV.

Influence of temperature upon the viscosity of solutions (dispersions) of euglobulin (horse) in dilute sodium chloride and alkali.

Concentration of sodium chloride = 1·82 %.
,, ,, hydrogen-ions = $10^{-8.03}$ normal.
,, ,, protein = 10·81 %.
Density of protein solution = 1·0479.
,, ,, 1·82 % NaCl solution = 1·0127.

Temperature, °C.	Mean time of flow in viscosimeter, secs.		Coefficient of viscosity (1·82 % NaCl solution at the same temperature = 1)
	Euglobulin solution	1·82 % solution of NaCl (from curve)	
2·1	939·8	36·3	26·79
9·1	532·4	29·5	18·67
17·4	288·7	24·1	12·39
25·0	198·1	20·8	9·85
32·1	138·6	18·6	7·71
41·3	93·6	16·2	5·97

The great influence of rise of temperature in lowering viscosity is characteristic of emulsoid colloids, perhaps the most striking example being that of solutions of gelatin. The difficulty experienced in filtering serum and other solutions containing proteins is also lessened to a surprising extent on raising the temperature owing to the fall in viscosity. With dilute solutions, of low viscosity, the effect may not be much in excess of that observed for water itself, or for solutions of crystalloids, but it becomes progressively greater with increase in concentration of the colloids and consequent rise in viscosity. For example, a solution of egg-albumin containing 7·04 % protein (coefficient of viscosity = 1·36 at 25°) showed a change of viscosity only about 5 % greater than that of distilled water when the temperature was raised nearly 40 degrees centigrade. When the strength of protein was increased to 20·1 % (coefficient of viscosity = 3·58 at 25·4°) and 28·15 % (coefficient of viscosity at 25·4° = 10·01) the change in the value of the coefficient was about 23 % and 54 % respectively for a similar change in temperature [see Chick and Lubrzynska, 1914, Table II]. In the case of whole serum (protein = 7·68 %; coefficient of viscosity = 1·87 at 25°), the coefficient of viscosity was decreased nearly 14 %, and with concentrated serum (18·1 % protein, coefficient of viscosity = 6·34 at 25°) the fall in value was about 51 % on raising the temperature about 40° [Chick and Lubrzynska, 1914, Table VII].

It is therefore quite consistent that solutions of pure euglobulin, with their very high viscosity, should suffer an even greater change in this respect with alteration of temperature. An interpretation of the phenomenon will be suggested later (see p. 277).

Pseudoglobulin.

Influence of concentration of protein.

The results of viscosity measurements with solutions of pure dialysed pseudoglobulin are set forth in Table V. The method by means of which it was freed from the water-insoluble material, formed during its lengthy preparation, has already been described (p. 263). The results are also graphically shown in curve *d* of Figure 1. It will be seen that, as regards viscosity, solutions of pseudoglobulin occupy a position intermediate between those of euglobulin and of serum albumin. For solutions of equal protein content, the viscosity of those of pseudoglobulin falls far below those of euglobulin and this is especially marked in the stronger solutions. For example, a solution containing 10 % protein, exhibits a viscosity less than one-third of that of a solution of euglobulin of equal strength. At the same time, the viscosity is considerably in excess of that of serum albumin, a 10 % solution being nearly twice and a 20 % solution about five times as viscous [see Chick and Lubrzynska, 1914, p. 65].

TABLE V.

Influence of concentration of the protein upon the viscosity of solutions of pseudoglobulin (horse) dialysed.

Temperature 25°.
Time of flow in viscosimeter for water = 48 secs.
Concentration of hydrogen-ions = $10^{-6.8}$ to $10^{-6.9}$ normal.

Concentration of protein, %	Mean time of flow in viscosimeter, secs.	Density of the solution (H_2O at 25° = 1)	Coefficient of viscosity ($H_2O = 1$)
20·37	1753·6	1·0619	38·79
18·45	1059·7	1·0558*	23·31
16·43	628·8	1·0490	13·74
14·06	370·8	1·0425	8·05
11·82	218·0	1·0341	4·70
10·04	160 1	1·0289*	3·43
8·32	121·5	1·0239*	2·59
5·95	87·5	1·0169	1·85
3·61	66·4	1·0102	1·40

* Interpolated values.

Influence of ammonium sulphate.

In Table VI are given the results of an experiment with un-dialysed material containing the salt which separated with the protein phase during precipitation by half-saturated ammonium sulphate. The insoluble protein present amounted only to a slight trace. The concentration of ammonium sulphate was equal to 10·4 %, and, in order to compare the results with those given in Table IV, the percentage concentration of protein is reckoned, not as grams per 100 grams of the system but as grams per 100 grams protein and water, see 3rd column Table VI.

TABLE VI.

Influence of concentration of protein upon the viscosity of solutions of pseudoglobulin (horse) containing ammonium sulphate.

Temperature 25°.
Time of flow in viscosimeter for 10·3 % $(NH_4)_2SO_4$ = 53·9 seconds.
Time of flow for water = 48 seconds.
Concentration of hydrogen-ions = $10^{-6.8}$ normal.
Concentration of $(NH_4)_2SO_4$ = 10·4 %.

Concentration of protein		Mean time of flow in viscosimeter, secs.	Density of solution (H_2O at 25° = 1)	Density of H_2O containing 10·3 % $(NH_4)_2SO_4$	Coefficient of viscosity, H_2O containing 10·3 % $(NH_4)_2SO_4$ = 1
G. in 100 g. total system	G. in 100 g. water + protein				
13·75	15·35	535·2	1·1025	1·0601	10·33
12·05	13·45	312·6	1·0972*	,,	6·00
10·26	11·45	198·8	1·0917*	,,	3·80
6·93	7·73	106·8	1·0814	,,	2·02
4·16	4·65	75·3	1·0729*	,,	1·41

* Interpolated values.

As a matter of fact, for accurate comparison, some allowance should also be made for the water appropriated by so large a proportion of salt, but that is impossible to estimate. While the effect of salt in lowering viscosity is much less than that obtaining in the case of euglobulin, its influence is evident from a comparison of Tables V and VI, or of curves *d* and *e*, Figure 1.

SIGNIFICANCE AND INTERPRETATION OF RESULTS.

From a survey of the foregoing results, it is seen that, as regards their viscosity, solutions of the three proteins of serum, albumin, pseudoglobulin and euglobulin, form a series varying from comparatively labile fluids, resembling those of crystalloids, in case of albumin, to liquids of considerable

viscosity, in the case of euglobulin. A concentration of protein of at least 10 % is necessary to reveal the colloidal nature of serum albumin and leads to excessively high viscosities with the other two proteins.

The interpretation of the characteristic phenomena displayed by solutions of these proteins may be found in the two-phase nature of the system. Proteins are "hydrophile emulsoids" and the actual volume of the disperse phase may be assumed to be much greater[1] than that indicated by the solution volume of the protein, as determined from the density of the solution.

Hatschek [1910, 1911] has developed a theory of the viscosity of two-phase systems based upon his own observations of the viscosity of oil-water emulsions. He has shown that when the volume of the oil approaches 70 % of the total volume, at which point the oil particles touch one another, there is an enormous increase in viscosity. This, in his opinion, is due to the inability of the oil particles to roll upon one another under the influence of a shearing force; as a result they suffer deformation. From mechanical considerations he has found that, when the volume of the oil is more than one-half the total volume, the viscosity of such a two-phase system can be expressed in terms of the phase ratio as follows:

$$\eta = \eta' \frac{\sqrt[3]{A}}{\sqrt[3]{A} - 1} \quad \ldots\ldots(1),$$

where η = the viscosity of the system,

η' = the viscosity of the continuous phase,

V = volume of the system,

v = volume of the disperse phase,

and $A = \frac{V}{v}$ = the phase ratio.

If the viscosity of the continuous phase be taken equal to 1·0

$$\eta = \frac{\sqrt[3]{A}}{\sqrt[3]{A} - 1} \quad \ldots\ldots(2),$$

or

$$A = \left(\frac{\eta}{\eta - 1}\right)^3 \quad \ldots\ldots(3).$$

According to this expression, the viscosity of the system is independent of the viscosity of the disperse phase and of the size of its particles, but depends upon the relative volume of the two phases.

The formula was tested by Hatschek [1911, 1913] in the case of oil-water emulsions, of which the composition was accurately known. When

[1] In this connection, it is of interest to note that Findlay and Creighton [1911] found that the solubility of oxygen, at atmospheric pressure, in de-aerated serum was only about one-fifth as great as in water; in the case of nitrogen, the difference was even greater.

the emulsion was sufficiently concentrated, the known volume of oil emulsified was found to be in close agreement with the value calculated by means of the above formula.

Hatschek [1912] has found the above formula also applicable to colloidal solutions of the emulsoid type, in cases where the disperse phase occupies more than one-half the total volume of the system, i.e. where A is less than 2. The two instances selected by him are solutions of glycogen, using the viscosity measurements of Bottazzi and d'Errico [1906], and those of caseinogen, using the results of Chick and Martin [1912]. The value of $A\left(=\frac{V}{v}\right)$ was reckoned by means of the formula (3) given above, V being the volume occupied by 100 grams of the system. The value of $A'\left(=\frac{V}{c}\right)$, where c is the weight of dissolved substance, could be calculated from the measured density of the system and the known concentration of the colloid. The ratio $\frac{A'}{A}\left(=\frac{v}{c}\right)$, expressing the volume occupied by unit weight of the dissolved substance, was then calculated and, in case both of glycogen and caseinogen, Hatschek found a very fair constancy in value, when A was less than 2.

One of the most important results of Hatschek's expression is that, given the viscosity, not only is a method afforded of determining the phase ratio, but from the latter, given the concentration of the colloid and the density of the system, a calculation can, for the first time, be made of (a) the volume occupied, and (b) the amount of water taken up, by unit weight of a hydrophile colloid.

In the case of caseinogen, the value of (a) is equal to 9·3[1], that is to say each gram of caseinogen in colloidal solution occupies a volume equal to 9·3 cc. having taken up 8·6 cc. water (solution volume of caseinogen = 0·70). It follows therefore that even in so comparatively dilute a solution as 6 % (100 grams of the system occupying 98 cc.) the caseinogen phase would occupy 56 cc. or 57 % of the total volume.

Using Hatschek's formula the values of $\frac{v}{c}$ for all the serum proteins used in the present investigation have been calculated and the results for euglobulin and pseudoglobulin are given in Tables VII and VIII and IX respectively. Table X contains a similar set of results for serum albumin, using the viscosity measurements published previously [Chick and Lubrzynska, 1913].

[1] The mean value given by Hatschek, 9·52, is slightly too high, the density of the system being neglected in evaluating A'.

TABLE VII.

Volume occupied by the euglobulin phase in solution at 25° C.

(*a*) Dispersed with salt and alkali (see Table I).
(*b*) Dispersed with alkali (see Table II).

	Concentration of protein, % $=c$	Concentration of hydrogen-ions, in terms of normality	Concentration of NaCl, %	Density of system $=\delta$	Coeff. of viscosity $=\eta$	$A=$ vol. of 100 g. system / vol. disperse phase $(=v)$ $=\left(\frac{\eta}{\eta-1}\right)^3$	$A'=$ vol. of 100 g. system / weight of dissolved substance $(=c)$ $=\frac{100}{\delta \times c}$	Volume occupied by 1 g. dissolved substance $=\frac{A'}{A}=\frac{v}{c}$	Mean value of $\frac{v}{c}$
(*a*)	12·95	$10^{-8.1}$	2·2	1·0582	21·69	1·152	7·298	6·335	
	10·81	,,	1·8	1·0479	10·86	1·336	8·828	6·608	6·51
	9·84	,,	1·7	1·0435	8·18	1·479	9·738	6·584	
	6·60	,,	1·1	1·0290	3·487	2·757	14·72	5·339	
	3·27	,,	0·5	1·0140	1·710	13·96	30·13	2·158	
(*b*)	5·68	$10^{-7.5}$	—	1·0164	12·17	1·294	17·32	13·39	
	,,	$10^{-9.3}$	—	,,	6·84	1·608	17·32	10·77	
	,,	$10^{-10.0}$	—	,,	3·62	2·638	17·32	6·57	

TABLE VIII.

Volume occupied by the euglobulin phase in salt solution at 25° C.
(See Table III, Exp. II.)

Concentration of protein, % $=c$	Density of the system $=\delta$	Coefficient of viscosity $=\eta$	$A=$ vol. of 100 g. of system / vol. of disperse phase $(=v)$ $=\left(\frac{\eta}{\eta-1}\right)^3$	$A'=$ vol. of 100 g. of system / wt. of dissolved substance $(=c)$ $=\frac{100}{\delta \times c}$	Volume occupied by 1 g. dissolved substance $=\frac{A'}{A}=\frac{v}{c}$	Mean value of $\frac{v}{c}$
13·20	1·0650	29·56	1·109	7·114	6·415	
12·32	1·0626	20·56	1·162	7·640	6·574	6·51
10·57	1·0574	10·87	1·336	8·948	6·698	
8·86	1·0522	6·23	1·690	10·73	6·346	
6·58	1·0451	3·32	2·930	14·54	4·964	
1·36	1·0298	1·19	245·5	71·40	0·291	

TABLE IX.

Volume occupied by the pseudoglobulin phase in solution at 25° C. (*dialysed*). (*See Table V.*)

Concentration of protein, % $=c$	Density of the system $=\delta$	Coefficient of viscosity $=\eta$	$A=$ Total vol. of system / vol. of disperse phase $(=v)$ $=\left(\frac{\eta}{\eta-1}\right)^3$	$A'=$ Total vol. of system / wt. of dissolved substance $(=c)$ $=\frac{100}{\delta \times c}$	Volume occupied by 1 g. dissolved substance $=\frac{A'}{A}=\frac{v}{c}$	Mean value of $\frac{v}{c}$
20·37	1·0619	38·79	1·082	4·623	4·272	4·50
18·45	1·0558	23·31	1·141	5·133	4·499	
16·43	1·0490	13·74	1·254	5·803	4·627	
14·06	1·0425	8·05	1·488	6·822	4·585	
11·82	1·0341	4·70	2·050	8·180	3·990	
10·04	1·0289	3·43	2·813	9·682	3·441	
8·32	1·0239	2·59	4·322	11·74	2·716	
5·95	1·0169	1·85	10·31	16·53	1·603	
3·61	1·0102	1·40	42·86	27·42	0·640	

TABLE X.

Volume occupied by the serum albumin phase in solution at 25° C. [*See Chick and Lubrzynska,* 1914, p. 65.]

Concentration of protein, % $=c$	Density of the system $=\delta$	Coefficient of viscosity $=\eta$	$A=$ vol. of 100 g. of system / vol. of disperse phase $(=v)$ $=\left(\frac{\eta}{\eta-1}\right)^3$	$A'=$ vol. of 100 g. of system / wt. of dissolved substance $(=c)$ $=\frac{100}{\delta \times c}$	Volume occupied by 1 g. dissolved substance $=\frac{A'}{A}=\frac{v}{c}$	Mean value of $\frac{v}{c}$
20·65	1·0593	7·538	1·534	4·572	2·980	2·81
19·24	1·0555	5·875	1·751	4·924	2·838	
17·85	1·0513	4·763	2·028	5·329	2·628	
14·54	1·0413	3·025	3·332	6·605	1·982	
10·45	1·0296	1·952	8·618	9·295	1·078	
5·19	1·0153	1·316	72·20	18·98	0·263	
2·59	1·0075	1·128	684·0	38·32	0·056	

In all cases, with the exception of the experiment set forth in Table VII *b*, which will be discussed separately later on, the value $\frac{v}{c}$ remained constant when the disperse phase occupied more than one-half the total volume. It was greatest in the case of euglobulin, one gram of which, in salt solution, was calculated to have a volume of 6·5 cc. while each gram of dissolved serum albumin was found only to occupy 2·81 cc., pseudoglobulin being intermediate between the two $\left(\frac{v}{c}=4{\cdot}52\right)$. The solution volume of these

TABLE XI.

Water taken up by various proteins in the formation of colloidal solution at 25° C.

Protein	Density of the protein in solution at 25° (H_2O at 25° = 1)	Solution volume (as calculated from the density of the system)	Volume occupied by 1 g. protein in solution, cc.	Water associated with 1 g. protein, when in solution, cc.
Serum albumin (crystallised)	1·38	0·72	2·81	2·09
Pseudoglobulin	1·39	0·72	4·50	3·78
Euglobulin (salt)	1·42	0·70	6·51	5·81
Sodium caseinogenate	1·43	0·70	9·33	8·63
Egg-albumin (crystallised)	1·36	0·73	2·30	1·57

proteins can be calculated from the density of their solutions and hence the actual amount of water associated with each gram of protein can be calculated. These values vary from 5·8 cc. in the case of euglobulin to 3·8 cc. and 2·1 cc. for pseudoglobulin and serum-albumin respectively. These figures are all collected in Table XI, those for caseinogen and egg-albumin being added for purposes of comparison.

In order to institute useful comparison with the other proteins in serum, solutions of euglobulin in presence of salt have alone been taken into account in the preceding paragraph, because, in normal serum, it is under those conditions that this protein exists. When euglobulin is dispersed by alkali alone, its viscosity shows very remarkable variations, as may be seen from the results of Exp. I in Table II, where the euglobulin was practically salt-free. Under these circumstances the degree of viscosity depends upon the amount of alkali employed, being comparatively high at first and rapidly falling with increasing concentration of alkali and hydroxyl-ions. Increase of the latter from near the neutral point to a concentration of about 10^{-4} N (hydrogen-ion concentration 10^{-10} N) was accompanied, in the case of a 5·68 % euglobulin dispersion, by a fall in the viscosity coefficient from 12·17 to 3·62, which latter number is near the figure obtained for salt-globulin of the same protein-concentration. This decrease in viscosity is accompanied by a visual change, the globulin mixture, which remains in the form of a precipitate at concentrations of hydrogen-ions from about 10^{-5} to 10^{-6} N, changing to a thick, opalescent fluid at the neutral point (conc. $H^+ = 10^{-7}$ N) and to a thin, clear liquid at a concentration of hydrogen-ions equal to about 10^{-10} N. The observed change in size of the protein particles would, according to the theory of Hatschek, be unaccompanied by any change in viscosity unless there were a concomitant change in phase-ratio. In other

words, we must suppose that, in the more alkaline solution, the globulin phase contains less water and that the amount appropriated by the protein steadily increases as the iso-electric point is approached, with a consequent great increase in the volume of the disperse phase. In Table VII *b* the calculated value of $\frac{v}{c}$, the volume occupied by one gram protein in solutions of varying alkalinity, is seen to vary from 13·4 at the neutral point to 6·6 in the most alkaline solution employed. The latter figure, which is practically the same as that obtained for salt-globulin, is probably too low, for, in this case, the volume of the disperse phase, as ascertained by Hatschek's formula, is less than one-half the total volume and the calculation is therefore not strictly permissible.

In the presence of a small concentration of salt, the water appropriated by the globulin phase appears to be nearly independent of the reaction, the calculated value of $\frac{v}{c}$ being the same in the case of the two experiments in Table I and Table III, when the hydrogen-ion concentration was varied from 10^{-8} N to 10^{-6} N (see Tables VII *a* and VIII respectively).

The fact that the addition of alkali to a protein, with the consequent formation of protein salt and increase of protein ions, should be accompanied by a *lowering* of viscosity, and the conception that this is due to a less degree of association of the protein with water is opposed to the results of similar investigations with other proteins. Lacqueur and Sackur [1903] showed that increase in alkalinity led to an *increase* in viscosity in case of sodium caseinogenate. Pauli and Handovsky [1909 and 1910], working with the proteins of ox-serum, have shown that an addition of either acid or alkali leads to a preliminary rise in viscosity, followed by a fall as the concentration of either acid or alkali is further increased. Pauli and Falek [1912] have demonstrated the same phenomenon in case of gelatin solutions, and the work of Spiro [1904], Fischer [1910] and Chiari [1911] has shown that the water imbibed by gelatin increases rapidly as the reaction is made either more acid or more alkaline than the iso-electric point.

The general influence of salts[1] in lowering viscosity of protein solutions (and imbibition of water by gelatin), presumably due to their water-withdrawing capacity, was also demonstrated by the above observers and has been confirmed in the present work. The effect in the case of euglobulin is of great importance and has been fully discussed above; it is also well marked in the case of pseudoglobulin.

[1] What is possibly an analogous phenomenon with alcohol has been demonstrated by Brailsford Robertson [1912]. He found that the viscosity of sodium caseinogenate solutions (about 3 %) was lowered when the proportion of alcohol present was raised from 50 % to 75 %.

Influence of temperature. In the light of the theory developed above, the very marked influence of temperature upon the viscosity of protein solutions (see Table IV above, and Tables II, V and VII, Chick and Lubrzynska [1914]) may be explained by a gradual loss of water from the protein phase as temperature is raised. It is usual, with most substances of crystalloid nature, to find less water needed for the solution of each gram of the solute at a higher temperature, and the same property may also be assumed for substances of a colloidal nature. On such an assumption, the greater the initial appropriation of water, either due to the nature of the colloid or to its high concentration, the greater will be the loss of water from the disperse phase on rise of temperature and the diminution in its volume; in consequence the greater will be the fall in viscosity.

The experimental facts are in accord with this conception. With solutions of egg-albumin, where a comparatively small amount of water is assumed to enter into the composition of the disperse phase, the effect of temperature upon the viscosity coefficient is found to be negligible except in the case of very concentrated solutions (20 % and upwards). On the other hand, comparatively dilute solutions of euglobulin, the most "hydrophile" of the proteins studied, show very marked changes in viscosity with alteration of temperature (see Table IV). In the following manner, I have attempted to estimate the volume occupied by one gram of (salt) euglobulin in solution at various temperatures. From curve *c*, Fig. 1, it is possible to determine the concentration of solutions which, at 25°, would possess the same viscosity coefficients as those determined for the 10·81 % solution at the various temperatures (see the 4th column, Table IV). In the case of these solutions, at 25°, the concentration (in %) multiplied by 6·51, gives the volume (in cc.) occupied by the disperse phase. The same values for the volume of the disperse phase will apply to the 10·81 % solution at the temperatures where the viscosity corresponds, if changes in viscosity due to alteration of temperature are assumed to be the result only of changes in phase-ratio (and if the differences in density of the solutions, caused by differences in temperature and concentration, are disregarded). In this way, the volume of one gram of euglobulin, in salt solution, at 2·1° has been estimated at 8·3 cc. and that at 43° at 5·3 cc.; at 25° the volume has already been calculated as 6·51 cc.

Application of the above results to the salting out of proteins.

The results of the preceding experiments, and the conceptions derived from them, throw a very interesting and illuminating light upon the phenomena displayed in the precipitation of proteins by neutral salts. They explain very clearly the influence of increased protein concentration, both in raising the proportion of protein thrown out by a given concentration of salt, and in lowering the limit of salt concentration at which precipitation occurs.

The values obtained, by the use of Hatschek's formula, for the amount of water presumably associated with unit weight of the various serum proteins in formation of colloidal solutions, yield a satisfactory explanation of the well-known precipitation limits of these proteins. The euglobulin, needing most water for its colloidal solution, is first driven out as water is withdrawn during any salting-out process; a higher concentration of salt is necessary to throw down the pseudoglobulin; while the albumin, which appropriates comparatively little water, is the last to be precipitated.

The current notion that albumin is less readily precipitated than the other proteins of serum, because it has a "greater affinity" for water, is thus seen to be erroneous. It is probably more consistent with the truth to affirm that the albumin, needing the exclusive use of less water for its own solution, is less liable to suffer precipitation when competition occurs.

Summary.

1. The viscosity of solutions of pseudoglobulin and euglobulin from horse serum has been investigated as regards the influence of:

(*a*) Concentration of protein.
(*b*) Temperature.
(*c*) Salt-content.
(*d*) Hydrogen-ion concentration.

The results of similar experiments with serum albumin and whole serum, published previously by Chick and Lubrzynska [1914], are included in the general survey of the results.

2. In all cases, increase in protein concentration is accompanied by a disproportionately great increase in the viscosity of the solution. The effect is greatest in case of euglobulin, solutions of which exhibit a high viscosity at a comparatively low protein content. It is least in case of serum albumin, which, for strengths of protein under about 10 %, behaves almost as a

crystalloid. Pseudoglobulin is intermediate between the other two proteins in this respect.

3. The viscosity of euglobulin solutions is dependent upon the manner in which solution (dispersion) of the protein is obtained. In case of "alkali globulin," where no salt is present, the viscosity of the solution depends upon the degree of alkalinity, falling rapidly with decrease in concentration of hydrogen-ions and distance from the iso-electric point. The viscosity of solutions of "salt-globulin" (NaCl) is considerably lower than that of alkali-globulin of equal protein and hydroxyl-ion content; in this case the viscosity is largely independent of the concentration both of salt (if above a small minimum, 0·5 to 1·0 %) and hydroxyl-ions. Euglobulin in serum is in the condition of salt-globulin.

4. The presence of a salt ($(NH_4)_2SO_4$) lowers the viscosity of pseudoglobulin solutions; it is without influence on the viscosity of solutions of albumin (egg) in concentrations up to 7 %.

5. The viscosity of protein solutions is decreased with rise of temperature frequently to a degree far in excess of that displayed by water or solutions of crystalloids. The greater the viscosity of the solution, the greater is the temperature effect, which is thus much enhanced in solutions of high protein concentration and most marked in case of euglobulin.

6. An interpretation of the above results is found in the two-phase nature of the systems studied. By means of Hatschek's formula, which gives an expression for phase-ratio in terms of viscosity, it is possible to calculate the relative volumes of protein- and water-phase in the more concentrated solutions employed. Hence values can be obtained for the volume occupied by one gram of the various proteins when in solution. These were found to show a satisfactory constancy and to be 2·8 cc., 4·5 cc. and 6·5 cc. for serum albumin, pseudoglobulin and salt-euglobulin respectively, at 25°. (The value obtained for alkali-euglobulin varied from 13·9 cc. to 6·6 cc. according to the alkalinity of the solution.) The solution volume of these proteins, reckoned from the density of their solutions, is equal to 0·7; the amount of water taken up by one gram of the protein at 25° would therefore be 2·1, 3·8 and 5·8 cc. respectively.

7. The conclusions given in no. 6 afford a satisfactory explanation of (*a*) the influence of protein concentration upon both the amount of protein precipitated, and the limit of salt concentration required to commence precipitation, in case of a pure protein; and (*b*) the relative order in which the above three proteins are precipitated from a mixture with increasing concentration of salt.

8. Hatschek's theory explains the observed disproportionate increase in viscosity of protein solutions with increase in concentration of the protein, the volume of the disperse phase being increased at the expense of the continuous phase. The relative magnitude of the phenomenon in case of the three proteins investigated is also interpreted.

9. The influence of temperature upon the viscosity of protein solutions would be explained by assuming a less degree of hydration of the colloid at higher temperature (an analogous phenomenon is encountered with crystalloids). A calculation has been made of the volume occupied by one gram of the protein at various temperatures in case of euglobulin. These volumes vary from 8·3 cc. at 2° to 5·3 cc. at 41°.

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XXXV. COLOURING MATTER CONTAINED IN THE SEED-COATS OF *ABRUS PRECATORIUS*.

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(*Received May 2nd, 1914*)

The toxic principle contained in the seeds of *Abrus precatorius* has been the subject of elaborate investigations by many workers, including Klein, De Wecker, Sattler, Warden, Waddell etc. But it appears that none has thought it worth his while to investigate the chemical nature or the properties of the colouring matters contained in the coatings of the seeds of *Abrus precatorius*.

Dr D. D. Cunningham, however, has given a description of the microscopical structure of the seeds of *Abrus precatorius* [1882], which I quote below so that the description may be recorded in a journal which is readily available for reference.

"The integument of the seed consists of no less than nine distinct layers of cells differing considerably from one another in their characters.

Proceeding from without inwards we find :—

(1) A layer of considerable thickness but containing only a single stratum of cells. They are thick-walled, columnar cells containing the colouring matter to which the seed owes its bright scarlet hue, and are arranged in radiant fashion around the deeper portions of the integument. Each cell is dilated peripherally and in many cases a slight basal bulbosity is also present. The peripheral dilated portion is cut up into a number of more or less cuneate portions which are closely adapted to one another. In many cases in the mature seeds the central point where the apices of the segment come into relation to one another really consist of a narrow canal leading directly into the cell-cavity. The surface of the seed-coat due to the close apposition of the cells, necessarily comes to present the appearance of being composed of a layer of cuneate facets arranged in rosetted fashion around central points—each rosette representing the extremity of one of the columnar cells, and the central points in many instances being openings, leading directly into the cell-cavities.

(2) A thick stratum of small cells with thick walls and irregular sinuous outlines.

(3) A thick stratum of small, also thin-walled cells.

(4) A thin stratum of small, also thin-walled cells.

(5) A stratum of elongated thin-walled cells.

(6) A stratum of thickened cells two or three layers deep.

(7) A single row of minute thin-walled cells of more or less cubical character.

(8) A stratum of thick-walled cells with dense yellowish granular contents.

(9) A stratum of thickened more or less parenchymatous cells with mere traces of cavities or contents.

The entire thickness of this stratified coating may be broken up in various ways, but apparently the line which may be taken as representing the transition from testa to tegumen runs through the thin-walled cells of the 7th stratum. The cells save in the 1st and 8th stratum appear to be almost or quite devoid of contents."

The above description throws some light on the following peculiar behaviour of these seeds towards certain reagents.

A few entire *Abrus precatorius* seeds are kept in a corked test-tube immersed in chlorine water, along with seeds broken into pieces and with those which have cracks or holes on the surfaces of the testa. The scarlet material contained in the testa of those seeds which are broken or which have cracks or holes in their surfaces is found to be decolourised, while the entire seeds are perfectly unaffected, though some of these were kept in the solution of chlorine water for several months.

When broken *Abrus precatorius* seeds or those which have cracks or holes on their surfaces are kept immersed in a dilute alkaline solution, e.g. of caustic potash or ammonia or simply water, the solutions become coloured owing to the dissolving out of the colouring matter contained in the testa of the seeds, which gradually lose their scarlet hue. The entire seeds without any hole or cracks on their surface, are not in the least affected when kept immersed in these solutions.

The above phenomenon is not due to the presence of any waxy material in the superficial part of the seed-coat, for, if the seeds having the seed-coats entire, i.e. having no cracks or holes in them, are kept immersed in a solution of ether, previously to their treatment with caustic soda solution etc., the seeds are not decolourised as before. The above experiments prove that

(1) there is a structure on the surface of the seeds which is perfectly impermeable to fluids;

(2) the colouring matter which gives a scarlet hue to the seeds is situated beneath this superficial structure;

(3) when any fluid can make its way, at any point, through the superficial impermeable structure this can readily find access to every part of the structure which contains the scarlet colouring matter of the seeds.

These phenomena can be readily understood by taking into account the fact that the superficial layer of cells on the coating of the seeds are not only elongated structures with thick walls, but really consist of two parts, viz. the peripheral portion and the central portion. The peripheral portion is dilated and cut up into cuneate portions, which are so closely applied to one another as to be perfectly impermeable to fluid.

To make the description quoted above consistent with the experiments described we are led to suppose that the openings, which according to Dr Cunningham lead directly into the cell-cavities, are merely blind pouches. In case of an entire seed being kept immersed in a fluid, which has the power of dissolving out the colouring matter contained in the seeds, no colouring matter comes out into the solution because the fluid cannot penetrate through these blind pouches into the portions of the cells which contain the colouring matter.

This property of impermeability of the superficial layer of cells to fluids and its power of thus protecting the internal layers of the seed, is evidently meant by nature for protection of the seeds so that the propagation of the species of the plant may be kept up. It is necessary for the propagation of the species of this plant that the seeds should be able to keep themselves intact for a long time without suffering any disintegration. For the plants die seasonally every year scattering their seeds all round and these germinate fresh plants next year. The above is also in agreement with the views expressed by many observers that the seeds are distributed by being eaten by birds, and passing out unchanged with their excreta. In the *Pharmaceutical Journal*, July 7, 1883, it has been stated that Dr Noura of Brazil, who, while a member of the government commission appointed to regulate the boundary between Brazil and Bolivia, found it in virgin forests where the husbandmen had never penetrated and where the seeds had very probably been carried by birds. C. Owatari in his article on "Botanical Excursion to Formosa (Taiwan)" published in *Botanical Magazine*, XI. Tokyo, 1897, holds however the opposite view. He considers that the attractive colouration of the seed in *Abrus precatorius* might be accounted for as imitating some beetles which are offensive to birds and are thus protected from being devoured." Extended observations are required to decide which

of these views are really correct. I carried on experiments with pigeons, by giving them entire *Abrus precatorius* seeds which they avoided taking while they greedily devoured paddy-grains. So this experimental evidence is in favour of the Japanese observer.

According to Dr Cunningham, as will be seen from the description quoted above, there are two kinds of colouring matter present in the seed-coats. The cells of the 1st layer contain the colouring matter which gives the scarlet hue to the seed-coats. The cells of the 8th layer contain a yellowish colouring matter. I have found by microscopical examination of the section of the seed-coats, that besides the dyes mentioned by Dr Cunningham a kind of black granule is present at the black spot of the seed-coat. This is however insoluble in acids and alkalis and is not a soluble dye but is in the nature of a pigment and so may be omitted from consideration.

For examination of the colouring matters contained in the seed-coats, a quantity of Abrus seeds are taken and the coatings are separated from the cotyledons and are then soaked in water for about four hours and the coloured solution filtered. The filtrate is shaken with ether which extracts traces of yellow colouring matter, which can be identified by the microscope with the yellow colouring matter contained in the 8th layer of the cell. This was obtained in very minute quantities and was not chemically examined any further. The aqueous extract on evaporation leaves a fine pink coloured residue. This is again dissolved in water and copper acetate solution is added in sufficient quantity to cause complete precipitation of the colouring matter. The mixture is left standing for a day and then filtered. The precipitate is repeatedly washed with distilled water, is dissolved in dilute hydrochloric acid, and decomposed by sulphuretted hydrogen. The filtrate from the copper sulphide is then evaporated to dryness in a steam-bath and the residue is extracted with water and filtered. This filtrate contains the colouring matter which gives the scarlet hue to the seed-coats.

The following reactions show that the colouring matter is a tannin substance.

(1) Dilute ferric chloride solution gives a blue black precipitate, the precipitation becoming complete in 24 hours. This reaction with iron salts is not only an evidence that the solution under the examination is a tannin substance, but it also gives indication as to the nature of the chemical composition of this tannin substance. For regarding tannin substances it may be stated as a general truth, that those which give blue-black colourations with ferric salts are derivatives of pyrogallol and gallic acid, while those which afford a green colouration are derived from catechol and protocatechuic acid.

(2) Lead acetate gives a copious blue coloured precipitate.

(3) Allen in his book on commercial analysis says:— "A delicate reaction for tannins (first noticed by the author and apparently general) is the deep red colour produced on treating a solution with potassium ferricyanide mixed with ammonia." When solutions of the colouring matter and of tannic acid are treated side by side with ammoniacal ferricyanide solution for comparison, exactly the same shade of colour is noticed in both cases.

(4) The solution is found to be gradually decolourised by strips of isinglass, which are found to acquire a reddish violet shade. This reaction proves that the reactions of tannin noticed in the solution are not due to any colourless tannin substance, but to the presence of the colouring matter, for it is the colouring matter which is attracted by isinglass.

(5) The colouring matter is precipitated by a solution of gelatin.

(6) On adding stannous chloride solution to the solution, it acquires a pinkish violet shade and after about 12 hours a copious precipitate having the same colour is formed.

(7) The residue obtained by evaporating the solution has an astringent taste like that of tannic acid, and is insoluble in ether, chloroform, benzene and carbon disulphide as is the case with tannins generally.

I should note here that I have carried on the above set of experiments as well as the experiments which will be described below, not only in test-tubes, but where possible by micro-chemical methods also. That is to say I have taken microscopical sections of the seed-coats of *Abrus precatorius*, have treated these with chemical reagents such as solutions of ferric chloride, stannous chloride, lead acetate etc., and have observed under the microscope changes in the colouring matter contained in the 1st layer of cells corresponding with those noticed in the test-tubes. For example, by adding ferric chloride solution in sufficient dilution, we can readily stain this colouring matter violet, leaving other portions of the seed-coats unaffected.

All these experiments, I think, conclusively prove that the colouring matter contained in the 1st layer of the seed-coats of *Abrus precatorius*, is a definite chemical substance of the nature of a tannin.

The reactions of this tannin substance were further studied with the view of finding out whether it can be identified with any known tannin or whether the substance is to be regarded as an altogether new tannin. Some of these reactions are stated below.

(1) Copper sulphate gives a bluish coloured precipitate, which dissolves in ammonia giving a brown coloured solution.

(2) On dissolving crystals of sodium nitrite in the solution on a

porcelain tile, the liquid is coloured from yellowish brown to reddish brown.

(3) With hydrochloric acid a red colour is produced.

(4) Addition of ammonia produces a brown colouration.

(5) Crystals of sodium sulphate added to the solution on a porcelain plate, after some time give yellowish red colour near the edges.

(6) Addition of lime-water causes a bluish coloured precipitate.

(7) Addition of ammonia after addition of ferric chloride solution, causes a violet coloured solution with a precipitate of the same colour.

(8) Tartar emetic solution slowly produces a yellowish brown coloured precipitate.

(9) Tartar emetic and ammonium chloride yield a clay-coloured precipitate.

(10) Nitrous fumes passed into the hot solution, give a yellow colouration and a yellow dye is left on evaporation.

Comparing the reactions stated above, with the reactions of the known tannins one will find that the colouring matter of *Abrus precatorius* seeds appears to be a new tannin substance.

In conclusion I beg to thank the authorities of the Indian Association for the Cultivation of Science for kindly allowing me to work at the laboratory of the Association.

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XXXVI. THE ACTION OF DIAZOMETHANE ON CASEINOGEN (Preliminary Communication).

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(*Received June 4th, 1914*)

Skraup and Krause [1909] methylated caseinogen with methyl iodide and boiling alcoholic potash with the object of showing which of the constituent amino-acids of the protein chain contained free amino- or hydroxyl-groups. Since their product on hydrolysis yielded no tyrosine or lysine and but little histidine and arginine they concluded that such free groups were present in the nuclei of these amino-acids. It is evident that such a vigorous methylation as they employed may be accompanied by an essential hydrolysis of the protein molecule. We have therefore methylated caseinogen with diazomethane in the hope that by this method results of a more conclusive character may be obtained. By the employment of such a mild reagent the possibility of decomposition is reduced to a minimum and it is therefore practically certain that the product obtained is a direct substitution product of caseinogen. By a comparison between the behaviour, on the one hand, of amino-acids and simple polypeptides and, on the other hand, of caseinogen, it is hoped that a direct view may be obtained into the protein molecule as it actually exists. Results have already been obtained which indicate clearly that the presence of carboxyl[1] groups may be proved and their number estimated. It may also be possible to estimate the number of free hydroxyl groups.

It is to be expected that our product, like that of Skraup and Krause, will on hydrolysis fail to give those amino-acids which when combined in the protein chain still contain free groups capable of methylation. It is further hoped that it may be possible to isolate in the form of their methyl

[1] According to T. B. Robertson [1912], for example, the acidity of proteins is not due to carboxyl groups but to groups of the type C(OH)=N.

derivatives amino-acids which in the free state may be unable to resist unchanged the vigorous hydrolysis usually employed.

In the present communication we include only the preliminary investigation of our product. The further investigation of this substance, and the study of the action of diazomethane on amino-acids and polypeptides are both in progress and will form the subject of further communications.

If an ethereal solution of diazomethane is poured over dry caseinogen, a moderate evolution of gas occurs. By the continued action of fresh portions of diazomethane a product is finally obtained which can in this way be no further methylated. The substance thus obtained is indistinguishable in appearance from caseinogen itself, but contains 3·5 % more methyl. In properties it differs most markedly in its insolubility. If a portion is added to an excess of dilute (N/50) caustic alkali a clear solution is obtained only after about 20 hours standing whereas caseinogen similarly treated dissolves in a few minutes. Towards acids methylo-caseinogen is even more stable, being dissolved by dilute sulphuric acid only after several days heating on a water-bath. By the addition of acid to the alkaline solution, a precipitate is formed containing less methyl than the original substance; this is readily re-dissolved by excess of acid.

Nitrogen estimations in our methylo-caseinogen by Kjeldahl's method gave consistently lower results than estimations by Dumas' method. It therefore seemed probable that by boiling with concentrated sulphuric acid part of the nitrogen was evolved as such. All attempts to estimate this nitrogen have however yielded only negative results.

Unlike Skraup and Krause's product our methylo-caseinogen readily gives Millon's reaction and it is therefore doubtful whether the tyrosine nucleus has been methylated. It may be pointed out that the failure of Millon's reaction in the case of their product is sufficiently accounted for by the iodine introduced by the action of methyl iodide, since it is known that iodo-proteins do not give this reaction. The Adamkiewicz-Hopkins reaction is more pronounced with methylo-caseinogen than with caseinogen. Skraup and Krause made the same observation but ascribed it to the presence of iodine.

It was found by Skraup and Krause that caseinogen when heated with hydriodic acid according to the Herzig-Meyer method gives a precipitate of silver iodide. We have been able to confirm this but obtain a lower value (0·36 %) than they (1·13 %) for the percentage of N-methyl; in this we are in agreement with the recent observation of Burn [1914]. Our

methylo-caseinogen contains about the same percentage of methyl (4·36 %) as Skraup and Krause's product (4·26 %).

Experimental Part.

Preparation of Methylo-caseinogen. 500 g. of Kahlbaum's "Casein nach Hammarsten" dried in a vacuum at 40° over phosphorus pentoxide were treated with diazomethane in ethereal solution in portions of about 5 g. The first few additions were followed by a vigorous evolution of gas. After each addition of diazomethane the reaction mixture was allowed to stand in the dark until it became colourless. The methylation was continued for four months and about 75 g. (a large excess) of diazomethane were used. The last few portions of diazomethane were only very slowly decolourised and no evolution of gas was observable. Methyl estimations from time to time by Herzig and Meyer's method showed that a maximum methyl content had been reached. The methylo-caseinogen was filtered from the ethereal mother-liquor and well washed with absolute ether. The ether was then removed by exposure to the air in shallow dishes for several weeks. All smell of ether had disappeared in a few days. For analysis the substance was further purified by extracting in a Soxhlet with absolute ether for a week. Only a minimal amount (less than 0·1 %) of fat was thus removed.

The ethereal filtrate and washings on evaporation yielded about 1 c.c. of a semi-solid mass. This probably consisted of fat or fatty derivatives and was not further investigated.

Methylo-caseinogen thus obtained is a light colourless powder indistinguishable in appearance from caseinogen. As has been mentioned above, it is only dissolved by acids and alkalies with difficulty; by the action of alkali methyl groups are lost. The alkaline solution is optically active.

Analysis. For analysis the pure fat-free methylo-caseinogen was allowed to stand for at least twelve hours exposed freely to the air of the balance room.

Water estimations were made whenever portions were weighed out for analysis. The results were calculated on the weight of water- and ash-free protein. [See Geake, 1914.]

It has been mentioned above that nitrogen estimations by Kjeldahl's method gave consistently lower results than by Dumas' method[1]. It was

[1] Such differences have been previously observed with proteins. Thus Pittom [1914] working with egg albumin obtained a difference between the two methods of more than 1 % nitrogen.

thought probable that this was due to a portion of the diazomethane having reacted with caseinogen to give a product containing diazo-nitrogen. Such a product when heated with concentrated sulphuric acid would give off part of its nitrogen as nitrogen gas. Estimations of diazo-nitrogen by the usual methods of heating with concentrated hydrochloric acid or with somewhat diluted sulphuric acid yielded negative results. A portion of the substance was therefore heated with the usual Kjeldahl mixture (30 g. conc. H_2SO_4, 10 g. K_2SO_4, 2 g. $CuSO_4$), and the gases evolved passed through a heated combustion tube containing copper oxide and a reduced copper spiral. Even in this way only a minimal amount of unabsorbed gas was collected.

The methylo-caseinogen gave the following results on analysis :

0·5086 g. (10·65 % H_2O) ; 0·0041 g. ash
0·5049 g. (10·65 % H_2O) ; 0·0034 g. ash
0·1957 g. (10·04 % H_2O) ; 0·3549 g. CO_2 ; 0·1322 g. H_2O
0·2118 g. (8·72 % H_2O) ; 0·3869 g. CO_2 ; 0·1429 g. H_2O
0·1657 g. (7·60 % H_2O) ; 0·3088 g. CO_2 ; 0·1143 g. H_2O
0·2728 g. (10·07 % H_2O) neutralised 25·89 c.c. N/10 H_2SO_4 (Kjeldahl)
0·2497 g. (10·07 % H_2O) „ 23·24 c.c. „ „
0·2967 g. (8·48 % H_2O) „ 28·58 c.c. „ „
0·2379 g. (8·48 % H_2O) „ 23·08 c.c. „ „
0·3066 g. (10·11 % H_2O) ; 36·1 c.c. N over water at 13·8° and 758 mm.
0·2767 g. (10·04 % H_2O) ; 32·8 c.c. „ „ 12·8° „ 744 mm.
0·2014 g. (8·48 % H_2O) ; 24·8 c.c. „ „ 11·8° „ 745 mm.
0·4998 g. (8·48 % H_2O) ; 0·0264 g. $BaSO_4$
0·4998 g. (8·48 % H_2O) neutralised 30·90 c.c. N/10 NaOH (Phosphorus estn. by Neumann's method.)

$$\text{Ash} = \frac{(0\cdot90)}{(0\cdot76)} = 0\cdot83\ \%$$

100 parts water and ash-free methylo-caseinogen contain

Found					
C	H	N		S	P
		Kjeldahl	*Dumas*		
55·43	7·21	14·90	15·43	0·800	0·755
55·03	7·26	14·62	15·31		
55·46	7·49	14·87	15·73		
		14·97			

Mean composition :

	Methylo-caseinogen	Caseinogen [Geake, 1914]
	%	%
C	55·31	53·20
H	7·32	7·09
N (Kjeldahl) ..	14·84	15·61
(Dumas) ..	15·49	15·63
S	0·800	1·015
P	0·755	0·731

Estimations of methyl were carried out by Herzig and Meyer's method. For convenience all results have been calculated for " CH_3," not for " OCH_3 " or " NCH_3." It was found by Skraup and Krause that caseinogen contains 0·40 % $(O)CH_3$ and 1·13 % $(N)CH_3$. Our results for the latter are considerably lower.

Estimation of methyl in caseinogen :

		AgI			
		HI at 140° C.	HI at 200° C.	$HI + NH_4I$ at 300° C.	
(I)	0·4090 g. (11·99 % H_2O)	0·0134 g.	0·0138 g.	0·0215 g.	
(II)	0·4265 g. (12·00 % H_2O)	0·0166 g.	0·0161 g.	0·0191 g.	
(I)	$(O)CH_3 = 0·24 + 0·25 = 0·49$ %;	$(N)CH_3 = 0·38$ %.	Total $CH_3 = 0·87$ %		0·88 %
(II)	$= 0·28 + 0·27 = 0·55$ %;	$= 0·33$ %	$= 0·88$ %		

Since the completion of this work Burn [1914] has obtained a similar result (0·97 % total methyl).

No great stress can be laid on these values since, as was observed by Skraup and Krause and confirmed by Burn, amino-acids themselves, though free from methyl groups, give a similar precipitation of silver iodide. We have observed that after heating to 140° C. till no further precipitate can be obtained more silver iodide is formed by heating to 200° C. and more again at higher temperatures. The distinction between $(O)CH_3$ and $(N)CH_3$ cannot therefore be taken as exact.

Skraup and Krause's methylo-caseinogen contained 0·90 % $(O)CH_3$ and 3·36 % $(N)CH_3$, making a total of 4·26 % methyl.

Our methylo-caseinogen gave the following results :

	AgI	
	HI at 140° C.	$HI + NH_4I$ at 300° C.
0·3960 g. (9·74 % H_2O)	0·1110 g.	0·1301 g.
0·3953 g. (9·76 % H_2O)	0·1105 g.	0·1286 g.
0·4340 g. (9·08 % H_2O)	0·1381 g.	0·1494 g.
0·4050 g. (9·08 % H_2O)	0·1210 g.	0·1185 g.

$(O)CH_3 = 1·99,\ 1·98,\ 2·24,\ 2·10.$ Mean 2·08 %
$(N)CH_3 = 2·33,\ 2·31,\ 2·42,\ 2·06$ 2·28 %
Total methyl = 4·36 %

Since caseinogen gave 0·88 % methyl, the increase of methyl on methylation is 3·48 %.

Colour Reactions. The following reactions are given equally by caseinogen and by our methylo-caseinogen : Biuret reaction, xanthoproteic reaction, lead sulphide reaction, Molisch reaction, Liebermann's reaction, tryptophane reaction with H_2SO_4, vanillin and KNO_2 or with HCl, vanillin and KNO_2, Reichl's reaction and Mörner's test for tyrosine. The Adamkiewicz-Hopkins

reaction and Denigé's test for tyrosine are given more strongly by methylo-caseinogen. The reddish violet colouration observed by Cooper [1913] when proteins are immersed in aqueous quinone solutions is also obtained much more rapidly and powerfully with methylo-caseinogen than with caseinogen.

Since the product gives Millon's reaction and the other tyrosine reactions it is doubtful whether the hydroxyl group of the tyrosine nucleus has been methylated[1]. As mentioned above, the absence of the reaction in the case of Skraup and Krause's product is sufficiently explained by the presence of iodine. Skraup and Krause also observed the more pronounced Adamkiewicz-Hopkins reaction, but ascribed this to the iodine in their substance.

[Note added July 30th. Since this paper was sent to the press we have noticed that Herzig and Landsteiner [1914] have also made experiments on the methylation of caseinogen by diazomethane. As their paper was only published on 27th April we did not see it until some time after the manuscript had left us on June 3rd.]

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[1] Diazomethane has no action on tyrosine in ethereal suspension but in methyl alcohol methylation takes place. Millon's reaction is not given by veratric acid but is given by trimethylgallic acid; the positive reaction is therefore not an absolute proof of the presence of a free hydroxyl group.

XXXVII. THE MECHANISM OF AGGLUTINATION OF BACTERIA BY SPECIFIC SERA.

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Flocculation of bacteria and other cells by specific antisera, being, if not the simplest, at least the most easily investigated of the "in vitro" phenomena of immunity, offers a suitable field for the study of the mechanism of serum tests in general, for if we knew the factors that control agglutination we might be able to correlate this phenomenon with others—e.g. lysis—which, while apparently very different therefrom, may in reality be closely allied thereto. The immediate purpose of the investigation was, then, to enquire into the factors determining flocculation of sensitised bacteria; while it was hoped that from a study of these an indication for the explanation of biological lysis and other laboratory serum tests on a basis of observed physiological fact might be obtained.

The experiments to be described in the present communication were carried out with a view to the corroboration and amplification of a statement made by Bordet [1899]: "That agglutination depends upon the presence of an electrolyte in the fluid used for suspending the organisms." This author showed that bacteria that have been exposed to the action of specific antiserum, then washed, and suspended in distilled water, form an homogeneous and stable suspension. The stability of this suspension of "sensitised" organisms depends upon the absence of electrolytes from the fluid as is shown by the fact that the addition of a small quantity of NaCl results in complete agglutination of the suspended organisms.

This experiment might be interpreted as indicating that the process of agglutination by antisera is divisible into two phases:

(*a*) A phase in which the antibody of the serum becomes united with its antigen—the organism;

(*b*) A phase in which the united " antibody-antigen couple " is flocculated by the electrolyte of the suspending fluid.

The second phase of the phenomenon is purely physical in character and is comparable with the sedimentation of sand at river estuaries and with the aggregation of particles of " denaturated " protein by the addition of small quantities of electrolytes. It is with the second phase of the reaction that the experiments to be described are mainly concerned.

Before the publication of Bordet's observation [1899] there had been a number of explanations offered of the nature of the reaction of specific agglutination. These were all founded upon chemical bases and failed to explain satisfactorily the clumping of organisms by specific antisera. Thus Gruber and Durham [1896] considered that the serum produced a chemical (?) change in the outer layers of the organismal protoplasm causing them to become sticky. This view of the phenomenon while offering adequate explanation of the mutual adhesion of sensitised bacteria or cells, when they had come into contact with one another, failed altogether to explain why they *should come together to form clumps.*

The next advance made in the investigation and explanation of specific agglutination is due to Krause [1897], who showed that the clear fluid obtained by filtration of an old broth culture of an organism produced a precipitate when mixed with an homologous antiserum. Basing their observations on this fact, Nicolle [1898] and Paltauf [1897] advanced the following hypotheses. The former considered that the phenomenon was really explicable by the theory advanced by Gruber and Durham, but he claimed that that moiety of the organismal protoplasm which was susceptible to the action of the agglutinating serum—" agglutinable substance "—was resident principally in the external layers of the organism and might in old cultures diffuse into the medium. Its precipitation in the organisms themselves led to agglutination while its flocculation from the culture filtrate led to the precipitum observed by Krause. It will be seen that Nicolle's theory involves the acceptance of Gruber's hypothesis and only differs therefrom in correlating the Krause phenomenon with agglutination. Paltauf considers that in all cultures a certain amount of agglutinable substance diffuses into the medium and is precipitated by the addition of antiserum, so forming a coagulum which entangles the suspended bacteria and subsequently contracting leads to the formation of agglutinated masses.

This view of Paltauf's is ingenious, but it presupposes that a coagulum is always formed in the suspending fluid and that this coagulum *is not*

discontinuous. As there is no evidence that these suppositions are valid, the explanation offered must be considered as inadequate. His hypothesis, however, seems to me to be of considerable import in that it draws attention, indirectly at least, to the physical state of the interacting bodies as an important factor in the phenomenon of agglutination.

From the experiments to be described it will be noted that a striking analogy may be drawn between the behaviour of sensitised organisms and that of suspensions of denaturated protein in presence of electrolytes. Furthermore, unsensitised bacteria behave in respect of electrolytes as does fresh protein, and Liefman [1913] has shown that in order to produce flocculation of unsensitised organisms a concentration of 70–80 % $(NH_4)_2SO_4$ is required. With different organisms different concentrations of the salt appear to give optimum results, therein exhibiting a striking resemblance to the fractional salting out of proteins from solution by similar reagents. Agglutination of sensitised bacteria on the contrary occurs in presence of, and is conditioned by low concentrations of, salts, e.g. physiological saline, and has, I think, its analogue in the aggregation of denaturated protein particles by low concentrations of salts. This subject of the aggregation of denaturated protein by the addition of salts has been fully investigated by Chick and Martin [1912], who show that flocculation under these circumstances depends upon the neutralisation of the surface electric charge carried, according to the Lippman-Helmholtz hypothesis, by particles or molecules in suspension.

If the hypothesis, then, " that aggregation of denaturated protein particles and agglutination of sensitised bacteria are similar phenomena " be correct, we should expect sensitised bacteria to exhibit certain of the features characteristic of denaturated protein. In respect of electrolytes, particles of denaturated protein recall, in certain respects, in their reactions those of rigid colloids ; thus the charge carried by such colloids is largely determined by the reaction of the suspending fluid, and the valency of the ions of the electrolyte in which the colloid is suspended is an important factor in its precipitation. We should expect then, if our hypothesis be correct, that the presence of hydrion and hydroxidion would markedly affect the process of agglutination and that in certain experimental conditions the valency of the kation of the electrolyte would determine the aggregation of the organisms while in other circumstances the clumping would be conditioned by the valency of the anion.

EXPERIMENTS TO SHOW THAT VARIATION OF THE INTRINSIC PRESSURE AND VISCOSITY OF THE SUSPENDING FLUID HAS NO MARKED INFLUENCE ON THE REACTION.

As the disposition of particles suspended in a fluid is largely determined by the electric charge carried by the particles and by the intrinsic pressure of the suspending fluid, one can alter the disposition of the particles by interfering with either of these factors. These are not infrequently interdependent, but without altering the intrinsic pressure of a fluid we may bring about flocculation of particles suspended in it by neutralisation of the surface charge carried by the particles, and I conceive of agglutination as being dependent on this mechanism. It seemed possible, however, that flocculation of organisms by antisera might depend upon alteration of the intrinsic pressure of the suspending fluid, and with a view to elucidating this point, agglutination reactions were carried out in presence of 0·9 % NaCl, 0·9 % NaCl dissolved in 10 % alcohol, and 0·9 % NaCl dissolved in 1 % gelatin.

Examination of these fluids by means of Traube's stalagmometer showed that for a given volume of the fluids at 37°—the temperature at which the reactions were carried out—

0·9 % NaCl equalled 58·5 " standard " drops,
0·9 % NaCl in 10 % alcohol = 89·5 drops,
0·9 % NaCl in 1 % gelatin = 65·2 drops,

thus indicating a very considerable difference in the intrinsic pressure of the solution in alcohol as compared with the others.

Employing the viscosimeter and working at the same temperature, it was found that for a given volume to pass through the tube

0·9 % NaCl required 2 min. 15 secs.,
0·9 % NaCl in 10 % alcohol required 3 min. 20 secs.,
0·9 % NaCl in 1 % gelatin required 3 min. 15 secs.,

thus showing a marked increase in the internal friction in both the alcoholic solution and the gelatin solution.

The following agglutination reaction was carried out, using the above fluids for diluting the serum and for suspending the organisms. Throughout the experiments the organism used was *B. paratyphosus* " *b.*"

TABLE I. 2 *hours at* 37°.

Fluid employed	Dilutions of agglutinating serum						
	1/100	1/500	1/1000	1/2000	1/4000	1/8000	1/16000
0·9 % NaCl	+	+	+	+	+	+	−
„ in 10 % alcohol..	+	+	+	+	+	−	−
„ in 1 % gelatin ..	+	+	+	+	+	+	−

Note. In this and the following experiments + indicates a positive result.

From this it is seen that notwithstanding considerable variation in the intrinsic pressure and internal friction of the fluids in which the reaction was carried out, the result is not markedly different in the case of any of the fluids.

Flocculation would appear then to be due to a direct interaction of "antibody-antigen" complex with electrolyte, and, within the limits of the experiment at least, to be independent of the intrinsic pressure and viscosity of the suspending fluid. This agrees with the findings of Gengou [1908], who states that the flocking out of red corpuscles from suspension by the addition of colloids is due "fundamentally to a direct interaction of the two bodies," *i.e.* colloid and cell.

It was shown by the same author that alteration of the physical state of the interacting bodies leads on the contrary to very different results. Thus if a suspension of $BaSO_4$ be added to fresh serum, the $BaSO_4$ is held in suspension, but its addition to the same serum previously heated to 65° results in sedimentation.

According to Gengou's views, when the $BaSO_4$ is added to fresh serum it remains dispersed because, adhering to the colloidal particles of the serum which show no tendency to spontaneous flocculation, the particles of $BaSO_4$ are kept apart from one another. Added to heated serum or to red blood cells the $BaSO_4$ does rapidly sediment, for both these colloids tend to flocculate of their own accord and naturally do so more rapidly when they have adsorbed the heavy $BaSO_4$.

I quote these experiments in order to emphasise the important influence that the physical state of the reacting bodies has upon the phenomena of immunity reactions and to call attention to the points that require special consideration in the investigation of these, particularly in respect of agglutination.

Influence of the Electrolyte upon the Titre of the Serum.

In view of the findings of the above experiments, I decided to proceed with the investigation of the reaction from the standpoint of its probable analogy with the aggregation of denaturated protein particles.

In the first series of experiments, agglutination tests were performed in presence of a variety of electrolytes in order to determine whether any of the electrolytes employed affected the titre of the agglutinating serum.

Technique. A 24 hours agar culture of the organism (*B. paratyphosus* "*b*") was washed off in distilled water and centrifuged, the supernatant fluid was pipetted off and to the deposit was added distilled water, the mixture well shaken and again centrifuged. This process was repeated twice and the deposit obtained after the last centrifuging was triturated and suspended in distilled water. To ensure the homogeneity of this suspension it was filtered through coarse filter paper.

This distilled water suspension was exposed to the action of varying dilutions of serum in presence of different electrolytes, the concentration of which was so arranged that with the addition of the distilled water suspension to each tube the electrolytes were equimolecular with 0·9 % NaCl. Employing this technique the following result was obtained:

TABLE II. 2 *hours at* 37°.

	Dilutions of Serum						
	1/100	1/500	1/1000	1/2000	1/4000	1/8000	1/16000
NaCl ..	+	+	+	+	+	+	−
NaF ..	+	+	+	+	+	+	−
Na_2SO_4 ..	+	+	+	+	+	+	−
K_2SO_4 ..	+	+	+	+	+	+	−
Na_2CO_3 ..	−	−	−	−	−	−	−
Na_2HPO_4	+	+	+	+	+	+	−
$BaCl_2$ ..	+	+	+	+	+	+	−
$Na_3Cit.$ ..	+	+	+	+	+	+	−
Aq. Dest.	+	−	−	−	−	−	−

Note. The positive result obtained with 1/100 dilution of the serum in water is probably due to the salts that the serum contains.

In the above reaction the concentrations of serum used were chosen with a view to showing whether any of the salts employed exercised a *marked* influence upon the titre of the serum.

From the above table the following points are made clear:

1. That for all the salts employed, in the above experimental conditions, the titre of the serum remains the same, provided that the solutions of the salts give an approximately neutral reaction.

2. That the clumping of sensitised organisms is independent of the presence of the Ca ion. If it were dependent on the presence of the Ca ion, agglutination would probably not occur in presence of fluoride.

I draw attention to this on account of the important function that Ca subserves in many vital phenomena.

3. That salts which hydrolyse to produce OH ions have not the property of agglutinating bacteria in presence of specific agglutinating serum.

This point is shown more strikingly in the following table in which are compared the actions of " acid " and " basic " carbonates and phosphates of sodium.

TABLE III. 2 *hours at* 37°.

	Dilutions of Serum						
	1/100	1/500	1/1000	1/2000	1/4000	1/8000	1/16000
NaCl ..	+	+	+	+	+	+	−
Na_2CO_3 ..	−	−	−	−	−	−	−
$NaHCO_3$ ..	+	+	+	+	+	−	−
Na_3PO_4 ..	−	−	−	−	−	−	−
Na_2HPO_4	+	+	+	+	+	+	−

Experiments on the Inhibition of Agglutination by Alkalis.

This inhibitory action of alkalis upon agglutination is of considerable interest in view of the influence that the reaction of the suspending fluid has upon the aggregation of particles of " denaturated " protein [Chick and Martin 1912; Hardy 1899], and the following series of tests was set up with a view further to elucidate the conditions that determined the inhibitory action of alkalis upon the flocculation of sensitised bacteria.

It might well be that the inhibition is essentially due to the same cause in each case and that this is probable is shown from the following results.

Technique. A 24 hours agar culture was washed off in saline and agglutinated at 22° in presence of 1/1000 agglutinating serum (titre—1/8000). The supernatant fluid was pipetted off and the material was washed twice with distilled water as in the previous series, then triturated, filtered and exposed to the action of varying dilutions of NaOH in presence of the following electrolytes each of which was, when all the reagents were added, equimolecular with 0·9 % NaCl.

TABLE IV. 2 *hours at* 37°.

	No NaOH Control	NaOH N/100. 1 cc.	0·9 cc.	0·8 cc.	0·7 cc.	0·6 cc.	0·5 cc.	0·4 cc.	0·3 cc.	0·2 cc.	0·1 cc.
NaCl ..	+	−	−	−	−	−	−	−	−	−	+
K_2SO_4 ..	+	−	−	−	−	−	−	−	−	−	+
KI ..	+	−	−	−	−	−	−	−	−	−	−
$NaHCO_3$ ..	+	+	+	+	+	+	+	+	+	+	+
Na_2HPO_4	+	+	+	+	+	+	+	+	+	+	+

This shows that until the OH ion is present in the suspending fluid in a concentration equal to that in N/500 NaOH it fails to inhibit agglutination. This concentration is presumably not reached in the case of $NaHCO_3$ and Na_2HPO_4 in the above experimental conditions, for the addition of the NaOH to the solutions leads probably to an increase in the quantity of ionised Na_2CO_3 and Na_3PO_4 in the solutions of these "acid" salts, this being necessarily accompanied by a removal from the fluid of OH ions available for the inhibitory action.

As this experiment showed that salts having a "replaceable" H group in the anion behaved in a manner different from other salts in respect of this alkali inhibition, it appeared of interest to show that salts containing an "irreplaceable" H group in the anion did not exhibit the same peculiarity.

Technique. A 24 hours agar culture was washed off with distilled water, then washed and centrifuged twice. The deposit obtained was suspended in distilled water and added to tubes containing serum and electrolyte of such concentration that with the addition of the emulsion the following dilutions of serum were obtained, the electrolytes being equal in concentration to 0·9 % NaCl.

TABLE V. 2 *hours at* 37°.

	Dilutions of Serum 1/100	1/500	1/1000	1/2000	1/4000	1/8000	1/16000
NaCl	+	+	+	+	+	+	−
NaCl in N/100 NaOH	−	−	−	−	−	−	−
CH_3COONa	+	+	+	+	+	+	−
CH_3COONa „ „	−	−	−	−	−	−	−
CCl_3COONa	+	+	+	+	+	+	−
CCl_3COONa „ „	−	−	−	−	−	−	−
$NaHCO_3$	+	+	+	+	+	+	−
$NaHCO_3$ „ „	+	+	+	+	+	−	−

The inhibition of agglutination by the hydroxidion is probably akin to the prevention of aggregation of protein particles in presence of an alkali first noted by Hardy and interpreted by him as a phenomenon depending

upon disturbance of the electrostatic condition of the particles in suspension. For optimum aggregation the reaction must occur in a solution that is approximately neutral, for protein particles acquire a positive charge when suspended in acid fluids and a negative charge when suspended in alkaline fluids.

To obtain the best conditions for precipitation, this charge must be neutralised, *i.e.* the solution must be so adjusted that it is " isoelectric " with the particles.

It appears probable then that the agglutination of sensitised bacteria also depends on their being suspended in an " isoelectric " fluid.

In what way the alkali produces the inhibition of clumping is by no means clear, for it might obviously act upon the serum, the organism, the union of the serum and organism, or upon the actual flocculation.

From what has been said above, the last of these is seemingly the most rational explanation of the dispersion.

Paal and Voss [1904] suggest that the inhibition of coagulation (aggregation) of protein by hydroxidion depends upon the alkali acting upon the protein and producing a degree of hydrolysis that results in the production of a layer of protective colloid around each particle in suspension. If such a colloid is produced by the action of the OH ion on bacterial protoplasm, its properties disappear when the organisms exposed to the alkali are well washed in distilled water as is shown by the following experiment.

Technique. A 24 hours agar culture of organisms was washed off with N/100 NaOH and exposed to this reagent for 2 hours at 37°. The suspension was then centrifuged, the supernatant fluid pipetted off and replaced by distilled water. The mixture was well shaken and again centrifuged. This washing process was repeated twice and the organisms were then exposed to the following dilutions of an agglutinating serum.

TABLE VI. 2 *hours at* 37°. *Electrolyte used,* 0·9 % NaCl.

	Dilutions of Serum						
	1/100	1/500	1/1000	1/2000	1/4000	1/8000	1/16000
Untreated organisms	+	+	+	+	+	?	–
Treated as above	+	+	+	+	+	?	–

From this it is seen that organisms that have been exposed to the action of alkali and then washed react in the same way as do organisms that have not been so treated. This shows that the OH ion has no permanent effect upon the protoplasm of the bacteria.

Further, that the alkali has no permanent effect on the serum is shown in the following experiment.

Technique. Serum was diluted 1/50 in N/100 NaOH in 0·9 % NaCl. This mixture was placed in the incubator at 37° for 2 hours and was then diluted with 0·9 % NaCl to obtain the following dilutions of serum when the suspension of organisms—in saline—was added to the tubes. In this way both serum and alkali were being diluted in the series.

TABLE VII. 2 *hours at* 37°.

	Dilutions of Serum						
	1/100	1/500	1/1000	1/2000	1/4000	1/8000	16000
Serum alone ..	+	+	+	+	+	?	–
Serum plus alkali	–	–	+	+	+	–	–

As is seen, the agglutination is negative in the tubes containing the higher concentrations of serum plus the higher concentration of alkali, the results become positive with dilution of the reagents. It is apparent then that the alkali has no permanent effect on the serum.

The inhibition of clumping in alkaline suspension is due then to the presence of the OH ion and does not depend upon any permanent effect that the alkali has upon either the organisms or the serum.

The probability is that the inhibition is dependent upon alteration of the electrostatic condition of the suspended organisms, and is therefore the exact counterpart of the dispersion of denaturated protein particles exposed to similar reagents. It is of interest to note in this connection that Chick and Martin [1912] failed to show evidence of hydrolysis of pure protein heated to 100° for 2 hours in presence of sufficient alkali to prevent coagulation[1].

[1] This influence of the OH ion may possibly account for certain cases of "negative phase" that occur in high concentrations of an agglutinating serum. Thus, one not infrequently finds (percentage = 6) that a typhoid serum that fails to give a positive Grünbaum-Widal reaction in a dilution of 1/30 may bring about agglutination in a dilution of 1/500 of the serum.

I merely offer this as a remotely possible explanation of certain of these negative phases, for phosphates with replaceable H groups are found in serum and as in presence of these a fair quantity of alkali is required to give the OH ion concentration necessary to produce inhibition, such an explanation is open to severe criticism, but the phosphates are present in small quantity only and constitute about 0·1 % of plasma by weight and as the alkalinity of the sera might be increased by contact with the soft glass of the capillary tubes in which the blood is collected, this explanation of the negative phase is quite possibly correct in a certain percentage of cases.

EXPERIMENTS ON THE INFLUENCE OF THE HYDROGEN ION ON AGGLUTINATION.

As the presence of hydroxidion has so marked an effect upon the phenomenon of clumping, we should expect, if the hypothesis that the inhibition is dependent upon alteration of the electrical state was correct, that hydrion would also induce an inhibition of agglutination.

That deviation from the isoelectric point leads also to inhibition if such deviation be on the acid side as well as if it be on the alkaline side is shown by the following experiments.

Technique. The procedure was much the same as that used in the previous series (Table IV), except that the concentrations of reagents employed were slightly modified. The organisms were sensitised with 1/100 serum, then washed twice, triturated, and agglutinated in presence of N/20 of the following electrolytes, varying proportions of N/50 HCl being added to each tube as shown in the following table.

TABLE VIII. 2 *hours at* 37°.

Electrolyte N/20	1 cc.	0·9 cc.	0·8 cc.	0·7 cc.	0·6 cc.	0·5 cc.	0·4 cc.	0·3 cc.	0·2 cc.	0·1 cc. N/50 HCl
NaCl	–	–	–	–	–	–	–	–	–	–
Na_2SO_4.. ..	–	–	–	–	–	–	–	–	+	+
Na_3Cit. ..	+	+	+	+	+	+	+	+	+	+
$BaCl_2$	–	–	–	–	–	–	–	–	–	–
Ce_2Cl_5 N/2000	–	–	–	–	–	–	–	–	–	–

These results, which are the converse of those obtained in the corresponding experiment in which alkali was used to inhibit agglutination (Table IV), show the following striking phenomenon. The result obtained with Na_2SO_4 differs from that obtained with NaCl in that a greater concentration of the H ion is required to inhibit agglutination when the former salt is used as the precipitating electrolyte than when the latter is so employed. As both these salts are neutral, there is obviously some other factor than mere concentration of hydrion that must be taken into consideration. This point will be considered in dealing with the relation of the valency of the anion to the flocculation of sensitised bacteria suspended in acid fluids. Attention, however, may be drawn here to the absence of inhibition when citrate is employed which is probably due to two factors: (1) neutralisation of the acid, and (2) the valency of the anion.

That the effect of the acid is not dependent upon any permanent action upon the protoplasm of the organism was demonstrated as follows:

Technique. A saline suspension of the organism was exposed to the action of N/50 HCl for 2 hours at 37°, then centrifuged and washed twice and exposed to the action of serum in saline in the following dilutions.

TABLE IX. 2 *hours at* 37°.

	Dilutions of Serum						
	1/100	1/500	1/1000	1/2000	1/4000	1/8000	1/16000
Untreated organisms	+	+	+	+	+	−	−
Treated as described	+	+	+	+	+	−	−

That the acid has no permanent effect on the serum is apparent from the following experiment.

Technique. Serum in a dilution of 1/25 was exposed to the action of N/25 HCl in saline. This was then diluted (with saline) to produce the following concentrations of serum and acid with the addition of the bacillary emulsion to each tube.

TABLE X. 2 *hours at* 37°.

	Dilutions of Reagents						
	1/100	1/200	1/400	1/800	1/1600	1/3200	1/6400
Serum alone	+	+	+	+	+	+	−
Serum plus acid	−	−	−	+	+	+	−

Note. If the above mixtures are left in the incubator longer than 2 hours there is decided sedimentation in all the tubes, the precipitum however differs from true agglutination in that the flocculi are very small while the supernatant fluid remains markedly turbid.

From the experiments in the two preceding sections, the following conclusions may be drawn:

1. Agglutination is markedly affected by the reaction of the suspending fluid, and deviation—but with certain limits—from the "isoelectric point" results in inhibition of agglutination.

2. The inhibition is conditioned by the presence of hydrion and hydroxid-ion and does not result from permanent change produced in the protoplasm of the organism or the character of the serum by the action of such ions.

3. Agglutination probably depends upon the neutralisation of charge carried by particles in suspension.

Experiments on the Influence of the Valency of Kation and Anion.

As previous experiments failed to show any variation in the activity—*i.e.* "titre"—of serum in presence of a variety of electrolytes whose solutions were approximately neutral and equimolecular with 0·9 % NaCl, the following experiment was carried out with a view to investigating the relative activity of a number of electrolytes acting upon sensitised organisms.

Technique. A 24 hours agar culture was washed off by saline and agglutinated at 22° for 18 hours in presence of 1/1000 agglutinating serum

(titre—1/8000). The supernatant fluid was pipetted off and the material was centrifuged and washed twice, then triturated and exposed to the action of the following salts.

TABLE XI. 2 *hours at* 37°.

	Dilution of Electrolytes					
	0·5 N	0·1 N	0·01 N	0·001 N	0·0001 N	0·00001 N
NaCl ..	+	+	+	−	−	−
Na_2SO_4 ..	+	+	+	−	−	−
$Na_3Cit.$ ..	+	+	+	−	−	−
$BaCl_2$ ..	+	+	+	+	−	−
$LaCl_3$ ..	0	0	0	+	+	−

0=not done.

From these results it would seem that the valency of the kation of the electrolyte is the most important factor in conditioning the agglutination of sensitised organisms in the above experimental conditions.

Lest however the result obtained might have been due to a specific action of the salts employed, the following experiment with the salts of the alkaline earths was carried out.

TABLE XII. 2 *hours at* 37°.

	Dilution of Electrolytes														
	0·005	0·004	0·003	0·002	0·001	0·0009	0·0008	0·0007	0·0006	0·0005	0·0004	0·0003	0·0002	0·0001	Normal
NaCl ..	+	+	+	−	−	−	−	−	−	−	−	−	−	−	
KCl ..	+	+	+	−	−	−	−	−	−	−	−	−	−	−	
$CaCl_2$ ..	+	+	+	+	+	+	+	+	+	+	+	−	−	−	
$SrCl_2$ ..	+	+	+	+	+	+	+	+	+	+	+	−	−	−	
$BaCl_2$ ..	+	+	+	+	+	+	+	+	+	+	+	−	−	−	
$MgCl_2$ ..	+	+	+	+	+	+	+	+	+	+	+	−	−	−	

This experiment shows that the result obtained cannot be explained on the hypothesis that it was due to any specificity of action of the salts employed.

These experiments agree with the findings of Coplans [1913] and of Beckhold [1904] that the agglutinating activity of an electrolyte depends upon the valency of its kation. The phenomenon is comparable too with the observations of Chick and Martin [1912] on the aggregation of particles of denaturated egg albumin. These authors show that egg protein dispersed by the addition of alkali to the suspending fluid is more susceptible to the action of salts with divalent kations than to those with monovalent kations while trivalent kations are more active than are divalent.

This, however, is not true of organisms that are suspended in acid fluids

and if agglutination be really comparable to the aggregation of denaturated protein one would not expect it to be so.

Moreover, if the process of sensitisation be akin to denaturation the anion should, in the case of acid suspensions, be the precipitating ion. To determine the validity of this hypothesis, the following experiment was carried out.

Technique. Organisms were agglutinated in saline for 2 hours at 37° in presence of 1/100 serum. These were then centrifuged and washed once in distilled water and then suspended in N/500 HCl. This suspension was added to equal volumes of the electrolytes so that the concentration of HCl in each tube was equal to N/1000, and the concentrations of the salts were such that with the addition of the suspension the following concentrations were obtained.

TABLE XIII. 2 *hours at* 37°.

	Dilutions					
	N/20	N/100	N/200	N/400	N/800	N/1600
NaCl	–	–	–	–	–	–
Na_2SO_4 ..	+	+	+	+	+	+
$Na_3Cit.$ ..	–	–	–	+	+	+
$BaCl_2$ ..	–	–	–	–	–	–
Ce_2Cl_5 ..	–	?	+	+	+	+

It will be seen that the result obtained is only in partial agreement with what one would expect, and the action of two of the salts requires special note.

(*a*) In the case of the citrate the absence of agglutination in presence of the higher concentration of the salt is probably due to the trivalent citrate ion conferring a strong negative charge upon the bacteria and so causing their dispersion.

That polyvalent ions may have such an effect is clear from the work of Burton [1906] on the influence of electrolytes upon the direction of movement of colloid particles exposed to the action of salts in an electric field. He found that the addition of aluminium ion to hydrosols of gold and silver in certain concentrations leads to the precipitation of the metal from its sol ; lower concentration of the salts of aluminium did not have this effect and the particles, which in the sols of these metals bear a negative charge, continued to move in the same direction as before but at a diminished rate. If a concentration of aluminium ion greater than that required to bring about precipitation were employed, the particles remained dispersed, but

now their direction of movement in the field was reversed, indicating a reversal of charge carried by the particles of the sol.

In the case of copper sols, which carry a positive charge, exactly the opposite was noted—polyvalent anions in this case leading to the reversal of direction.

In the experiment described the effect of the citrate is apparently that of a polyvalent anion on a suspension bearing a negative charge. A positively charged and therefore dispersed protein (protein in acid suspension carries a positive charge) is precipitated in presence of certain concentrations of a salt with a trivalent anion, but with greater concentration of the same salt, owing to an overplus of negative charge being conferred upon the particles, they again become dispersed.

(*b*) The other point of special interest is the occurrence of a positive result with the cerium salt in acid suspension. I cannot suggest an adequate explanation of this peculiar finding but it will be seen from further experiments that salts with polyvalent kations behave in a manner very different from salts with mono- and di-valent kations in respect of their precipitation of bacterial suspensions.

In acid suspension as in alkaline suspension, then, sensitised bacteria behave as do denaturated protein particles and the results obtained agree in principle at least with those obtained by the authors above quoted [Hardy 1899; Chick and Martin 1912].

This finding further correlates the two phenomena, viz. aggregation of denaturated protein and agglutination of sensitised bacteria, so adding weight to my thesis that the process of sensitisation is similar to that of "denaturation."

Further Investigation of the Influence of the Valency of the Kation.

The comparative action of Na, Ba, and La ions was next examined in further detail to determine if possible the quantitative relation existing between these ions in respect of their power of producing agglutination.

Technique. The organisms were sensitised as in the previous experiments and the concentrations of the salts were such that with the addition of the suspension the following concentrations were obtained.

TABLE XIV. 2 *hours at* 37°.

Sensitised Organisms

	·1	·01	·005	·004	·003	·002	·001	·0009	·0008	·0007	·0006	·0005	·0004
NaCl	+	+	+	+	?	−	−	−	−	−	−	−	−
$BaCl_2$	+	+	+	+	+	+	+	+	+	+	+	+	+
$LaCl_3$	0	0	+	+	+	+	+	+	+	+	+	+	+

Unsensitised Organisms

NaCl	−	−	−	−	−	−	−	−	−	−	−	−	−
$BaCl_2$	−	−	−	−	−	−	−	−	−	−	−	−	−
$LaCl_3$	0	0	?	+	+	+	+	+	+	+	+	+	+

Sensitised Organisms

	·0003	·0002	·0001	·00009	·00008	·00007	·00006	·00005	·00004	·00003	·00002	·00001	Normal
NaCl	−	−	−	−	−	−	−	−	−	−	−	−	
$BaCl_2$	?	−	−	−	−	−	−	−	−	−	−	−	
$LaCl_3$	+	+	+	+	+	+	+	+	+	+	−	−	

Unsensitised Organisms

NaCl	−	−	−	−	−	−	−	−	−	−	−	−	
$BaCl_2$	−	−	−	−	−	−	−	−	−	−	−	−	
$LaCl_3$	+	+	+	+	+	+	+	+	+	+	−	−	

0 = not done.

This result is interesting for it bears some resemblance to the formula

$$1 : X : X^2$$

suggested by Linder and Picton [1895] for expressing the relative precipitating activity of mono-, di- and tri-valent kations acting upon colloidal As_2S_3. The particles of this colloid, like organisms suspended in saline without previous treatment, carry a negative charge. The relative values of the kations as precipitating ions in the experiments of these investigators using the above colloid were

$$1 : 35 : 1023,$$

while in the above experiment taking the value for NaCl (0·003) as unity, the values of barium and lanthanum being 0·0003 and 0·00002 respectively, we obtain approximately

$$1 : 10 : 100.$$

Although this result is of considerable interest, I hesitate to lay any stress upon it, for the action of the salts with polyvalent kations is so different from that of other salts that they are not really comparable.

Investigation of the Action of Polyvalent Kations.

Gengou in an article on molecular adhesion [1908] points out that bacteria which have been exposed to the action of aluminium salts and washed in distilled water are agglutinated on the addition of NaCl. He says, " We

have also found that bacteria that have been agglutinated by moderate doses of alum and then washed in distilled water are well agglutinated on the addition of NaCl (this fact may be compared with the agglutination of bacteria by specific sera and with the observations of Beckhold [1904] on the flocculation of bacteria by the salts of heavy metals). If we treat bacteria that have been mixed with alum in a citrated medium in the same way, the subsequent agglutination by NaCl fails to occur. It is evident then that the mode of action of the citrate in this instance seems comparable to its action on substances in aqueous suspensions (barium sulphate, calcium fluoride, or mastic); but, owing to the fact that chemical reactions may take place between alum and citrate, and thus prevent the manifestations produced by the alum, we think it better to reserve any interpretation of this fact."

As Beckhold [1904] and other observers also call attention to similar phenomena, it seemed of interest to examine the action of salts with polyvalent kations on sensitised and unsensitised organisms.

Technique. (*a*) For the preparation of sensitised organisms, the growth was washed off into saline and exposed to the action of 1/500 serum (titre—1/8000) at 22° for 18 hours. The suspension was then centrifuged and washed with distilled water twice, then suspended in water and exposed to the action of the following electrolytes.

(*b*) For preparation of unsensitised organisms, the 24 hours growth was washed off with distilled water, then allowed to stand and centrifuged to obtain a deposit of organisms. The deposit was washed twice and a suspension in distilled water made as before.

Exposing these to the action of the following salts for 2 hours at 37°, agglutination took place thus :

TABLE XV. 2 *hours at* 37°.

	N/20	N/40	N/100	N/200	N/500	N/1000	N/2000	N/5000	N/10000	N/20000	N/40000
Sensitised Bacilli											
NaCl ..	+	+	+	+	−	−	−	−	−	−	0
$BaCl_2$ ..	+	+	+	+	+	+	+	−	−	−	0
$LaCl_3$ ..	0	0	0	+	+	+	+	+	?	−	0
Ce_2Cl_5 ..	0	0	0	+	+	+	+	+	+	−	0
$AlCl_3$ ..	−	−	−	−	−	+	+	+	+	−	−
Unsensitised Bacilli											
NaCl ..	−	−	−	−	−	−	−	−	−	−	−
$BaCl_2$ ..	−	−	−	−	−	−	−	−	−	−	−
$LaCl_3$ ..	0	0	0	p	+	+	+	+	−	−	−
Ce_2Cl_5 ..	0	0	0	sl	sl	p	+	+	+	sl	0
$AlCl_3$ ..	−	−	−	−	−	−	+	+	+	+	−

0 = not done . p = partial sl = very slight

The salts with polyvalent kations, then, behave in a manner very different from the salts of mono- and di-valent kations—for they are apparently as active in agglutinating unsensitised organisms as in agglutinating sensitised organisms.

I hesitate to offer an explanation of this phenomenon, but it probably depends partly at least on the alteration of the sol form into the gel form of the hydroxides, as pointed out by Coplans [1913], for it is possible that the suspension of bacteria when prepared in the above manner has a slightly alkaline reaction, and this causing the formation of a gel form of the hydroxide gives rise to clumping by entanglement of the organisms in the contracting gel.

This would explain, in part at least, the negative phase obtained with the higher concentrations of $AlCl_3$. The solution of this salt is acid owing to its hydrolytic decomposition, and the physical state of the hydroxide formed by adding the suspension will vary with the dilution of the salt, and as the agglutination by aluminium salts in all concentrations is inhibited by the presence of acid in the suspending fluid, this factor must be considered as a possible cause of the negative phase in the above experiment.

But the negative phase occurring with the higher concentrations of a polyvalent kation may be explained otherwise. It is possible that the polyvalent kations may produce a reversal of charge carried by organisms in the same way as it caused a reversal of direction of movement of the particles of metal sols of gold and silver in the experiments of Burton.

In order further to investigate this point $TaCl_5$ was made use of. This salt is very sparingly soluble in water and does not undergo hydrolysis with any concentration of the salt, therefore the addition of organism suspension should lead to the production of a hydroxide in the same physical state in each case, unless the concentration of the salt itself has a marked influence on the physical state of the hydroxide formed.

Employing the saturated solution of the salt, the following result was obtained.

TABLE XVI. 2 *hours at* 37°.

	1 cc	0·5 cc.	0·2 cc.	0·1 cc.	0·05 cc.	0·02 cc.	0·01 cc.
	Sensitised Organisms						
$TaCl_5$ Sat. ..	−	−	+	+	−	−	−
	Unsensitised Organisms						
$TaCl_5$ Sat. ..	−	p	+	+	−	−	−

This result shows that the negative phase in the case of polyvalent kations is not *wholly* explicable on the grounds that it is due to acid inhibition and it seems improbable—though I make the suggestion with reservation—that it is due in the case of tantalum to the formation of a hydroxide in a special state determined by the conditions of the experiment. In this connection the experiments of Gengou are of importance. The results obtained by that author suggest that, notwithstanding the fact that with $TaCl_5$ a negative phase does occur, the precipitating action of the polyvalent kations is partly explicable on the hypothesis that it is due to the formation of a hydroxide in accordance with the views of Gengou [1908].

The dispersion with the higher concentrations of the polyvalent kations may however be due to a reversal of charge produced by these reagents apart from that due to hydrolytic decomposition of the salts used.

In order to obtain further information on this point I decided to carry out the following experiment.

Technique. A large culture of the organism was washed off with saline and the suspension was divided into two parts, "*a*" and "*b*," each of 5 cc. volume. To "*a*" was added agglutinating serum 1/500, while "*b*" was left unsensitised. After 18 hours at 22° the liquids were centrifuged, the deposit washed in distilled water, again centrifuged and the supernatant fluid decanted and replaced by N/250 $AlCl_3$. Both were then allowed to interact for 2 hours at 37°. The liquids were again centrifuged and the deposit washed as before, and suspensions of the organisms were made in distilled water.

These suspensions were mixed (1) with NaCl, and (2) with NaCl in presence of N/200,000 $Na_3Cit.$, so that the concentrations shown in the following table were obtained in the tubes, when all the reagents were mixed.

Agglutination was allowed to proceed for 2 hours at 37°. The following results were obtained.

TABLE XVII. 2 *hours at* 37°.

	N/20	N/100	N/200	N/400	N/800	N/1600	NaCl
Sensitised Organisms							
NaCl	+	+	+	+	+	+	
NaCl plus citrate ..	−	−	−	−	−	−	
Control	−	−	−	−	−	−	
Unsensitised Organisms							
NaCl	+	+	+	+	+	+	
NaCl plus citrate ..	−	−	−	−	−	−	
Control	−	−	−	−	−	−	

Whether the polyvalent kations have a double action on the organisms is not clear. It might be that they act both as denaturating agents and as precipitating agents, and if this were so the agglutination by the polyvalent kations would be comparable to specific agglutination by sera in presence of mono- and di-valent ions.

If they do not act in this dual capacity, it is highly probable that the sedimentation is dependent on the formation of hydroxides.

I had hoped to obtain some light on the matter by performing the above test, but inasmuch as the sensitised and unsensitised organisms react in the same way when they have been exposed to the action of aluminium and subsequently agglutinated in presence of NaCl and of NaCl in citrate, no information can be gained upon the point.

Had the sensitised organisms agglutinated in presence of citrate after they had been exposed to aluminium, one could have stated that the action of the aluminium alone differed greatly from the action of serum in presence of NaCl or other electrolyte with mono- or di-valent kation.

The chief difficulty arises from the fact that the organism suspension adsorbs both serum and aluminium and the dispersion of the latter by the citrate masks or is more potent than the agglutination of the sensitised bacilli by the salt.

Apart then from the meagre indication obtained from the experiments in which $TaCl_5$ was made use of, evidence is lacking that the phenomenon of sedimentation by the polyvalent kations is not due to the formation of hydroxides of the metals in a peculiar physical state, and therefore agglutination of unsensitised organisms with these reagents is probably not comparable to agglutination in presence of specific serum.

Beyond calling attention to this phenomenon and submitting the above suggestions, I wish to advance no further hypothesis concerning the action of these bodies in producing agglutination of sensitised and unsensitised organisms, as it is highly probable that even a slight alteration in the conditions of the experiment would lead to very different results, for we are dealing with a number of reagents whose affinities for one another, whose physical state, and whose " concentration " under any circumstances are all mutually interdependent.

The only conclusion then that can be definitely deduced from this section of the investigation is that the salts with polyvalent kations behave in respect of both sensitised and unsensitised organisms in a manner very different from the salts with mono- and di-valent kations. The mechanism of their

action is obscure and one experiences difficulty in correlating the result obtained with aluminium and other trivalent ions with that obtained with the pentavalent tantalum.

Experiments to investigate the Relation between the Concentrations of Serum and of Electrolyte.

Having determined that: (1) the precipitating value of an electrolyte acting on sensitised organisms is dependent on the valency of the kation of the electrolyte, and

(2) The titre of an agglutinating serum is no higher when a divalent kation is present in the precipitating fluid ($BaCl_2$ equimolecular with 0·9 % NaCl) than when a monovalent kation is employed (0·9 % NaCl) it seemed of interest to examine the relation of serum to electrolyte. It might well be that a low concentration of electrolyte would produce agglutination in presence of a given quantity of serum but that the reaction would not take place with the same concentration of the electrolyte in presence of less than that given quantity of the same serum.

To investigate this point, the following experiment was carried out.

Technique. An emulsion of organisms in distilled water was added to serum that had been diluted in NaCl and $BaCl_2$ to obtain the following concentration of the salts and dilutions of the serum when all the reagents were added to each tube.

TABLE XVIII. 2 *hours at* 37°.

NaCl					
N/20	N/40	N/100	N/200	N/400	Serum
+	+	+	+	+	1/100
+	+	+	+	+	1/500
+	+	+	+	?	1/1000
+	+	+	?	−	1/2000
+	+	?	−	−	1/4000
−	−	−	−	−	1/8000
$BaCl_2$					
N/400	N/500	N/666	N/1000	N/2000	
+	+	+	+	+	1/100
+	+	+	+	?	1/500
+	+	+	−	−	1/1000
+	+	−	−	−	1/2000
+	−	−	−	−	1/4000
−	−	−	−	−	1/8000

This shows that a relation of the nature indicated above does exist within certain limits; but the relation is probably not so simple as would appear from the above results, for $BaCl_2$ N/400 agglutinates in presence

of 1/4000 of the serum while in presence of $BaCl_2$ in concentration equal to N/6·5 (experiments of series 1) it only produces flocculation in 1/8000 of the same serum.

It is probable that as one approaches the dilution of serum which might be termed the " threshold value," the electrolyte has to be added in greatly increasing concentration in order to obtain aggregation of the bacteria.

Suggested Application of the above to the Differentiation of Bacterial Species.

It is possible that use might be made of the relation of serum to electrolyte for the purpose of differentiating closely allied bacterial species. Thus while the agglutinating " titre " of an anti-aertrycke serum might be the same for both *B. aertrycke* and for *B. paratyphosus b*, it seemed possible that if emulsions of these organisms were sensitised each in presence of 1/50 anti-aertrycke serum that the washed deposit in the case of the aertrycke bacilli sensitised with the anti-aertrycke serum might be flocculated by a lower concentration of the electrolyte employed than would the paratyphosus b. bacilli sensitised with similar concentration of the same serum. Or employing the serum as a constant throughout a series one might find that the anti-aertrycke serum in presence of aertrycke bacilli agglutinated with a lower concentration of electrolyte than did anti-aertrycke serum in presence of paratyphosus and *vice versa*.

The latter technique was adopted in the experiments. Employing anti-aertrycke serum in a dilution of 1/500 in presence of varying dilutions of NaCl to which were added emulsions of (1) typical *B. aertrycke*, (2) *Bacillus " D "*—a paratyphosus having peculiar reactions—and (3) typical *B. paratyphosus b*, the following result was obtained.

TABLE XIX. 1 *hour at* 37°.

Serum	NaCl	N/20	N/40	N/80	N/100	N/200	N/400
Anti-aertrycke	*B. aertrycke*	+	+	+	+	−	−
	Bacillus " D " ..	?	−	−	−	−	−
	B. paratyphosus b ..	?	−	−	−	−	−
	(titre of serum = 1/2000)						
Anti-paratyphosus b. ..	*B. aertrycke*	+	+	+	−	−	−
	Bacillus " D " ..	+	+	+	+	+	+
	B. paratyphosus b ..	+	+	+	+	+	+
	(titre of serum = 1/8000)						
Anti-" D "	*B. aertrycke*	+	+	+	−	−	−
	Bacillus " D " ..	+	+	+	+	+	+
	B. paratyphosus b ..	+	+	+	−	−	−
	(titre of serum = 1/4000)						

These results show that the specific antibody of the serum brings about agglutination in presence of a lower concentration of electrolyte than do the non-specific antibodies. Experiments with $BaCl_2$ corroborate the above results and I have applied the same technique in the examination of a number of strains of *B. aertrycke* and *B. paratyphosus b*, obtaining fairly constant results. The technique of the test, however, requires further elaboration before it could be satisfactorily applied to the differentiation of bacterial species.

Influence of Acid and Alkali on the Process of "Sensitisation."

The inhibiting action of alkalis and acids has been shown to be due to an interference with actual flocculation but it is possible that hydrion and hydroxidion also affect the process of sensitisation.

To investigate the action of acid in this connection a mixture of organisms suspended in saline, with HCl present in concentration equal to N/100, was sensitised with specific agglutinating serum 1/100. The mixture was allowed to stand at room temperature for 48 hours, it was then centrifuged, the supernatant fluid drawn off and the deposit washed with distilled water. This procedure was repeated twice and the material obtained was then suspended in distilled water.

This emulsion was added to a series of tubes containing the following reagents of such concentration that with the addition of the bacillary emulsion the dilutions shown in the following table were obtained.

TABLE XX. 2 *hours at* 37°.

	N/20	N/100	N/1000	N/10000	N/100000
NaCl ..	–	–	–	–	–
Na_2SO_4 ..	–	–	–	–	–
$Na_3Cit.$ ..	–	–	–	–	–
$BaCl_2$ ..	–	–	–	–	–

The above result appears to show that the presence of hydrion interferes with the union of antibody with antigen in addition to inhibiting the actual process of clumping.

The converse of the above experiment was carried out thus :

Technique. A saline suspension of organisms was rendered alkaline by the addition of N/10 NaOH till the concentration of NaOH was equal to N/100. To this was added agglutinating serum sufficient to make the serum

equal to 1/50 of the fluid. The mixture was allowed to stand for 48 hours, and was then centrifuged, decanted and distilled water added. It was afterwards shaken and the washing process repeated twice. The suspension was then exposed to the action of NaCl of the following concentrations.

TABLE XXI. 2 *hours at* 37°.

	N/20	N/50	N/100	N/200	N/400
NaCl ..	–	–	–	–	–

From these results it would appear that the reaction of the fluid has a most important influence on the process of sensitisation and that if it be too alkaline or too acid, the process is inhibited just as flocculation is prevented by the presence of hydrion and hydroxidion in certain concentrations.

I do not wish to lay too great stress on this series of experiments since, while the above appears to be true in the experimental conditions of the test, it is quite possible that a slight alteration of the conditions might have a profound effect upon the result obtained, and as I have so far not had the opportunity of repeating the experiments of this series, I hesitate to draw any definite conclusion from them.

Discussion of Results.

Reviewing the experiments that have been described, we see that, in the main, unsensitised organisms exhibit characters that are similar to those of fresh egg-white protein ; sensitised organisms on the other hand recall in their properties certain features characteristic of " denaturated " egg-white. Unsensitised organisms and fresh egg protein behave as do " non-rigid " colloids, while the reactions of sensitised organisms and " denaturated" egg-white in presence of electrolytes recall those of " rigid " colloids under the influence of these reagents.

I advance the hypothesis then that the action of antisera is probably akin to that of denaturation and this raises the question—" Why is it that in the case of certain bacterial species sensitised (by specific serum) the organisms are flocculated by electrolytes, while in the case of other species they are not so flocculated ? "

I cannot completely answer this question but if sensitisation be really akin to denaturation, we should almost expect that certain sensitised organisms would not be flocculated, for while denaturated egg-white is easily

flocculated by the addition of small quantities of electrolytes, serum protein on the contrary is very easily dispersed by the same reagents. The dispersion of egg-white is obtained only with difficulty by the use of salts and that only with polyvalent ions. I propose to investigate further this relation which I suggest exists between denaturation and sensitisation by performing a series of experiments similar to the above but employing anti-protein serum prepared by inoculating an animal with egg-white.

The fact that only certain serum-organism complexes show this peculiarity of flocculation is very strikingly shown in the case of the Bordet-Gengou bacillus of whooping cough [Bordet, 1909], in the case of which Bordet has, I think, shown conclusively that only certain antibody-antigen " complexes " are flocculable by electrolytes.

Looking at the question of bacteriolysis from the same standpoint, a number of important questions arise.

Thus, if the sensitisation of organisms to the agglutinating activity of electrolytes be a " denaturating " process, is the sensitisation of bacteria by specific serum to the dissolving activity of complement also a similar phenomenon ? In fact does a process similar to denaturation precede solution in the process of bacteriolysis or cytolysis ?

Arising out of the previous question a further problem presents itself—Is " complement " not a peculiar electro-physical state of some constituent or constituents of serum rather than a chemical " entity " ?

I am at present engaged on the investigation of this problem, but so far I have not sufficient evidence for or against the hypothesis to permit me to draw any conclusions from the work done, beyond stating that salts with divalent ions inhibit the dissolving action of complement upon red corpuscles while they certainly have no inhibitory effect upon the process of sensitisation of these by haemolytic serum.

In conclusion I should like to call attention to the important question of specificity of action of sera in connection with what one might call the colloidal theory of immunity. Specificity is well explained certainly by the side-chain hypothesis of Ehrlich, but it seems to me that it is capable of explanation otherwise.

I hesitate to advance any theory on this point, but we see from the above experiments that in one respect certainly—that of flocculation—immunity reactions (in vitro) are very delicate indeed and in view of the fact that an electrolyte becomes, in general, a more potent disturbing factor with increase in complexity of the molecule, may it not be that with bodies of very large

molecule, like bacterial protoplasm and serum, the adjustment of the physical conditions of the interacting substances is exceedingly delicate and that upon the delicacy of this adjustment depend the phenomena of immunity ?

Conclusions.

The following conclusions seem permissible from the experiments described.

(1) That in presence of salts with mono- and di-valent kations unsensitised organisms behave in a manner similar to " non-rigid " colloids, but that when sensitised they behave in respect of these reagents in a manner similar to " rigid colloids."

(2) That the factors determining specific agglutination are markedly altered by the presence of hydrion and hydroxidion in the suspending fluid.

(3) That the inhibitory action of these ions may be twofold, for they certainly inhibit flocculation and there is some evidence to show that they also interfere with the union of antigen with antibody.

(4) That—*caeteris paribus*—the character of the electrolyte has but little influence on the " titre " of the serum, provided always that it is sufficiently soluble and does not undergo hydrolysis.

(5) That when emulsions of bacteria that have been washed off directly in saline are used, the precipitating value of the electrolyte varies with the valency of the kation.

(6) That in acid suspensions, on the contrary, it varies with the valency of the anion.

(7) That the action of salts with polyvalent kations is peculiar in that these salts are as active in precipitating unsensitised organisms as they are in flocculating sensitised organisms.

No definite explanation of this is offered but it is suggested that the phenomenon of flocculation in these conditions depends upon a peculiar physical state of the metal hydroxides.

(8) That as unsensitised organisms behave in respect of the above points towards electrolytes as does fresh egg protein, but when sensitised have characters that recall those of denaturated egg-white, the process of sensitisation is akin to that of denaturation.

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XXXVIII. THE MODE OF OXIDATION OF CERTAIN FATTY ACIDS WITH BRANCHED CHAINS.

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(*Received July 6th, 1914.*)

Our knowledge of the mechanism by which the simpler fatty acids with branched chains are oxidised in the body is still very meagre. This is no doubt due to the difficulty in interpreting the experimental results so far obtained, in any way that will lead to a generalisation such as we possess for the normal fatty acids; namely, β-oxidation followed by removal of the two end carbon atoms of the acid. This difficulty may be illustrated by reference to the behaviour of α-methylbutyric acid and α-ethylbutyric acid. In the diabetic organism the former gives rise to increased "acetone body" production [Baer and Blum, 1906] whereas the latter yields, in part at any rate, methyl propyl ketone [Blum and Koppel, 1911]. Similarly α-methylpropionic acid (isobutyric acid), gives rise to extra glucose formation [Ringer, Frankel and Jonas, 1913] and traces of *d*-lactic acid [Baer and Blum, 1906], a result interpreted by Baer and Blum as indicating that lactic acid is a probable intermediate stage in the oxidation of isobutyric acid, the methyl group being replaced by a hydroxyl group, thus:

$$CH_3 \cdot CH(CH_3) \cdot COOH \rightarrow CH_3 \cdot CH(OH) \cdot COOH.$$

This view gains support from the fact that lactic acid gives rise to extra glucose formation in diabetics. Replacement of the methyl group by hydroxyl is not general however, since α-hydroxybutyric acid, which might be expected by the above hypothesis as an intermediate product in the oxidation of α-methylbutyric acid, does not give rise to "acetone body" formation whereas α-methylbutyric acid does.

Another explanation of the behaviour of isobutyric acid has been advanced by Ringer [Ringer, Frankel and Jonas, 1913]. This observer showed that propionic acid, like isobutyric acid, produces increased glucose formation

in phlorizinised dogs. He suggests therefore that demethylation takes place by hydrolysis, methyl alcohol and propionic acid being formed. This is a type of reaction however that is at present unknown in organic chemistry and had therefore better be reserved as an explanation until proof is supplied that it can take place.

A third possibility has been suggested, namely, that the α-methyl group in such acids as isobutyric and α-methylbutyric, is oxidised to a carboxyl group. This view was abandoned by Baer and Blum, who first put it forward, when they discovered that ethylmalonic acid, which under this scheme would be produced from α-methylbutyric acid, does not cause increased "acetone body" formation in diabetics.

The object of the present communication is to suggest a mechanism of oxidation which applies to the α-substituted fatty acids and will explain the facts so far known about the catabolism of these acids. It is merely an application of the rule of β-oxidation advanced by Knoop and postulates that the carbon atom of the methyl group, which in these acids is in the β-position to the carboxyl group, is selectively oxidised, rather than the β-carbon atom of the main chain. Instead of a β-ketonic acid being produced therefore, as with the normal fatty acids, a derivative of the half aldehyde of malonic acid would result:

$$\begin{array}{l} \mathrm{CH_3} \\ | \\ \mathrm{R-CH\cdot COOH} \end{array} \rightarrow \begin{array}{l} \mathrm{CHO} \\ | \\ \mathrm{R-CH-COOH.} \end{array}$$

Malonic semi-aldehyde has been prepared by Wohl [1900] who showed that it rapidly lost carbon dioxide, especially on warming, producing acetaldehyde. In this way it behaves like the β-ketonic acids, which also easily lose carbon dioxide but give a ketone instead. No derivatives of malonic semi-aldehyde have been described but it is very probable by analogy with the derivatives of the β-ketonic acids, that they would be equally unstable. The second reaction therefore in the scheme suggested would be the loss of carbon dioxide with production of the *normal* aldehyde of an acid containing one carbon atom less than the original methylated acid. This aldehyde on further oxidation, or by the Cannizzaro reaction, could then give rise to the corresponding normal fatty acid:

$$\begin{array}{l} \mathrm{CHO} \\ | \\ \mathrm{R-CH-COOH} \end{array} \rightarrow \mathrm{R-CH_2-CHO} \rightarrow \mathrm{R-CH_2-COOH.}$$

In this way, isobutyric acid would give rise to propionic acid, α-methyl butyric to *n*-butyric acid and α-methylvaleric to *n*-valeric acid. In support of this view is the fact that the fate of these methylated acids in the body is exactly that of the corresponding normal acids which would be produced from them by demethylation. In the case of α-ethylbutyric acid there is no methyl group in the β-position for selective oxidation, and this acid appears to undergo β-oxidation in the usual fashion producing α-ethyl-acetoacetic acid and then methyl propyl ketone. The scheme of oxidation suggested thus provides an explanation of the fact that α-methylbutyric acid on oxidation in the body gives rise to acetone formation and not methyl ethyl ketone, which might be expected by analogy with α-ethyl butyric acid [Blum and Koppel, 1911].

The hypothesis put forward above, to explain the mode of oxidation of the α-methylated acids, will also apply to those acids, such as isobutyl-acetic, which contain a methyl group in the γ-position, since by undergoing the usual β-oxidation they yield α-methylated acids. The mode of oxidation of β-methylated acids, such as isopropylacetic acid, cannot yet be explained by a simple scheme such as that suggested for the α-methylated acids. The reactions involved in their catabolism must be left for further experiment.

It has been shown by Dakin [1908] that on warming the ammonium salts of the normal fatty acids with dilute hydrogen peroxide, β-oxidation is regularly observed in all the cases investigated from butyric to stearic acids. A ketone can be isolated from the products of oxidation which is derived from a β-ketonic acid. Since in hydrogen peroxide we have an oxidising agent known to be capable of producing β-oxidation it has been employed in the first instance in this investigation to determine whether the type of oxidation represented above can actually take place with iso-butyric acid and α-methylbutyric acid. If the scheme suggested for the oxidation of these acids be correct, then isobutyric acid should give propion-aldehyde as an oxidation product and similarly α-methylbutyric acid should give butyraldehyde. Evidence has been obtained that the ammonium salts of these acids on oxidation with hydrogen peroxide do yield propionaldehyde and butyraldehyde respectively. Direct identification of the aldehydes was not possible, because of the simultaneous production of ketones resulting from oxidation at the α-carbon atom, acetone being obtained in the first case and methyl ethyl ketone in the second. The aldehydes were therefore oxidised to the corresponding acids and these isolated as silver salts. In addition to butyraldehyde, acetaldehyde was regularly observed as an

oxidation product of α-methylbutyric acid. The mechanism of its production appears to be somewhat obscure.

It is worthy of note that propionic acid is similarly constituted to the α-methylated fatty acids in that its β-carbon atom forms part of a methyl group, and it might be expected therefore to undergo the same kind of oxidation as the α-methylated acids. In that case it would yield malonic semi-aldehyde:

$$CH_3 - CH_2 - COOH \rightarrow CHO - CH_2 - COOH.$$

We already possess a certain amount of evidence in support of this view. It is certain for instance that β-phenylpropionic acid undergoes β-oxidation yielding benzoylacetic acid, and not α-oxidation which would yield phenyl-pyruvic or phenyl-lactic acid. Further, on oxidation with hydrogen peroxide, propionic acid yields a large amount of acetaldehyde [Dakin, 1908], although this might equally well be explained by α-oxidation, with the intermediate production of lactic acid, and not malonic semi-aldehyde. Ringer has observed that in phlorizinised dogs, propionic acid gives rise to extra glucose formation. No experiments have been performed with malonic semi-aldehyde but quite recently Ringer and Frankel [1914] have stated that acetaldehyde produces extra glucose formation in diabetic dogs. Since the administration of acetaldehyde causes a marked depression in the nitrogen excretion and the calculation of the extra glucose formed depends on the G/N ratio, the interpretation of these authors cannot be accepted unreservedly. It is noteworthy however that in spite of this variation an absolute rise in the glucose excretion was observed.

Experimental.

Oxidation of Ammonium Isobutyrate. Kahlbaum's isobutyric acid was used. Portions of ten grams were neutralised with ammonia, the solution diluted with about 100 cc. of water and twenty grams " perhydrol " added, the volume being then finally brought to about 200 cc. by further dilution with water. The oxidation was carried out by warming this solution in a flask connected with a well cooled condenser, the receiver being immersed in ice and salt. During the experiment a gentle current of air was aspirated through the apparatus in order to assist in the removal of the volatile products of oxidation from the flask in which the oxidation was carried out. The temperature was raised fairly rapidly to 70° and thereafter more slowly to boiling point. The heating was continued for half to one hour at this point

and the flame was maintained at such a height that distillation was very slow. The distillate, which was always alkaline, owing to the ammonia present, was redistilled to free the volatile oxidation products from possible traces of isobutyric acid. Direct isolation of a pure *p*-nitrophenylhydrazine derivative from the distillate was impossible, no substance with a sharp melting point being obtainable. The presence of aldehydes was easily detected by Tollen's reagent which was immediately reduced in the cold. Dakin [1908] using a slightly different method has shown that acetone is produced when ammonium isobutyrate is distilled with hydrogen peroxide. In order therefore to identify the aldehyde or aldehydes present, the distillate was oxidised with ammoniacal silver oxide containing a trace of sodium hydroxide. Oxidation was carried out at the ordinary temperature for several hours and then overnight in the incubator at 37°. The precipitated silver was filtered off, the filtrate nearly neutralised with phosphoric acid and distilled to remove the ketone. The distillate still contained a little aldehyde. This was removed by adding a few cc. of permanganate solution and redistilling. On treating the distillate with *p*-nitrophenylhydrazine acetate an immediate precipitate of hydrazone was obtained, which melted sharply at 148–9° after two recrystallisations from alcohol. This is the melting point of acetone-*p*-nitrophenylhydrazone. Dakin's observation of the formation of acetone was thus confirmed. The alkaline liquid from the oxidation with silver oxide, after removal of the acetone by distillation, was acidified with phosphoric acid and the volatile acids, corresponding to the aldehydes in the original distillate, distilled off. The distillate was neutralised with baryta and evaporated to dryness. The barium salts were washed with a little watery alcohol to remove barium acetate if present, then dissolved in a small amount of water and the solution left to crystallise. The crystalline barium salt was converted into the silver salt by precipitation with silver nitrate, washed with a little distilled water and dried in vacuo over sulphuric acid. The silver salt on analysis proved to be silver propionate.

0·1938 g.; 0·1165g. Ag = 60·1 %.
Calculated for $C_3H_5O_2Ag$ 59·7 %.

Propionic aldehyde was therefore one of the oxidation products of isobutyric acid.

Non-volatile Oxidation Products. The residual liquid from the peroxide oxidation contained much unchanged isobutyric acid. It was tested specially for formic acid by the mercuric chloride reaction and this acid was

present in traces. After removal of the isobutyric acid by continuous extraction with petroleum ether, the liquid was extracted with ether but no methylmalonic acid could be isolated from this extract.

Oxidation of Ammonium α-Methylbutyrate.

The acid used was prepared from methylethylmalonic acid and partly from methylethylacetoacetic ester. The oxidation was carried out in the same way as that of the isobutyrate. The distillate containing the volatile oxidation products did not yield a pure *p*-nitrophenylhydrazine derivative so that a separation of the aldehydes and ketone by means of ammoniacal silver oxide was undertaken. In this way a ketone was obtained which gave a *p*-nitrophenylhydrazone crystallising in orange-coloured needles and melting sharply at 124–124·5°. This was found to be the melting point of the *p*-nitrophenylhydrazone of methyl ethyl ketone prepared from methylacetoacetic ester[1]. A mixture of the two derivatives showed no change in the melting point. The acids obtained from the aldehydes by oxidation with silver oxide were isolated as barium salts which did not crystallise but were easily soluble in 95 % alcohol. The acids were therefore liberated by means of phosphoric acid, distilled off and converted into the calcium salts by neutralising the distillate with lime water. These crystallised readily on evaporating the solution and had the characteristic flocculent appearance of calcium acetate. The calcium salt was transformed into the silver salt and analysed.

0·1286 g.; 0·0781 g. Ag = 60·7 %.
∴ Mean molecular weight of acids = 70.

The mean molecular weight of the acids lay therefore between that of propionic acid (74) and acetic acid (60). Propionic acid was not present in any considerable amount however as the barium salts of the mixed acids were easily soluble in 95 % alcohol, whereas barium propionate is almost insoluble. The acetic acid undoubtedly present was therefore mixed with a higher acid. That butyric acid was present was easily demonstrated by warming a small portion of the calcium salts with sulphuric acid and methyl alcohol when the characteristic odour of methyl butyrate was obtained. It was thought desirable to obtain more exact evidence of the presence of butyric acid. A trial with equal amounts of calcium acetate and butyrate

[1] Dakin [1908] gives 128—9° as the melting point of the *p*-nitrophenylhydrazone of methyl ethyl ketone, prepared by him, but does not state the source of the ketone.

dissolved in water and evaporated, showed that separation could not be obtained satisfactorily with 95 % alcohol, in which calcium acetate is practically insoluble and calcium butyrate easily soluble, due no doubt to the formation of a mixed salt with intermediate solubilities. Distillation of the free acids was therefore used as a means of separation. The acids obtained from several experiments were obtained as sodium salts, and the free acids then liberated by sulphuric acid and taken up in a small amount of petroleum ether. The petroleum ether and acetic acid were then removed by distillation up to a temperature of 155°. The residual acid was dissolved in water and converted into the calcium salt by boiling with calcium carbonate. The calcium salt, which was easily soluble in 95 % alcohol, was converted into the silver salt which was dried in vacuo over sulphuric acid and on analysis proved to be silver butyrate probably still containing traces of the acetate.

0·2245 g.; 0·1255 g. Ag = 55·9 %.
Calculated for $C_4H_7O_2Ag$ 55·4 %.

Non-volatile Oxidation Products. As in the case of isobutyric acid traces of formic acid were detected by the mercuric chloride reaction. After removal of unchanged α-methylbutyric acid by distillation in steam, the residual liquid was neutralised, concentrated on the water bath, then acidified with phosphoric acid and extracted with ether for twelve hours. The ether extract contained a crystalline acid which, on recrystallisation from benzene containing a little acetone, and then from water, melted at 115°. This proved to be methylsuccinic acid. On heating with ammonia and zinc dust the pyrrole reaction was easily obtained.

0·1196 g. required 17·89 cc. 0·1 N. NaOH for neutralisation. Equivalent = 66·8.
Calculated for $C_3H_6(COOH)_2$ 66.

No evidence of the presence of ethylmalonic acid was obtained.

Summary.

1. A scheme of oxidation is suggested which explains the results so far obtained in studying the catabolism of α-methylated fatty acids such as isobutyric, α-methylbutyric and α-methylvaleric acid, and also acids such as γ-methylvaleric acid which by β-oxidation would yield α-methylated acids.

2. The rule of β-oxidation is applied to these acids with the proviso that the carbon atom of that methyl group which is in the β-position undergoes oxidation first. The derivative of malonic semi-aldehyde produced,

would, by loss of carbon dioxide give rise to the *normal* aldehyde of the demethylated fatty acid and by subsequent oxidation, or the Cannizzaro reaction, to the normal acid itself.

3. In support of this view as a possible biochemical process it is shown that ammonium isobutyrate on oxidation with hydrogen peroxide yields amongst other products propionaldehyde, and that ammonium α-methylbutyrate yields similarly butyraldehyde.

4. The possibility of this scheme being applied to the catabolism of propionic acid is suggested, since this acid is similarly constituted as regards the position of its methyl group to the α-methylated acids.

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XXXIX. THE CHEMICAL NATURE OF A BACTERIAL HAEMOLYSIN.

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I. The relation between the velocity of Heat-destruction of a Bacterial Haemolysin and the True Reaction of the Substrate.

Our present knowledge of the effect of heat, and also of acid and alkali on the velocity of destruction of bacterial haemolysins, is due to Famulener and Madsen [1908]. They were able to show that in all cases, whether by altering the temperature or the degree of acidity or alkalinity of the medium, the process of destruction proceeded as a reaction of the first order—that is to say, the destruction at any instant was proportional to the amount of haemolysin then present. They found that as they increased the quantity of acid or alkali, the velocity constant increased and there was a minimum velocity somewhere in the neighbourhood of the neutral point. The exact relationship of this minimum to neutral reaction of the medium, they did not determine however, as the hydrogen ion concentration was not measured. Since that time the technique of the determination of the hydrogen ion concentration of biological liquids by the electrical method has greatly developed and at the present day we can find its value with a very considerable degree of accuracy. It is therefore interesting to investigate how the velocity of destruction varies with the hydrogen and hydroxyl ion concentration. The fact that the velocity increases both with acidity and alkalinity is strongly in favour of the view that the substance producing haemolysis is an amphoteric electrolyte and that both anion and cation are thermolabile, while the undissociated molecules are probably not at all so, under the conditions of the experiment. On this assumption the velocity should be at a minimum when the anions and cations are present in the fewest possible numbers, that is at the isoelectric point. From such experiments therefore

we ought to obtain information about the relative magnitudes of the acidic and basic dissociation constants of the haemolysin. We shall then know whether the haemolysin functions as a weak acid or base in pure watery solution.

The haemolysin I have used is that produced in broth cultures of *Vibrio nasik*. After growing this micro-organism in alkaline broth (such as is employed for the production of diphtheria toxin) for a period of nine or ten days at 37°, it is killed by toluene and the dead bacteria removed by a centrifuge at a speed of about 5000 revolutions per minute. The clear liquid is separated and toluene added. The haemolysin keeps well at room temperature. It is lytic for the red corpuscles of many different animals. I have used a 1 % suspension of sheep red cells in 0·9 % NaCl. The relative amounts of haemolysin present in a series of samples were estimated by Madsen's colorimetric method [Kraus and Levaditi, 1908].

The reaction of nasik lysin was altered by the addition of HCl or NaOH according as an acid or alkaline medium was required. Small amounts of acids simply had the effect of neutralising or lessening its degree of alkalinity —the lysin being alkaline to begin with. In each case a definite amount of acid or alkali was added to 90 cc. of lysin and the whole made up to 100 cc. with distilled water. It was then left overnight in the cold room and next day hydrogen was bubbled through for three hours. This procedure dissipated the traces of toluene which were still dissolved in the lysin, and which Sörensen has shown may adversely affect the electrodes in the electrical method of estimating hydrogen ion concentration. It also had the effect of removing any excess of CO_2 or NH_3. Carbon dioxide is not an electrically active gas, that is to say, it does not give rise to an electromotive force in a platinum electrode dipping in a liquid, but when dissolved in water it forms H_2CO_3 which is slightly dissociated into $H^{\cdot}$ and HCO_3' ions and therefore increases the acidity of the solution. This would not matter except for the fact that the CO_2 easily diffuses out of the solution, notably into the atmosphere of hydrogen during electrical measurement of the reaction, thereby altering the reaction of the liquid and the saturation of the electrode. Ammonia on the other hand is electrically active and would modify the potential of the hydrogen electrode. In the most alkaline samples of lysin with which I worked there was a very distinct smell of NH_3, due no doubt to decomposition of ammonium salts by the added NaOH and so it was very necessary to get rid of this. The lysin was therefore left in contact with hydrogen for another day in the cold room. The most suitable temperature at which the destruction of the lysin could be followed, was shown by

Famulener and Madsen's experiments to be about 46·0° and this temperature was therefore chosen. Immediately before the heating of the lysin took place, its hydrogen ion concentration [H˙][1] was measured by the electrical method. The particular type of apparatus used was that described by Michaelis and Rona [1909] the characteristics of which are the *still* atmosphere of hydrogen combined with *minimum immersion* of the electrode. When the electrodes only just dip into the liquid the E.M.F. becomes constant very quickly and this is often of great importance in biological work where the reaction may be altering during the operation. The measurements took place at 46·0°—the same temperature as that at which the destruction of lysin was followed. As normal electrode I used a hydrogen electrode dipping into N/10 HCl. The [H˙] of this solution at 46° is only slightly less than at ordinary room temperature and it was estimated by graphic interpolation from data given by A. A. Noyes and others [1910] to be dissociated to the extent of 91·4 %. As connecting liquid KCl was used and readings taken with two strengths, namely, 1·75 N and 3·5 N solutions and the true value extrapolated by addition of the difference of the two potentials to the latter according to the method suggested by Bjerrum [1905]. About two minutes was allowed for the cells to attain the temperature of the water bath and then readings taken immediately. Potential equilibrium is practically established by this time as one would expect, especially as the hydrogen electrodes have been in contact with the liquids to be tested for some minutes previous to immersion in the water bath. By continuous observation of the E.M.F. at 46° a very gradual change is perceived due to slight alterations of the reaction of the lysin, as will be mentioned later. An immediate reading as soon as the lysin has reached 46° is necessary therefore and although an error may creep in here it can only be trifling.

The reaction of a given sample of lysin being ascertained, it was next heated to 46·0°. For this purpose a water bath with a toluene regulator was employed. During the heating the temperature never varied more than ± 0·025° and often was practically constant, the thermometer used being an accurate one graduated in tenths of a degree and read with the naked eye. Arrhenius has introduced an empirical formula for the relation between velocity constants and temperature, namely,

$$\frac{k_1}{k_2} = e^{\frac{\mu}{2}\left(\frac{T_1 - T_2}{T_1 T_2}\right)},$$

[1] H˙ stands for hydrogen ion, the brackets for concentration, and p_H. for the negative exponent when the normality is expressed as a power of 10.

k_1 and k_2 being the velocity constants at the absolute temperatures T_1 and T_2 and μ a constant for a given substance. Famulener and Madsen [1908] have shown that this equation is valid for nasik haemolysin, where $\mu = 128{,}000$. We can therefore calculate what would be the change in the velocity constant for a rise in temperature from 45·975° to 46·025°. Under these conditions the ratio $\frac{k_1}{k_2} = 1{\cdot}032$, which represents the maximum error and is so small as to be negligible. The lysin was placed in a large test tube containing the thermometer, which was also used for stirring, and fitted with a large cotton wool plug. It was previously heated to about the required temperature and quickly transferred to the water bath. As soon as the thermometer registered exactly 46·0° a sample was removed by insinuating a narrow bore glass tube at the side of the wool plug, the top of which was fitted to a 10 cc. pipette with a piece of rubber tube. From the pipette 7 cc. were measured off into a tube containing sufficient acid or alkali to neutralise the added alkali or acid, and immediately cooled down in ice-cold water. By this manœuvre the temperature of the lysin in the water bath did not appreciably alter. The attempt to bring the reaction back to its original value by adding the calculated amount of acid or alkali as the case might be, was only partially successful as can be easily imagined, but this is not of great importance since the haemolytic action of the lysin on red cells, within fairly wide limits, is independent of the [H·]. After seven samples had been removed at definite intervals of time the [H·] of the remainder was determined as before. It was found that in practically every case the reaction had changed during heating to a greater or less extent. It might be thought that it would have been better to alter the reaction by means of regulator mixtures which keep the reaction constant, that is, weak acids or bases in conjunction with their salts, instead of HCl and NaOH. There are however objections to this. In the first place I was desirous of introducing, if possible, only ions of the kind already present. Evidence is accumulating that other anions and cations besides H ions and OH ions, influence the chemical actions of biological substances and so may have altered the chemical relation of the lysin to H and OH ions. In addition, the amount of regulator necessary would be relatively large, and the dilution of the lysin would be such as materially to limit the range over which the destruction could be studied. As can be seen from Fig. 1 the alteration of reaction during heating is in most instances relatively slight and in any case insufficient to vitiate the main results of this investigation. Fortunately the greatest changes took place

in the neighbourhood of the neutral point, where considerable alterations in the reaction produce relatively small changes in the velocity constant.

The following will serve as a type of a single determination of a velocity constant :

1 N.HCl	H_2O	Nasik lysin	$[H^{\cdot}]$
2·2 cc.	7·8 cc.	90 cc.	$p_{H^{\cdot}}$ at 46·0°
			5·47

Heated at 46·0°.

Minutes	Haemolytic Equivalents. Volumes of lysin producing equal haemolysis	$\left(k=\frac{1}{t_2-t_1}\log_e\frac{A-x_1}{A-x_2}\right)$ $k\times 0{\cdot}4343$
0	0·055	
15	0·065	0·00484
30	0·07	0·00214
45	0·072	0·00082
60	0·075	0·00118
75	0·09	0·00528
90	0·11	0·00581
	Mean	0·00335

To 7 cc. of each sample after heating were added 0·154 cc. of N.NaOH and 0·846 cc. of water. After heating at 46°, $p_{H^{\cdot}}$ was found to be 5·51.

TABLE I. *Velocity of Destruction of Nasik Lysin at Different Reactions.*

$\left(k=\frac{1}{t_2-t_1}\log_e\frac{A-x_1}{A-x_2}\right)$ $k\times 43{\cdot}43$	$p_{H^{\cdot}}$ at 46·0°	
	before heating	after heating
28·0	8·43	8·49
18·93	8·30	8·23
15·7	8·01	8·17
9·84	8·19	8·10
5·2	7·55	7·62
2·95	7·37	7·17
0·72	7·06	6·95
0·4	7·06	6·62
0·08	6·54	—
0·22	6·46	6·18
0·16	5·82	5·49
0·335	5·48	5·5
2·05	5·17	5·07
3·95	5·04	5·04
6·4	5·10	4·88
10·6	5·00	4·82
16·95	4·77	—

In this way a number of values for k were obtained between two limits of $[H^{\cdot}]$ in each case. The results have been tabulated and charted. An inspection of Fig. 1 will reveal the points of interest.

The lines through which the curve has been drawn represent the limits of the change of reaction during heating. The velocity of destruction rises

with increasing acidity and also with increasing alkalinity though not so abruptly in the latter case. The minimum does not correspond with the neutral point but is slightly on the acid side.

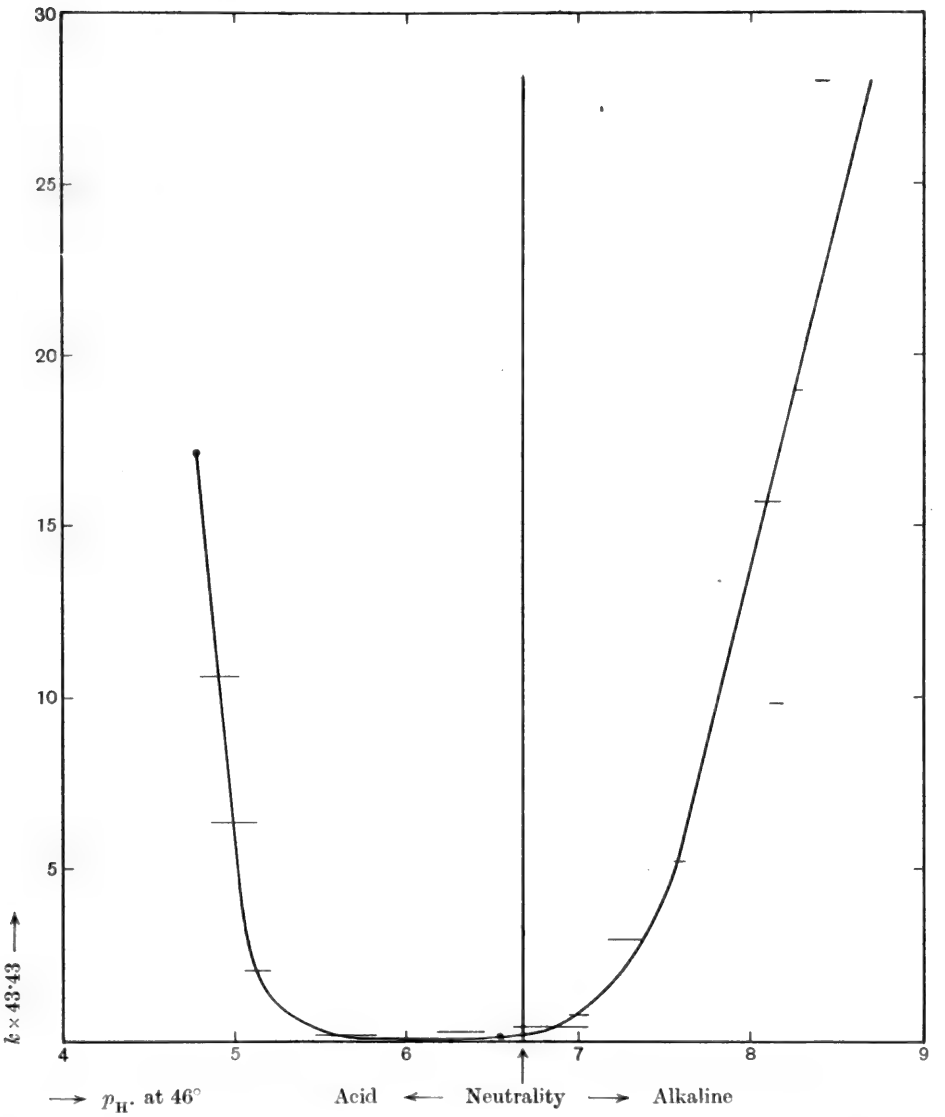
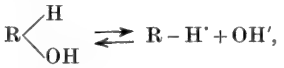


Fig. 1. Relation between Velocity of Destruction of Nasik Haemolysin and the Reaction of the Substrate at 46·0°.

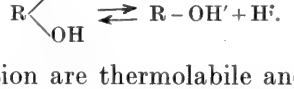
Interpretation of Results.

These facts can best be explained on the assumption that the substance causing haemolysis is an amphoteric electrolyte with an acid dissociation constant greater than the basic, that is $k_a > k_b$. If we represent an ampholyte (amphoteric electrolyte) in the usual way

then in strongly acid solution it will be ionised practically entirely thus:



and in strongly alkaline solution



Both the anion and cation are thermolabile and we should expect that the velocity of destruction would be at a minimum at the isoelectric point (I.P.) because here the total number of anions and cations is a minimum. The I.P. is by definition the [H˙] of the medium when the anions and cations of the ampholyte are present in equal numbers and it can also be shown that their total concentration is the least possible at a given temperature and in a given solvent—in other words at the I.P. the molecules of an ampholyte suffer least dissociation in solution. It is easy to see that if the H˙ and OH′ have the same tendency to split off from the molecule ($k_a = k_b$) the I.P. will correspond exactly with the neutral point; but if, for example, there is a greater tendency for the H˙ than the OH′ to dissociate, as is the case with so many protein substances, it will be necessary, in accordance with the law of mass action, to add an acid to limit the splitting off of H ions in order that an equal number of anions and cations may be present. Now the neutral point at 46·0° obtained by graphic interpolation from data for the dissociation product of water at different temperatures given by Lundén [1908] is $p_{H^\cdot}$ 6·69. On reference to Fig. 1 the minimum of the curve appears well on the acid side of the neutral point, and the explanation is that the lysin has a larger acid than basic dissociation constant. These results are consistent with the view that the lysin is an ampholyte comparable with the serum proteins.

There is another possibility, however, namely that the lysin is a dibasic acid. A minimum velocity of destruction would then correspond with a

maximum number of monovalent anions present, and the velocity would increase on the one hand with the number of undissociated molecules of lysin, and on the other hand with the divalent anions, according as the solution was made more acid or less acid respectively.

Trypsin is an ampholyte in which only the monovalent anion has a ferment action, but it seems probable that divalent anions which are inactive are formed by splitting off of another $H^{\cdot}$ on further reducing the acidity [Michaelis and Davidsohn, 1911]. Similar considerations would apply in the case of a di-acid base. It is only possible to choose between the two alternatives by testing the direction of movement in an electric field, in solutions of varying $[H^{\cdot}]$.

II. Cataphoresis experiments with Nasik Lysin.

The lysin already employed was not suitable for this purpose on account of its high electrical conductivity. The original broth on which the vibrio was cultivated contained 0·5 % NaCl in addition to salts from the meat and peptone. I first attempted to produce a lysin in a salt-poor medium—peptone water alone or with a little broth added—but I only succeeded in obtaining a weak lysin in this way. Dialysis was next tried. Parchment paper proved useless as the lysin disappeared almost as quickly as the salts from the solution. Much better results followed the use of an animal membrane as dialyser (the so-called Fischblase), which has been successfully used to obtain electrolyte-free ferment solutions. By dialysing for 24 hours against distilled water, I was able to get a fairly strong lysin with an electrical conductivity about quarter that of N/100 NaCl. Reduction of lytic power occurred here also, but to a much less extent than in the case of the parchment membrane.

The apparatus used was that designed by Landsteiner and Pauli, with unpolarisable electrodes of Ag in NaCl, and Cu in $CuCl_2$ which have been recommended by Michaelis. In order to vary the $[H^{\cdot}]$ a regulator mixture was used as it did not seem probable that the reaction would remain sufficiently constant during the experiment by the simple addition of HCl. It was not necessary to make the solution more alkaline than it already was, as the lysin migrated to the positive pole without any addition.

The most suitable mixture to produce the required range of $[H^{\cdot}]$ was lactic acid and its sodium salt. This regulator has been used several times in the case of ferments and the lactate ion has not been found to have

any specific effect. This regulator was added to the lysin in the ratio of regulator : lysin = 1 : 9. It is not practicable to dilute the lysin more as its strength would be insufficient to make it easy to detect when it had moved to one or other pole in small quantity. Its content of added salt (sodium lactate) was 0·02 N. This is not very concentrated for a regulator but it seemed desirable to keep the electrolyte content as low as possible. The slaking power of the lysin on the [H˙] of the regulator was considerable. After several mixtures had been made and the values of their [H˙] estimated it was easy to construct a curve from which to predict fairly accurately in advance the [H˙] of any mixture. Such mixtures were put in the middle compartment of the cataphoresis apparatus, and in the side pieces a regulator mixture of as nearly as possible the same [H˙], with the lysin replaced by distilled water. The latter mixture was therefore of slightly lower specific gravity than the lysin mixture, but had the same amount of added electrolyte, if we neglect the slightly dissociated anions of the lactic acid. The electrodes were filled with 0·01 N NaCl in addition to the small amount of concentrated salt solutions—NaCl on the positive side, and $CuCl_2$ on the negative side—which formed layers at the bottom. The whole apparatus when filled was air-tight, and was sunk in a water bath at 10°. A pressure of 200 volts was then applied for four hours. The current passing was of the order of 0·25 milliampere, but varied of course with different conditions of the solutions, the concentration of electrolyte at the electrodes and the size of the electrodes. At the end of the time allowed, 0·4 cc. of liquid was removed from the bottom of each side tube of the apparatus with a Jena glass pipette, which was drawn out to a very fine capillary and was fitted to a syringe by means of a small rubber cork. Very evenly regulated slow suction was produced in this way, so that the bottom layer of liquid just above the taps in the side tubes could be abstracted without mixing with the supernatant layers. To this liquid was added 0·2 cc. of a 3·6 % solution of NaCl, to bring its tonicity up to the level which the red blood cells can tolerate without becoming haemolysed. It was then carefully neutralised with 0·2 N NaOH. The technique of neutralisation presented some difficulties at first owing to the fact that it was not feasible to waste more than a minute amount of liquid in the process, and it was very necessary to bring the solution near neutrality or else haemolysis would be produced by the excess of acid or alkali present. Very small drops were taken and tested with equally small drops of suitable indicators such as methyl-red, rosolic acid, α-naphthol-phthalein, on a white porcelain plate. It was found

absolutely essential to use Jena glass for the pipettes for removal of the liquid and indicators. In this way it was easy to neutralise with a very small total loss of liquid. Lastly 1·0 cc. of a 1 % suspension of sheep red cells in 0·9 % NaCl was added, and the whole incubated for three hours at 37°. The tubes were then shaken and examined next day for haemolysis. A similar sample was always taken from the middle compartment at the end of cataphoresis to show that the lysin had not been destroyed by excessive acidity or other circumstances (see later). In all the mixtures of lysin and regulator a turbidity appeared immediately which gave rise to more or less precipitate as time went on, according to the degree of acidity.

This precipitate is presumably mainly composed of denaturated protein coming from the meat used in the preparation of the original broth, which only completely flocculates when brought to its isoelectric point. At any rate a similar precipitate can be obtained from a meat broth (without peptone) with its optimum at about the same [H·]. The presence of this protein impurity in the lysin solution was a considerable complication. At certain degrees of acidity it had the power of removing much of the lysin from the solution, in the process of precipitation, leaving only a weak lysin to work with. Before discussing its effect on the direction of migration of the lysin I will indicate the experimental results.

Table II shows the direction of movement of the lysin in an electric field at varying [H·]. The temperature was 10° and the [H·] was determined at the same temperature using in this case a calomel electrode as standard. The π_0 value (0·3383) at 10° was obtained by graphic extrapolation from values given by Sörensen [1912]. It will be seen that in most cases there is not very much difference between the [H·] of the lysin before and after cataphoresis. The liquid on the + side in every case became more alkaline ; that on the − side sometimes increased in acidity but showed less tendency to change from the original reaction of the regulator. It is obvious that these small deviations from the reaction of the lysin in the middle compartment will not militate against the definition of the limit of movement to either pole. For instance a lysin anion travelling towards the + pole on coming in contact with a more alkaline medium will not tend to have its charge reversed as it would if it met a more acid medium, and it will proceed on its way. Similarly on the acid side increased acidity will not interfere with the progress of the lysin cations. The charge and therefore the direction of movement of the ions will thus be determined by the [H·] of the lysin. It will be seen that movement to both poles has been obtained

TABLE II. *Cataphoresis of Nasik lysin*

Temperature 10°
Duration, 4 hours

E.M.F. 200 volts.

k_w at 10°, $10^{-14.51}$

No.	Lysin, cc.	0·1 N. Sod.lactate, cc.	0·1 N. Lactic acid, cc.	H_2O cc.	p_H· Before cataphoresis: Lysin	Before cataphoresis: Regulator	After cataphoresis: Lysin (middle compt.)	After cataphoresis: +side	After cataphoresis: −side	Movement Pole	Remarks
1	9	0·5	0·075	0·425	3·92	4·00	3·87	4·23	3·88	+	
2	,,	,,	0·1	0·4	3·77	3·94	3·85	4·28	3·92	+	
3	,,	,,	0·15	0·35	3·60	3·60	3·59	3·85	3·63	+	+liquid turbid.
4	,,	,,	0·2	0·3	3·29	3·26	3·23	3·55	3·25	0	No movement of visible particles. Strength of lysin after exp. much reduced.
5	,,	,,	0·4	0·1	3·01	2·87	3·01	3·02	2·9	0	
6	,,	,,	0·5	0	2·94	2·99	2·92	3·10	2·97	−	−liquid turbid.
7	,,	,,	0·5	0	2·94	2·70	—	2·9	2·78	−	
8	,,	,,	0·7	0	2·75	2·73	2·82	3·02	2·74	?	All lysin destroyed at end of exp., due to extreme acidity.

with a zone of no movement between them, according to the degree of acidity. This points to the fact that the lysin is an ampholyte and lends additional support to the interpretation of the experiments on velocity of destruction of the lysin by heat.

Discussion.

The early experiments to determine the electric charge of antigens (toxins, etc.) were all vitiated by neglect to exclude sharp changes of reaction at the electrodes. Field and Teague [1907] used platinum electrodes and in addition complicated their work by the presence of agar, through which they designed the toxin should travel and lend itself to easy detection. They determined that both the toxin and antitoxin of diphtheria and tetanus had positive charges which were not reversed by altering the reaction of the solvent—a most unlikely result in the light of later research. Teague and Buxton [1907] believed that they had shown that haemagglutinins moved to the cathode and considered that "all the active principles of serum concerned in the anti-reactions have a tendency to migrate towards the cathode." Their experiments were carried out in a similar way to those of Field and Teague except that they used unpolarisable electrodes. Bechhold [1907] used membranes with the idea of excluding the products formed at the poles from the substance being examined.

Landsteiner and Pauli [1908] in the case of abrin, ricin, and the haemagglutinin found in normal fowl serum, obtained a movement to the negative pole in each. A determination of the [H˙] of the solution was here lacking, but it was presumably not far from the neutral point. These experiments are an advance on the previous ones as the fallacies of the method were eliminated.

Landsteiner [1913] states that he has some unpublished results showing that staphylolysin and arachnolysin are amphoteric. The only details given are that in weak acid reaction the lysins migrated to the negative pole, and in weak alkaline to the positive pole.

Let us now attempt to co-ordinate the two series of results, namely, the curve of velocity of destruction and the movement in an electric field. The temperature in the two cases was different because the minimum velocity of destruction of the lysin had to be reduced for the cataphoresis experiments, so that the lysin should not be appreciably weakened by the end of the time allowed. Suppose for the present we estimate the I.P. at 10° (Table II) at about p_H 3·3. At 46° the minimum velocity of destruction

can be read from the curve at roughly p_H· 6·1. Is it reasonable to suppose such a difference in the I.P. would occur at the two temperatures? In the first place it must not be forgotten that values of [H˙] for different temperatures are not directly comparable. For instance at 46° the [H˙] of neutral reaction is p_H· 6·69, but at 10° the [H˙] of neutral reaction is p_H· 7·26. It is obvious that these two values are thermodynamically equivalent. It is in fact the ratios H˙/OH′, which correspond at two different temperatures, this ratio being of course equal to unity at neutrality. A simple calculation shows that the ratios of H˙ to OH′ will be the same at the two temperatures when the p_H· is 6·1 at 46° and 6·67 at 10°. In other words there is a difference of I.P. corresponding to the difference between p_H· 6·67 and p_H· 3·3 to be accounted for. We have now to see what is the effect of temperature on the other amino-acids. The only data I have been able to find in the literature are those of Lundén [1906]. He determined the acid and basic dissociation constants of three simple amino-acids at 25° and 40°. I have calculated the shift of I.P. in each case, by means of the equation $R = \frac{I^2}{k_w}$ [Michaelis and Rona, 1910].

Ampholyte	Temperature	k_a	k_b	$\frac{k_a}{k_b} = R$	Iso-electric Point $R = \frac{I^2}{k_w}$ p_H·	k_w at 25° = 10^{-14} at 40° = $10^{-13\cdot49}$
Acetoxime	25°	$6\cdot0 \times 10^{-13}$	$6\cdot5 \times 10^{-13}$	0·92	7·02	
	40°	$9\cdot9 \times 10^{-13}$	$19\cdot0 \times 10^{-13}$	0·52	6·89	
β-i-Asparagine	25°	$1\cdot35 \times 10^{-9}$	$1\cdot53 \times 10^{-12}$	882·4	5·53	
	40°	$3\cdot22 \times 10^{-9}$	$4\cdot23 \times 10^{-12}$	761·2	5·56	
o-Amino-benzoic acid	25°	$1\cdot06 \times 10^{-5}$	$1\cdot38 \times 10^{-12}$	$7\cdot68 \times 10^{6}$	3·56	
	40°	$1\cdot35 \times 10^{-5}$	$3\cdot16 \times 10^{-12}$	$4\cdot37 \times 10^{6}$	3·68	

The basic constant in each case increases more than the acidic with rise of temperature, which leads to a displacement of the I.P. towards the alkaline side. In the case of acetoxime the basic constant is greater than the acidic and the I.P. is consequently on the alkaline side of neutrality, but in the last two the I.P. is on the acid side. Allowing again for the fact that the values of I.P. need correction as they are determined at different temperatures, which correction as we have already seen still further increases the difference, we find that the nett result of a rise of temperature on the I.P. in the case of ampholytes like β-i-asparagine and o-amino-benzoic acid, is to shift it towards the neutral point. This is exactly what has taken place with the lysin. It is true that the difference in the last case is much greater than

the more extended range of temperature would account for and it is quite possible that there are other factors concerned. It must be remembered, for instance, that the salt content of the lysin solution in the heat destruction experiments was very different from that in the cataphoresis experiments. However the lysin is in all probability much more complicated than such simple ampholytes as the ones cited, so that it is unwise to expect too close a parallel. From this point of view therefore the experimental facts are in harmony with the theory that the lysin is an ampholyte.

We have still to consider the effect of the presence of other substances on the behaviour of the lysin. I have already drawn attention to the fact that it is not pure. In biochemistry we are constantly faced with the difficulty that the body we are investigating is mixed with other substances which may influence its action. There is some experimental evidence bearing on this point. Firstly there are some anomalous instances of heat destruction of haemolysins. Madsen, Famulener, and Walbum [1904] found that the haemolysin of staphylococcus was greatly reduced in strength at 70°, but almost completely returned on raising the temperature to 100° for five minutes. Landsteiner and Rauchenbichler [1909] found the same thing and attributed it to a union of staphylolysin at a medium temperature with some other substance in the solution, which compound was dissociated again at 100°. Working with megatheriolysin I was able to show [Atkin, 1910] that the velocity of destruction increased up to a temperature of about 55°, then it decreased up to a temperature of 75°, after which it increased up to 100°. This phenomenon is probably due to the same cause as that operating in the case of staphylolysin. I also repeated Madsen and Famulener's experiments without being able to confirm their results—the staphylolysin showed only a gradually increasing destruction with rise of temperature. This seems further to substantiate the view that the return of activity at a high temperature is not a characteristic of the lysin itself, but due to the influence of some foreign substance. Naturally in different samples the quantitative if not the qualitative relations of the several constituents will vary. No such property exists in the case of nasik haemolysin however, possibly because of its relatively great thermolability, for it is completely destroyed at a temperature considerably less than that at which the supposed union takes place in the case of staphylolysin and megatheriolysin. Similar considerations also apply to tetanolysin. If the hypothesis that megatheriolysin undergoes a union with some other body at certain temperatures, which modifies its destruction by heat, is correct, the process of destruction

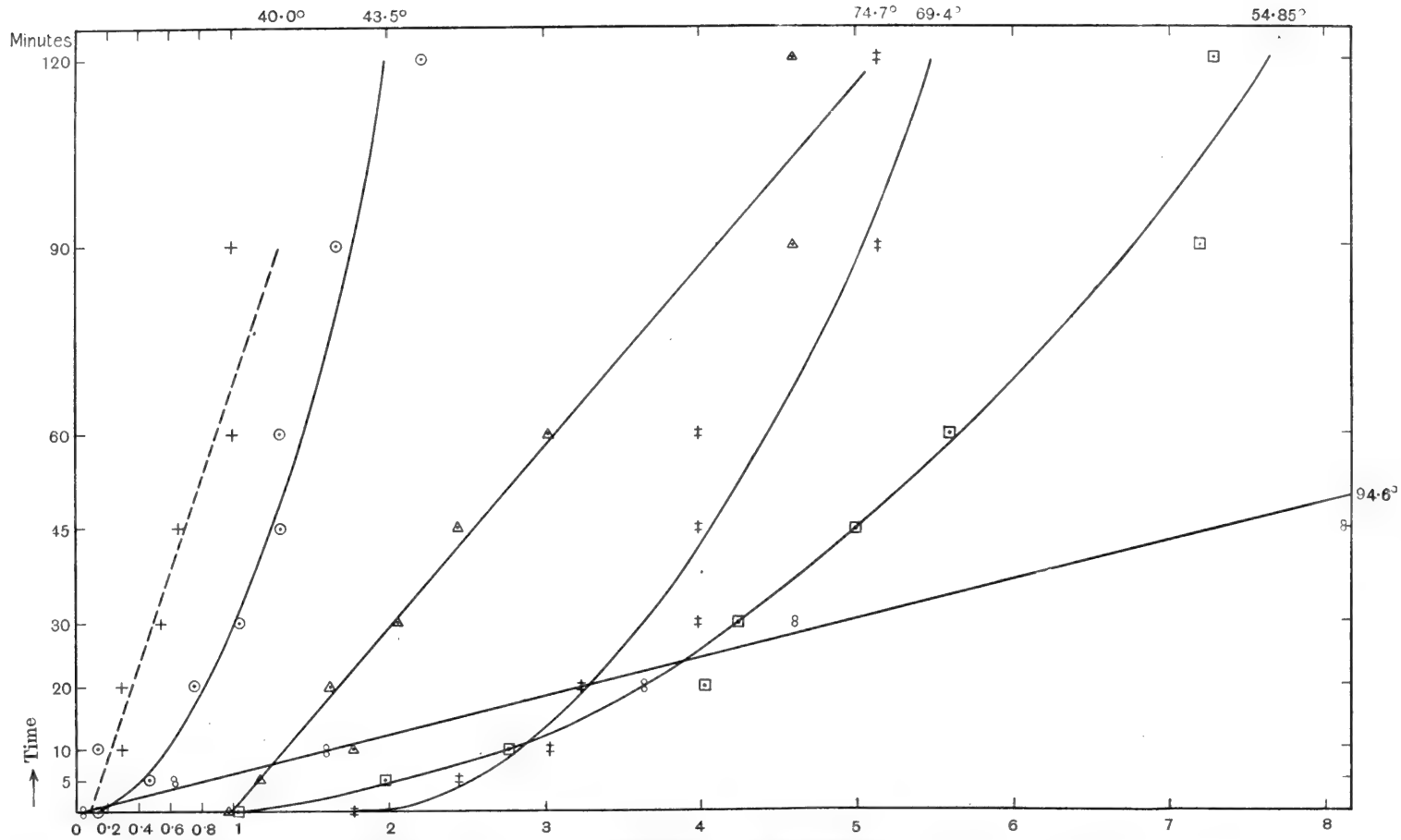


Fig. 2. Destruction of Megatheriolysin.

The points of origin of the curves are arbitrarily chosen for convenience of presentation.

should not be a unimolecular one. In other words, if we plot the logarithms of lysin concentration against time we ought not to get a straight line. If, on the other hand, at higher or lower temperatures such a combination either does not take place or is disrupted, it is reasonable to expect the process to be then a logarithmic one. I have applied this test to my experimental results in the case of megatheriolysin [Atkin, 1910] and find it actually to be so.

As the curves show (Fig. 2) the process of destruction is only unimolecular, firstly from a temperature of about 74°, upwards, which is near the point at which the rate of destruction again begins to increase with temperature; secondly, below a temperature of about 43°. It is not quite certain that the destruction at 40·0° is logarithmic, but at any rate it approximates closely to a straight line, if it is in reality a curve. It has been drawn in the figure as a dotted line. This last point is interesting as it shows that the influence of the foreign substance is being felt at a temperature considerably lower than that at which it is able to decrease the velocity of destruction with rising temperature. It will be noticed that the hypothetical union with another substance is not a reversible process, otherwise the decomposition which presumably takes place at high temperatures would result in a re-union on cooling down, for we must remember that the actual test on red blood cells is carried out at 37°. In fact the observation that we are able to detect the phenomenon at all renders it highly probable that it is irreversible, for if this were not so we should expect the union at medium temperatures to be split up again on cooling to 37°. We seem to have a criterion therefore with regard to the abnormal destruction of lysins by heat, namely, that if at any temperature it can be shown that the destruction is *not* unimolecular, the velocity of destruction will not rise regularly with temperature, or what is another phase of the same phenomenon, the strength of the lysin will decrease at a certain temperature, and reappear again at a higher one.

In my present experiments with nasik lysin the temperature was kept constant at 46°, the reaction alone being changed. It is hardly to be expected that a union with another body would take place having the same effect as in the case of megatheriolysin, seeing that this effect requires a considerably higher temperature, but nevertheless I have subjected the figures relating to the velocity of destruction to the same test and find that in every case the process is unimolecular. This fact is in favour of the chemical freedom of nasik lysin, under the conditions of these experiments.

Work bearing on the influence of impurities on the movements of enzymes in an electric field has been carried out by Pekelharing and Ringer [1911]. One of them devised a method of purifying crude pepsin by dialysing and thus reducing the acidity, so that the enzyme partially separates out. They do not claim that their product is absolutely pure, but at any rate it is more nearly pure than any commercial preparation. With it they obtained migration to the anode only, within the limits of acidity they tried. Addition of caseinogen, crystallised serum albumin, or albumose (Witte's peptone), caused migration more or less to the cathode. The pepsin was in fact carried by the albumin, etc. They were of opinion that the value $5{\cdot}5 \times 10^{-3}$ found by Michaelis and Davidsohn [1910] for the I.P. of pepsin was not reliable.

In the present case the lysin is mixed with, at any rate, one other protein substance which flocks out at a definite [H·]. It is difficult to say whether the lysin also flocks out at its I.P. as its absolute concentration must be extremely small and at 10° the I.P. apparently nearly coincides with that of the protein impurity. A glance at Table II, No. 4, shows that at this point there is no movement of the visible particles and at the same time the solution has become depleted of most of its lysin. We may here have a condition of things where the lysin and visible precipitate have opposite electric charges and consequently a union has taken place. The lysin would then presumably move with the precipitate. If this were so this boundary of the I.P. or rather isoelectric zone of the lysin would be rendered obscure. When however the sign of the charge of the lysin changed, the two bodies would again have similar charges and the lysin would be free to move independently. Similar considerations apply to the other boundary because the visible particles may become negatively charged before the lysin. A rough approximation to an isoelectric zone is therefore all that can be attained. If we take this zone as p_H 3·0 to 3·6 and assume that the geometric mean represents the I.P. then this will be the arithmetic mean of the p_H· values, namely p_H· 3·3. Therefore approximately,

Isoelectric point at 10° p_H· 3·3.
" " 46° p_H· 6·1.

This represents a large decrease in acid character with rise of temperature. It is interesting to attempt a determination of the acid and basic dissociation constants. Michaelis and Menten [1913] have devised a graphic method of obtaining these constants. Using the velocity constants I have constructed

dissociation-residue curves but find that they do not correspond with any theoretical curve, the steepness being much greater. The action of the H· and OH′ cannot be one of simple control of the dissociation of the lysin, but they must also take part in the destruction by heat. It is impossible therefore from the data to determine the dissociation constants, but the I.P., where H· and OH′ are in equal numbers, gives us a measure of the ratio of acid to basic constant.

From this investigation we can conclude that the experiments relating to heat destruction are in favour of the view that nasik haemolysin is an ampholyte. This is confirmed by the cataphoresis experiments. I must admit, however, that the presence of other proteins may influence the results, at any rate of the cataphoresis experiments. Similar objections may be raised to the whole series of the many determinations of the direction of movement in an electric field in the case of ferments, which have been investigated in the last few years, and it is quite possible that all these results will have to be revised.

Summary.

1. The velocity of destruction of nasik haemolysin at 46·0° is at a minimum slightly on the acid side of the neutral point. This is in favour of the view that the lysin is an ampholyte with its acid dissociation constant greater than its basic, and the point of minimum destruction is the isoelectric point. The anion and cation are therefore thermolabile, and the undissociated molecules not so.

2. Cataphoresis experiments at 10° confirm this view.

3. Approximate values for the isoelectric points are

$$p_{H\cdot} \text{ at } 10^\circ = 3{\cdot}3\,; \quad p_{H\cdot} \text{ at } 46^\circ = 6{\cdot}1.$$

This decrease of acidity with rising temperature is also found in the case of simple ampholytes, but to a smaller extent.

4. Incidentally a criterion is given for the anomalous heat destruction of certain haemolysins, for instance, megatheriolysin, namely, that the process is not unimolecular at temperatures where the velocity of destruction decreases with rising temperature, but it can be shown to be so at temperatures considerably removed where the velocity of destruction follows the more ordinary course of increase with rise of temperature.

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XL. THE RELATIONS OF VITAMINE TO LIPOIDS.

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(*Received July 8th*, 1914.)

Although vitamine, i.e. the substance which prevents beri-beri and avian polyneuritis, has been shown to be present in higher concentration in various tissues rich in lipoid, e.g. liver, cardiac muscle, brain (ox) and egg-yolk, than in voluntary muscle (ox, fish) which is poor in lipoid [Cooper, 1913, 1] it is now generally believed that the active substance as isolated from foodstuffs is not a lipoid. It contains no phosphorus or carbohydrate [Cooper and Funk, 1911; Funk, 1911, 1914; Edie, Evans, Moore, Simpson, Webster, 1912] and is therefore neither a phosphatide nor cerebroside. MacLean [1912] furthermore showed that lecithin, which had previously been found by Schaumann [1910] to be slightly curative, had still less effect after purification, and he concluded that the anti-neuritic substance is not a lipoid but is present in lipoids as an impurity extractable by simple methods. Cooper [1913, 2] subsequently found that when the fats and lipoids (alcoholic extract) of horse voluntary muscle were treated with ether the bulk of the anti-neuritic substance contained therein was precipitated and by a complex fractionation of the active precipitate a substance was isolated, small doses of which readily cured polyneuritis in pigeons. 4 g. of the original alcoholic extract exerted a rapid curative action in neuritic pigeons, but after one extraction with ether as much as 12 g. of the fats and lipoids (ether-soluble fraction) had only a slight ameliorative effect. More than 66 per cent. of the total amount of active material present in the original extract could thus be removed by simple treatment with ether and was thus apparently not in chemical union with the lipoids. Similar results were obtained when the flesh was not air-dried at 30° before extraction with alcohol [Cooper, 1913, 1]. This showed that the anti-neuritic substance was not split off from a lipoid complex during the process of air drying.

The above facts however do not exclude the possibility that, although

the anti-neuritic substance is preformed in the diet, in the nervous system it in part becomes chemically combined with other substances to form a lipoid.

Experiments on similar lines have therefore been carried out with cardiac muscle and brain, and the results obtained are set forth in the present communication. The work with heart muscle was undertaken primarily with the object of isolating a sufficient amount of the anti-neuritic substance for a study of its chemical nature, the cardiac muscle being substituted for voluntary muscle on account of its higher content of active material [Cooper, 1913, 1].

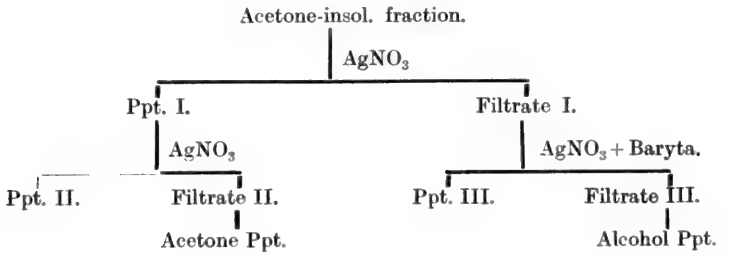
It must first be pointed out however that according to Vedder and Williams [1913] dry and wet beri-beri are caused by a deficiency in the dietary of distinct substances. In birds as a rule only dry beri-beri occurs, and, although infantile beri-beri is almost exclusively of the wet type, no signs of this condition were observed by the author even in very young chickens fed on polished rice. In this paper we are therefore only concerned with the substance which prevents and cures dry beri-beri and avian polyneuritis.

I. Preparation of an anti-neuritic substance from cardiac muscle.

150 lbs. of ox-heart were minced, dried at 30° by means of an electric fan, ground, and thoroughly extracted with 95 % alcohol. The filtered extract, from which the alcohol was removed by evaporation *in vacuo*, was treated with an excess of ether, and the material precipitated possessed marked anti-neuritic properties, 0·2 g. given orally rapidly curing pigeons affected with polyneuritis. The procedure subsequently followed was a modification of that employed in the fractionation of horse-flesh and is briefly described below.

The ether-insoluble precipitate was taken up with 95 % alcohol which dissolved a considerable amount of material. The insoluble residue contained both inorganic and organic matter, but was inactive. The soluble fraction however was strongly curative and was next treated with excess of ether, which completely precipitated the anti-neuritic substance. The precipitate was dissolved in water and allowed to stand in a vacuum desiccator for several days. A white crystalline substance slowly separated, but this had no curative action. The supernatant liquor was then treated with basic lead acetate until no more precipitate was formed. As in the case of horse-flesh, the active substance was not precipitated by this reagent, and the filtrate

possessed marked curative properties. So far the method was identical with that employed in the case of horse-flesh, but at this stage a departure from the original process was made. It was found in the previous work that the addition of silver nitrate after the lead acetate precipitation only partially precipitated the curative substance. This manipulation was therefore postponed in the subsequent technique with the object of ascertaining what influence intermediate fractionations might have upon the precipitability of the substance by the silver salt. The lead acetate filtrate after the removal of excess of lead by dilute sulphuric acid was therefore evaporated *in vacuo* and the concentrated solution treated with excess of 95 per cent. alcohol. By this procedure some material was separated, but all the anti-neuritic substance remained in solution. The filtrate was evaporated to a syrup and treated with acetone, which precipitated practically all the active substance. The filtrate possessed only slight curative properties, whilst 0·15 g. of the precipitated substance given orally rapidly cured pigeons affected with polyneuritis. The substance was insoluble in benzene, ether, and chloroform, but dissolved readily in water. The aqueous solution was next treated with silver nitrate solution, which however still only precipitated a portion of the active substance, and the latter could furthermore only be partially precipitated from the filtrate by the addition of baryta. The first precipitate (with silver nitrate only) was decomposed with dilute hydrochloric acid and silver nitrate again added to the filtered solution. On this occasion the substance was not precipitated to any appreciable degree and the filtrate was strongly curative. The filtrate after removal of excess of silver as the chloride was concentrated *in vacuo* and was then treated with acetone. A substance was thereby precipitated, 0·04 g. of which given orally quickly cured polyneuritis in pigeons whilst 0·008 g. cured by subcutaneous injection. The filtrate from the third precipitate (obtained with silver nitrate and baryta) was concentrated and treated with 95 per cent. alcohol, and a substance was precipitated, 0·04 g. of which given orally also cured polyneuritis. The manipulations are set forth schematically below.



(The thick lines represent the main course of the active substance during the fractionations.)

The substances separated in the above way from filtrates II and III and the solution obtained by decomposition of precipitate III were now combined and treated with 95 per cent. alcohol, which precipitated a small amount of material possessing no curative properties. The filtrate was evaporated and acetone added. This caused the precipitation of a substance (5 g.) 0·04 g. of which administered orally readily cured neuritic pigeons. The filtrate was only slightly curative.

On standing a few days in a sulphuric acid desiccator the substance however ceased to have any curative action, and even large doses only prolonged the lives of neuritic birds. It was thus of little interest to carry out a chemical analysis of the substance.

It is evident therefore that the anti-neuritic substance is extremely labile and with the chemical methods at present at our disposal its isolation will prove a very difficult matter. Further work must be carried out to ascertain the conditions favourable to the existence of the active substance. For this reason and also on account of cost it is doubtful if highly purified preparations will be of much therapeutical value.

An inexpensive method however of preparing a non-toxic highly curative solution suitable for use in the treatment of human beri-beri was described in a previous communication [Cooper, 1914, 2].

II. The relations of Vitamine to Lipoids.

The curative power of the original alcoholic extract of cardiac muscle was determined before and after treatment with ether. It was found that about 3 g. of the extract readily cured polyneuritis in pigeons, but after the extract was treated with ether 4 to 8 g. of the fats and lipoids (ether-soluble fraction) had little or no curative action. The small amount of active substance still retained by them could not be removed by a second extraction with ether, but by treating the ether-soluble fraction with acetone the phosphatides were precipitated and as much as 10 g. had not the slightest effect upon neuritic pigeons. These observations support the conclusion of MacLean (p. 347).

Different observations however were made in the case of brain. 13 kilos. of ox-brain were minced, dried at 30° for 16 hours, and extracted with alcohol on the shaking machine. The filtered extract was concentrated

in vacuo to remove the alcohol, and when dry weighed 730 g. The extract was next treated with excess of ether, which precipitated 100 g. of a white substance. 0·6 g. of this rapidly cured polyneuritis in pigeons, but 0·4 g. had only a slight action.

This fraction from brain was less curative than the corresponding fractions from voluntary muscle and cardiac muscle, 0·2 g. of which was quite sufficient to bring about recovery. It also differed from the voluntary muscle preparations in not being completely soluble in water. The ether-soluble fraction from brain, on the other hand, unlike the analogous fractions from muscle, possessed marked curative properties, 2-3 g. being sufficient to cure pigeons affected with polyneuritis. The fats and lipoids still retained their activity even when extracted three times with ether.

On treating the ether-soluble fraction with acetone however the active substances were present in the acetone-soluble fraction, 1–3 g. of which readily cured, and, as was observed in the case of cardiac muscle, the precipitated phosphatides in doses of 5 to 10 g. possessed no detectable curative power. The phosphatides had no curative effect even after hydrolysis with 5 per cent. sulphuric acid, a process which does not affect the active substance. This shows that the negative results were not due to the indigestibility of the lipoids.

Similar results were obtained when alcoholic extracts of sheep-brain and egg-yolk, were treated with ether. There would thus appear to be present in brain and egg-yolk an ether- and acetone-soluble substance which possesses a marked affinity, chemical or adsorptive, for vitamine, and which may function as a carrier in the living organism.

The residues left after extracting the ox- and sheep-brain with alcohol were retained, and experiments carried out to ascertain to what extent the anti-neuritic substance contained in brain was extracted by alcohol.

13 kilos. of the ox-brain yielded 1680 g. of residue after exhaustion with alcohol. It was previously found [Cooper, 1914, 1] that the addition of 6 g. of ox-brain to the polished rice diet (1/20th body-weight ration daily) was sufficient to prevent polyneuritis in pigeons for at least 50 days.

Two pigeons were therefore fed daily on the above ration of polished rice and an amount of the brain residue equivalent to 12 g. of brain (1·5 g.). Both birds developed polyneuritis in 30 days. It is evident from this result that the greater part of the anti-neuritic substance contained in ox-brain is extracted by 95 per cent. alcohol.

1900 g. of the sheep-brain yielded 238 g. of alcohol-insoluble material.

It was previously observed [Cooper, 1913, 1] that the addition of about 8 g. of sheep-brain to a 1/20th body-weight ration of polished rice daily prevented polyneuritis in pigeons for 50 days. Three pigeons were therefore fed daily on the above ration of rice and an amount of the brain residue equivalent to 15 g. of the original brain (2 g.). All the birds developed polyneuritis within 30 days. As in the case of ox-brain, the bulk of the anti-neuritic substance is evidently extracted along with the fats and lipoids by 95 per cent. alcohol.

It having been demonstrated that brain phosphatides when purified possessed no curative power, experiments were next carried out to ascertain if the anti-neuritic substance were more firmly combined with certain other constituents of brain. Cholesterol prepared by crystallisation from an acetone extract of brain was first tested on neuritic pigeons, but 1 g. was found to exert no curative action.

1 kilo. of ox-brain was next minced and without drying repeatedly extracted with absolute alcohol at 40°. The extract was filtered and kept at 0° for 12 hours. By that time a yellowish precipitate (protagon) separated out. This was filtered off and washed with cold alcohol, the residue weighing 15 g. 2 to 5 g. of this deposit however had no effect at all upon neuritic pigeons.

5 g. were then treated with ether. The insoluble residue (3 g.) containing cerebroside (cerebrone) had no curative action, and kephalin (1 g.) precipitated from the ethereal extract by the addition of absolute alcohol also proved to be inactive. The original alcoholic extract after removal of the " protagon " was concentrated *in vacuo*, and the residue treated with ether. As before, the precipitate was curative. It was extracted with absolute alcohol at 0°, and the undissolved substance (1·5 g.) containing cerebrosides was again inactive. The alcohol-soluble fraction was concentrated *in vacuo* and when dry weighed 5 g. This was highly curative, 0·25 to 0·5 g. being adequate to cure polyneuritis.

The original ether-soluble fraction containing the phosphatides and fats of the brain was freed from ether and treated with acetone. The precipitated phosphatides were dissolved in a little chloroform and again reprecipitated with acetone. The phosphatides in doses of from 4 to 8 g. were inactive but 1 to 2·5 g. of the acetone-soluble fraction readily cured pigeons, thus confirming the observations detailed earlier in the paper.

This work was then repeated on a larger scale, 3 kilos. of ox-brain being used. Similar results were obtained, protagon (even when hydrolysed)

kephalin, cerebrone, and the phosphatides again being found to be ineffective when given to neuritic pigeons in approximately the above mentioned doses.

The failure of protagon, kephalin, cerebrone, cholesterol and phosphatides to cure polyneuritis is of interest in relation to the researches of Stepp [1909, 1911, 1913] who has found that food extracted with alcohol-ether is deleterious to the health of mice, and that the harmful effect of the diet is not checked by the addition of lecithin, cholesterol, kephalin, cerebrone, and phytin. When however egg-yolk or alcoholic extracts of egg-yolk or brain are added the animals remain healthy. Of these substances cholesterol, kephalin, and cerebrone as stated above do not contain vitamine, and lecithin [MacLean, 1912 ; Cooper, 1913, 2] and phytin [Schaumann, 1910 ; Cooper and Funk, 1911] when purified are also practically free from the active substance.

Egg-yolk and alcoholic extracts thereof on the other hand contain the essential substance [Cooper, 1913, 1], as also do alcoholic extracts of brain [Funk, 1912 ; Cooper, 1913, 2]. It is therefore probable that the harmful effects of diets consisting of food extracted with alcohol-ether is due not to the deficiency in the diets of lipoids, but to a deficiency of vitamine which is extracted by alcohol along with the lipoids.

The main conclusion that emerges from these observations is that vitamine does not apparently enter into the constitution of the lipoids of the brain, but may possibly be stored therein until required in the nutrition of the nervous system.

To what extent lipoids can adsorb the active substance from an *aqueous* medium is a point to be settled by further experiment. Meanwhile it may be noted that asbestos (Ural) does not appreciably adsorb vitamine from solutions of autolysed yeast (acid reaction).

Summary.

1. Although infantile beri-beri is almost exclusively of the wet type, young chickens fed on polished rice develop symptoms of the dry form only, like adult birds.

2. A method is described for the preparation from cardiac muscle of a substance small amounts of which cure polyneuritis in pigeons. The substance however was extremely unstable and lost its activity in a few days after isolation.

3. As in the case of voluntary muscle, the bulk of the anti-neuritic

substance contained in the fats and lipoids (alcoholic extract) of cardiac muscle can be separated therefrom by means of ether.

4. In the case of brain, however, the anti-neuritic substance can only be partially precipitated from the alcoholic extract by treatment with ether, but can be entirely removed from the phosphatides by the subsequent addition of acetone. An explanation of this is offered.

5. Purified brain phosphatides possess no curative power even after hydrolysis with acid. This shows that their inability to cure polyneuritis is not due to incomplete digestion.

6. Protagon (even when hydrolysed), kephalin, cholesterol, and cerebrone similarly have no curative action.

7. The bulk of the anti-neuritic substance contained in sheep- and ox-brain can be extracted by 95 per cent. alcohol.

Conclusion.

The results show that vitamine does not enter into the constitution of the lipoids of brain and muscle, but may possibly be adsorbed in the lipoids and so stored, until required by the organism.

It would thus appear that the deleterious effect of lipoid-free diets observed by Stepp is due not to the deficiency of lipoids, but to the mechanical removal of vitamine during the alcohol-ether extractions.

I desire to express my best thanks to Dr Hugh MacLean for kindly help in the course of this investigation.

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XLI. THE RELATIVE AMOUNTS OF β-HYDROXY-BUTYRIC ACID AND ACETO-ACETIC ACID EXCRETED IN ACETONURIA.

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(*Received July 9th*, 1914.)

Various workers have shown that a reversible reaction occurs in the body between aceto-acetic acid and β-hydroxybutyric acid [for references see Dakin, 1912]

$$\underset{+\,O}{CH_3 . CHOH . CH_2 . COOH} \rightleftarrows \underset{+\,H_2O}{CH_3 . CO . CH_2 . COOH}.$$

Neubauer [1910] showed that when aceto-acetic acid was administered to a diabetic, not only this acid, but also β-hydroxybutyric acid was excreted in increased amounts, and he confirmed the older statement that the administration of β-hydroxybutyric acid in diabetes increased the output of both acids. He pointed out further that the relative amounts of the two acids in the urine in cases of acetonuria should indicate the equilibrium point of the reversible reaction within the body. He found that the amount of β-hydroxybutyric acid, expressed as a percentage of the total acetone bodies, was remarkably constant, this percentage being from 60 to 80 in well-marked cases of acetonuria, and somewhat lower than this when the total output of acetone bodies was small. In spite of the enormous numbers of estimations of acetone bodies which have been made, no other writers appear to have paid much attention to the relative amounts of these substances. The cases given below serve to provide some illustrations of Neubauer's statement.

It is necessary to have some short term by which to express the relative amounts of β-hydroxybutyric acid and aceto-acetic acid in urine. The term

" β-ratio " is used below to denote the percentage of the total acetone bodies which is made up by β-hydroxybutyric acid, each substance being calculated in the form of acetone.

No attempt is made here to establish with any great accuracy the proportions in which the different acetone bodies are produced in the body. To do this in a satisfactory manner, one would require firstly an accurate method for the estimation of β-hydroxybutyric acid. The method used in nearly all these observations gives results which are from 4 to 10 % too low [Shaffer and Marriot, 1913 ; Kennaway, 1914]. If the β-ratio in a urine were actually 75, and the result of the β-hydroxybutyric acid estimation were 10 % too low, the β-ratio found would be 72·96. Secondly, one would require estimations of the amount of acetone in the expired air. Since there is a considerable fall in molecular weight in the formation of acetone (from 102 to 58), the comparatively small amounts of this substance in the breath are of importance in ascertaining the total production of acetone bodies.

The estimations on Case 1 below were made by the Messinger-Huppert and polarimetric methods, and all the others by a combination of the Shaffer and Scott-Wilson methods [Kennaway, 1914]. The pathological urines were obtained with two exceptions from the wards of Guy's Hospital, and I wish to express my thanks for the material to the physicians in charge of these cases. I am greatly indebted also to Drs Pembrey, Poulton, and Graham for permission to include in the tables their estimations of alveolar carbon dioxide, and to Drs Poulton and Graham for the opportunity to make observations in the course of their numerous experiments upon themselves [Graham and Poulton, 1913, 1914, 1, 2].

Cases of Diabetic Acidosis.

If a person is excreting two molecules of β-hydroxybutyric to one of aceto-acetic acid, the β-ratio will be 66·6 ; if five molecules to one, it will be 83·3, and so on. In Fig. 1, all the β-ratios obtained from Cases 1–14 of diabetes are arranged in order of magnitude ; Case 15, which showed a much smaller output of acetone bodies and lower β-ratio than any of the others, is omitted to save space (see Table IV).

It is hardly necessary to point out that this figure shows merely the range observed in these cases ; it does not of course show the frequency of occurrence of any particular ratio, the number of points near the level of 75 being due to the number of estimations made on Case 4.

The results given in the tables below indicate that Case 1 was excreting chiefly two molecules of β-hydroxybutyric acid to one of aceto-acetic acid; in Case 4, the proportion was about three to one, and in Case 7 probably five to one.

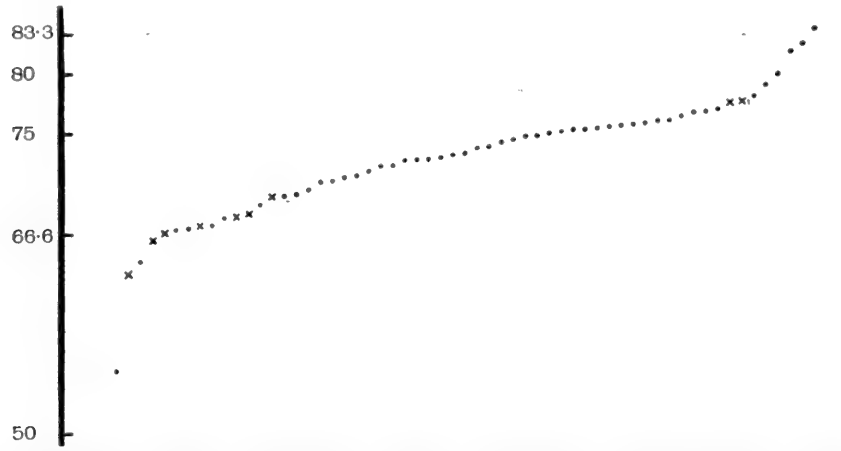

Fig. 1. β-ratios of Cases 1–14 (Diabetes), in order of magnitude. × indicates urine at autopsy.

TABLE I.

Cases 1-3. *Diabetes.*

	Date	Urine c.c.	G. acetone per day: Aceto-acetic acid + acetone	G. acetone per day: β-hydroxy-butyric acid	G. acetone per day: Total	β-ratio
Case 1.	27 vii.	1650	1·29	3·22	4·51	71·4
	29 vii.	2460	1·01	2·056	3·066	67·1
	30 vii.	1700	0·51	1·037	1·547	67·0
	1 viii.	1500	0·472	0·975	1·447	67·4
	2 viii.	1210	0·454	0·970	1·424	68·0
Case 2.	28 i.	3380	5·24	14·13	19·37	73·0
	6 ii.	5000	6·41	20·85	27·26	76·5
Case 3.	1 v.	4145	5·43	20·67	26·10	79·2
	7 v.	4125	6·04	21·55	27·59	78·1
	9 v.	3030	4·85	19·37	24·22	80·0

Case 4 (Table II and Fig. 2) had received before coming under observation large doses of sodium bicarbonate, on account of signs of incipient coma. The alveolar carbon dioxide pressure was found by Dr Pembrey to be abnormally high (49·3 mm.), no doubt as a result of the ingestion of alkali. In the table, the doses of bicarbonate are calculated as acetone derived from the

amounts of β-hydroxybutyric acid which could be neutralised by the bicarbonate, in order to show the assistance given to the neutralising powers of the body. On two occasions (Aug. 9th and 18th) the dose of alkali was doubled, and the alveolar carbon dioxide pressure showed a slight increase.

TABLE II.

Case 4. *Diabetes.*

Date	Urine c.c.	Alveolar CO_2 mms. Hg.	$NaHCO_3$ as g. acetone per day	G. acetone per day: Aceto-acetic acid + acetone	G. acetone per day: β-hydroxy-butyric acid	G. acetone per day: Total	β-ratio
18 vii. ..	—	—	62	—	—	—	—
19	—	—	92	—	—	—	—
20 ..	—	—	32	—	—	—	—
21 ..	4640	49·3	16	6·39	14·72	21·11	69·8
22 ..	4000	46·4	,,	5·37	13·19	18·56	71·1
23 ..	4040	39·1	,,	3·99	10·68	14·67	72·8
25 ..	4250	39·4	,,	3·59	12·01	15·60	76·9
28 ..	5830	35·7	,,	4·54	13·71	18·25	75·1
30 ..	6670	34·2	,,	6·57	19·21	25·78	74·5
31 ..	6510	—	,,	7·82	21·24	29·06	73·1
1 viii. ..	4560	33·3	,,	4·96	13·28	18·24	72·8
3 ..	2710	31·4	,,	3·96	11·86	15·82	75·4
6 ..	4270	33·9	,,	4·00	12·26	16·26	75·4
7 ..	4600	—	,,	4·15	13·96	18·11	77·1
8 ..	3130	36·0	,,	1·94	5·99	7·93	75·6
9 ..	7390	40·3	32	5·36	16·84	22·20	75·9
10 ..	4810	—	32	3·56	9·58	13·14	72·9
11 ..	9290	37·8	16	4·85	12·20	17·05	71·5
12 ..	7790	38·1	,,	6·87	21·96	28·83	76·2
13 ..	6770	33·3	,,	5·82	18·25	24·07	75·8
17 ..	5820	—	,,	7·03	19·95	26·98	73·8
18 ..	7640	34·3	32	7·83	21·49	29·32	73·3
19 ..	9670	36·6	32	7·89	21·80	29·69	73·4
20 ..	7260	35·2	16	4·21	12·21	16·42	74·3
						mean 20·34	mean 74·1

In Cases 5–13, the urine present in the bladder at autopsy was examined; the results obtained are enclosed in brackets in the tables. The β-ratio in this urine is in most cases low, and in those in which the comparison is possible (Cases 5, 7, 8, 9)[1], it is lower than it was on the previous days; a similar change was observed in a non-diabetic condition (Case 26). The explanation of this is not clear. One would not expect the oxidative change of the

[1] In case 6 the comparison cannot be made owing to the week's interval after the last estimation before death.

reversible reaction to be favoured in the hours preceding death. Possibly a diminished output of acetone from the lung may be the cause of the difference.

TABLE III.

Cases 5–13. *Diabetes.*

	Date	Urine c.c.	Alveolar CO_2 mm. Hg.	G. acetone per day: Aceto-acetic acid + acetone	G. acetone per day: β-hydroxy-butyric acid	G. acetone per day: Total	β-ratio
Case 5. ..	25 vi.	1860	27·4	3·47	15·77	19·24	81·9
	2 vii.	2200	27·0	4·42	14·72	19·14	76·9
	3 vii.	2000	—	5·06	15·74	20·80	75·7
	4 vii.	1860	26·9	3·92	11·11	15·03	73·9
	5 vii.	3600	24·5	8·21	24·49	32·70	74·9
	7 vii.	—	17·6	—	—	—	—
Not 24 hours urine (Coma	8 vii.	—	8·8	—	—	—	71·0
„	9 vii.	—	—	—	—	—	70·0
Death on ..	9 vii.	—	—	—	—	—	—
Urine at autopsy ..	—	—	—	—	—	—	[63·4]
Case 6.	3 iii.	3820	—	3·33	10·30	13·63	75·5
	4 iii.	3860	—	4·12	12·31	16·43	74·9
	5 iii.	3090	—	3·94	12·60	16·54	76·2
	10 iii.	5305	—	5·99	18·18	24·17	75·2
	11 iii.	4830	—	4·58	11·97	16·56	72·3
	12 iii.	6050	—	5·98	15·67	21·65	72·4
Death in coma on	20 iii.	—	—	—	—	—	—
Urine at autopsy ..	—	—	—	—	—	—	[77·6]
Case 7.	10 iii.	5580	13·8	4·30	20·33	24·63	82·5
Coma on ..	12 iii.	—	—	—	—	—	83·6
Death on ..	13 iii.	—	—	—	—	—	—
Urine at autopsy ..	—	—	—	—	—	—	[77·7]
Case 8.	30 v.	4800	29·7	10·56	23·66	34·22	69·1
Coma. Death on	2 vi.	—	—	—	—	—	—
Urine at autopsy ..	—	—	—	—	—	—	[68·4]
Case 9.	30 i.	—	17 8	—	—	—	70·3
No coma, death on	31 i.	—	—	—	—	—	—
Urine at autopsy ..	—	—	—	—	—	—	[68·1]
At autopsy, females: *Case* 10 ..	—	—	—	—	—	—	[69·7]
At autopsy, females: *Case* 11 ..	—	—	—	—	—	—	[66·6]
At autopsy, females: *Case* 12 ..	—	—	—	—	—	—	[66·2]
At autopsy, females: *Case* 13 ..	—	—	—	—	—	—	[67·4]

Cases 14 and 15 showed a much smaller output of acetone bodies, and the β-ratios are low. In Case 15, the urine was almost free from sugar; the amount of acetone from aceto-acetic acid decreased to 11 mg. per day, while normal urine yields from 2 to 4 mg. [Scott-Wilson, 1911].

TABLE IV.

Cases 14 *and* 15. *Diabetes.*

	Date	Urine c.c.	Alveolar CO_2 mm. Hg.	G. acetone per day: Aceto-acetic acid + acetone	G. acetone per day: β-hydroxy-butyric acid	G. acetone per day: Total	β-ratio
Case 14.	13 v.	1240	36·1	0·347	0·627	0·974	64·4
	20 v.	1350	35·2	0·226	0·278	0·504	55·1
Case 15.	7 v.	1425	—	0·103	0·087	0 190	45·8
	20 v.	1580	38·7	0·0114	0·0077	0·0191	40·2

The largest change in the β-ratio within three days observed in this series of diabetics is that from 71·5 to 76·2 in Case 4.

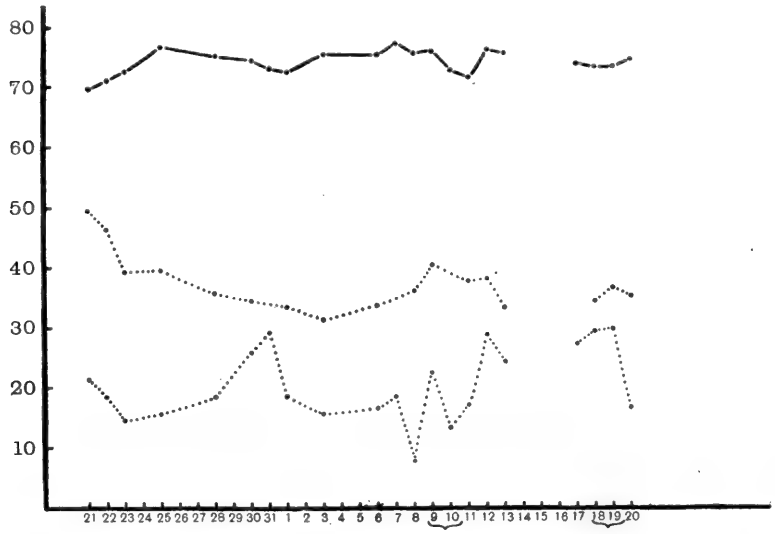

Fig. 2. Case 4 (Diabetes). Continuous line represents β-ratio. Upper dotted line represents alveolar CO_2 pressure in mm. Hg. Lower dotted line represents daily output of total acetone bodies as grams of acetone. The days on which the dose of sodium bicarbonate was doubled are enclosed in brackets.

Cases of Non-diabetic Acidosis.

In the first days of carbohydrate starvation in a healthy person no constancy of the β-ratio, such as is seen in cases of diabetes, is to be expected, since the degree of acidosis and the whole state of metabolism are changing

rapidly. The ratio rises with the increasing formation of acids, and may reach the value seen in cases of severe diabetes (Table V and Fig. 3).

TABLE V.

Acidosis due to lack of carbohydrate in healthy persons.

	Urine c.c.	Alveolar CO_2 mm. Hg	G. acetone per day: Aceto-acetic acid + acetone	G. acetone per day: β-hydroxy-butyric acid	G. acetone per day: Total	β-ratio
Case 16.						
Diet of protein and fat (Graham)						
2nd day	700	33·4	0·280	0·303	0·583	52·0
3rd day	1310	30·8	0·754	1·539	2·293	67·0
Case 17.						
Diet of protein and fat (Poulton)						
2nd day	670	38·3	0·402	0·521	0·923	56·4
3rd day	1070	37·0	0·446	0·556	1·002	55·4
Case 18.						
Diet of protein and fat (Donaldson)						
2nd day	758	36·5	0·437	0·299	0·736	40·7
Case 19.						
Starvation (Poulton)						
1st day	410	—	0·087	0·101	0·188	53·8
2nd day	1230	36·1	0·584	1·007	1·591	63·3
3rd day	730	35·3	0·539	1·383	1·922	72·0
Case 20.						
Starvation (Graham)						
2nd day	1240	36·1	0·477	0·692	1·169	59·2
3rd day	1680	33·0	0·527	0·905	1·432	63·2
Diet of fat						
4th day	1300	32·5	0·462	1·126	1·588	70·9
5th day	1390	—	0·882	2·638	3·520	74·9

Cases 21, 22, and 23 (Table VI) show low ratios with the excretion of very small amounts of acetone bodies. Some of the samples obtained from cases of vomiting (24 to 28) in young children were very similar as regards percentage and proportions of acetone bodies to the urine in severe forms of diabetes. Case 24 was subject to attacks of cyclical vomiting, in which condition well-marked acid intoxication occurs, but whether the illness on this occasion was of that nature was uncertain. Case 26 showed a condition of hyperpnoea and drowsiness such as is seen before the onset of coma in diabetes; the administration of dextrose and sodium bicarbonate had no beneficial effect, and the child died on the fifth day of the illness. The volume of urine was of course small as a result of the vomiting, and the percentages of acetone

bodies in this case are the highest in the whole series. Unfortunately the 24 hours output could not be ascertained. Cases 27 and 28 (Table VII) show

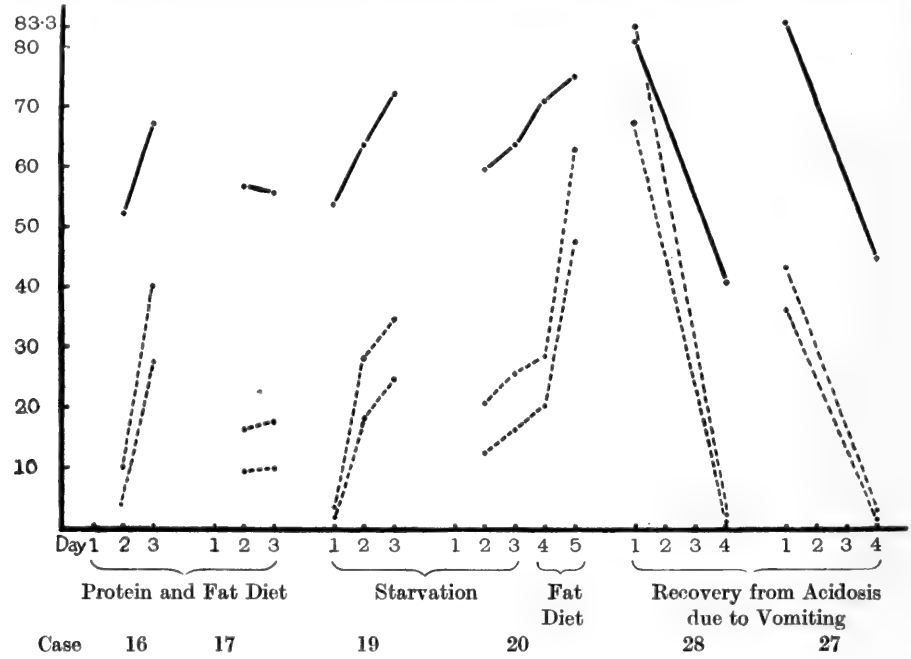

Fig. 3. Cases of non-diabetic acidosis. Continuous line represents β-ratio. Lower dotted line represents day's output of β-hydroxybutyric acid in decigrams. Upper dotted line represents day's output of β-hydroxybutyric acid + aceto-acetic acid in decigrams.

TABLE VI.

			G. acetone in 100 c.c. urine			
			Aceto-acetic acid + acetone	β-hydroxy-butyric acid	Total	β-ratio
Case 21. Carcinoma of Oesophagus						
after about 36 hours fasting			0·016	0·0094	0·0254	37·0
4 hours later			0·0147	0·0123	0·027	45·6
Case 22. After anaesthesia			0·0181	0·0083	0·0264	31·6
Case 23. „ „			0·054	0·077	0·131	58·8
Case 24 (age 2 years). Vomiting			0·058	0·149	0·207	71·5
Case 25 (age 5 years). „			0·139	0·468	0·607	77·1
Case 26 (age 22 months). „ ..	19 vi.		0·193	0·766	0·959	80·0
	21 vi.		0·316	0·925	1·241	74·6
	Death on 22 vi.		—	—	—	—
Urine at autopsy			0·357	0·956	1·313	[72·8]
		Urine c.c.	Amounts in 24 hours			
Case 27 (age 7 years). Vomiting	22 v.	520	0·393	2·033	2·42	83·8
Recovery	25 v.	550	0·086	0·069	0·155	44·6
	27 v.	550	trace	trace	—	—
Case 28 (age 6 years). Vomiting	24 vi.	575	0·917	3·76	4·67	80·4
Recovery	27 vi.	1210	0·062	0·042	0·104	40·7

the fall in the β-ratio which occurs when the total amount of acetone bodies decreases (Fig. 3). The high proportion of β-hydroxybutyric acid in these cases is noteworthy. Case 28 excreted on one day 0·3 g. acetone bodies per kilo. body-weight (15·5 kilos.); in a patient with severe diabetes, excreting 35 g. acetone bodies and weighing 70 kilos., the output would be 0·5 g. per kilo.

Discussion of Results.

The cases of non-diabetic acidosis show much the same range of β-ratio (from 31·6 to 83·8) as do the cases of diabetes (from 40·2 to 83·6). The value 31·6 indicates about one molecule of β-hydroxybutyric acid to two of aceto-acetic acid, while a ratio of 83·6 corresponds to a proportion of about five molecules of the former to one of the latter acid. These are then the extreme limits of composition of the mixtures observed in this series.

The results given in the tables above show that the higher β-ratios occur in the cases excreting the larger total amounts of acetone bodies, but there is no approach to any close parallelism between the two sets of figures. The data given here are of course much too few for the establishment of any rules, but they indicate that a person excreting daily acetone bodies yielding more than about one and a half grams of acetone produces two or more than two molecules of β-hydroxybutyric acid to one of aceto-acetic acid (Table VII).

TABLE VII.

		β-ratio	Daily output of acetone bodies as grams acetone	Alveolar CO_2 mm. Hg.
Diabetes.	*Case* 3	mean 79·1	mean 26·0	—
„	„ 5	„ 76·7	„ 21·2	mean 26·4
„	2	„ 74·7	„ 23·3	—
„	„ 6	„ 74·4	„ 18·2	—
„	„ 4	„ 74·1	„ 20·3	„ 37·3
„	„ 1	„ 68·2	„ 2·4	—
„	„ 14	„ 59·7	„ 0·74	„ 35·6
„	„ 15	„ 43·0	„ 0·104	„ 38·7
Results of all estimations on 24 hours urine.				
Diabetic		over 75	„ 20·5	„ 32·4
		75 to 66	„ 17·8	„ 35·6
		below 66	„ 0·42	„ 36·7
Non-diabetic		over 66	„ 2·73	„ 32·9
		below 66	„ 0·78	„ 35·8
All cases		over 66	maximum 34·2	—
			minimum 1·42	—
		below 66	maximum 1·59	—
			minimum 0·019	—

A shifting of the equilibrium point in the direction of lessened oxidation along with increase in the total production of acetone bodies is evident in Fig. 3, which shows the rapidly developing acidosis in the cases of carbohydrate starvation, while the reverse change is seen in recovery from attacks of vomiting.

The cases of severe diabetes behave somewhat differently in this respect. The results tabulated under Cases 4 and 6 show that fluctuations of 100 % or more in the amounts of acetone bodies produced leave the β-ratio almost unaffected[1]. In these conditions of persistent acidosis, the two acids seem to be produced in very constant proportions which are unaffected by large variations in their total amounts.

One might suppose that the higher proportion of β-hydroxybutyric acid in the urine in the cases of more severe acidosis was due to the washing-out of acetone from the blood into the expired air by the increased lung-ventilation which occurs in such cases [Beddard, Pembrey, and Spriggs, 1908]. If this were the whole explanation, the β-ratio should show an inverse relationship to the alveolar CO_2 pressure, the former rising as the latter falls. The cases given in the tables show that on the whole the higher β-ratios do accompany the lower CO_2 pressures (Table VII), but in detail the relation between the two quantities is very irregular, and there are some quite definite exceptions. Thus Case 4 (diabetes) showed an alveolar CO_2 pressure within or even above normal limits at a time when the β-ratio was above 70, and in Case 19 (starvation) the ratio rose on the third day to about this level, while the alveolar CO_2 was but slightly lowered. Changes in lung-ventilation do not then provide a sufficient explanation of the variations in the proportions of acetone bodies in the urine.

The rise in the β-ratio with increasing acidosis can hardly be due to a shortage of oxygen. If one atom of oxygen were required to convert one molecule of β-hydroxybutyric acid into aceto-acetic acid, the amount of oxygen required to lower the β-ratio for the fifth day in Case 20 (Table V) from the actual value of 75 to 33, would be about 280 c.c., an amount which would be absorbed in less than a minute. Moreover the production of the acid itself increases the supply of oxygen to the lung, by action on the respiratory centre.

The increase in the proportion of β-hydroxybutyric acid when the total amount of the two acids rises suggests that it is the hydroxy-acid which is

[1] Magnus-Levy [1908] has drawn attention to the large variations in the output of acetone bodies which occur even when all the conditions of diet and treatment are kept as constant as possible

the primary product; the evidence upon this point obtained in other ways is not sufficient to decide this question [Dakin, 1912].

The expenses of this work were defrayed by a grant from the Science Committee of the British Medical Association.

Summary.

Estimations are given of the relative amounts of β-hydroxybutyric acid and aceto-acetic acid in the urine in conditions of acidosis. The cases of diabetes show the constancy in the proportions of the two acids to which Neubauer drew attention. In carbohydrate starvation the proportion of β-hydroxybutyric acid rises with the increasing formation of acetone bodies. The estimations on cases excreting more than about two and a half grams of the two acids daily indicate the production of one molecule of aceto-acetic acid to from two to five molecules of β-hydroxybutyric acid.

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XLII. SOME NEW PHYSIOLOGICALLY ACTIVE DERIVATIVES OF CHOLINE.

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A short time ago I described [Ewins, 1914, 1] the isolation from extract of ergot, of a new active principle which was identified as acetyl-choline. The action of this base was shown by Dale [1914] to be closely related to that of muscarine, the active principle of the fungus *Amanita muscaria*, to which Harnack [1875] assigned the formula $C_5H_{15}O_3N$, and suggested that it was an oxidation product of choline. "Synthetic" muscarine (pseudo-muscarine), originally obtained by Schmiedeberg and Harnack [1877], and considered by them to be identical with muscarine, but shown by later observers to differ from natural muscarine in physiological action, I have recently shown to be the choline ester of nitrous acid [Ewins, 1914, 2]. The constitution of the natural muscarine, however, still remained (and remains) unsolved, but from the fact that acetyl-choline and pseudo-muscarine, both of which are choline esters, very closely resemble the natural base in physiological action, we were led to suppose that muscarine might also be an ester of choline. Some support was given to this idea when we found that the nitric acid ester of choline, originally prepared by Schmidt and Wagner [1904], still more closely resembled muscarine in its action. It was found, however, that the action of extracts of *Amanita muscaria* was not appreciably lessened by boiling with dilute acid or alkali, so that it seems highly improbable that the natural base is a choline ester.

Since the amount of *Amanita muscaria*[1] at our disposal was too small to admit of isolation of the natural base in a state of purity, or in any quantity,

[1] For an extract of *Amanita muscaria* we are indebted to Dr O. Rosenheim, and for a quantity of dried material to Prof. W. Wiechowski, to both of whom we wish to render our best thanks.

I prepared a number of the simpler choline derivatives which might possibly give a clue to the constitution of the natural base. So far the investigation has not led to the identification of muscarine itself, but certain of the derivatives prepared were of some considerable interest, the ethyl ether of choline $OH . N(CH_3)_3 . CH_2 . CH_2 . O . C_2H_5$ in particular being very closely related indeed in its action to that of the natural muscarine. The bases were in all cases physiologically examined by Dr H. H. Dale, whose results will be published shortly.

The following is a table of the substances examined :

(1)	Acetyl-choline	$OH . N(CH_3)_3CH_2CH_2O . OCCH_3$.
(2)	*Formyl-choline*	$OH . N(CH_3)_3CH_2CH_2O . OCH$.
(3)	Lactyl-choline	$OH . N(CH_3)_3CH_2CH_2O . CHCH_3$. | $OH . N(CH_3)_3CH_2CH_2O . CO$.
(4)	Choline nitrous acid ester	$OH . N(CH_3)_3CH_2CH_2O . NO$.
(5)	Choline nitric acid ester	$OH . N(CH_3)_3CH_2CH_2O . NO_2$.
(6)	Choline methyl ether ..	$OH . N(CH_3)_3CH_2CH_2O . CH_3$.
(7)	Choline ethyl ether ..	$OH . N(CH_3)_3CH_2CH_2O . C_2H_5$.
(8)	*Choline propyl ether* ..	$OH . N(CH_3)_3CH_2CH_2O . C_3H_7$.
(9)	*Choline ether*	$OH . N(CH_3)_3CH_2CH_2O . CH_2CH_2N(CH_3)_3OH$.
(10)	Trimethyl-β-bromo-ethyl-ammonium bromide	$BrN(CH_3)_3CH_2CH_2Br$.
(11)	Trimethyl-β-chloro-ethyl-ammonium chloride	$ClN(CH_3)_3CH_2CH_2Cl$.
(12)	*Trimethyl-β-cyano-ethyl-ammonium hydroxide*	$OH . N(CH_3)_3CH_2CH_2CN$.
(13)	*Trimethyl-β-amino-ethyl-ammonium hydroxide*	$OH . N(CH_3)_3CH_2CH_2NH_2$.
(14)	*Dimethyl-oxazolium hydroxide*	$(OH)\ N(CH_3)_2—CH_2$ | | . $CH_2 . CH_2 . O$
(15)	*Formocholine methyl ether*	$OH . N(CH_3)_3CH_2OCH_3$.
(16)	*Formocholine ethyl ether*	$OH . N(CH_3)_3CH_2OC_2H_5$.
(17)	*Formocholine propyl ether*	$OH . N(CH_3)_3CH_2OC_3H_7$.
(18)	*Formocholine butyl ether*	$OH . N(CH_3)_3CH_2OC_4H_9$.

All of these bases showed considerable physiological activity, those approaching most nearly to muscarine being choline ethyl ether, choline nitric acid ester, and trimethyl-β-amino-ethyl ammonium hydroxide. The curare-like action of all of these is, however, more pronounced than in the case of natural muscarine.

The ethers of formocholine were found to possess an action closely resembling that of the corresponding choline ethers, but were quantitatively much less powerful. Formocholine propyl ether was found to be the most active of the series whereas the isomeric choline ethyl ether was the most active of the choline ethers.

The compounds printed in italics in the above table are new substances. Their preparation and properties are described below. The remaining bases were obtained by the methods previously employed by other workers, notably Schmidt and Wagner [1904] and Nothnagel [1894].

Experimental.

(*a*) *Choline derivatives.*

Formyl-choline, $OH . N(CH_3)_3CH_2CH_2O . OCH$. The base was obtained by boiling a solution of choline chloride in formic acid (D 1·22) under reflux for three hours. The excess of formic acid was removed by distillation *in vacuo*. The residue was dissolved in absolute alcohol and converted into the platinichloride by precipitation with an alcoholic solution of platinic chloride. The precipitate was filtered off and crystallised from hot water in which it is readily soluble. On standing, the crystalline *platinichloride* was obtained in orange-red octahedra. M.p. 255–256°.

0·1111 ; 0·0840 CO_2 ; 0·0450 H_2O. C = 20·6 ; H = 4·5.
0·1087 ; 0·0313 Pt. Pt = 28·8.
Calculated for $(N(CH_3)_3CH_2CH_2O . OCH)_2PtCl_6$. C = 21·4 ; H = 4·2 ; Pt = 29·0.

The *aurichloride* crystallised from hot water in thin golden yellow hexagonal plates. M.p. 175°.

0·1168 ; 0·0488 Au ; Au = 41·8.
Calculated for $N(CH_3)_3CH_2CH_2O . OCH . AuCl_4$; Au = 41·8.

Choline propyl ether, $OH . N(CH_3)_3CH_2CH_2O . C_3H_7$. This was obtained by acting on β-iodo-ethyl propyl ether [Karvonen, 1909] with alcoholic trimethylamine solution at the ordinary temperature. The crystalline iodide of choline propyl ether separated on standing, and was filtered off, converted into the chloride in the usual manner and then into the *platinichloride*, which was obtained crystalline from hot aqueous solution.

Orange red prisms. M.p. 246°.

0·1205 ; 0·1197 CO_2 ; 0·0626 H_2O ; C = 27·1 ; H = 5·75.
0·0922 ; 0·0262 Pt ; Pt = 28·4.
Calculated for $(C_8H_{20}ON)_2PtCl_6$. C = 27·4 ; H = 5·7 ; Pt = 27·9.

Choline ether, $OH . N(CH_3)_3CH_2CH_2O . CH_2CH_2(CH_3)_3N . OH$. ββ-Diiodo-ethyl ether [Sand, 1901] was treated with a slight excess of alcoholic trimethylamine solution at the ordinary temperature. On standing, the crystalline choline ether iodide rapidly separated, and was filtered off after about twenty-four hours.

The *iodide* crystallises from 95 per cent. alcohol in thin rectangular plates melting at 275°. The salt is very readily soluble in water, but sparingly soluble in absolute alcohol.

0·0736 ; 0·0781 Ag I ; I = 57·3.
Calculated for $C_{10}H_{26}ON_2I_2$; I = 57·2.

The *chloride* forms rhombic plates melting above 280°.

The *platinichloride* crystallises from water in clusters of small prisms melting at 226° (with decomposition). It is sparingly soluble in cold water and almost insoluble in alcohol.

0·1680 ; 0·0534 Pt ; Pt = 31·8.
Calculated for $(C_{10}H_{26}ON_2)$ Pt Cl_6 ; Pt = 32·6.

The *aurichloride* is very sparingly soluble in hot water. On cooling it separates as golden yellow rhombic plates melting at 269°.

0·1300 ; 0·0591 Au ; Au = 45·46.
Calculated for $C_{10}H_{26}ON_2 . 2AuCl_4$; Au = 45·34.

Trimethyl-β-cyano-ethyl-ammonium hydroxide, $OH . N(CH_3)_3CH_2CH_2CN$. β-Chloropropionitrile, $ClCH_2CH_2CN$ [Henry, 1898] was treated with an alcoholic solution of trimethylamine. On standing at the ordinary temperature, the crystalline chloride of the base separated.

The *chloride* was recrystallised from hot absolute alcohol in which it is fairly soluble, whilst it is very little soluble in cold alcohol. The salt separates as prisms melting at 228–229° (with decomposition) and is hygroscopic.

0·1329 ; 0·1272 Ag Cl ; Cl = 23·7.
0·1329 ; 20·8 c.c. N_2 at 19° and 765 mm. ; N = 18·2.
Calculated for $C_6H_{13}N_2Cl$; Cl = 23·9 ; N = 18·8.

The *platinichloride* crystallises from hot water, in which it is moderately easily soluble, in brownish yellow aggregated octahedra, melting at 249°–250° with decomposition.

0·1728 ; 0·0542 Pt ; Pt = 31·3.
Calculated for $(C_6H_{13}N_2)_2PtCl_6$; Pt = 30·8.

The *aurichloride* separates from a hot aqueous solution as thin golden yellow needles melting at 213–214°.

0·1376 ; 0·0601 Au ; Au = 43·7.
Calculated for $C_6H_{13}N_2 . AuCl_4$; Au = 43·6.

Trimethyl-β-amino-ethyl-ammonium hydroxide, $OH . N(CH_3)_3CH_2CH_2NH_2$. This compound was obtained by heating choline nitric acid ester with an alcoholic solution of the calculated amount of ammonia, for several hours at 100°. The solution was then evaporated to dryness, dissolved in a little water and precipitated with a slight excess of an aqueous solution of gold chloride. The precipitated *aurichloride* was filtered off and crystallised from hot, very dilute, hydrochloric acid solution. On cooling, the salt separated as yellowish brown rectangular prisms melting at 263°, very little soluble in cold, moderately soluble in hot water.

0·0999 ; 0·0507 Au ; Au = 50·7.
Calculated for $C_5H_{15}N_2 . 2AuCl_4$; Au = 50·5.

N-Dimethyloxazolinium hydroxide, $OH(CH_3)_2N{-}CH_2{-}CH_2$ with CH_2 bridging to O. This substance was obtained in an attempt to prepare an oxy-choline, the constitution of which would be denoted by the formula

$$OH(CH_3)_2N\begin{matrix}\diagup CH_2CH_2OH \\ \diagdown CH_2OH\end{matrix}.$$

On analysis, however, the compound obtained was found to contain one molecule of H_2O less than required by the above formula ; hence it is concluded that ring formation has occurred. This is supported by the fact that heating the chloride of the base with acetyl chloride at 100° for 3–4 hours leaves the compound unchanged. Under similar conditions choline is readily acetylated. The compound was obtained as follows. Dimethylaminomethyl alcohol was prepared by the method due to Henry [1898] and was then treated with one molecular proportion of ethylene chlorohydrin. A vigorous reaction ensued, so that the reaction mixture had to be cooled from time to time during addition of the chlorohydrin. The product was allowed to stand for some time, and then dissolved in a little absolute alcohol. The solution was precipitated by alcoholic mercuric chloride, the very voluminous precipitate filtered off, and crystallised from a large volume of hot water. The crystalline mercury compound was then suspended in water and decomposed by sulphuretted hydrogen. The filtrate was evaporated to dryness, and from the residue, which showed indications of crystallising, the following salts were obtained. The *platinichloride* was readily crystallised from hot water, in which it is moderately soluble (but much less soluble than the corresponding salt of choline). It was obtained as orange red rectangular prisms, melting at 237–238°.

0·1750 ; 0·1257 CO_2 ; 0·0662 H_2O ; C = 19·6 ; H = 4·2.
0·1151 ; 0·0817 CO_2 ; 0·0385 H_2O ; C = 19·36 ; H = 3·7.
0·2134 ; 0·0685 Pt ; Pt = 32·1.
Calculated for $(C_5H_{12}ON)_2\,PtCl_6$; C = 19·6 ; H = 3·9 ; Pt = 31·8.

The *aurichloride* is sparingly soluble in cold, moderately soluble in hot water. It forms golden yellow prisms melting at 279°.

0·1267 ; 0·0567 Au ; Au = 44·8.
Calculated for $C_5H_{12}ON\,AuCl_4$; Au = 44·7.

The *mercuric chloride compound* crystallises from water in hexagonal prisms melting at 244–245°. It appears to have the formula $C_5H_{12}ON.2HgCl_2$.

(*b*) *Formo-choline derivatives.*

Formo-choline itself was obtained by Schmidt and Litterscheid [1904] who were, however, unable to obtain either acetyl, benzoyl, or lactyl derivatives. Attempts by the writer to prepare the nitric ester of the base by methods similar to those employed for the preparation of the corresponding choline ester proved unsuccessful. It was found, however, that the ethers of formo-choline could readily be obtained by the action of trimethylamine on the corresponding iodomethyl ethers.

Formo-choline methyl ether, $OH . N(CH_3)_3CH_2O . CH_3$. Iodomethyl ethyl ether $I . CH_2O . CH_3$ was prepared according to the method employed by Henry [1893]. For purification it was washed with a very little ice water, dissolved in ether and dried over $CaCl_2$. The ether was removed and the residue distilled *in vacuo*. It boiled constantly at 39° under 20 mm. pressure.

To the ethereal solution of the iodo-ether was added alcoholic trimethylamine, the mixture being kept cool by immersion in ice. A crystalline product consisting of a mixture of formo-choline methyl ether iodide, and trimethylamine iodide, separated immediately. The mixture was suspended in cold absolute alcohol. The bulk of the trimethylamine iodide remained undissolved and was filtered off. The filtrate was evaporated to dryness, and the residue crystallised from a little alcohol by addition of ether. Formo-choline ether iodide was thus obtained as white glistening hygroscopic plates, melting at 84°.

0·1421 ; 0·1428 Ag I ; I = 54·5.
Calculated for $CH_3OCH_2N(CH_3)_3I$; I = 54·98.

The *platinichloride* crystallised from water in columnar aggregates of orange-red rhombic prisms, melting with decomposition at 234°. The salt was anhydrous and sparingly soluble in cold water, but readily soluble in hot water.

0·1028 ; 0·0322 Pt ; Pt = 31·3.
Calculated for $(C_5H_{14}ON)_2PtCl_6$; Pt = 31·6.

The *aurichloride* is moderately soluble in hot water, sparingly in cold. It crystallises in golden yellow needles melting at 135–136°.

0·1212 ; 0·0536 Au ; Au = 44·2.
Calculated for $C_5H_{14}ON\ AuCl_4$; Au = 44·4.

Formo-choline ethyl ether, $OH . N(CH_3)_3CH_2O . C_2H_5$. Iodo-methyl ethyl ether, $I . CH_2O . C_2H_5$, was prepared by a method analogous to that employed by Henry to obtain the corresponding methyl ether. On distillation *in vacuo*

a liquid was obtained which boiled constantly at a temperature of 44° at 30 mm. The product, however, on analysis was found to be still somewhat impure, but owing to the highly reactive nature of the liquid it was not further purified, but treated at once with trimethylamine, and the product worked up in the same manner as described for the corresponding methyl ether.

Formo-choline ethyl ether iodide was thus obtained as thin lustrous deliquescent plates, melting at 94°.

0·1100 ; 0·1066 AgI ; I = 52·3.
Calculated for $C_2H_5OCH_2N(CH_3)_3I$; I = 51·8.

The *platinichloride* crystallised from hot water in large regular crystals orange-red in colour and melting at 241–242°. It is moderately soluble even in cold water, but quite insoluble in alcohol.

0·1052 ; 0·0321 Pt ; Pt = 30·5.
Calculated for $(C_6H_{16}ON)_2PtCl_6$; Pt = 30·3.

The *aurichloride* crystallised from water in golden yellow leaflets melting at 138–139°.

0·1426 ; 0·0618 Au ; Au = 43·3.
Calculated for $C_6H_{16}ONAuCl_4$; Au = 43·1.

Formo-choline propyl ether, $OH . N(CH_3)_3CH_2O . C_3H_7$. *Iodomethyl propyl ether* $I . CH_2O . C_3H_7$ (b.p. 61–62° at 28 mm.) was obtained by the method employed in the previous preparations, treated with trimethylamine, and the product worked up as before.

Formo-choline propyl ether iodide was thus obtained crystalline from alcohol by addition of ether as long fine needles melting at 108°. The salt was not very deliquescent.

0·1201 ; 0·1112 AgI ; I = 50·0.
Calculated for $C_3H_7OCH_2N(CH_3)_3I$; I = 49·0.

The *platinichloride* was obtained crystalline from hot water, in which the salt is very easily soluble. It is insoluble in alcohol and very little soluble in cold water ; long thin orange-red needles melting at 236–237°.

0·1165 ; 0·0342 Pt ; Pt = 29·35.
Calculated for $(C_7H_{18}ON)_2PtCl_6$; Pt = 29·0.

The *aurichloride* is only moderately soluble even in hot water. It crystallises in golden yellow leaflets melting at 114°.

0·1094 ; 0·0460 Au ; Au = 42·0.
Calculated for $C_7H_{18}ONAuCl_4$; Au = 41·6.

Formo-choline butyl ether, $OH . N(CH_3)_3CH_2O . C_4H_9$, was prepared by the action of trimethylamine on iodomethyl butyl ether $I . CH_2O . C_4H_9$ (b.p.

78–80° at 20 mm.). The *iodide* crystallised in glistening colourless plates melting at 98°.

0·1812 ; 0·1610 AgI ; I = 48·0.
Calculated for $C_8H_{20}ONI$; I = 46·5.

The *platinichloride* crystallises from hot water as orange-red octahedra melting at 243–244°. It is insoluble in alcohol, sparingly soluble in cold, but fairly readily soluble in hot water.

0·0992 ; 0·0276 Pt ; Pt = 27·7.
Calculated for $(C_8H_{20}ON)_2PtCl_6$; Pt = 27·8.

The *aurichloride* is little soluble in cold water, moderately so in hot. It crystallises in golden yellow leaflets melting at 81°.

0·1110 ; 0·0450 Au ; Au = 40·5.
Calculated for $C_8H_{20}ONAuCl_4$; Au = 40·6.

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XLIII. ON THE RESPIRATORY EXCHANGE IN FRESH WATER FISH.—Part I: ON BROWN TROUT.

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In the course of an inquiry, commenced some time ago by one of us, on the toxic effects of various impurities in river water on fish life, data were required on the relative amounts of oxygen absorbed by different varieties of fresh water fish, on the least quantities of dissolved oxygen compatible with healthy fish life, and on the influence of temperature on these quantities. On searching through the literature we were unable to find the systematic information required, and though a number of observations had been made with carp, perch, roach, etc., respiratory exchange had been mainly studied on marine animals. We therefore decided to investigate systematically the common British fresh water fish, and in this paper we give an account of some of our experiments with brown trout (*S. Fario*).

The respiration of fishes was first studied by G. Humboldt and Provençal [1807]. The fishes were placed in a flask of water, the gaseous contents of which had been analysed, and then after an interval a sample of the water was examined and the alteration in its gases determined. The quantity of water was measured, and thus it was possible to estimate the amount of gases absorbed and discharged by fish. A similar method has been used by Vernon [1896] for the measurement of the respiratory exchange in marine invertebrates. Vernon was the first we believe to make use of pumps to determine the oxygen, nitrogen and carbon dioxide. Baumert [1855] improved Humboldt and Provençal's method by passing a stream of water through the flask containing the animals; the gases contained in samples of the water entering and leaving the flask were determined. In recent years a somewhat similar method has been made use of by August Pütter [1908, 1909].

The first elaborate apparatus for measuring the respiratory exchange of fish was devised by Jolyet and Regnard [1876], and was based on the principle of the well-known apparatus of Regnault and Reiset for land animals. This apparatus in improved form has also been made use of by J. P. Baunhiol [1905]. The largest and most complicated apparatus is that devised by Zuntz [1901], who also describes an ingenious apparatus for automatically supplying oxygen as it is used up by the fish.

The method we employed does not differ much in principle from those already used by various observers.

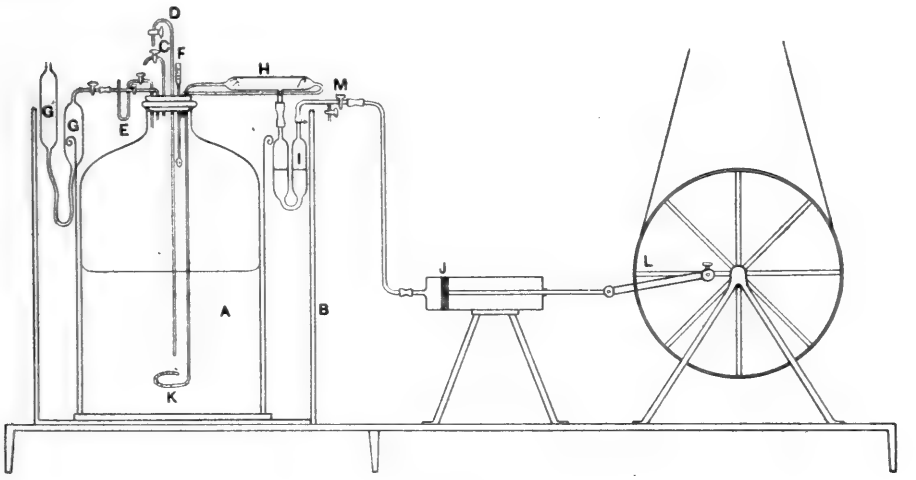

Fig. 1.

The animals were placed in a hermetically closed vessel containing known volumes of water and air, both of which were carefully analysed at the beginning of the experiment. The air was then pumped through the water continuously for a definite period, the oxygen absorbed by the animal being gradually replaced from a reservoir. At the end of the period the air and water were again analysed. The differences in carbon dioxide and oxygen content give the oxygen used by the animal and the carbon dioxide produced in the given time. The apparatus used is simpler than some of the more complex forms described and presents points of novelty.

Apparatus employed.

The apparatus is shown in diagrammatic form in Fig. 1. The receiver *A*, in which the animal was placed, consisted of a large glass bottle of some

50 litres capacity, and with a neck sufficiently large to admit of the animals being easily introduced. This was fixed on a lead base sufficiently heavy to sink it in the water of the thermostat, and provided on the outer edge with stout upright steel rods, which served the double purpose of keeping the bottle firmly in position and affording convenient handles for lifting the apparatus. The bottle was carefully calibrated by weighing it empty and full of water on a delicate machine capable of reading to a gram. The bottle was placed in a thermostat consisting of a large wooden tub *B* containing water. The temperature of the water in the tub was regulated by means of ice or warm water circulating in a coil of pipe, or by an electric lamp—whichever happened to be most convenient for any particular experiment. The water was stirred by bubbling air through from a cylinder. These accessories are not shown in the diagram for the sake of clearness.

The neck of the bottle was closed by a stout rubber bung, through which passed (1) a capillary tube *C*, for withdrawing samples of air, fitted with a three-way tap, so that the capillary could be filled with mercury ; (2) a capillary tube *D* for withdrawing samples of water, also fitted with a tap and reaching to the middle of the water in the bottle ; (3) a delicate manometer *E* which could be closed by a tap, shown diagrammatically; (4) a thermometer *F*, of the kind used in incubators, for recording the temperature of the air ; (5) a tube leading to the oxygen reservoir *G* ; (6) two tubes communicating with the pumping mechanism *H*, *I* and *J*. One of these tubes connected the end of the valve chamber *H* with the air in the bottle and the other, connected to the opposite end of *H*, reached to the bottom of the water and terminated in a perforated glass spray *K*. The mechanism for pumping the air in the bottle *A* through and through the water consisted of a cylindrical valve chamber *H* of about 80–100 cc. capacity containing two valves opening as indicated. Each valve was made by a disc of thin sheet rubber fitting over a brass nose, and proved very effective. The body of the valve chamber was connected to one limb of a large U-tube of the shape indicated containing mercury, the other limb of which was connected by a brass tube to an accurately made single action brass pump *J*. By means of this pump the mercury was made to rock up and down in the U-tube, and by means of the valves in *H* ultimately sucked air from the upper part of *A* and forced it through the spray *K* in the lower part. The amount of displacement of mercury was regulated by the length of stroke of the pump, which was varied by clamping the end of the connecting rod further from or nearer to the axis of the wheel *L*. The wheel *L* was geared by means of a reducing pulley

(not shown in diagram) to main shafting. In most experiments it was found convenient to arrange so that the piston of the pump gave 25 strokes per minute, each stroke displacing approximately 100 cc. of mercury in the U-tube. The side tube *M*, fitted with a tap, afforded a convenient fine adjustment. The oxygen reservoir *G* consisted of an accurately measured tube containing the amount of oxygen required in any particular experiment. Reservoirs of various sizes were provided. The oxygen can be as a rule most conveniently delivered by hand, by raising the companion tube *G'* containing water and opening the tap, a few cc. at a time at regular intervals, or if desired the tap can be left open and the oxygen delivered continuously at any desired rate by dropping water into *G'* by means of a water clock or other arrangement. Usually, however, it was found quite satisfactory to do this by hand.

The volume of the gas in the valve chamber *H*, with its various tubes, and in the left hand limb of the U-tube *I*, when the mercury was drawn, as described later, to a certain mark *Z* on the narrow upper part of the right hand limb, was accurately determined, and also the volume of the glass of the various tubes dipping into the bottle.

In performing an experiment, the bottle *A* was weighed, and water poured in and its volume determined by weighing again. The bottle was then placed in the thermostat and left for the temperature to adjust itself. The temperature of the water was then read, also that of the thermostat, which should be approximately the same. The fish to be experimented on were then taken from their tank, washed with pure water and weighed. They were then put into the bottle. If the temperature of the water in the tank is much different from that to be used in the experiment, the temperature of the fish must be slowly brought to that required in the experiment while in the pure water serving to wash them. This precaution seems to be very necessary. The stopper was then adjusted as in the diagram, and samples of air and water withdrawn from *C* and *D* by displacement of mercury. The apparatus for collecting the sample of water is given in Fig. 2. It consisted of a cylindrical vessel *a* fitted with a three-way tail tap at *b*, and at the other end with a capillary delivery tube *c*: *a* and its tubes were filled with mercury and connected at *b* with the tube *D* of the fish bottle. By suction through the tail of the tap *b* the capillaries were filled with the water and the taps adjusted. The bottle *d* was then fixed to the delivery tube *c* by a rubber tube and by sucking gently at *e* the mercury flowed from *a* and was displaced by the water from the fish bottle. The sampling tube *a* was

accurately calibrated and thus a known sample of the water was obtained quite free from air bubbles and protected from contact with air. After taking samples, the tap *D* (Fig. 1) was closed, and by suction at the tap *M* by means of a small hand pump (not shown) the mercury in *I* was drawn to the point *Z*. The manometer tap was opened to see that the gas in the bottle was at atmospheric pressure, and the tap of the air tube *C* was closed. The thermometer *F* was then read, and the time taken. The pump *J* was now started, and the air of the bottle *A* sprayed through the water. During the experiment the oxygen used by the fish was renewed from *G* as described, care being taken that the pressure in *A* as recorded on the manometer remained approximately the same. At the end of the experiment the pump was stopped,

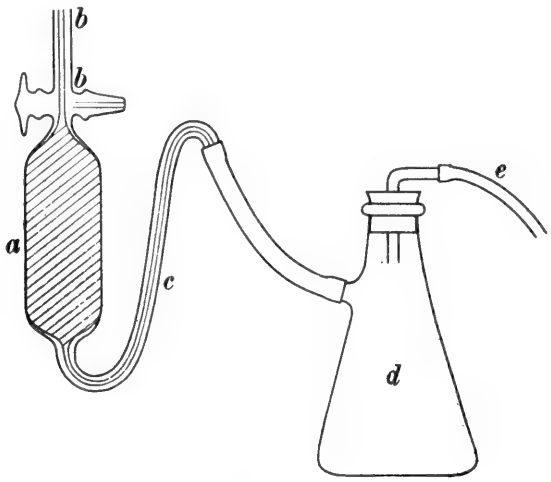

Fig. 2.

and the mercury in *I* sucked again to *Z* and the manometer read. The temperature recorded by *F* was again taken, and samples of air and water withdrawn as before. The time at which the water sample was taken was noted, and the temperature of the water in *A* and in the thermostat read. The gases in the water samples were pumped out by means of the modified form of Toepler pump without taps or joints, devised by Buckmaster and Gardner [1910] for blood-gas work. The samples of water were introduced into the pump through the barometer direct from the sampling tube (Fig. 2) by displacement of mercury in the manner described in the paper referred to. As it was found better to determine the whole of the carbon dioxide, free and combined, in the water samples, 10–15 cc. of 80 % phosphoric acid were

introduced into the pump before the water samples. This pump, with some further modifications introduced by Buckmaster and Gardner [1912], proved very suitable and convenient for this work, as it was not found necessary to cut the apparatus down after each experiment for cleaning purposes as in the case of blood work.

The gas analysis apparatus employed for the analysis of the air samples and the gases pumped from the water was the well-known form of Frankland, as modified by W. A. Bone.

Various difficulties were encountered in perfecting the apparatus, and of these we may mention one, an apparent "diffusion" of nitrogen through the rubber cork and rubber joints of the apparatus. This was more noticeable when there was any difference of nitrogen partial pressure on different sides of the rubber, as for instance in experiments in which oxygen, or mixtures of oxygen and nitrogen richer in oxygen than air, were used in the bottle. This was obviated by covering the upper and lower faces of the bung with tin foil, and by thickly painting the bung and all rubber joints with the acid proof enamel used by electricians for painting the connections of accumulators. We also found it very necessary to take the utmost care in the measurement of the temperature and pressure of the air in the bottle, and to arrange that the temperatures of the inner water and the thermostat were very nearly the same all through. In some of our earlier experiments we modified the chamber *H* by introducing a device by which the carbon dioxide could be absorbed from the air passing through, and thus the accumulation of carbonic acid in the water prevented. This carbon dioxide was afterwards estimated chemically. It was found however that comparatively little carbon dioxide was swept out of the water by the air current, and that we had merely introduced another estimation of somewhat doubtful accuracy with little if any beneficial effect. Further the experiments of Krepzis [1901], and also of Hans Winterstein [1908], have shown that fish such as perch and roach are not apparently affected by far larger quantities of this gas than were ever produced in our experiments. The procedure was therefore discontinued.

An example will make clear the method of calculation:

Weight of water taken 29526 g.; volume of air above the water at the beginning of experiment 17299·7 cc.; temperature 21·2° C., barometer 762·5 mm. The volume of dry air at 0° and 760 mm. was therefore 15710·9 cc. 13 fish were used weighing 300 grams and the experiment was commenced at 11.15 a.m.

A sample of the air taken for analysis measured 20·09 cm. at 20·42° and constant volume; after treatment with potash it measured 20·00 cm. at 20·40°; and after alkaline pyrogallate 15·86 cm. at 20·32°. The total air therefore contained 69·3 cc. of carbon dioxide, 3234·4 cc. of oxygen and 12407·2 cc. of nitrogen at 0° and 760 mm.

53·5 cc. of the water yielded to the pump 8·39 cm. of gas at 20·25° and constant volume (48·65 cc.); after potash the pressure was 1·53 cm. at 20·20° and after alkaline pyrogallate, 1·13 cm. at 20·20°.

The total volume of water (29526 cc.) therefore contained 2257·2 cc. of carbon dioxide (free and combined), 130·4 cc. of oxygen and 371·7 cc. of nitrogen at 0° and 760 mm.

The experiment was continued until 6.15 p.m., and during this time 278·96 cc. of commercial oxygen, measured at 21·2° and barometric height 762·5, were added. 13·97 cm. of this oxygen at 20·3°, after treatment with pyrogallate, measured 0·42 cm. at 20·3°. The dry gas added contained therefore 245·7 cc. and nitrogen 7·6 cc. at 0° and 760 mm. After the end of the experiment the volume of the gas above the water was 17299·7 cc., the temperature was 21·2° and the barometric height 762·5. The manometer showed a positive pressure of + 0·15 cm. The volume of the dry gas at 0° and 760 mm. was therefore 15698·1 cc.

11·9 cm. of the air at 20·88° measured after treatment with potash 11·74 cm. at 20·7°, and after alkaline pyrogallate 9·42 cm. at 20·8°.

The total volume of gas therefore contained 201·6 cc. of CO_2, 3066·5 cc. oxygen and 12429·9 cc. of nitrogen. 53·5 cc. of the water yielded to the pump 9·01 cm. gas at 20·8° and constant volume (48·65 cc.), which after treatment with potash measured 1·38 cm. at 20·7°, and after alkaline pyrogallate 1·09 cm. at 20·55°.

The total water therefore (29526 cc.) contained 2504·5 cc. CO_2, 95 cc. oxygen and 358·1 cc. nitrogen at 0° and 760 mm.

Adding therefore the volumes of carbon dioxide, oxygen and nitrogen together we get:

	At beginning of experiment	At end of experiment	Difference
CO_2 ..	2326·5	2706·1	+379·6 cc.
Oxygen ..	3610·5	3161·5	−449·0 „
Nitrogen ..	12786·5	12788·0	+ 1·5 „

$$\text{Oxygen consumed per kilogram per hour} = \frac{449}{0{\cdot}3 \times 7} = 213{\cdot}8 \text{ cc.}$$

$$\text{Oxygen consumed per fish per hour} = \frac{449}{13 \times 7} = 4{\cdot}63 \text{ cc.}$$

$$\text{Respiratory quotient} = \frac{379{\cdot}6}{449} = 0{\cdot}84.$$

Sources of error. In respiration experiments care should be taken that towards the end of the experiment there should be no oxygen deficiency. It is well-known that fish can stand a moderate diminution of the oxygen content of the water without harm, but it is not advisable to allow the oxygen to diminish during the experiment by more than one-third of its original value, and even this should be avoided if possible. It might be thought that a rapid spraying of the air, in which oxygen was continually renewed, through the water, as in our experiments, would effectively prevent this diminution of the oxygen content of the water. This was not however the case, as diffusion of a gas through water is a slow process. If the oxygen content of a bulk of water such as was used in our experiments is allowed to diminish, it takes a surprisingly long time before equilibrium is again established by even a very vigorous spray. It is important therefore not to place too many fish in a given bulk of water. The experiment quoted in detail above illustrates this difficulty. The number of fish was rather too large, and they used up the oxygen at a somewhat greater rate than could be counteracted by the spraying. Other and more serious sources of error might arise from the consumption of oxygen by organic matter and bacteria in the water, and bacteria introduced into the water by the bodies of fish. The observations of Knauthe [1898] on the oxygen destruction in water in which fish have lived lend strong support to this fear, and any investigation on the oxygen consumption of water animals must reckon with this error and either dispose of it in some way or estimate it quantitatively.

In a botanical metabolic investigation, for instance with germinating seeds, it might be necessary to collect the metabolic products during the course of, say, a week, and from analysis of such products argue as to the changes in the seed. In such a case, obviously, rigidly sterile conditions would be necessary. If, however, in an animal investigation, the necessity for such rigid conditions be postulated, no metabolic researches would be possible, since there are no germ-free animals. Fortunately in our experiments the duration was not so long, and in one, two or three hours and particularly at lower temperatures it did not seem likely that sufficient development of bacteria could take place to introduce any serious error into the results. This conclusion is borne out by the extended series of observations of August Pütter [1909], Zuntz [1901], and others.

To ascertain however, for ourselves, how far serious error due to these causes entered into our results, we made a number of blank experiments under conditions rigidly similar to those in our actual fish experiments, using

both fresh London tap water, and tap water in which trout had been living for from 1·5 to 2·5 hours. The figures are given in the following Table I. As the duration of different experiments varied considerably, the results are reduced to one hour.

TABLE I.

Kind of water	Temperature of water	Duration of expt. in hours	Oxygen lost or gained p. hr. in cc. at 0° and 760	Carbon dioxide lost or gained per hr. in cc. 0° and 760	Nitrogen lost or gained per hr. in cc. at 0° and 760
Tap	10°	3·5	−2·18	+3·94	− 1·03
Tap	15	4·7	−0·25	−7·32	− 6·60
Tap	16	4·5	−2·13	−5·67	− 0·11
Tap	9	2·75	+4·10	+5·21	−33·60
Tap	16·5	3·42	−5·30	+0·63	+13·00
Tap in which fish had lived	15	3·0	−0·07	−6·78	−23·44
„	10	4·17	−2·08	+1·50	+ 4·80
„	10	3·70	−3·30	−8·00	− 4·84
„	10	3·0	+1·97	—	+ 1·97
„	12	2·32	+3·70	−2·02	− 1·84

The differences should of course be nil, but a glance at the table will show that they are irregularly + and −, so that the source of error in question did not seriously affect our experiments. Nevertheless we adopted all the precautions we could think of to reduce such error. London water, as is well known, is very efficiently filtered and is remarkably free from bacteria, so much so that probably the bulk of those in a sample drawn from the tap are post-filtration organisms. It contains of course a good deal of organic matter in solution, but this appears to be in stable conditions, and on the whole it proved a very suitable medium for the work. In all experiments at summer temperatures we took the precaution to keep the water used in fully oxygenated condition for a day or two in closed vessels before use. The fish were always washed by being allowed to swim about in a considerable bulk of pure water before being placed in the experimental bottle.

The method of pumping out the gases from a small sample of water by means of the blood-gas pump and analysis by the method described proved very satisfactory, and the results of consecutive experiments were sensibly identical. The errors set out in the above table may appear to be very large, but this must of necessity be the case in metabolic experiments of this kind, however accurate the analytical methods adopted. A very minute error in the analysis of the gas from 50–100 cc. of water appears of considerable magnitude when the results are multiplied up to give the gas in 30 litres.

The errors in the oxygen and nitrogen we believe are largely due to the difficulty of determining the exact average temperature of the gas above the water. The carbon dioxide determinations, as will be seen from the table, are less satisfactory than those of oxygen. This is mainly due to the difficulty of estimating very exactly the small quantities of carbon dioxide in the air above the water. Further, in nearly all our experiments the second sample of water was put into the same pump which had been used for the first sample, and which still contained the pumped out water of that sample. The second sample was thus diluted, prior to pumping by its own volume of water, and thus the difficulty of pumping out every trace of carbon dioxide was somewhat increased.

In most of the experiments, as in the one quoted above, when a considerable volume of oxygen was absorbed and carbon dioxide produced, the errors are not serious and make very little difference in reckoning the respiratory quotients. In some of the low temperature experiments, however, when the volume of carbon dioxide produced is small the effect is considerably more marked.

Other chemical methods of estimating carbon dioxide did not seem likely to give as satisfactory results, and indeed are rather put out of court by the fact that brown trout appear to secrete some acid substance which affects the composition of hard water. We have not yet investigated the nature of this substance, nor are we aware of any experiments by other observers. We repeatedly noticed, however, that four or five eight inch trout in some 30–40 litres of water, kept well aerated for a couple of hours, produced enough of this substance markedly to interfere with the action of potassium chromate used as indicator in the estimation of chlorides by silver nitrate, an effect not observed in the case of fish such as dace. The production of this acid appears to be well known to the trout breeders, and Mr F. G. Richmond informs us that our observation fits in with his experience of travelling fish in hard and soft water. The hard water apparently neutralises acids given off by the fish, and this secretion from brown trout is more acid than that from other varieties. We have not yet had an opportunity of experimenting with other varieties than brown trout.

Experimental Results.

Two series of fish were taken for experiment, approximately four inches and eight inches long respectively. The small fish were fairly uniform in weight, 20–24 grams each, but the large fish showed very considerable variations,

70–150 grams. The small fish were kept in a tank in the laboratory, and the temperature of the water was pretty constant—about 14°–15° C. They were fed on a commercial fish food, consisting mainly of ground up fish, and appeared to eat well.

The large fish were kept in a tank outside, and as the experiments were performed during the winter months, the average temperature of the water was lower and varied considerably. They were fed on live minnows, and this was the only food they would take at this period.

The results of our experiments are gathered together in the following Table II. We do not give the full protocol of each experiment, since, as the same apparatus was used all through, the details do not differ very much from those in the example already given in full.

Discussion of Results.

Influence of temperature. The oxygen absorbed appears within limits to be proportional to the temperature, and is approximately doubled for an increase of ten degrees. This is best seen in the case of the small four inch trout, partly because under the conditions of experiment each small animal had a freer range of movement, but mainly because the same animals were used in each experiment and in relatively large numbers, so that the influence of individual idiosyncrasies was to a considerable extent got rid of.

In the case of the larger fish, experiments 5–20, the results are very variable. The eight inch trout differed from one another much more in weight than the four inch, only two or four animals were used in each experiment, and in different experiments different animals were of necessity used. These were taken from the stock in the tank indiscriminately, the only precaution being that only healthy fish were used. Our tank accommodation and water supply did not permit of our keeping more than eight or ten of these fish at a time in healthy condition, in fact it was found better to have a smaller number than this. The fish were therefore frequently renewed from the farm as they died off or were killed for experimental purposes. Some of the fish used were therefore fresh from the farm and had not yet got used to their surroundings, others had been in the tank for a considerable time. This would no doubt have an influence. Most of the experiments were done in the winter months, and the animals were kept in an open tank with running water outside. On the very cold days the fish were sluggish and did not feed much, but even in the warmer periods when they appeared more ravenous they would only take live minnows. In the spring and summer they would

TABLE II.

No. of experiment	Temperature of water °C.	No. of fish	Total weight of fish in grams	Average weight per fish in grams	Oxygen absorbed per kilo per hour in cc. at 0° and 760	Oxygen absorbed per fish per hour in cc. at 0° and 760	Carbon dioxide evolved per kilo per hour in cc. at 0° and 760	Respiratory quotient	Remarks	
1	7·5– 7·8°	12	276·6	23·0	89·04	2·05	49·4	0·55	Experiments performed end of May and beginning of June, and fish kept indoors and fed on commercial fish food.	
2	15·8–16°	11	264·0	24·0	117·40	2·82	129·0	1·09		
3	21·2–21·4°	13	300	23·0	213·80	4·63	180·8	0·84		
4	25 –25·1°	11	240	21·8	258·87	5·64	209·2	0·81		
			Experiments with eight inch brown trout						Large fish in a tank kept outside in running water.	
5	4·1– 4·5°	4	423	106	144·36	15·26	32·18	0·22	Fresh fish.	Dec.
6	6·3– 6·6°	2	235	118	93·21	10·95	11·43	0·12		Dec.
7	5·9– 7·2°	2	235	118	105·26	12·37	1·13	0·01	Same fish as in (6).	Dec.
8	6·3– 6·5°	3	285	95	130·6	12·40	17·79	0·14		Nov.
9	6·3– 6·4°	3	322	107	71·58	7·68	27·78	0·39		Dec.
10	6·1– 7·0°	4	354	88·5	96·44	8·53	93·19	0·96	Fish in tank several days and tank at same temp. as experimental water	Dec.
11	6·3– 7·4°	5	403	80·6	76·20	6·14	74·3	0·98		Jan.
12	10°	3	285	95	230	21·85	172·4	0·75		Nov.
13	12·5–12·6°	3	322	107	149·76	16·07	129·3	0·86		Dec.
14	16 –16·6°	4	325	81	206·45	16·77	131·5	0·64	New fish.	Nov.
15	16·8°	3	267	89	224·78	20·45	213·6	0·95		Nov.
16	16·1–16·6°	4	384	96	188·44	18·09	118·1	0·63	Taken on warm day following a spell of cold in which they had not been feeding.	Jan.
17	20·1–20·4°	3	307	102	189·5	19·39	147·8	0·78		Dec.
18	22 –22·7°	3	267	89	218·1	19·40	103·8	0·47	Same as (15).	Nov.
19	21·4–22·2°	4	410	102	186·06	19·0	160·3	0·87	One fish died during experiment towards end	Nov.
20	21·9–22·3°	4	367	92	223·3	20·5	204·5	0·91		Nov.

readily take other food such as worms, flies, etc., but not in the winter. If we take experiments 5–11, done below 7° C. the results are very variable and show nothing as to the influence of temperature, similarly if we take 14, 15 and 16 at 16°, or 17–20 at 22°. If, however, we take the average value of each series, as shown in the following Table III, it will be seen that the

TABLE III.

Number of experiments	Temp.	Average oxygen consumption cc. per kilo per hour
7	4·1– 7·4°	102·5
3	16·1–16·8°	206·5
4	20·1–22°	204·2

oxygen consumption at 16° is double that at about 6°, i.e. it is doubled for a rise of 10° as in the case of the four inch fish. On the other hand the increase appears to stop at this temperature, and the average at 20–22° is approximately the same. It would appear from this that the organism in the case of the larger fish ceases to react to temperature above the limit of 16 or 17°, but it would seem probable that this is more apparent than real, for as is well known, and as indeed we shall ourselves show in a subsequent paper, fish can live and keep well for long periods at a very much lower level of oxygen partial pressure than that corresponding to full saturation; and further the large fish were somewhat restricted in their movements in the experimental tank compared with the small animals, and were consequently less favourably situated for assisting the pumping of water through the gills by swimming movements. If we make the assumption that a fish absorbs the whole of the oxygen from the water passing through its gills, then calculating on the basis of Winkler's figures [1905] for the oxygen content of water saturated with air at various temperatures, a four inch trout at 7–8° would need to pump 242–248 cc. of water through its gills per hour, or about 4 cc. per minute, while at 21° he would need 743 cc. per hour or 12·4 cc. per minute, and at 25°, 977 cc. per hour or 16 cc. per minute. On the same basis an eight inch trout would pump, at 6°, 1207 cc. per hour or 20 cc. per minute, while at 16° he would require 2676 cc. per hour or 44–45 cc. per minute. The above assumption is perhaps scarcely warrantable, so that these figures must be regarded as minimum values. It is therefore perhaps not surprising that the large fish should apparently cease to react to increases of temperature at a lower temperature than the small fish. On the other hand higher temperatures appear to be dangerous or fatal to trout. On

attempting to determine the oxygen absorption for eight inch trout at 25° C., to compare with result No. 4 for four inch trout, of the three fish used in the experiment one died in about two minutes, though the water was fully oxygenated, and the other two turned over on their backs in about ten minutes, and would no doubt have died, but on adding cold water they recovered completely and rapidly and appeared quite well the next day. This result was not due to any sudden change of temperatures as the fish were carefully warmed up beforehand during the course of one hour. It must be noted, however, that the small fish which stood the 25° temperature during an experiment of several hours duration without apparent harm had been living for some days in a tank in the laboratory in relatively warm water (14–18°), whereas the large trout had been living in a tank outside at ordinary winter temperatures of 9–10° or thereabouts. It is possible that had they been living under summer conditions for a long time they might have stood the high temperature better. In another experiment owing to the accidental use of an imperfect thermometer, four eight inch fish were plunged into well oxygenated water at 33° C. The fish gave a few violent leaps and collapsed, as one might perhaps imagine a warm blooded animal doing on falling into boiling water, and were all quite dead in under one minute. The water afterwards was found to be quite frothy as though it contained some saponaceous material.

Influence of size of animal. The oxygen absorption appears to be approximately proportional to the body weight at a given temperature but we have not sufficient data for small animals to show precisely what the average relation is. As far as the experiments go, the rate of oxygen used per kilo per hour at 7° for four inch and eight inch fish is 1 : 1·13, and at 21–22°, 1 : 0·95 ; at 16° the ratio is different 1 : 1·75, but probably the average of a larger number of experiments would bring this mean to 1 : 1.

Respiratory quotients. If we except experiments 5–9 done at low temperature, the average value of the respiratory quotient is 0·81. The variations lie between 0·5 and 1·01, but the majority lie round 0·8. This value is of much the same order as that found for carp and tench, and for various kinds of marine fish by Jolyet and Regnard, and others, and the variations fall within similar limits (*vide* table in Schäfer's *Text-book of Physiology*, vol. I, p. 703).

In experiments 5–9 done at low temperatures, and on fish which had been living in a tank outside during a spell of cold weather, the respiratory quotient shows a remarkable drop, and its average value in the five experiments is only 0·18. We do not profess that any of the five individual values

possesses any high degree of accuracy, for the quantities of carbon dioxide produced are very small and therefore the error of experiment would be very large, but we think there is no doubt about the drop in the value of the quotient, if for no other reason than that after the first anomalous result the utmost care was taken with the other experiments. The change appears to take place somewhere about 6–6·5° C., and below this temperature the fish appear to be in a condition akin to that of a hibernating mammal. We noticed that the fish in our tanks during cold weather were very sluggish and as far as we could tell did not eat. They left minnows in the tank severely alone, whereas on somewhat warmer days they took them readily. We do not know whether this is the experience of other observers, or whether fish in a state of nature get into this condition. We have, however, made many inquiries amongst fishermen, river watchers and others interested in fish, but have not been able to obtain any definite evidence on the point.

In the hope of ascertaining whether this phenomenon had anything to do with the nitrogen metabolism, estimations were made of the saline and "albuminoid ammonia" contained in the water before and after fish had been kept in it for definite periods at various temperatures (by the conventional methods of water analysis). The results are given in the following table:

TABLE IV.

Temp. of water	Wt of fish used in g.	Saline ammonia given off per kilo per hour	Albuminoid ammonia per kilo per hour
3–4°	329	0·0038	0·002
4–5°	345	0·0058	0·0007
1·8–2·2°	354	0·0058	0·003
	mean	0·0051	0·0019
6·4–6·8°	341	0·0071	0·0045
6·9–7°	383	0·0087	0·0012
	mean	0·0079	0·0028
13–15°	352	0·0064	0·0049
13·7–13·8°	351	0·0118	0·0042
	mean	0·0091	0·0045
21·6°	366	0·0162	0·0039
20·6–21·2°	346	0·0187	0·0061
	mean	0·0117	0·0050

The figures in the last column do not represent of course the total nitrogen excreted, other than saline nitrogen, but merely that portion of the soluble organic nitrogen evolved as ammonia on boiling with alkaline permanganate under conventional conditions. The figures throw no light on the cause of

the low respiratory quotients at low temperatures, though they generally confirm the conclusion that the metabolism of fish increases with rise of temperature.

Estimations were also made of the saline ammonia, urea and water-alcohol soluble substances containing amino-nitrogen in the whole fish in case of animals kept for about a week at definite temperatures. For this purpose the animal was rapidly cut into lumps which were dropped into hot water, rendered faintly acid with acetic acid (1 cc. 50 % acetic per litre). After heating on a water bath for about half an hour, the lumps were pounded up in a mortar and repeatedly extracted with hot acid water, after each extraction the solid matter being filtered on the pump. The aqueous extracts, usually about a litre in volume, were evaporated to small bulk *in vacuo* at a temperature below 40° and made up to a definite volume. Aliquot portions were taken for ammonia and urea estimations which were made by the urease method as described by Plimmer [1914]. The remainder of the solution was mixed with ten times its volume of absolute alcohol and allowed to stand overnight. The alcoholic liquor was then filtered, the insoluble residue washed with alcohol, and after being rendered alkaline, the alcohol was distilled from the filtrates *in vacuo*. The alkaline aqueous solution so obtained was then made up to a definite volume and analysed for amino-nitrogen by van Slyke's method. The results are given in Table V.

TABLE V.

Temp. average during week	Amino-nitrogen of alcohol soluble substances in g. of N. per 100 g. of fish	Urea-nitrogen in g. per 100 g. of fish	Saline-nitrogen as ammonia in g. per 100 g. of fish
4°– 5° C.	0·082	0·00028	0·0045
4°– 5° C.	0·077	0·0033	0·0194
8°– 9° C.	0·0886	0·0007	0·0139
9°–10° C.	0·081	nil	0·015
10° C.	0·0798	0·0027	0·0226

How far the urea and ammonia figures have any value we cannot say, owing to the risk of hydrolysis during the prolonged vacuum distillations.

The amino-nitrogen figures are very constant at the various temperatures, and the ammonia figures fairly so. We think it is evident from these experiments that low respiratory quotients have no direct connection with the nitrogen metabolism or the quantities of soluble nitrogenous substances in the tissues.

A possible explanation may perhaps be found in the fat. If at the low temperatures the animals are in a state of hibernation or starvation, they may be living on their fat and partially converting it into glycogen and sugar. Some such process might result in a low respiratory quotient, as indicated in the following equation :

$$2C_3H_5(C_{18}H_{33}O_2)_3 + 64O_2 = 16C_6H_{12}O_6 + 18CO_2 + 8H_2O$$
$$CO_2/O_2 = 18/64 = 0{\cdot}281.$$

Owing to the fact that we had no spell of frosty weather this year after the end of January we were unable to test the validity of this hypothesis, but we hope to be able to investigate this question more closely during the coming winter.

We take this opportunity of expressing our thanks to the Government Grant Committee of the Royal Society for aid in carrying out this work, and also to Mr F. G. Richmond of the Surrey Trout Farm for help in obtaining supplies of fish and for much valuable advice.

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XLIV. A METHOD FOR THE ESTIMATION OF SUGAR IN BLOOD WITH OBSERVATIONS ON SOME MODERN METHODS.

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Until recent years, exact determinations of the sugar content of blood have been attended with great difficulty. The high protein content of blood rendered the separation of the sugar a tedious and uncertain process and even when this was accomplished, there was no very accurate method for the quantitative estimation of small amounts of sugar. In order to ensure fair accuracy, large amounts of blood had to be taken, and this fact in itself prevented complete investigation of the blood in many cases. A few years ago Rona and Michaelis [1908] made use of dialysed iron to separate the blood proteins and a combination of this procedure with Bertrand's [1906] method for the estimation of sugar gave excellent results. The disadvantage, however, was that twenty grams or so of blood were required and the method was rendered tedious by the filtering and evaporation necessary; on this account the number of observations that could be carried out in a day was very limited. In many cases in which the estimation was of the greatest importance it was difficult to obtain the necessary amount of blood; this often happened in the case of the human being, and in smaller animals in which the total blood volume is low. In cases where the necessary amount of blood was available the method gave very good results, and although many other methods have been suggested and used from time to time, our experience of them suggests that, on the whole, the combination of dialysed iron and Bertrand's sugar estimation is undoubtedly the best for blood.

Attempts have been made to estimate the sugar in much smaller amounts of blood than the twenty grams or more used in the above method. Recently Michaelis [1914] has published an account of a method by which he claims to

be able to estimate the sugar in 1 cc. of blood. We have tried to use this, but although the directions given were scrupulously followed, the results in our hands were not satisfactory. The principle is the same as that of the older method, but in this case the precipitate of cuprous oxide is separated by the centrifuge. In the case of small amounts of sugar we were often unable to get any precipitate, and sometimes in the case of two parallel experiments carried out in exactly the same way a precipitate would be obtained in one of them while not a trace was evident in the other one. As we were quite unable to overcome these obstacles, we did not persevere with the method, but this process of sugar estimation might be recommended, if some satisfactory means of separating the reduced cuprous oxide could be found, both on account of the rapidity with which results are obtained and the small volume of blood necessary.

Another method based on a different principle is that of Bang [1913, 1914]. This method we have investigated and used with very many specimens of blood in which the total sugar present was estimated by other means. Using a few modifications which will be described later, we obtained very good results and consider this method of Bang a very valuable one where only traces of blood can be obtained. It is obvious however that the small amount of blood used in the estimation (an average of 0·1 gram) tends to exaggerate any error when the total sugar is expressed in percentage; again a special torsion balance is required and in practice the method takes considerable time.

In certain investigations of the blood sugar of patients—both normal and diabetic—which we have been carrying out, it was necessary to procure samples at frequent intervals and to obtain sufficient blood to secure controls. What was required was some method by which the amount of sugar in about 2 cc. of blood could be estimated quickly and accurately.

Various attempts were made to evolve some method or modification of a method whereby this could be done, but at first the results were not very satisfactory. By using a very dilute solution of permanganate in Bertrand's method and working out a special table, fairly accurate results could be obtained with a few cc. of blood by diluting and clearing with dialysed iron, but the process was tedious owing to the time taken for evaporating the comparatively large volume of fluid to a small bulk; here again it sometimes happened that the fluid which appeared quite clear in large volume showed a distinct turbidity and colouration on evaporation. After many experiments we have now evolved a modification by which the percentage of sugar in 2 cc. of blood may be estimated in from 15 to 20 minutes. No special

apparatus is necessary except a filter pump and even this can be dispensed with. The principle of the method is based on the fact that when blood is treated with a small volume of water and haemolysed it forms a thick paste on the addition of dialysed iron and sodium sulphate. This paste will not filter, but the greater part of the liquid can be expressed by placing a piece of strong linen or cotton material in a funnel and putting a filter paper in position above it, pouring the pasty mass on to the filter paper, bringing the cloth over the latter and gradually pressing out the contents, if possible without bursting the filter paper. In this way a great deal of the liquid is procured, and since experiment shows that the concentration of sugar in the expressed fluid is the same as that in the original paste, an estimation of the sugar in an aliquot part of the fluid gives the factor necessary for estimating the percentage in the blood.

In carrying out the method it generally happens that the fluid so expressed is somewhat turbid and unsuitable for direct estimation of its sugar content; in order to obtain a clear fluid it is necessary to press very gently and carefully and even then the result is often unsatisfactory. There is however no necessity to obtain a clear fluid at this stage; no matter how turbid the fluid is, it is only necessary to pass it through a small filter paper in order to get a water-clear liquid. This filtration requires only a minute or two, so that in practice one presses out as much fluid as possible, folding the cloth over the filter paper and wringing it hard with the hands towards the end of the process. The more or less turbid fluid obtained is then filtered. The whole operation does not take more than about six minutes; in fact we have occasionally measured the blood, performed the necessary manipulations and had the liquid ready for direct estimation of the sugar in five minutes. This compares very favourably with the older methods where long periods are required for filtration. Besides, the only apparatus necessary is a pipette for measuring the blood, a funnel, a mortar and pestle and the requisite flasks and filtering materials, and this is important from the clinical point of view.

After devising means by which an aqueous solution ready for the estimation of sugar could be quickly prepared from blood, the next point that arose was the determination of the amount of sugar in the liquid. Bertrand's method was tried and gave good results with high sugar values, but with small amounts of sugar the method as usually carried out with the filter pump was found to be absolutely unreliable. With the traces of sugar present in these experiments (varying from 0·25 mg. to 6 mg.) it was

comparatively easy to obtain accurate results with the higher values, but, in the case of the lower amounts, great difficulties were encountered. When sugar in very low concentration is boiled with alkaline copper sulphate solution, some of the precipitate is present in a more or less colloidal form so that separation of these particles presents a very difficult problem. In these cases the values obtained depended, among other factors, on the particular sample of asbestos used, while a very slight variation in the size of the perforations of the crucibles produced a marked effect. Using two samples of " asbestos for Gooch crucibles " which seemed quite efficient, a difference of over 40 % of the total amount was obtained. Some samples again absolutely failed to retain any of the precipitate even when a thick layer of asbestos was used. Good values were only obtained when the layer of asbestos was of such thickness that filtration was so exceedingly slow as to render it useless for practical purposes.

As a result of many experiments, we have arrived at the conclusion that in the case of very weak sugar solutions, it is quite impossible to obtain reliable results by using asbestos and the suction pump for separating the reduced sub-oxide in the manner suggested by Bertrand. In ordinary circumstances where the concentration of sugar is fairly high no doubt the method works admirably. With a suitable sample of asbestos, however, we found that fairly rapid filtration could be accomplished by simply packing the asbestos in the crucible and pouring on the liquid to be filtered without using the pump. By this means we obtained excellent results but had great difficulty in procuring a sample of asbestos sufficiently fine to retain the precipitate and yet sufficiently porous to let the liquid through fairly quickly. One excellent specimen for this purpose we found in a sample of " asbestos wool specially prepared for filtering[1]." Since a suitable sample of asbestos is so very important we would suggest the use of this particular variety to anyone making use of the method. The time required for filtration is very short—only about 2 minutes—and the same pad can be used over and over again without being removed from the crucible.

When filtration is complete, the cuprous oxide is washed with water, dissolved in Bertrand's ferric sulphate solution and the sugar estimated in the ordinary way, the special table being used. The steps and details of the method are given below. Since the method is capable of being used clinically we have gone into considerable detail in describing the different manipulations. In order to keep the volume of liquid as small as possible

[1] Sold by Baird and Tatlock.

concentrated solutions of copper sulphate and alkali are used. As weak solutions of potassium permanganate do not keep well, an N/5 stock solution is made and this diluted when required with ten times its volume of water to give an N/50 solution.

Details of Method for estimating Sugar in Blood.

Solutions and substances required:

No. 1. Copper sulphate, 20 g.
Distilled water to 100 cc.

No. 2. Rochelle salt, 90 g.
Caustic soda, 66 g.
Distilled water to 300 cc.

No. 3. Ferric sulphate, 25 g.
Concentrated sulphuric acid, 100 g.
Distilled water to 500 cc.

No. 4. N/5 potassium permanganate solution (6·3200 g. per litre) from which N/50 may be prepared for the experiment.

No. 5. Dialysed iron. Liq. ferri dialysatus, B.P. 1885.

No. 6. Saturated aqueous solution of sodium sulphate.

No. 7. Asbestos. "Asbestos wool specially prepared for filtering."

Together with some pieces of linen about 6–7 inches square, which must be free from starch.

I. *Preparation of Blood.*

In carrying out the method, blood is obtained and if not used immediately is prevented from coagulating by the addition of a trace of potassium oxalate. Two cc. of the blood are then carefully measured into a small mortar by means of a graduated pipette and to this 7 cc. of distilled water are added. The mixture is then stirred gently with the pestle, allowed to stand for half a minute or longer, and 14 cc. of the dialysed iron solution added from a burette. The mixture is then stirred up thoroughly with the pestle for about 10 seconds and 1 cc. of a saturated solution of sodium sulphate run in. On again stirring thoroughly with the pestle a thick pasty mass is obtained which on continued stirring becomes much more fluid. It is now ready for separation of the liquid; the total volume of the mass amounts to 24 cc.

II. *Separation of Fluid Part.*

A small filter paper of about 11 to 12 cm. in diameter is placed in a funnel and rests on a piece of cloth fitted into the funnel so that the cloth which is next to the glass is roughly shaped like the filter paper. As much as possible of the thick mixture in the mortar is transferred to the filter paper, the pestle being used to rub off any adhering to the sides. The upper part of the cloth is then brought over the filter and pressure gradually exerted. After a few seconds the pressure is increased till ultimately the cloth is twisted round and sufficient strength exerted to press out as much liquid as possible. In this way, from 16·5 to 18 cc. of a slightly turbid fluid are generally obtained. Exactly 16 cc. of this are refiltered through a small filter into a graduated 25 cc. cylinder. This fluid, which amounts to $\frac{2}{3}$ of the original volume, and contains $\frac{2}{3}$ of the total sugar content of 2 cc. of blood, is now ready for estimation of the sugar.

III. *Estimation of Sugar in* 16 *cc. Fluid from II.*

(*a*) *Reduction to cuprous oxide.* The 16 cc. fluid are carefully poured into a small Erlenmeyer Jena flask of 100 cc. capacity. Then 1 cc. of No. 1 copper sulphate solution is measured into the cylinder together with 3 cc. of No. 2 alkaline mixture. This is shaken up and added to the contents of the flask. In this way any sugar remaining in the cylinder is washed out. The mixture is then boiled over a strong flame, using an asbestos wire gauze, for exactly 3 minutes. The flame must be of such a strength that it brings the mixture to the boiling point in a period of from 1 min. 10 sec. to 1 min. 20 seconds. The boiling point is taken as that point at which ebullition becomes general throughout the fluid. Isolated bubbles appear in from 5 to 7 seconds before this, but the boiling point as thus defined is very distinct. After 3 minutes the flask is removed and the liquid is poured on to a specially prepared crucible containing asbestos.

(*b*) *Preparation of crucible and filtration.* By far the most suitable arrangement for the filtration is a Murman crucible[1]. This crucible resembles a Gooch, but a narrow tube leads off from the bottom of the apparatus so that the filtered fluid is delivered directly into the flask ; it is virtually a funnel in which is placed a perforated plate. The stem of the crucible is passed through a

[1] Listed by Gallenkamp and Co.

rubber stopper which fits into a small filtering flask of about 150 cc. capacity. The specially prepared asbestos mentioned above is thoroughly washed in water and then rubbed up gently in a mortar. Some of it is then taken and put into the crucible, a glass rod being used to spread it out evenly; if a pump is available it may be fixed in position in the usual way by suction. On adding some water it swells up, and if the layer be not too thick the water soon begins to trickle through. The necessary thickness is obtained when water at room temperature filters through at the rate of about 10 to 20 drops per half minute. When the crucible is prepared, a little water is poured on, the flask containing the cuprous oxide shaken round a few times and the whole of the contents carefully poured while still hot into the crucible. If the asbestos is of the correct thickness the blue copper solution filters quickly, the whole contents of the flask passing through in about 2 minutes. If less than this time is required more asbestos should be added. The asbestos and sides of the crucible are then washed with a few cc. of hot water with which the boiling flask has already been washed. *During all these manipulations the suction pump must on no account be used.* The crucible is now removed, the contents of the filtering vessel thrown away, the vessel thoroughly washed with cold water and the crucible replaced. The reduction flask is then cooled, 5 cc. of Bertrand's ferric sulphate solution added and the flask shaken to dissolve any traces of cuprous oxide adhering to the glass. About 3 cc. of the ferric sulphate solution is poured carefully on to the sides of the crucible and the asbestos; the asbestos is gently pressed with a glass rod and after a few seconds the last trace of iron solution sucked through by the pump. The remainder of the iron solution is then added and the process repeated. The flask is now washed with exactly 10 cc. of distilled water and this is poured on to the asbestos; after careful manipulation with the glass rod the last drop is sucked through. As with the iron sulphate, the washing here can be advantageously carried out in two parts. The solution is now ready for titration with the N/50 potassium permanganate, which is carefully added from a small 10 cc. burette, until a faint but distinct reddish tinge appears. The number of milligrams of glucose which corresponds to the amount of permanganate required is found on reference to the table. The table was compiled using all the above precautions with the special asbestos described. To be quite comparable with the blood solutions, sodium sulphate was added in each case.

Table showing the amount of glucose equivalent to a certain amount of N/50 Potassium Permanganate solution.

Total volume including alkaline copper sulphate was in each case 20 cc. Filtration under exact conditions described. 5 cc. ferric sulphate used for dissolving cuprous oxide; 10 cc. distilled water used for washing asbestos.

N/50 Perm. cc.	Gluc. mg.	N/50 Perm. cc.	Gluc. mg.	N/50 Perm. cc.	Gluc. mg.	N/50 Perm. cc.	Gluc. mg.	N/50 Perm. cc.	Gluc. mg.
0·53	= 0·25	2·59	= 1·40	4·67	= 2·55	6·28	= 3·70	8·00	= 4·85
0·62	= 0·30	2·66	= 1·45	4·73	= 2·60	6·35	= 3·75	8·08	= 4·90
0·71	= 0·35	2·72	= 1·50	4·80	= 2·65	6·42	= 3·80	8·16	= 4·95
0·82	= 0·40	2·82	= 1·55	4·87	= 2·70	6·49	= 3·85	8·24	= 5·00
0·91	= 0·45	2·93	= 1·60	4·94	= 2·75	6·56	= 3·90	8·31	= 5·05
1·00	= 0·50	3·03	= 1·65	5·00	= 2·80	6·63	= 3·95	8·38	= 5·10
1·09	= 0·55	3·13	= 1·70	5·07	= 2·85	6·70	= 4·00	8·44	= 5·15
1·18	= 0·60	3·24	= 1·75	5·14	= 2·90	6·77	= 4·05	8·51	= 5·20
1·27	= 0·65	3·33	= 1·80	5·21	= 2·95	6·85	= 4·10	8·58	= 5·25
1·37	= 0·70	3·43	= 1·85	5·28	= 3·00	6·92	= 4·15	8·65	= 5·30
1·48	= 0·75	3·53	= 1·90	5·35	= 3·05	7·00	= 4·20	8·72	= 5·35
1·58	= 0·80	3·62	= 1·95	5·42	= 3·10	7·07	= 4·25	8·78	= 5·40
1·69	= 0·85	3·72	= 2·00	5·50	= 3·15	7·15	= 4·30	8·85	= 5·45
1·81	= 0·90	3·81	= 2·05	5·56	= 3·20	7·22	= 4·35	8·92	= 5·50
1·92	= 0·95	3·90	= 2·10	5·64	= 3·25	7·30	= 4·40	8·99	= 5·55
2·05	= 1·00	3·99	= 2·15	5·71	= 3·30	7·37	= 4·45	9·05	= 5·60
2·12	= 1·05	4·08	= 2·20	5·78	= 3·35	7·45	= 4·50	9·12	= 5·65
2·18	= 1·10	4·17	= 2·25	5·85	= 3·40	7·53	= 4·55	9·19	= 5·70
2·25	= 1·15	4·26	= 2·30	5·92	= 3·45	7·60	= 4·60	9·26	= 5·75
2·32	= 1·20	4·35	= 2·35	6·00	= 3·50	7·69	= 4·65	9·33	= 5·80
2·39	= 1·25	4·44	= 2·40	6·07	= 3·55	7·76	= 4·70	9·39	= 5·85
2·46	= 1·30	4·53	= 2·45	6·14	= 3·60	7·84	= 4·75	4·96	= 5·90
2·52	= 1·35	4·62	= 2·50	6·21	= 3·65	7·92	= 4·80	9·53	= 5·95
								9·60	= 6·00

Results of Method.

In investigating the accuracy of the method, a known amount of glucose was added to a blood which had stood at laboratory temperature for several days. The whole of the sugar in this blood had disappeared and on the addition of 0·1 gram glucose per 100 cc. of blood, the following results were obtained:

1. 0·101 %
2. 0·099 %
3. 0·094 %
4. 0·099 %
5. 0·105 %
6. 0·110 %

Average 0·101 %.

Some fresh bloods were also tested; three or four specimens of each were examined as parallel experiments and in all cases the results agreed very well

among themselves. The total sugar concentration was usually almost identical with that obtained by Bang's method or by the old dialysed iron method; the figures generally did not differ by more than about 0·01. Parallel experiments carried out on two different specimens of human blood by this method gave the following values:

(*a*) 0·064 %, 0·067 %, 0·060 % = Average 0·063 %.
(*b*) 0·096 %, 0·101 % = Average 0·098 %.

The total sugar content of two other human bloods was found by this method to be:

(*a*) 0·022 % (diabetic),
(*b*) 0·095 % (normal),

while in the same bloods Bang's method gave (*a*) 0·227 %, (*b*) 0·095 %.

The method as described is not sufficiently delicate to estimate the sugar in blood containing only traces of glucose, *i.e.* less than 0·02 %. In the examination of fresh blood such a contingency never arises, in human blood at any rate, as from 0·05 % to 0·15 % seems to be always present. In experiments on glycolysis, however, it is useful to be able to determine very small traces of sugar, and in order to do this, it is only necessary to add a known amount of sugar beforehand to the solution to be tested. This is most conveniently done by having in stock a special No. 1 copper sulphate solution containing in addition exactly 0·1 % of glucose. Such a solution keeps well and by the addition of the usual 1 cc. to the blood extract, 1 mg. of sugar is added to that actually present in the blood. This plan of adding sugar to increase the sensitiveness of the reaction has been often used in estimating traces of sugars in fluids. Among others, Korowsky [1913] adopted it in the case of blood and Flückiger [1885] in estimating the reducing power of urine.

In carrying out the estimation by this means, one proceeds exactly as before to the point at which the 16 cc. of fluid are poured out of the cylinder into the boiling flask. To ensure exactness, however, the 1 cc. of copper solution is added directly to the liquid in the flask, the cylinder being washed out with the 3 cc. of alkaline solution. The flask is then thoroughly shaken to dissolve the precipitate of copper hydroxide formed and the remainder of the process carried out as described.

In estimating the effects of glycolysis in blood it is often advisable to employ the special copper sulphate solution for the blood when a disappearance of much of the sugar is expected. It can also be used for ordinary

blood but this is not necessary. The only calculation required is the deduction of 1 mg. sugar from the amount actually found.

Using these modifications, it is easy to calculate exactly the percentage of sugar in blood when this amounts only to 0·01 % or less. The table given above extends from 0·25 mg. to 6 mg. of glucose, but the higher values are only obtained in bad cases of diabetes. Normal blood with an average of about 0·1 % glucose requires 2–3 cc. of the permanganate solution which is a convenient amount to work with.

Bang's Micro-method.

As already mentioned, we have found this method exceedingly satisfactory. In the main, we have followed Bang's directions but have introduced certain modifications which in our hands have proved of much value. In this method a small amount of blood is soaked up by a small filter paper and the filter paper extracted with hot potassium chloride solution containing a trace of acetic acid. According to Bang, the protein is coagulated by the hot potassium chloride solution and remains in the paper, whilst the sugar diffuses out into the liquid. We found it impossible, however, to prevent some of the protein passing out into the fluid, and since the presence of traces of protein interferes materially with the result, many of our experiments were rendered valueless.

After various attempts, this difficulty was entirely overcome by putting the filter paper containing the blood into a hot oven (90°–100° C.) for from three to five minutes before extraction. This coagulates the protein so that only on very rare occasions did the fluid show turbidity on subsequent extraction with the potassium chloride solution. If any turbidity did occur it was exceedingly slight and usually insufficient to affect the result of the estimation. This apparently trifling modification has made a marked difference in the practical use of the method and we can now be certain that trouble from protein will not be encountered.

Since the protein is so firmly coagulated we have prolonged the time of extraction to 45 minutes, or better, one hour, in order to allow for complete removal of the sugar. The fluid is brought to boiling point at least twice during each period.

Again, the alkaline copper solution is freed as much as possible from dissolved gases by shaking up and exhausting with a suction pump for a minute or two at the commencement of each series of estimations.

At first we had great difficulty in procuring suitable blotting paper, as many varieties contained something which absorbs iodine even after prolonged treatment with water and acetic acid. The amount of this substance varied even in pieces which had been purified in the same way and all taken from the same sheet. The special samples supplied for the purpose, which are specially purified and ready for use, we have, however, found most satisfactory, and all our later experiments have been carried out with these.

For a few experiments the weight of the blood can be ascertained by any ordinary sensitive balance, but when many estimations are undertaken a special torsion balance is essential.

It is desirable to carry out at least four parallel determinations of each sample of blood and if time allows five or six tubes may with advantage be set up. The method is so extremely delicate that safety is only to be found in numbers and little extra time is involved in setting up a few more tubes.

The mean of three or four satisfactory estimations may be taken as the correct figure.

Frequently it is possible to obtain a series of values which vary very little from the mean. As an example we quote the results of such a case where seven parallel determinations were made. The figures obtained were :—

(1)	0·074 % sugar	(5)	0·076 % sugar
(2)	0·077 % ,,	(6)	0·075 % ,,
(3)	0·074 % ,,	(7)	0·077 % ,,
(4)	0·074 % ,,		

Mean 0·0753 %.

In all cases 0·01 % is subtracted from the final result since experience shows that blood contains a trace of something which reduces iodine and in the case of about 0·01 g. of blood the results obtained are invariably too high by almost exactly 0·01 %.

We have carried out a considerable number of experiments in which the same sample of blood was tested simultaneously by this and by another method. For the control either the old dialysed iron process or the new modified method described in this paper was employed.

The comparative values thus obtained agree within fairly narrow limits, the difference between the estimations being generally not more than 0·01 % to 0·015 %.

	Diabetic blood.		*Normal Blood.*	
	Bang's method	New modified method	Bang's method	Old method (dialysed iron)
	0·227 %	0·221 %	0·095 %	0·09 %
	0·237 %	0·224 %	0·092 %	0·10 %
	0·220 %		0·099 %	
	0·225 %	Mean 0·222 %		Mean 0·095 %
	0·226 %		Mean 0·098 %	
Mean	0·227 %			

The method can therefore be said to be quite accurate when carefully carried out if the mean of a number of observations is taken.

The figures given below are picked out at random from a series of observations and show the average deviation of the control experiments.

	Blood (*a*) Normal	Blood (*b*) Normal	Blood (*c*) Diabetic	Blood (*d*) Diabetic	Blood (*e*) Normal
	0·0620 %	0·0636 %	0·334 %	0·300 %	0·0820 %
	0·0507 %	0·0610 %	0·334 %	0·360 %	0·0758 %
	0·0610 %	0·0565 %	0·337 %	0·337 %	0·1020 %
	0·0665 %	0·0489 %	0·329 %	0·369 %	0·0821 %
Mean	0·0600 %	0·0575 %	0·333 %	0·332 %	0·0833 %

Bang's method is indispensable for cases in which only very small amounts of blood are obtainable but for routine purposes it is neither so speedy nor so convenient as the new modified method described.

These two methods fulfil all requirements for the estimation of sugar in blood.

Conclusions.

1. A method is described by which the estimation of sugar in a sample of blood can be carried out in about 20 minutes.

The amount of normal blood required is 2 cc., but in the case of diabetic blood less suffices.

To ensure accuracy it is necessary to follow very carefully the directions given.

2. Bang's micro-method gives good results; coagulation of the blood beforehand as described in the text entirely overcomes the difficulties of interference by protein.

It is advisable to carry out at least four parallel determinations for each sample of blood investigated.

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XLV. THE APPARENT FORMATION OF EU-GLOBULIN FROM PSEUDO-GLOBULIN AND A SUGGESTION AS TO THE RELATIONSHIP BETWEEN THESE TWO PROTEINS IN SERUM.

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Introduction.

A great difficulty in completely separating water-insoluble protein has been the common experience of all who have attempted to prepare pure salt-free samples of pseudo-globulin. In the course of fractionating horse-serum for viscosity measurements [Chick 1914], I found that, however carefully the euglobulin of the serum had been previously separated, the final product invariably contained more or less water-insoluble protein. Haslam [1913] devoted much attention to this question and came to the conclusion that the presence of the latter was due to incomplete removal of the euglobulin in the original serum. This is to some extent true, but my experience has led me to believe that the bulk of the water-insoluble material cannot be accounted for in this manner, for a protein, having the characteristics of euglobulin, was found to increase progressively in amount as the purification of the pseudo-globulin proceeded. A small amount of water-insoluble protein can usually be detected immediately after the first separation of the pseudo-globulin by salt precipitation and this does appear to consist of euglobulin originally present in the serum. The comparatively large quantity (in one case, Exp. IV, Table III, nearly one-fifth of the total protein) present at the end of a lengthy dialysis appears, on the other hand, to be derived, at least in part, from a transformation of the pseudo-globulin itself. The experiments about to be described show that, under certain circumstances, pseudo-

globulin may undergo change into a protein possessing the properties of euglobulin and suggest that such may very likely be the origin of the euglobulin present in serum.

Origin of the water-insoluble protein found present in pseudo-globulin.

In one set of experiments (Table I) pseudo-globulin was prepared from diluted horse-serum by the following method, rapidly executed. Ammonium sulphate was first added to one-third[1] saturation, the precipitate separated and the filtrate brought to half saturation of ammonium sulphate, this second precipitate being pressed free from mother liquor and afterwards dissolved in a small amount of water. The presence of a small amount of insoluble protein was always apparent on dilution, especially if the solution were adjusted to a definite, slightly acid, reaction.

This insoluble protein we may regard as euglobulin not thrown out from serum by one precipitation in one-third saturated ammonium sulphate for if, in addition to this process, as much as possible of the euglobulin is previously removed by careful acidification of the diluted serum, the water-insoluble protein which can be detected in the precipitated pseudo-globulin is considerably reduced (see Table II).

When pseudo-globulin (horse), after undergoing one or more re-precipitations, is subjected to a lengthy dialysis to remove ammonium sulphate, it is found to contain a comparatively large amount of water-insoluble protein. As a general rule from one-tenth to one-fifth of the total protein is found to be in this condition. This insoluble material exhibits a striking likeness to euglobulin. It is dispersed by small quantities of dilute acid or alkali and when first separated is dissolved by neutral salts, which latter property, as is the case with euglobulin, becomes impaired with increasing age of the preparation.

It has not been possible to determine with certainty the origin of the whole of this "euglobulin." One view is that it is derived from the pseudo-globulin by some process of "denaturation"[2] or degradation taking place

[1] "One-third" and "one-half" saturation indicate the concentrations of ammonium sulphate obtained when a saturated solution of ammonium sulphate is added to twice its volume or an equal volume of water.

[2] "Denaturation" of proteins cannot at present be identified with any chemical changes in the material. The term "denaturated" (German, *denaturiert*) is applied to proteins possessing a certain group of solubility characteristics, including a total insolubility in water at the iso-electric point, in absence of electrolytes. Solution (dispersion) in water occurs in the presence of acid, alkali or electrolytes, and is associated with the acquisition of an electric charge by the protein particles, while the system displays a high degree of viscosity. The best-known example is that

after separation from the serum and the results of Exps. I–IV set forth in Tables I–III may, on the whole, be said to support this view. On the other hand, it may be urged that the results of these experiments do not necessarily prove that dialysis of salt-precipitated pseudo-globulin is accompanied by its "denaturation," but that removal of all salt may be essential to permit complete precipitation of any "denaturated" protein (euglobulin) present from the beginning. If this is the case, the concentration of euglobulin in serum must be far higher (two or three times) than is generally believed and the usual method of separation, by dilution and acidification, very inadequate.

In order to throw light upon this point, some experiments were made in order to ascertain with what degree of accuracy water-insoluble protein can be estimated in presence of salts and other soluble protein. In one experiment euglobulin was mixed with a sample of thoroughly dialysed pseudo-globulin (from which all insoluble protein had been separated) and ammonium sulphate added; the mixture contained 0·462 % euglobulin, 2·02 % pseudo-globulin and 1·2 % ammonium sulphate. The concentration of insoluble protein was then estimated by the dilution and acidification method described in detail below. The value obtained was 0·337 %, or 73 % of that added. The concentrations of protein and salt in the final acidified, and diluted, solution were 0·048 % and 0·024 % respectively.

A second experiment was made with a thoroughly dialysed pseudo-globulin solution containing 4·77 % total protein, of which about one-seventh was in the "denaturated" condition and present as a precipitate. Estimations of the insoluble protein present in 10 cc. of the above solution were made both before and after addition of 5 cc. of strong ammonium sulphate (10·3 %). In the control, dilution 50-fold, the proportion of insoluble protein was found to be 0·70 %, in the presence of ammonium sulphate the estimated concentration was only 0·23 % and 0·26 % when diluted 50-fold and 100-fold respectively. In this experiment the final concentration of protein and ammonium sulphate was 0·10 % and 0·093 % respectively in the 50-fold dilution and half these amounts in the 100-fold dilution. The proportion of the total insoluble protein which could be precipitated under these conditions was 33 % and 37 % in the two cases.

of proteins which have been acted upon by hot water [see Chick and Martin 1910, 1911 and 1912]. The vegetable globulins and the euglobulin naturally occurring in serum may be regarded as "denaturated" proteins [see Michaelis and Rona 1910, 2]; their property of forming electrically-neutral solutions with certain salts is, however, a special characteristic not shared by heat-"denaturated" proteins.

When whole horse-serum is diluted ten times, as is usual when euglobulin is separated from serum, the total concentration of protein is from 0·6 % to 0·8 % and that of sodium chloride from about 0·08 % to 0·07 %. Under these circumstances, it is quite possible for some euglobulin to escape precipitation when the diluted serum is adjusted to the iso-electric point of euglobulin by the addition of acetic acid. On the other hand, it must be remembered that a chloride is a less efficient solvent for euglobulin than a sulphate [see Mellanby 1905]. Further, there is, as a rule, no suggestion of incompleteness about the process. The euglobulin usually settles with comparative ease, leaving a clear liquid on top.

The results of Exp. III, Table II also throw some light upon this question. In this case a solution of pseudo-globulin made immediately after its precipitation from the horse-serum contained 5·2 % ammonium sulphate, 12·86 % total protein, and only a trace of water-insoluble protein, when subjected to analysis by the method described below. Part of the material was kept in the laboratory as a control and part was dialysed, toluene being present in both cases to prevent decomposition. At the end of two months the water-insoluble material in the control was equal to 8·6 % of the total protein. In the portion which had been dialysed for 51 days the proportion estimated was 14·1 %. This experiment shows that water-insoluble protein, if present, can be detected in presence of electrolytes and other soluble protein. Therefore, a large proportion, at least 61 % (8·6 parts out of a total of 14·1) of the euglobulin found present at the close of dialysis must be regarded as formed from the pseudo-globulin by some process of denaturation taking place after separation from the serum. The remaining 39 % may, on the other hand, be regarded as euglobulin which was present from the first, although it could not be separated in presence of salt. But, from the fact that immediately after preparation only a trace of insoluble protein could be detected in the salt-containing pseudo-globulin, it seems probable that part of this may also be derived from the pseudo-globulin, showing that the denaturation of this protein is accelerated by dialysis.

Experimental methods.

In the experiments set forth in Tables I to III pseudo-globulin was prepared in various ways by salt-precipitation from horse-serum, the details of themethod employed being given at the top of each table. Estimations of the denaturated protein were made before, after, and, in some cases

(Experiments I and II, Table I), during the dialysis, by determining the amount of protein which was precipitated by dilution and adjustment to the iso-electric point.

The method of estimation was as follows. A small portion of the material was taken for a preliminary trial, and largely diluted, so that the protein-content did not exceed 0·1 or 0·2 %. If electrolytes are present the dilution should be as large as possible. 10 cc. of this diluted solution were then placed in each of a series of test tubes and various small quantities of N/100 acetic acid added. If any denaturated protein were present an opalescence or slight turbidity was usually apparent on mere dilution and, when the solution was adjusted to the iso-electric point of the protein by addition of the required amount of acid, a precipitate was formed which aggregated and settled. The series of trial test tubes was always allowed to stand several hours and the composition of the material in the tube in which the precipitate was largest and agglutination most perfect was taken as the pattern for the estimation.

5, 10, or 15 cc. (according to the protein-content) were taken for analysis, diluted suitably and addition made of dilute (N/10 or N/100) acetic acid in the amount necessary for complete precipitation of the denaturated protein, as calculated from the results of the preliminary trial. The whole was allowed to stand 24 hours and then centrifuged, the deposit being transferred to a small, weighed, centrifuge tube, spun down again, washed with distilled water slightly acidified and centrifuged a third time. The final deposit was dried in the weighed tube at 105° until constant in weight.

It is not advisable to permit the estimation to extend over more than one day, as, in some cases, the denaturation of the pseudo-globulin seems to be continued at an appreciable rate in the dilute solution and, when several days are spent over the estimation, higher values are frequently obtained.

If the material to be analysed is slightly acid, small quantities of dilute ammonia must be substituted for the dilute acetic acid. In cases where the material has been dialysed for a long time and the reaction is approaching that of the iso-electric point, dilution with distilled water containing about one-thirtieth of its volume of water saturated with carbon dioxide is often found to be a quick and efficient method of precipitating all the denaturated protein.

If electrolytes are present, denaturated protein may be detected by the above method, but precipitation, under certain circumstances, is incomplete (see above p. 405). In any case it is advisable to make the dilution as great as is possible.

The above method has been described in detail, because the estimation of denaturated protein in presence of other, water-soluble, protein is by no means an easy matter even in the absence of electrolytes, the difficulty being to ensure complete precipitation of the former. When this has not been accomplished, faint turbidity can be detected in the supernatant fluid after centrifuging; in a successful estimation the aggregation of the precipitate should be complete and the top liquor clear. Some of the earlier values for concentration of "denaturated" proteins given in Tables I to III were obtained when the method was not perfected and may be a little inaccurate; the later ones were more satisfactory, as may be seen in some cases where the results of duplicate analysis are inserted.

It was possible that this water-insoluble protein, like euglobulin [Freund and Joachim 1902, Gibson 1906, Banzhaf 1911], might be thrown out by addition of sodium chloride to saturation, and this would have proved a convenient method of separation and estimation. It was tried in Exp. II,

Table I, but without success. After diluting the material 10-fold and saturating with sodium chloride the residual protein (calculated on the original, undiluted, material) was 10·78 %, the total being 11·12 % ; that is to say only 0·34 % protein had been thrown down. The concentration of "denaturated" protein as separated by the above method was, however, found to be 0·96 %.

Precipitation by saturated sodium chloride is of little value for the accurate separation of proteins, the amount precipitated depending, to a greater degree than is the case with other salts, upon external conditions, such as the total concentration of protein and the reaction of the material. For example, on one occasion, with horse-serum, the precipitate obtained on saturation with sodium chloride was four times greater when the serum was diluted 1 in 10 than when the dilution was only 1 in 2 ; in the latter case the higher concentration of total protein protected more than three-quarters of the otherwise precipitable protein. Further, if diluted serum is made slightly acid in reaction, the whole of the pseudo-globulin and albumin is precipitated as well as the euglobulin.

Discussion of Results.

The results of the experiments in Tables I–III suggest that the pseudo-globulin undergoes a progressive denaturation after its separation from the serum. For example, in Exp. I, Table I, the pseudo-globulin immediately after precipitation from the serum showed only a small amount of water-insoluble protein capable of separation by dilution and adjustment of the reaction to the iso-electric point. At the end of dialysis for 10 and 18 days, however, 9·1 % and 12·9 % respectively, of the total protein was found to be in the insoluble condition. It is probable that the ammonium sulphate present interfered with a complete separation of any euglobulin present at the beginning of the experiment, but it is unlikely that the difference in amount of the traces of salt present after 10 and 18 days' dialysis respectively could be responsible for the large difference in amount of insoluble protein found on these two occasions.

Experiment IV, Table III is a second instance. Here material dialysed for five days against tap water contained 11·1 % denaturated protein ; after a further dialysis against distilled water lasting 29 days this proportion was increased to 18·1 %.

TABLE I.

Spontaneous formation of euglobulin from pseudo-globulin at ordinary temperature; pseudo-globulin prepared by half-saturation of undiluted horse-serum with ammonium sulphate, after separation of euglobulin by one-third saturation with ammonium sulphate.

Exp.	Material	Date of analysis	Length of dialysis, days	Total protein, %	Water-insoluble protein, %	G. water-insoluble protein per 100 g. total protein	Phosphorus-content, g. P per 100 g. protein: in water-soluble fraction	Phosphorus-content, g. P per 100 g. protein: in water-insoluble fraction
I.	Prepared 2. 12. 13; re-precipitated twice; final precipitate dissolved in water. Dialysed in presence of toluene against distilled water, changed daily, from 5. 12. 13 to 23. 12. 13	5. 12. 13			small amount			
		15. 12. 13	10	14·56	1·33	9·11		
		14. 3. 14[1]	18	13·55	1·75	12·9	0·018	0·171
II.	As in Exp. I but not re-precipitated	6. 12. 13	3	11·17	present			
		26. 1. 14[2]	3	11·12	0·964	8·67		
	(*a*) dialysed against dist. water from 13. 2. 14 to 25. 3. 14	3. 4. 14[3]	43	4·77	0·654	13·7		
		9. 7. 14	43	4·77	0·700	14·7		
	(*b*) as above, but dialysed against dist. water saturated with CO_2 from 13. 2. 14 to 25. 3. 14	26. 3. 14	43	5·51	{0·652 / 0·660}[4]	11·9		

[1] Kept in cold room from 23. 12. 13 until 14. 3. 14.
[2] Kept in cold room from 6. 12. 13 until 13. 2. 14; dialysis then recommenced.
[3] Kept in cold room from 25. 3. 14 until 9. 7. 14.
[4] Duplicate analyses.

TABLE II.

Spontaneous formation of euglobulin from pseudo-globulin at ordinary temperature; pseudo-globulin prepared from diluted (1 *in* 20) *horse-serum by half-saturation with ammonium sulphate, after successive removal of euglobulin precipitates obtained* (1) *by acidification of the serum* (1 *in* 10 *dilution*), *and* (2) *by one-third saturation of the serum* (1 *in* 15 *dilution*) *with ammonium sulphate: once re-precipitated and dissolved in a small quantity of water.*

Exp.	Material	Date of analysis	Length of dialysis, days	Concentration of $(NH_4)_2SO_4$, %	Total protein, %	Water-insoluble protein, %	G. water-insoluble protein per 100 g. total protein	Phosphorus-content, g. P per 100 g. protein: in water-soluble fraction	Phosphorus-content, g. P per 100 g. protein: in water-insoluble fraction
III.	Control, immediately after preparation	4. 2. 14	—	5·23	12·86	trace	—	—	—
	(*a*) Control, allowed to remain at laboratory temperature, in presence of toluene	3. 4. 14	—	5·23	2·86	1·107	8·61	—	—
	(*b*) Dialysed against distilled water, changed daily, in presence of toluene from 2. 2. 14 to 25. 3. 14	30. 3. 14	51	—	4·86	{0·688 / 0·681}[1]	14·1	0·012	0·262

[1] Duplicate analyses.

TABLE III.

Spontaneous formation of euglobulin from pseudo-globulin at ordinary temperature; pseudo-globulin prepared from horse-serum (diluted 1 in 4) by precipitation with one-half saturated ammonium sulphate, this first precipitate extracted with saturated brine, brine-extract precipitated by addition of acetic acid to a concentration of 0·25 %[1]; this second precipitate drained, pressed and mixed with washing soda to a concentration of 3 % and dialysed for five days against tap water.

Exp.	Material	Date of analysis	Length of dialysis, days	Total protein, %	Water-insoluble protein, %	G. water-insoluble protein per 100 g. total protein	Phosphorus-content, g. P per 100 g. protein: in water-soluble fraction	Phosphorus-content, g. P per 100 g. protein: in water-insoluble fraction
IV.	Control	3. 3. 14	(5)[2]	11·72	1·301	11·10	—	—
	(*a*) Dialysed further against dist. water, changed daily, in presence of toluene from 6. 3. 14 to 4. 4. 14	4. 4. 14	29 (+5)	5·87	1·055	17·98	0·0062*	0·156
	(*b*) as (*a*) the distilled water being saturated with CO from 6. 3. 14 to 4. 4. 14	4. 4. 14	29 (+5)	6·40	1·161	18·14	0·0066*	0·098

[1] Gibson's [1906] process.
[2] Against tap water.
* Approximate only (less than 1 cc. N/10 NaOH neutralised).

The results of Experiments I to IV also suggest in every case that the degradation of the pseudo-globulin is approaching a limit. For example, in Exp. II, Table I, after three days' dialysis 8·7 % of the protein was found to be water-insoluble. After an interval of more than two months, including a second dialysis lasting 40 days, the proportion had only risen to 13·7 % and, after having been kept in the refrigerator for a further period of over three months, to 14·7 %. A similar result is shown in Exp. I. In many cases, the experiments were continued beyond the date of the last analysis given in the tables, and the denaturation process was found to be practically at an end. The degraded material was separated as completely as possible by a modification of the method described above, and the clear, top liquor adjusted to the iso-electric point of the denaturated protein and kept for a considerable time in the cold room under observation. In the case of Exps. III and IV (*a* and *b*), only a trace of insoluble material was deposited after periods of two months and one month respectively.

Phosphorus-content of this water-insoluble pseudo-globulin.

The resemblance between the denaturated pseudo-globulin obtained in these experiments and euglobulin led me to make estimations of the phosphorus contained respectively in the insoluble and unchanged pseudo-globulin.

Hardy [1905] demonstrated the presence of phosphorus (0·07 to 0·08 %) in euglobulin (ox-serum) and considered it to be one of the characteristics marking off this protein as a chemical entity. The pseudo-globulin (separated from the serum by saturation with magnesium sulphate) he found to contain a trace only (about 0·009 %) of phosphorus. Haslam [1913] also found phosphorus to be a constant constituent of euglobulin (ox-serum), even after repeated purification, and to be absent from the purest samples of pseudo-globulin which he was able to obtain. In consequence, he used the absence of phosphorus as a criterion of the purity of pseudo-globulin.

In the present case phosphorus was determined by Neumann's method, using N/10 alkali and acid for the final titration, the quantity to be estimated being very small. In some cases where the ammonium molybdate precipitate was only just visible and the total amount of standard sodium hydrate neutralised less than 1 cc., the values obtained must be regarded as approximate only; such values are indicated in the tables by an asterisk.

The results of the phosphorus estimations showed the resemblance between

the denaturated pseudo-globulin and euglobulin to be maintained in this respect also. The former was found to remove almost the whole of the small amount of phosphorus contained in the original pseudo-globulin preparation. In Exp. I, Table I, the water-insoluble protein at the close of the experiment contained nearly 10 times, in Exp. III, Table II, more than 20 times, as much phosphorus as the unchanged pseudo-globulin; in both cases that present in the latter was reduced to a mere trace. In Exp. IV the contrast was even greater; the denaturated pseudo-globulin contained 0·16 % phosphorus, while that remaining with the soluble protein was too small in amount to be estimated with any degree of accuracy.

Two different explanations might be advanced to resume these facts. According to the first, the process described in the last paragraph is merely the separation of the last traces of phosphorus-containing euglobulin derived from the original serum and present from the beginning. On this theory the cessation of the gradual precipitation of insoluble protein would naturally coincide with the disappearance of the phosphorus originally present in the solution. On the other hand, we may regard this precipitate containing protein and phosphorus, which gradually makes its appearance, as the product of a gradual "denaturation" of the pseudo-globulin which requires the co-operation of some phosphorus-containing body (probably a serum-lipoid) which is present in small quantity in the preparations employed. When this serum-lipoid is exhausted, the process is at an end.

The second explanation would appear to be the correct one for the following reason. After the removal of phosphorus from the pseudo-globulin solution, by the separation of the insoluble protein precipitate, addition of a weak emulsion of lecithin, in presence of salt, causes additional formation of a protein resembling euglobulin which can be precipitated on subsequent dialysis.

Handovsky and Wagner [1911] showed that, when emulsions of lecithin, and other lipoids extracted from serum, were added to dialysed serum, a precipitation of the protein took place, which was prevented if salts were present. I have been able to confirm these observations. Addition of a dilute lecithin emulsion produces only slight turbidity when added to solutions of pure egg- or serum-albumin, a fact also noticed by Handovsky and Wagner. In the case of a dialysed pseudo-globulin, an immediate precipitation of protein takes place, which is prevented if a small concentration of salt (1 % sodium chloride), acid, or alkali is present.

I have prepared artificial euglobulin from pseudo-globulin in the following

manner. A small amount of a watery emulsion of lecithin [1] was added to the salt-free material obtained at the close of Exps. III and IV, in which the denaturation process had ceased and from which the insoluble protein had been separated. An immediate precipitate occurred, which was prevented by the presence of alkali in minute proportion or of salt (NaCl), to a concentration of about 1 %. On subjecting the solution to dialysis for 14 days precipitation occurred of a protein, containing phosphorus, and with the characteristics of euglobulin.

Suggestions as to the origin of euglobulin in serum.

On consideration of the results of the foregoing experiments it seems not improbable that euglobulin in serum is a complex material, formed from pseudo-globulin by association with some serum-lipoid, to the presence of which it owes its phosphorus-content.

Hardy [1905], on the other hand, has regarded phosphorus as an integral part of the euglobulin molecule. He [1905, p. 331] was unable to remove all the phosphorus from heat-coagulated euglobulin (ox-serum) by treatment with strong acetic acid and subsequent extraction with alcohol and ether. He therefore concluded that it could not be "due to entangled lecithin." Haslam [1913, p. 514] found that extraction of euglobulin (ox-serum) with boiling alcohol removed a yellow, fatty substance which contained phosphorus, the proportion of the latter remaining in the euglobulin being reduced to about one-half the original. It is, however, quite possible that complete extraction with alcohol or ether is rendered very difficult by the state of aggregation of the protein and that if fresh surfaces could in succession be exposed to the action of these solvents a larger proportion of phosphorus could be removed.

In this connection it is worthy of note that the available analyses of euglobulin, some of which are collected in Table IV, show no approach to constancy in the proportion of phosphorus present. Hardy found 0·07 to 0·08 % phosphorus in euglobulin prepared from ox-serum by dilution and acidification; Haslam found 0·108 % and 0·105 % respectively present in two different samples. In three specimens prepared by the same method from three separate samples of horse-serum, I found 0·12 %, 0·032 %, and 0·065 % phosphorus respectively.

[1] For this preparation I am indebted to the kindness of Dr H. Maclean. The lecithin was prepared by his own method [1914] from heart muscle.

TABLE IV.

Phosphorus-content of euglolubin.

No.	Description of material and mode of preparation from serum	Method	G. phosphorus per 100 grms. protein	mean	Authority
1.	Euglobulin (ox-serum) by dilution and acidification	Neumann	0·11 0·11	0·11	Haslam
2.	Euglobulin (ox-serum) by dilution and acidification	Carius	0·07 0·08	0·075	Hardy
3.	Euglobulin (horse-serum *A*) by dilution and acidification once re-precipitated and washed	Neumann	0·122 0·117	0·119	Chick
4.	Euglobulin (horse-serum *A*) after three subsequent re-precipitations	Neumann	0·092 0·096	0·094	Chick
5.	Euglobulin (horse-serum *A*) as (4), but after extraction of the euglobulin suspension with acetone followed by dry ether	Neumann	0·019* 0·020	0·020	Chick
6.	Euglobulin (horse-serum *B*) by dilution and acidification, twice re-precipitated	Neumann	0·033 0·032	0·032	Chick
7.	Euglobulin (horse-serum *C*) by dilution and acidification, four times reprecipitated	Neumann	0·069 0·062	0·065	Chick

* Less than 1 cc. of N/10 NaOH neutralised.

TABLE V.

Proportion of phosphorus contained in a sample of whole horse-serum and in the various proteins after separation by different methods.

No.	Material	G. P. per 100 g. protein	Mean
1.	Horse-serum; whole serum dried in vacuo at 37°	0·098 0·103	0·100
2.	Total proteins of horse-serum, precipitated by dropping into a large volume of absolute alcohol; washed with alcohol and ether; and dried at 105°	0·027 0·018*	0·027
3.	Total proteins coagulated in hot water, coagulum washed with water, alcohol and ether; dried at 105°	0·004* 0·008*	0·006*
4.	Euglobulin prepared from No. 6 by one-third saturation with ammonium sulphate (re-precipitated six times)	0·071	0·071
5.	Euglobulin prepared by dilution and acidification of the original serum (re-precipitated four times and washed twice with distilled water)	0·069 0·062	0·065
6.	Total globulin, prepared by one-half saturation with ammonium sulphate (re-precipitated six times)	0·023	0·023
7.	Serum albumin precipitated in the filtrate from No. 6 by saturation with ammonium sulphate (re-precipitated five times)	0·032	0·032

* Approximate only, less than 1 cc. N/10 NaOH neutralised.

A further set of analyses, see Table V, was made of the total globulin, and euglobulin of horse-serum, as well as the total protein precipitated by various methods. The specimens used were all prepared by Dr P. Hartley with great precautions and carefully purified and I am greatly indebted to him for generously placing this valuable material at my disposal. The results showed that none was free from phosphorus. In some cases the proportion contained showed great variation according to the method employed in preparation (*e.g.* nos. 1, 2, and 3, Table V). The euglobulin contained the greatest amount of phosphorus, and the content was about the same whether precipitated with ammonium sulphate or by dilution and acidification of the serum. This would indicate that the phosphorus contained in euglobulin was in closer association than the traces found present in the purified samples of the other proteins.

Any direct evidence regarding the character of the union of phosphorus in euglobulin is at present scanty, but the following results are worthy of consideration. Haslam [1913] was able to remove about half the total phosphorus from a sample of euglobulin by means of extraction with alcohol and ether. Absolute alcohol must, however, in case of proteins be regarded as a powerful reagent, causing serious changes; all are rendered permanently insoluble if contact is long enough and the temperature is allowed to rise above a low maximum. If previous to extraction with ether, acetone[1], in place of alcohol, is employed to remove the water, no damage appears to be suffered.

The material can then be extracted with ether, three or four changes being employed to remove completely all acetone, and the euglobulin remaining in contact with ether at room temperature for one or two days. The last ether extract being removed, the euglobulin is shaken up once more with ether, which is evaporated by pouring the euglobulin suspension on to a warmed porcelain basin and allowing a gentle current of warm air to play over it, the whole operation taking place in a hot room (about 36°). The extracted euglobulin is then obtained as a fine, white powder, which is exceedingly hygroscopic. The solvents employed should all be carefully purified and freed from water.

The extracted euglobulin was found to have lost nearly all its phosphorus; on one occasion it was reduced to less than one-quarter of the original (see Table V, original phosphorus-content 0·094 %; final 0·02 %). It is possible that the care taken to free all materials from water may account for the

[1] Acetone can be employed to remove water from the proteins of whole serum, withou impairing their solubility in water.

large proportion of the phosphorus removed from the euglobulin in these experiments when compared with those of Haslam.

The ether-extracted euglobulin, like that treated by acetone, was readily soluble in water containing a small amount of acid or alkali. Its solubility in dilute salt solution was, however, always found to be reduced to a greater or less extent. On one occasion about 13 % was found to be soluble in dilute sodium chloride (0·85 %); in most cases the ether-extracted material was totally insoluble in salt solution.

The acetone and ether extracts, when taken to dryness, were found to contain fatty substances, presumably the lipoids contained in the original euglobulin. These fatty materials, which contained a considerable proportion of phosphorus, amounted in one instance to as much as 13·3 % by weight of the protein taken for the experiment. On one occasion, when the extracted euglobulin was rubbed up in a mortar with the fatty residue and salt solution, the power of salt solubility was found to have been restored to some extent, and a similar result was obtained with petrol-extracted euglobulin and the residue from the petrol extract. These two experiments were made with the same sample of euglobulin. With a second sample of euglobulin the experiment was unsuccessful and with a third a trace only was rendered soluble in dilute salt solution by this means. In the case of the first, successful, experiment a large proportion of the lipoid extracted from a comparatively large quantity of euglobulin was devoted to a small fraction of the treated protein and this procedure appears to be necessary to obtain any union and consequent restoration of salt solubility.

Some experiments with ether-extracted euglobulin were also made in which a lecithin emulsion was substituted for the fatty substance extracted from the euglobulin itself. In some instances the lecithin-treated material had distinctly greater solubility in sodium chloride, in others little or no difference from the control could be demonstrated. The question is still under investigation.

The evidence contained in the preceding paragraphs in favour of regarding euglobulin as a mechanical complex consisting of a protein (pseudo-globulin) and a lipoid may briefly be summarised as follows. The phosphorus-content is quite inconstant and shows wide variation in specimens prepared from different samples of serum. By extraction with acetone and ether, under certain circumstances, almost all the phosphorus can be removed together with a small amount of a fatty body. By this procedure the euglobulin, otherwise unchanged, loses its property of salt solubility, which characteristic

has, in some cases, been restored by addition of a lipoid to the ether-extracted material.

As regards the nature of this complex, euglobulin in serum must, in my opinion, be regarded as the product of the interaction of two colloidal systems, viz. the colloidal solution of pseudo-globulin and the emulsion of some lipoid present in the serum. The process is not reversible. The serum-lipoid remains firmly associated with the protein, and cannot be separated by repeated dispersion and re-precipitation, but can be removed by extraction with acetone followed by ether. The freed protein is in the "denaturated" condition, the pseudo-globulin having apparently undergone this irreversible change in the first instance under the influence of the lipoid.

Walpole [1914] has pointed out that, in some respects, euglobulin shows analogies with certain artificial colloidal systems which he has investigated, as, for example, solutions of gelatin to which suspensions of mastic or emulsions of oil have been added. In these mixtures of two colloidal constituents with widely separated iso-electric points, the dual nature of the system is apparent in the want of accurate coincidence between the iso-electric point and that of optimum flocculation. The same phenomenon has been demonstrated in the case of euglobulin [Chick 1913] and may doubtless be similarly explained. Feinschmidt [1912] determined the iso-electric point of six samples of lipoid from various sources to lie at a concentration of hydrogen-ions between about 10^{-2} and $10^{-3\cdot7}$ normal. The iso-electric point of the protein, on the other hand, is at a concentration of hydrogen-ions somewhere between 10^{-5} and 10^{-6} normal. [Michaelis and Rona 1910, 1, Michaelis and Davidsohn 1911, Sörensen and Jürgensen 1911, Chick and Martin 1912.]

The identification of the protein in euglobulin with pseudo-globulin is justified on chemical grounds by some recent work of Hartley[1]. He has shown that on examination of the three proteins (euglobulin, pseudo-globulin and albumin) of serum (horse and ox) by van Slyke's method, the composition of pseudo-globulin and euglobulin is found to be practically the same while a marked difference exists in case of serum albumin.

Summary.

1. Pseudo-globulin, prepared from diluted serum by precipitation with ammonium sulphate, the euglobulin having been previously removed as far as possible by dilution and bringing the solution to the iso-electric point,

[1] Communicated to the Physiological Society, Feb. 14, 1914 (Lister Institute, London) and to the Biochemical Society, July 11, 1914 (Oxford).

was always found, at the end of a long dialysis, to contain large quantities of a water-insoluble protein, resembling euglobulin.

2. Evidence is adduced to show that this insoluble material is not merely euglobulin present in the original serum and precipitated on dialysis, but is largely derived from the pseudo-globulin by a gradual process of "denaturation."

3. The gradual precipitation of the insoluble protein is accompanied by a disappearance from the solution of the small amount of phosphorus originally present in the pseudo-globulin preparation, after which there is no further separation of water-insoluble protein.

4. On addition of a watery emulsion of lecithin to thoroughly dialysed pseudo-globulin, from which insoluble protein and phosphorus had been removed as above, the "denaturation" process could be re-initiated.

5. The presence of minute amounts of acid and alkali or of a small concentration of salt (e.g. 1 % sodium chloride) prevents the precipitation of pseudo-globulin by a watery lecithin emulsion but, on dialysis, an insoluble protein, containing phosphorus and displaying the properties of euglobulin, is separated.

6. This artificial euglobulin appears to be a mechanical complex resulting from the interaction and mutual precipitation of the two colloidal systems : (*a*) the solution of pseudo-globulin and (*b*) the lipoid emulsion.

7. It is suggested that the euglobulin in serum is a protein-lipoid complex of similar origin. In support of this view the following facts are brought forward :

(*a*) The inconstant phosphorus-content.

(*b*) The readiness with which phosphorus can be removed from euglobulin by extraction with acetone and ether, during which process the property of salt solubility is lost. This can, in some cases, be restored by addition of the extracted fatty substance or of lecithin to the extracted euglobulin.

(*c*) The want of accurate coincidence between the iso-electric point of euglobulin and the point of optimum flocculation, as pointed out by Walpole [1914].

(*d*) The similarity in chemical composition between pseudo-globulin and euglobulin, as shown by Hartley.

In conclusion I desire to express my indebtedness to Prof. C. J. Martin, F.R.S. for valuable criticism.

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XLVI. THE HYDROLYSIS OF GLYCOGEN BY DIASTATIC ENZYMES. Part III. FACTORS INFLUENCING THE END-POINT OF THE HYDROLYSIS.

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In a previous communication [Norris, 1913, 1] the author confirmed the result previously obtained by Philoche [1908] that in the hydrolysis of glycogen by diastatic enzymes, the hydrolysis is as a rule far from complete, and also pointed out that the final degree of hydrolysis, as represented by the maltose production, increases with rising concentrations of enzyme though at a very much slower rate. This is therefore rather a different case from the breaking down of starch, where, although the hydrolysis is not complete, the equilibrium point according to most workers is the same with different concentrations of enzyme, viz. about 80·8 per cent. maltose and 19·2 per cent. dextrin. In this connection however Maquenne and Roux [1906, 1, 2, 3] suggest that the reaction only comes to an end owing to an insufficient activity of the enzyme and state that if the diastase be activated by the addition of traces of acid the hydrolysis proceeds to completion, a result which has been confirmed by Bayliss [1914].

It was also found by Brown [1904] that if the maltose formed were removed by fermentation with yeast the whole of the dextrin was hydrolysed, a result which suggested that the products exerted a retarding influence. As however acid is produced from the yeast this result may be really due to the activation of the enzyme and not to the removal of the products.

Under these circumstances it seemed of interest to examine the causes limiting the hydrolysis of glycogen by the glycogenase of the pancreas and some experiments carried out from this point of view are described in the present communication.

The glycogen used was prepared from oysters by Pflüger's method and

purified by repeated precipitation with alcohol. In those experiments where the influence of salts was under investigation the solutions were dialysed before use for several days against frequent changes of distilled water in presence of toluene.

The enzyme was as a rule prepared from dried pigs' pancreas which had previously been rendered fat free, by extraction with 50 per cent. alcohol and precipitation of the filtered extract with alcohol and ether. The precipitate was again taken up in 50 per cent. alcohol, filtered and reprecipitated, this process being repeated two or three times.

The course of the hydrolysis was followed by estimations from time to time of the reducing power of the solutions by Bertrand's method, the assumption being made that the reduction was due to maltose alone. While this is probably not entirely correct, the method is far more convenient than estimations of the optical rotation. The latter is open to the same objection as the former method and in addition necessitates the employment of very dilute solutions whereby further inaccuracies are in all probability introduced. In many cases the iodine reaction was also followed during the experiment.

Exp. 1. *Influence of enzyme concentration on the hydrolysis of glycogen.*

A. 105·5 cc. solution containing 1 g. glycogen, 0·1 cc. enzyme and 1 cc. toluene.

B. 105·5 cc. solution containing 1 g. glycogen, 0·5 cc. enzyme and 1 cc. toluene.

C. 105·5 cc. solution containing 1 g. glycogen, 5·0 cc. enzyme and 1 cc. toluene.

The above were incubated at 37° and samples taken from time to time for analysis, the results being shown in the following table.

TABLE I.

Duration of experiment Hours	Percentage hydrolysis		
	A.	*B.*	*C.*
1	36·65	52·90	69·8
2	40·45	54·00	77·1
3	42·80	54·30	82·0
4	43·70	54·55	84·8
5	44·60	54·70	87·4
6	45·10	—	89·0
7	45·40	—	90·2
8	45·60	—	91·0
9	45·60	55·10	91·5

In the above experiment it will be seen that while with the highest concentration of enzyme rather over 90 per cent. of the glycogen was completely hydrolysed to maltose, with 0·1 cc. enzyme only about half this quantity of maltose was formed. At the same time all the glycogen had disappeared, having been converted into a dextrin which remained to a large extent unhydrolysed. The intermediate products in the hydrolysis of glycogen have not received a great amount of attention. Tebb [1898] however found that a dextrin was formed which was extremely resistant to further hydrolysis and identified this with the dextrin previously described by Seegen [1879] and termed by him dystropodextrin. This dextrin gives no iodine reaction, in fact with pancreatic glycogenase no erythrodextrins appear to be formed, or if they are produced they are hydrolysed so rapidly that it is impossible to detect them.

It is difficult to believe however that the resistant nature of this dextrin is the cause of the stopping of the hydrolysis. For considering the experiment just described, the rates fell off when the reduction indicated a hydrolysis of rather over 40 per cent. in *A*, of 50 per cent. in *B*, and over 80 per cent. in *C*. In other words the slower the initial rate of hydrolysis, the greater was the quantity of resistant dextrin apparently formed. In the case of *C* the slowing off in the rate could be quite well explained by the exhaustion of the substrate, but in *A* and *B* this explanation cannot hold. Another possible solution is that two enzymes may be concerned. This view in the case of diastase has been advanced by several investigators [Brown and Heron 1879; Kjeldahl 1879; Duclaux 1899; Fraenkel and Hamburg 1906]. Now if the enzyme preparation employed in these experiments consists of a large proportion of glycogenase which only carries the hydrolysis to the dextrin stage and a very small amount of dextrinase the results obtained above are to a certain extent what would be expected. For in this case with the low enzyme concentration there might be enough glycogenase to carry out the first stage of the hydrolysis but insufficient dextrinase to hydrolyse the dextrin thus formed at a corresponding rate. In this way an accumulation of dextrin would take place. With the high concentration of enzyme the rate of the first stage of the hydrolysis would be limited by the concentration of glycogen employed and hence would not increase proportionally with rising enzyme concentrations. The second stage of the hydrolysis however would proceed at a much greater rate and hence the accumulation of dextrin would be less as the concentration of enzyme increased. Now while this is actually the final result found experimentally an examination of Table I shows that

when the slow stage of the hydrolysis is reached, *B* and *C* are proceeding not very much more quickly than *A*. Hence some other factors must be concerned and the two which first suggest themselves are (1) destruction of the enzyme during the course of the experiment, and (2) retarding influence of the products. Before considering these points however it will be as well to describe some further experiments in which the results indicated above were reproduced by other methods.

It is well known that the activity of a glycogenase preparation is almost completely lost on dialysis but can be restored by the addition of certain salts, notably chlorides. A certain minimum concentration of such salts varying with the concentration of enzyme is required to produce the optimum rate of hydrolysis [Cole 1906 ; Starkenstein 1910, 1 ; Norris 1913, 2].

Now if a series of dialysed glycogen solutions is made up, each containing the same quantity of dialysed glycogenase but increasing quantities of KCl, not only does the initial rate of hydrolysis increase with rising concentration of salt but the final degree of hydrolysis also rises. These results only obtain of course until the salt concentration reaches the optimum required for the particular strength of enzyme employed.

Exp. 2. *Influence of salt content on the total degree of hydrolysis.*

A. 100 cc. 1 per cent. dialysed glycogen containing 1 cc. toluene and 0·5 cc. dialysed enzyme.

B. 100 cc. 1 per cent. dialysed glycogen containing 1 cc. toluene, 0·5 cc. N/20 KCl and 0·5 cc. dialysed enzyme.

C. 100 cc. 1 per cent. dialysed glycogen containing 1 cc. toluene and 2·0 cc. N/20 KCl and 0·5 cc. dialysed enzyme.

The solutions were incubated at 37° and the reducing power estimated at frequent intervals.

In this experiment *A* which contained no added salt had still a low activity, the dialysis not having been carried sufficiently far to render the solution entirely salt free. *A* therefore represents a solution with very low salt content.

Results :

TABLE II.

Duration of experiment Minutes	Percentage hydrolysis		
	A.	*B.*	*C.*
15	2·07	9·2	13·2
30	3·22	14·6	22·7
60	5·07	21·6	—
75	—	—	30·18
120	10·14	25·68	—
135	—	—	33·71
240	12·89	28·4	—
255	—	—	37·44
360	—	29·28	—
420	16·58	—	38·34
480	—	29·73	39·24

The results are therefore exactly similar to those obtained when different concentrations of enzyme are employed and indicate that a certain amount of the enzyme remains entirely inactive in presence of insufficient salt. This is of some interest as Starkenstein [1910, 2] has shown that even in the absence of any salt, adsorption of diastase by starch still takes place though no chemical change results until salt is added. The salt presumably must have some specific activating effect on the enzyme and its only function cannot be to assist the combination between enzyme and substrate. It also shows that if two enzymes are present they are equally dependent on the presence of salts.

In view of the above experiment it was of interest to see what would be the effect of adding further salt to a solution such as *A* or *B* above when the rate of hydrolysis had become stationary and hence the following experiment was carried out.

Exp. 3. *Addition of salt at "stationary point."*

A. 50 cc. 2 per cent. glycogen + 0·25 cc. N/20 KCl + 1 cc. toluene + 0·5 cc. enzyme + 48·25 cc. H_2O.

B. 50 cc. 2 per cent. glycogen + 10 cc. N/20 KCl + 1 cc. toluene + 0·5 cc. enzyme + 38·5 cc. H_2O.

C. As *A*.

The glycogen and enzyme solutions were dialysed before use. When the rate of hydrolysis in *A* and therefore in *C* had become extremely slow, further salt was added to *C* so that the concentration of KCl became equal to that in *B*.

The course of the hydrolysis in each case is shown in Table III below.

TABLE III.

Duration of experiment Minutes	Percentage hydrolysis		
	A. Low salt content	*B.* High salt content	*C.* Low salt content. Salt added at stationary point
15	3·95	10·14	—
30	7·39	17·40	7·39
240	24·80	33·50	24·90
315	26·50	35·20	26·54
			KCl added here
345	26·80	35·80	28·60
405	27·50	—	31·40
465	27·70	—	32·32
585	28·30	37·80	34·16
705	28·78	38·20	34·63

The results show that the addition of salt to *C* after 315 minutes resulted in an increase in the rate of hydrolysis, *C* increasing 8·1 per cent. during the remainder of the experiment as against the 2·2 per cent. increase shown by *A*. Compared with *B* however, which contained the high salt concentration all the time, *C* still showed a deficit of 3·6 per cent. when the two rates had become equal. This however would be expected as even assuming that all the excess of enzyme not combined with salt were intact when further salt was added, there was at this point already present in *C* a considerable percentage of the hydrolytic products which might retard the freshly activated enzyme. At the same time if the curves shown in Fig. 1 representing this experiment be observed it will be noticed that the rate of action in *C* after the addition of the salt was slightly less than that obtaining in *B* when at the same stage of the hydrolysis, this suggesting that slight injury to the enzyme had occurred.

The case when further enzyme is added at the stationary point is also of interest and will be considered next.

Exp. 4. *Addition of enzyme at "stationary point."*

A. 50 cc. 2 per cent. dialysed glycogen + 0·25 cc. N/20 KCl + 1 cc. toluene + 0·5 cc. dialysed enzyme.

D. 50 cc. dialysed 2 per cent. glycogen + 10 cc. N/20 KCl + 1 cc. toluene + 2 cc. dialysed enzyme.

E. 50 cc. 2 per cent. dialysed glycogen + 0·25 cc. N/20 KCl + 1 cc. toluene + 2 cc. enzyme.

F. As *A*. Enzyme added at stationary point so that enzyme concentration then became equal to *E*.

G. As *A*. Enzyme and salt added at stationary point so that the mixture was then similar in composition to *D*.

The hydrolyses were carried out in the usual manner till *F* and *G* had become very slow, which occurred after about five hours, when the additions were made, the results being shown in Table IV.

TABLE IV.

Duration of experiment minutes	Percentage hydrolysis				
	A. Low salt, low enzyme content	*D.* High salt, high enzyme content	*E.* Low salt, high enzyme content	*F.* Addition of enzyme	*G.* Addition of enzyme + salt
300	26·2	42·1	36·0	26·2	26·2
					Enzyme and salt added
315	26·5	42·4	36·2	26·54	—
				Enzyme added	
330	26·65	42·6	36·3	—	32·32
345	26·80	42·8	36·4	29·36	—
390	27·30	—	—	—	37·90
405	27·50	—	—	31·86	38·50
585	28·30	43·15	37·8	35·11	41·60
705	28·78	43·69	38·3	35·60	42·05

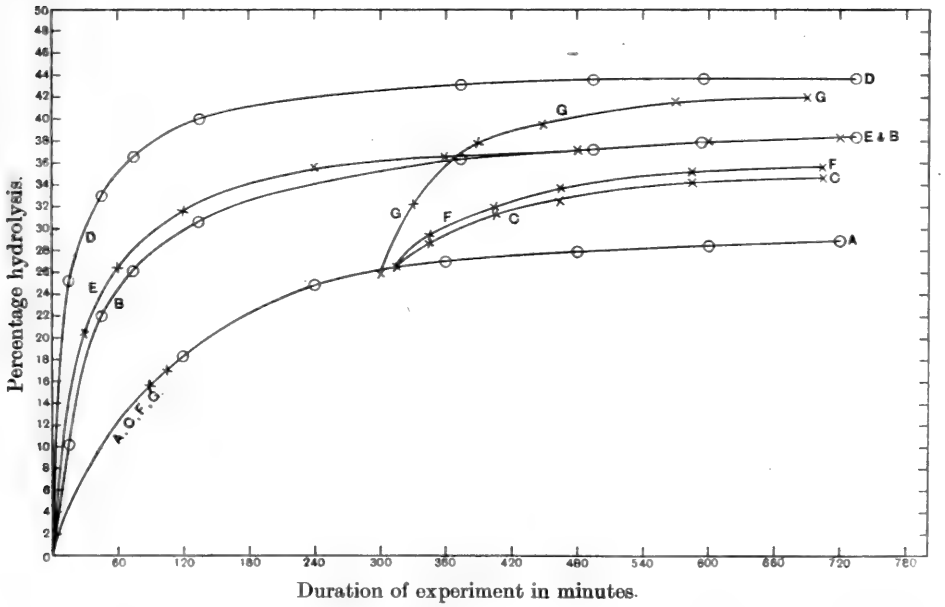

Fig. 1.

The first point of interest is that addition of further enzyme to *F* produced an acceleration although the concentration of salt employed was quite insufficient to permit the enzyme initially present to produce its maximum

effect. There is evidently then an equilibrium established between free salt, free enzyme and the salt-enzyme complex which is changed by the addition of further enzyme, more of the complex being formed and the hydrolysis thereby accelerated. The same point is indicated by the two controls *A* and *E*, *E* proceeding at a much greater rate than *A* although having the same low salt content. The results are shown graphically in Fig. 1, together with those of Experiment 3.

The addition of salt and enzyme naturally produces a greater acceleration than the addition of either separately, and this acceleration is in fact greater than the sum of the two components as one would expect, the enzyme in *F* being retarded by an insufficiency of salt. At the same time the rate in *G* after the addition is less than that obtaining in the control *D* when at the same stage of the hydrolysis. Examining the total degree of hydrolysis produced it will be seen that while *G* never quite reached the control *D*, it approached it very nearly and this also applies to *F* and its control *E*, the deficiency being however slightly greater in this case.

All these results are against the view that the stopping of the action is caused by the resistance of the residual dextrin to further hydrolysis, the addition of further enzyme producing initially a fairly rapid rate of hydrolysis which quickly falls off again, thus suggesting an alteration of an equilibrium between enzyme and the hydrolytic products. The only other obvious factor is the possible destruction of the enzyme during the course of the experiment and the degree to which this is concerned was examined in the following experiment.

Exp. 5. *Addition of further glycogen at the "stationary point."*

A. 100 cc. 1 per cent. glycogen + 1 cc. toluene + 1 cc. enzyme.

B. 100 cc. 2 per cent. glycogen + 1 cc. toluene + 1 cc. enzyme.

C. Initially as *A*. Further glycogen added after 4 hours hydrolysis.

Hydrolysis was allowed to proceed at 37° until the rate in *A* and *C* became slow and then further glycogen was added to *C* so that the total concentration became equal to that originally present in *B*, and the further course of the hydrolysis observed.

The results are shown graphically in Fig. 2, the abscissae representing the duration of the experiment in minutes and the ordinates the amount of hydrolysis which had taken place expressed in percentages of one gram, the latter being calculated as previously explained on the assumption that the reducing power of the solution is due to maltose alone.

The glycogen was added to *C* at 240 minutes and as will be observed was rapidly hydrolysed, showing that a large proportion of the enzyme was still in an active condition although the rate was much lower than that at which the first quantity of glycogen was initially hydrolysed, the relative values for 60 minutes being 9·5 and 17 respectively. As the final degree of hydrolysis observed was practically the same however in *B* and *C* it is extremely improbable that any of the enzyme had been destroyed during the experiment and the difference in the two rates must be attributed to the influence of the products present when the addition of glycogen was made. This view is supported by the next experiment.

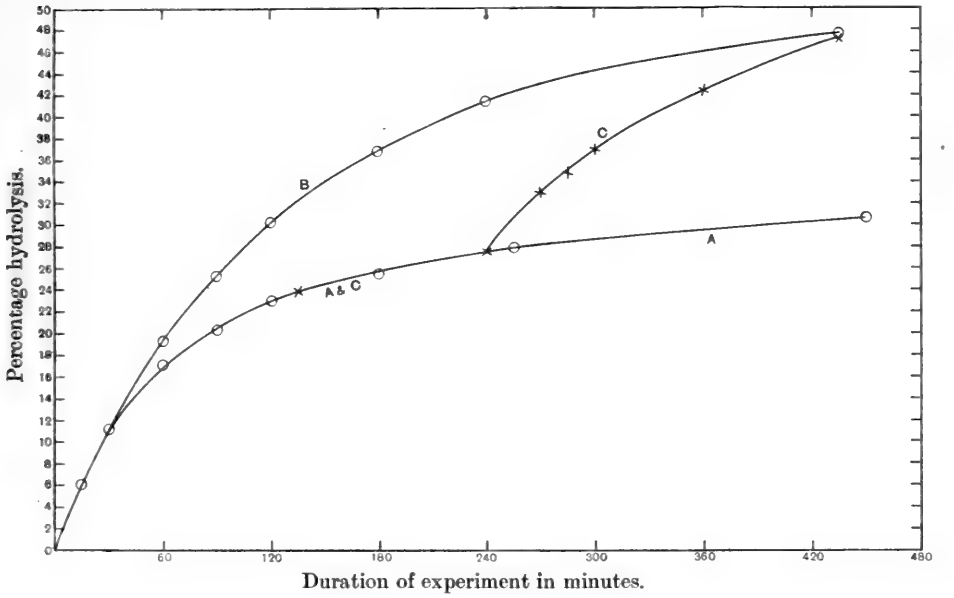

Fig. 2.

Exp. 6. *Addition of glycogen and enzyme at the "stationary point."*

A. 100 cc. 1 per cent. glycogen + 1 cc. enzyme + 1 cc. toluene.

B. 100 cc. 2 per cent. glycogen + 2 cc. enzyme + 1 cc. toluene.

C. Initially as *A*. When the action had slowed down, enzyme and glycogen were added to make the proportion of these similar to those obtaining in *B*.

It was to be expected that the glycogen added to *C* would be hydrolysed with a considerable initial velocity but that this rate would fall off quickly owing to the influence of the products on the freshly added enzyme. For the

same reason it was unlikely that the final degree of hydrolysis would be as great in *C* as in *B*.

An examination of the results shown in Fig. 3 indicates that these hypotheses were verified. Here again the ordinates represent the degree of hydrolysis expressed in the percentages of one gram hydrolysed to maltose.

The glycogen and enzyme were added to *C* after 300 minutes and while the glycogen was readily hydrolysed the rate was actually less than that obtaining initially in *A* which had only half the concentration of enzyme. This result can only be explained by the influence of the products as whatever

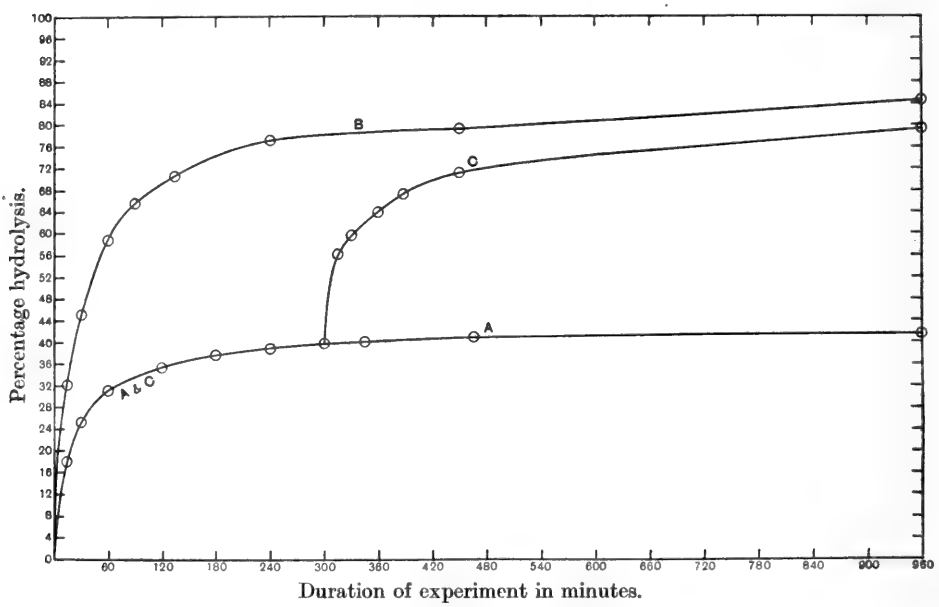

Fig. 3.

may have happened to the first quantity of enzyme during the course of the experiment, that added later was in an active condition and yet could not give such a high rate of hydrolysis as a similar quantity of enzyme acting on a solution containing none of these products. Doubtless for the same reason the final degree of hydrolysis in *C* was less than that observed in *B*.

Influence of the hydrolytic products. (*a*) *Maltose.*

Exp. 7. *A*. 100 cc. 1 per cent. glycogen solution + 1 cc. toluene + 0·5 cc. enzyme.

B. 100 cc. 1 per cent. glycogen solution + 1 cc. toluene + 0·5 g. maltose + 0·5 cc. enzyme.

The only difference between the two experimental mixtures is that *B* contains initially an amount of maltose corresponding to that found at the conclusion of a hydrolysis with this strength of enzyme. The rates at which hydrolysis proceeded in *A* and *B* were then compared, the results being shown in Table V.

TABLE V.

Duration of experiment	mg. Cu from 20 cc. solution	
	A.	*B.* Corrected for added maltose
15 mins.	27	28
30 „	46	46
1 hr.	66	65
2 hrs.	80	80
4 „	92	91
8 „	100	99
20 „	108	104
Percentage hydrolysis	49·6	47·6

This quantity of maltose had then very little depressing action on the rate of hydrolysis.

(*b*) *Influence of mixed products.*

Exps. 8 and 9. 200 cc. of 1 per cent. glycogen solution containing 2 cc. enzyme and toluene were incubated at 37° for 24 hours. The solution was then boiled and the degree of hydrolysis accurately determined.

The following experiments were then carried out.

A. 100 cc. 1 per cent. glycogen solution + 1 cc. toluene + 1 cc. enzyme.

B. 100 cc. boiled solution containing hydrolytic products + 1 g. glycogen + 1 cc. toluene + 1 cc. enzyme.

Two similar experiments were carried out, the results being shown in Table VI.

A control was carried out in which the boiled solution of products alone was incubated with enzyme but practically no further change took place during the course of the experiment.

In Experiment 9 there was a slightly higher initial proportion of products than in 8. In both cases however there is a well marked retarding influence exerted by the hydrolytic products, the differences observed at the end of the experiment being as follows :

Exp. 8. 5·69 in a hydrolysis of 46·55 = 12·2 per cent.

Exp. 9. 6·26 in a hydrolysis of 47·10 = 13·2 per cent.

It is possible that the freshly formed products may have an even greater influence than those examined in the experiments where the solutions had been boiled.

TABLE VI.

Duration of experiment Minutes	Experiment 8		Experiment 9	
	Percentage hydrolysis			
	A.	*B.*	A_1.	B_1.
15	21·40	19·0	18·0	15·2
30	29·49	26·6	27·1	21·87
60	33·77	29·55	33·28	27·65
120	38·57	33·37	38·57	32·78
175	—	—	39·03	33·35
240	40·93	34·3	—	—
360	42·78	36·2	—	—
510	43·25	37·15	—	—
675	—	—	43·72	37·9
1375	—	—	47·1	40·84
1380	46·55	40·86	—	—

Summary and Conclusions.

1. A solution of glycogen is not completely hydrolysed to maltose by the glycogenase of the pancreas unless very high concentrations of enzyme are employed, although, even with low enzyme concentrations, the whole of the glycogen may disappear.

2. The total degree of hydrolysis produced rises with increasing concentration of enzyme but not proportionally.

3. In dialysed solutions the same result is obtained by increasing the salt content up to a certain optimum concentration.

4. Evidence is brought forward to show that the resistance of the residual dextrin to further hydrolysis is not the main factor in the stopping of the reaction.

5. There is little evidence indicating that destruction of the enzyme takes place to any considerable extent during the course of the experiment.

6. The mixed products formed have a marked retarding influence on the velocity of the reaction and are probably the most important factors in the stopping of the hydrolysis. Maltose alone however has a very small effect.

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XLVII. ON THE PURINE METABOLISM OF RATS.

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(*Received July 15th, 1914.*)

Folin and Morris [1913] make the statement that "The purine metabolism of rats is like that of man and unlike that of other mammals hitherto investigated." If this were true the rat would be a most valuable animal on which to investigate the problems of gout.

This statement rests on the following evidence:

1. Rats excrete about 0·5 mg. of uric acid nitrogen a day. This *in proportion to body weight* is about as much as is found in human urine.

2. 100 g. of rat's blood contains 2·4–2·5 mg. of uric acid, "substantially the same figures as Folin and Denis found for human blood."

3. From 50 cc. of rat's urine enough uric acid was isolated for complete identification.

No attempt was made to estimate the allantoin in rat's urine or to observe the effect on the purine and allantoin excretion of adding purine substances to a purine free diet.

The essential difference between the purine metabolism of man and other mammals, except perhaps the chimpanzee, is not in the amount of uric acid excreted in proportion to body weight or the percentage of it contained in the blood, but rather in the fact that in man the purines are not further converted to allantoin as an end product while in other mammals this is the case. The observations recorded in the experimental part of this paper show that the relatively large amount of uric acid excreted by rats is an expression of a very high rate of purine metabolism, the main end product of which is allantoin, this latter representing about 15 times as much nitrogen as that of the total purine bases and uric acid.

Moreover when hypoxanthine is given by the mouth to rats about 60 per cent. of its nitrogen is excreted in the form of allantoin while only about 3 per cent. is excreted as purine base + uric acid nitrogen. This latter observation is also of interest from the fact that Jones and Rhodé [1909] were unable to demonstrate the existence in rat's organs of a ferment capable of destroying hypoxanthine.

Experimental Part.

The rats were kept in pairs in a modified form of the cage described by Völtz [1912]; the urine and faeces were separated by the ball method originally used at the Cancer Hospital, Fulham.

The cages were washed down daily and the urine + washings from each pair of rats preserved with toluene for seven days. The toluene was driven off on the water bath and the seven days' urine made up to 500 cc.

5 cc. were used for total nitrogen by Kjeldahl's method, 275 cc. for purine base + uric acid nitrogen by the Camerer-Arnstein method and 200 cc. for allantoin by Wiechowski's method. In this latter the original method was carried out exactly as described [Wiechowski 1907], and the figures for allantoin nitrogen, calculated from the weight obtained, as well as those given by the Kjeldahl analysis of the mercury acetate precipitate, are given. Most writers seem to consider the latter more accurate and I am inclined to agree with this view from subsequent observations when the diet was less simple in character than bread and water. The allantoin obtained melted in every case at 232°–234°; its solution was not precipitated by silver nitrate but gave a copious precipitate on further addition of ammonium hydrate, soluble in excess of the latter. Boiled with caustic soda it gave the glyoxylic reaction. A collection of the allantoin obtained once recrystallised from water gave the following analysis:

Found		Calculated	
N	= 35·0 %	N	= 35·44 %
C	= 29·73 %	C	= 30·3 %
H	= 3·82 %	H	= 3·8 %

The rats were fed on white bread and water for two weeks before and throughout the experiments. The amount of bread eaten was not limited, for although white bread contains traces of allantoin [Ackroyd 1911] the quantity in the bread eaten by a rat is negligible in comparison with the amount excreted.

All the figures given are calculated as excretion of one rat for one day and represent an average of seven days' excretion for two rats.

During the second week 0·45 g. of hypoxanthine was given to each pair of rats; this was given in equal portions with the first food in the morning of the first five days of the period, the last two days were intended to cover any delayed excretion. This would be equivalent to 0·013 g. purine nitrogen per rat per day calculated for the whole seven day period.

Rats A. Two males weighing 158 *g. and* 174 *g.*

Date	Total N mg.	Purine N mg.	Allantoin N mg. by weight	Allantoin N mg. by Kjeldahl	Diet
Jan. 19–26	191	0·62	10	9·5	Bread, water
Jan. 26–Feb. 2	182	0·95	18·3	17·4	Bread, water. 13 mg. hypoxanthine N
Feb. 2–9	137	0·51	9·4	8·7	Bread, water
Feb. 9–16	156	0·5	8·7	8·5	Bread, water

The average excretions of the first, third and fourth periods are: purine nitrogen 0·54 mg.; allantoin nitrogen 8·9 mg. The increased excretions over these averages in the second period are 0·41 mg. purine N and 8·5 mg. allantoin N equivalent to a recovery of 3·1 % as purine N and 65 % as allantoin N of the extra hypoxanthine N given.

Rats B. Two males weighing 133 *g. and* 165 *g.*

Date	Total N mg.	Purine N mg.	Allantoin N. mg. by weight	Allantoin N. mg. by Kjeldahl	Diet
Jan. 20–27	172	0·66	8·7	8·5	Bread, water
Jan. 7–Feb. 3	191	1·06	17·4	16·3	Bread, water 13 mg. hypoxanthine N
Feb. 3–10	180	0·6	9·8	10	Bread, water
Feb. 10–17	145	0·63	7·5	7·7	Bread, water

The average excretions of the first, third and fourth periods are purine N 0·63 mg., allantoin N 8·4 mg. The increased excretions over these averages in the second period are: purine N 0·43 mg., allantoin N 7·9 mg. equivalent to a recovery of 3·3 % as purine N and 60·7 % as allantoin N of the extra hypoxanthine N given.

Conclusion.

The purine metabolism of rats is like that of other lower mammals and unlike that of man[1].

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[1] Since this work was finished (February 1914), Hunter and Givens (Proceedings of the American Society of Biological Chemists, *J. Biol. Chem.* **17**, xxiii) published an analysis of rats' urine; they find that of the purine + allantoin nitrogen 93 per cent. is present as allantoin, 4 per cent. as uric acid and 3 per cent. as purine bases.

XLVIII. THE PRODUCTION OF ω-HYDROXY-*s*-METHYLFURFURALDEHYDE FROM CARBOHYDRATES AND ITS INFLUENCE ON THE ESTIMATION OF PENTOSANS AND METHYLPENTOSANS.

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(*Received July 13th, 1914.*)

In the method for the estimation of pentosans devised by Kröber and now extensively in use, the substance is boiled with a 12 per cent. solution of hydrochloric acid. The conditions are regulated so that 30 cc. are distilled over every fifteen minutes, 30 cc. of fresh acid being then added to the contents of the flask. The furfuraldehyde contained in the distillate is precipitated by the addition of phloroglucinol, and from the weight of the greenish black precipitate obtained the amount of furfuraldehyde, and consequently of pentosan present, may be calculated with the help of tables given by Kröber [1901]. In the case of methylpentosans, methylfurfuraldehyde is obtained which gives a red precipitate with phloroglucinol. This, unlike the black compound given by furfuraldehyde, is easily soluble in alcohol and on this difference Ellet and Tollens [1905] have based a method for the separation of the two phloroglucides and the consequent estimation of pentosan and methylpentosan groupings.

The phloroglucinol method has long been recognised as a " conventional " one in that (i) a definite procedure of working must be followed, (ii) the relation of the phloroglucide formed to pentosan present is empirical, (iii) other substances may be present in the distillates which also give a precipitate with phloroglucinol.

The difficulty last mentioned was investigated by Unger [1902], who noticed that various pure substances, and also natural products which contained no pentosan groupings, such as dextrose and starch, yielded, on

distillation with hydrochloric acid, volatile substances which could be precipitated with phloroglucinol. Tollens [1902] determined the percentage of " pentosan " given in this way by substances expected to contain very few or no pentosan groupings as follows :

	Per cent.		Per cent.
Cane sugar	1·2	Filter paper	1·5
Potato starch	0·8	Cotton wool	1·6

Grund [1902] also in an investigation of the content of " combined pentoses " in various animal tissues obtained an orange red precipitate from hexoses and from glycogen, and remarks that " apparently with hexoses in addition to furfuraldehyde, another and, till now, unknown body is formed." The complications brought about by the presence of these unknown substances are mentioned in many researches dealing with technical applications of the phloroglucinol method. Reed and Schubert [1914] for example, criticising methods in use for the testing of tanning materials, mention that the red phloroglucide precipitate indicating methylfurfuraldehyde given by various substances which are capable of yielding rhamnose on hydrolysis, such as xanthorhamnin, hesperidin, etc., is not absolutely reliable as a test for these, since dextrose gives a very similar, brown coloured precipitate.

A series of experiments described below has led us to the conclusion that, in the majority of cases, the substance giving this brown precipitate and the superficial reactions of methylfurfuraldehyde is ω-hydroxy-*s*-methylfurfuraldehyde, and that it is a constant product of the action of dilute hydrochloric acid on hexoses and on polyoses, such as starch and cellulose, which are capable of yielding hexoses on hydrolysis.

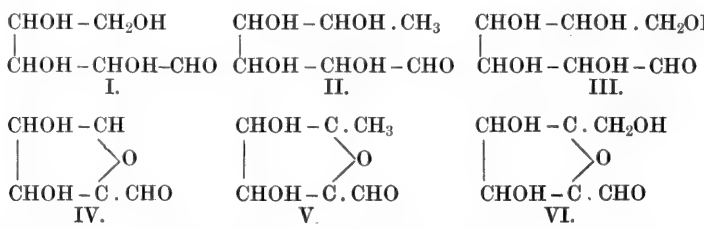
Method of Experiment.

The Kröber method prescribes that the distillation is finished when a drop of the liquid gives no red colour when brought on to aniline acetate paper. As methylfurfuraldehyde does not give this reaction, a solution of phloroglucinol in hydrochloric acid is used when methylpentosans are to be estimated. A solution of aniline in alcohol is also a very sensitive reagent for the detection of furfuraldehyde and its derivatives, and in some estimations we made use of it. We found however that the rose colour given by aniline-alcohol persisted in portions of the distillate long after the aniline acetate paper had ceased to indicate the presence of furfuraldehyde. As is well known in the case of vegetable tissues which contain a considerable proportion of pentosan groupings, the formation and elimination of the furfuraldehyde

are comparatively rapid, *e.g.* with 2 grams of jute (which yielded 9 per cent. of furfuraldehyde) 210 cc. of distillate were sufficient. After this stage no colour with aniline acetate could be obtained. A further 950 cc. however were distilled over before the colouration with aniline-alcohol had ceased to be given.

A number of experiments on these lines was therefore carried out on cellulose and other carbohydrates (see Table III). In each case the aniline-alcohol reaction was obtained during a more or less prolonged period of distillation. In some instances the aniline acetate test for furfuraldehyde could not be observed, in others it was possible to separate the distillate into (*a*) a portion giving the reaction with aniline acetate and containing furfuraldehyde, and (*b*) a portion reacting only with aniline-alcohol.

This second portion in every case gave several of the tests prescribed as characteristic of methylfurfuraldehyde, including the absorption spectrum test devised by Oshima and Tollens [1901] for the detection of this aldehyde in the presence of furfuraldehyde, and a brown precipitate with phloroglucinol soluble in alcohol. Seeing however that pentoses (I) and methylpentoses (II) readily yield furfuraldehyde (IV) and methylfurfuraldehyde (V) respectively, it seemed more probable that hexoses (III) would yield ω-hydroxymethylfurfuraldehyde (VI) thus:



van Ekenstein and Blanksma [1910] have already shown that this substance is formed by the action of dilute oxalic acid, under pressure, on hexoses, and they have proved that many of the colour tests given by the hexoses are due to the formation of ω-hydroxymethylfurfuraldehyde and not to furfuraldehyde as previously supposed. We therefore made a careful comparative examination of the hydrochloric acid distillates obtained from dextrose, starch, cellulose and lignocellulose and satisfied ourselves that these contained besides furfuraldehyde small quantities of ω-hydroxymethylfurfuraldehyde. This derivative is attacked by dilute acids, being converted into laevulic and formic acids thus:

$$C_6H_6O_3 + 2H_2O = C_5H_8O_3 + H \cdot COOH,$$

so that no very large proportion of the theoretical yield could be expected. A recent note of E. Fischer [1914] shows, however, that if the action of the acid be controlled and stopped before this secondary decomposition has begun, comparatively large yields of ω-chloromethylfurfuraldehyde may be obtained.

PROPERTIES AND REACTIONS OF FURFURALDEHYDE AND ITS DERIVATIVES.

For purposes of comparison methylfurfuraldehyde was prepared from rhamnose [Fromherz, 1906] and ω-hydroxymethylfurfuraldehyde both by the action of oxalic acid on cane sugar [Kiermayer, 1895] and from the chloro-derivative obtained from laevulose by Fenton's reaction. Our observations made from distillates from about 0·2 gram of each aldehyde are given in the following table.

TABLE I.

Reactions of furfuraldehyde and its derivatives.

	Furfuraldehyde	Methyl-furfuraldehyde	ω-Hydroxymethyl-furfuraldehyde
1. Aniline acetate	pink	yellow	pink
2. Aniline-alcohol	bright red	yellow-red	bright red
3. Phloroglucinol-ppt.	black	red	red-brown
4. ,, -filtrate ..	green	red-brown	orange-red
5. Absorption spectrum of (4) ..	none	band in blue green	band in blue green
6. α-Naphthol + H_2SO_4*	grey red greenish	colourless scarlet greenish	violet crimson emerald green
7. Thymol in alcohol + H_2SO_4 ..	red, violet above	red-pink, yellow above	scarlet with violet above
8. Barbituric acid	yellow ppt.	yellow ppt.	No ppt.

* Colours given in order from the top.

The second portion of the distillates from all the substances mentioned in Table IV gave reactions indistinguishable from those shown by ω-hydroxymethylfurfuraldehyde. The Maquenne test for methylfurfuraldehyde was obtained only with rhamnose. In order further to confirm the presence of the hydroxyaldehyde 64 grams of viscose silk were boiled under a reflux for 2 hours with 600 cc. of the hydrochloric acid. The filtered liquid was neutralised and extracted with ether. From the extract a brown oil giving the reactions of furfuraldehyde was obtained. The extracted water solution was distilled in a current of steam and gave a distillate which answered all the tests for ω-hydroxymethylfurfuraldehyde. This observation is in keeping with the fact that this substance is not extracted by cold ether from aqueous solution.

The Composition of the Precipitates given by Phloroglucinol with Furfuraldehyde and its Derivatives.

Kröber [1901] stated that the phloroglucide was formed by the condensation of one molecule of furfuraldehyde and one molecule of the phenol with elimination of two molecules of water thus:

$$C_5H_4O_2 + C_6H_6O_3 - 2H_2O = C_{11}H_6O_3,$$

and that the composition of the precipitate (C = 71·0; H = 3·2 per cent.) was independent of the conditions of precipitation. Tollens and Goodwin [1904] showed that the compound is considerably oxidised on drying in the air, but that preparations dried in hydrogen gave figures agreeing exactly with those required by the equation:

$$C_5H_4O_2 + C_6H_6O_3 - H_2O = C_{11}H_8O_4.$$

Assuming that a similar reaction takes place with the derivatives we have the following theoretical values:

TABLE II.

	Phloroglucide of:	Formula	requires
(I)	Furfuraldehyde	$C_{11}H_8O_4$	C = 64·7; H = 4·0
(II)	Methylfurfuraldehyde	$C_{12}H_{10}O_4$	C = 66·0; H = 4·6
(III)	Hydroxymethylfurfuraldehyde	$C_{12}H_{10}O_5$	C = 61·5; H = 4·2

The alcohol soluble part of a phloroglucide which we obtained from dextrose, after drying as far as possible with exclusion of air, gave results agreeing to some extent with the composition of the hydroxymethyl derivative:

0·1260; 0·2856 CO_2; 0·0550 H_2O.
Found C = 61·8; H = 4·3 per cent.

The formula $C_{12}H_{10}O_5$ gives the factor 0·54 for conversion of the weight of phloroglucide to weight of hydroxymethylfurfuraldehyde. In calculating Table III, the factor 0·6 was employed.

Quantitative Experiments.

In order to ascertain whether the constant formation of hydroxymethylfurfuraldehyde involves any error in the estimation of furfuraldehyde by the phloroglucinol method, and to obtain an idea of the amount produced, the experiments summarised in the table below were made. The precipitate given by that portion of the distillate which reacted to aniline acetate, if it could be collected separately, or if not, the portion of the total precipitate

insoluble in alcohol, was calculated as furfuraldehyde. The soluble portion was calculated as ω-hydroxymethylfurfuraldehyde (factor 0·6). A comparative value only can, however, be attached to these latter numbers. For the purification of the celluloses employed see Dorée [1913].

TABLE III.

No.	Substance	Weight (anhydrous)		Distillate cc.*	Weight of phloroglucide	Weight soluble in alcohol	Furfuraldehyde per cent.	ω-hydroxymethylfurfuraldehyde per cent.	Remarks
1.	Rhamnose	0·424		760	0·2630	all	—	—	Ppt. red and consists of methylfurfural phloroglucide
2.	Dextrose	2·123		610	0·0565	0·0550	0·18	1·6	No aniline acetate reaction; ppt. brown
3.	Laevulose	3·770		240	0·0843	0·0712	0·20	1·1	After 60 cc. no aniline acetate reaction; ppt. red
4.	Starch (potato)	3·774		680	0·0981	0·0902	0·15	1·4	After 250 cc. no aniline acetate reaction; ppt. red
5.	Cellulose (cotton wool)	2·021	(*a*)	100	0·0073	none	0·20		Ppt. black
			(*b*)	600	0·0344	0·0115	(0·67)	0·34	Ppt. red
6.	Cellulose (filter paper)	5·090	(*a*)	250	0·0296	—	0·35	—	
			(*b*)	1080	0·0580	—	—	0·69	
7.	Cellulose (No. 5 mercerised)	1·662		1025	0·0227	all	0·00	1·0	
8.	Cellulose (structureless from the xanthate)	2·142	(*a*)	200	0·0358	—	1·04	—	After 180 cc. no aniline acetate reaction
			(*b*)	300	0·0317	—	—	0·9	
9.	Cellulose ,,	4·753		1560	0·2466	0·1862	0·76	2·4	
10.	Oxycellulose (from No. 7 by ozone)	2·00		660	0·0550	0·0193	1·11	0·66	After 300 cc. no aniline acetate reaction
11.	Lignocellulose (Jute)	1·869	(*a*)	210	0·3291	0·0010	9·4	—	Part soluble was red
			(*b*)	950	0·0357	0·0127	—	0·90	
12.	Lignocellulose (Beech Wood)	2·600	(*a*)	400	0·6909	—	12·68	—	
			(*b*)	660	0·0148	—		0·50	

* (*a*) represents portion of distillate reacting to aniline acetate, (*b*) is the portion subsequently obtained active to the aniline-alcohol reagent.

Discussion of the Results.

(1) These results show that the condensation which produces furfuraldehyde takes place rapidly and is almost completed before the hydroxymethyl derivative begins to distil over. In experiments 5 and 11 for example the

phloroglucides obtained from the first portion of the distillates were practically insoluble in alcohol, consisting entirely of the furfuraldehyde compound. The aniline acetate paper test therefore determines the separation of the two aldehydes and practically no error is made in pentosan estimations if this indicator is used. The possible error is further minimised in the case of bodies such as jute and beech wood which contain a large proportion of pentosan constituent, for the results show that these typical substances give the smallest yields of hydroxymethylfurfuraldehyde (Nos. 11 and 12).

(2) The hydroxyaldehyde is produced at a very slow rate. Its total amount is small, varying between 1 and 2 per cent. in the case of the hexoses and those celluloses which contain little or no pentosan. The probable reason for this is the ready hydrolysis of the hydroxymethylfurfuraldehyde to formic and laevulic acids already mentioned. This property no doubt explains the observations of Fraps [1901]. He found that if the distillates obtained from a number of natural products, food-stuffs, etc. were distilled again a considerable loss of " furfural " was shown. With pure furfuraldehyde this did not occur. The reduction in the amount of pentosan constituent varied from 7 per cent. in the case of oak wood to 22·6 in the case of bran. He considered that this portion could not be regarded as true pentosan, and, in the absence of further knowledge, designated it as the " furaloid " constituent. In all probability however the " furaloid " is a hexose-yielding constituent which gives hydroxymethylfurfuraldehyde and this on a second distillation is largely decomposed.

(3) The constant presence of ω-hydroxymethylfurfuraldehyde in the distillates from cellulose and starch renders determinations of methylpentosan, previously made, open to considerable suspicion. In the only method available, that of Ellet and Tollens [1905], the alcohol-soluble portion of the phloroglucide is calculated as methylpentosan. The work of Fromherz [1906] is interesting in this respect. In order to carry out an examination of the variation in the pentosan and methylpentosan constituents of wood under various alkaline and hydrolytic treatments and anticipating the presence of unknown substances likely to give a precipitate with phloroglucinol he used the method of precipitation with barbituric acid, first suggested by Unger [1902], as a check on the results. Fromherz worked out the application of this reagent to the estimation of methylfurfuraldehyde, finding that combination took place according to the equation :

$$C_6H_6O_2 + C_4H_4O_3N_2 - H_2O = C_{10}H_8O_4N_2.$$

Some of his results with phloroglucinol are as follows :

TABLE IV.

	Furfuraldehyde per cent.	Methyl-furfuraldehyde per cent.
Wood (extracted with alcohol-ether)..	12·68	0·43
„ (6 times treated + NaOH) ..	2·29	0·50
„ (9 times „ „) ..	2·27	0·50

It will be observed that while the pentose constituent represented by the furfuraldehyde obtained is rapidly dissolved by treatment with sodium hydroxide, the methylpentosan remains unchanged in amount. This in itself is exceedingly improbable, but it will further be noticed that the amount of " methylfurfuraldehyde " obtained agrees closely with the amount obtained by ourselves from purified beech wood and shown to consist in all probability of ω-hydroxymethylfurfuraldehyde derived from the cellulose. Unfortunately the barbituric acid method does not permit of a separate estimation of the methylfurfuraldehyde, but calculating by an indirect method Fromherz found that the total of the furfuraldehydes given by phloroglucinol was always higher than that given by barbituric acid, the figures in one case for example being 2·78 and 1·92 per cent. respectively. In a series of experiments we found that in the dilutions commonly obtained in the distillates, ω-hydroxymethylfurfuraldehyde did not give a precipitate at all with barbituric acid. This observation would seem to confirm our view that it is not a case of methyl- but of hydroxymethyl-furfuraldehyde of quite different origin.

(4) Experiments do not throw any light on the remarkable fact that the ketoses give comparatively large yields of hydroxymethylfurfuraldehyde by the action of acids, while the aldoses give little or none. Some of these values calculated as the hydroxy-derivative are given in the following table:

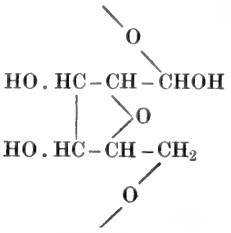
TABLE V.

Yield of ω-hydroxymethylfurfuraldehyde per cent.

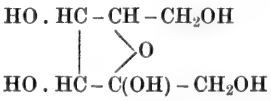
	HBr in chloroform solution[1]	Aqueous oxalic acid[2]	40 % aqueous HCl (sp. g. 1·21)[3]	Aqueous HCl (sp.g. 1·06)[3]
Dextrose ..	2	1	1	1·6
Lævulose ..	15	20 to 25	—	1·1
Cane Sugar	6	—	15 to 20	—
Cellulose ..	20	—	5	0·6

[1] Cross and Bevan.
[2] van Ekenstein and Blanksma.
[3] Dorée and Cunningham.

Although by the action of 40 per cent. aqueous hydrochloric acid cellulose is converted quantitatively into dextrose [Willstätter, 1913], yet with an ethereal solution of the acid it gives a high yield of ω-chloromethylfurfuraldehyde. It has been argued from this that cellulose must have a structure similar to that of laevulose and probably therefore contains a carbonyl grouping in the complex. But on theoretical grounds it seems to us that under anhydrous conditions the transition of laevulose to hydroxymethylfurfuraldehyde is far more likely to take place if the sugar functions in the γ-oxide form (II), and if this is so the cellulose formula (I) given by Green [1906], modified to show a connection between the unit groupings, explains very simply the conversion of cellulose, through laevulose, to ω-hydroxymethylfurfuraldehyde.



I. Cellulose formula (Green) modified for an aggregate of groupings.



II. Laevulose; γ-oxide formula.

Summary.

(1) ω-Hydroxymethylfurfuraldehyde is formed by the action of dilute hydrochloric acid on hexoses, starch and the celluloses. Its amount varies from one to two per cent.

(2) Owing to its slow formation it does not interfere with the accuracy of pentosan estimations made by the Kröber phloroglucinol method, if aniline acetate is used as the indicator.

(3) Its occurrence, however, renders previously made estimations of methylpentosan of doubtful value.

(4) It is probably the unknown substance giving a precipitate with phloroglucinol referred to by previous workers, and its presence explains many of their observations.

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XLIX. THE UREASE CONTENT OF CERTAIN INDIAN SEEDS.

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During preliminary work on the nitrogenous constituents of certain Indian seeds a number were tested for urease.

Owing to the fact that they contain urease, soy beans have been utilised by Marshall [1913, 1, 2), and by Plimmer and Skelton [1914] for the estimation of urea in urine. In the present work it was thought worth while to test certain well defined varieties of soy-beans in order to see if there were any large variation in their urease activity. During the course of the work a seed was found whose urease activity was many times greater than that of soy-bean and this short paper has chiefly been written to bring this fact to notice, since it should be very useful in the estimation of urea.

Zemplèn [1912] has tested the seeds of many plants for urease. He found the enzyme in most papilionaceous but not in graminaceous ones.

In the work here described the following preliminary test for urease was applied to the seeds. They were finely powdered in a Maercker mill and about 0·5 g. was then placed in a test-tube containing 10 cc. of 1 per cent. urea solution with 3 drops of phenolphthalein and a few drops of toluene. Each tube was left 24 hours. If urease be present in the seed, ammonium carbonate should be formed and shew its presence by the reddening of the liquid.

By this method the following seeds appeared to contain urease :

Soy bean yellow, Soy bean spotted, Soy bean chocolate, Soy bean common, Soy bean black, *Sida spinosa*, Gram (*Cicer arietinum*), Rice (*Oryza sativa*), Cowa (*Setaria italica*), Sword bean (*Canavalia ensiformis*), (both a sample from U.S.A. and a sample grown at Dacca), *Urana lobata*, Khulti kalai (*Dolichos biflora*), Castor bean (*Ricinus communis*).

The method indicated the absence of urease in the following seeds: French beans (*Phaseolus vulgaris*), Velvet beans (*Mucuna pruriens*), Sona mung (*Phaseolus radiatus*), *Sida* sp. (from Burmah), *Sida cordifolia*, White til (*Sesamum indicum*), Black til (*Sesamum indicum*), Mati kalai (*Phaseolus mungo*), Cow pea (*Vigna catiang*), White mustard (*Brassica campestris* var. *sarson*), Red mustard (*Brassica campestris* var. *dichotoma*), Niger (*Guizotia abyssinica*), Taramira (*Eruca sativa*), Sweet pea (*Lathyrus sativus*).

The above test is by no means a reliable one, however, as it was found later (see below) that certain seeds which by the above test appeared to contain urease actually contained none at all, *e.g.*, cowa and rice. The shell of castor bean is strongly alkaline and hence itself colours the test solution red.

A number of the above seeds were next taken for determination of their comparative urease activity.

The following method was used:

10 g. of the powdered seed were treated with 100 cc. of distilled water and allowed to stand with occasional shaking for one hour at room temperature in presence of toluene. 2 cc. of this extract were then added to 50 cc. of a 1 per cent. urea solution together with 0·5 cc. of toluene. 5 cc. of the liquid were then immediately removed and titrated, using methylorange as indicator, with N/10 HCl. Thence onward, generally at half hour intervals, successive portions of 5 cc. were titrated. The tests were carried on at room temperature, about 27°.

We thus have a small amount of enzyme acting on a large amount of substrate and therefore the method is a fairly accurate comparative test of the urease activity. The tables shew that there are considerable differences in the urease activity of different seeds.

The following were in this way found to contain no trace of urease: *Sida spinosa*, Rice (*Oryza sativa*), Cowa (*Setaria italica*), Peas (*Pisum arvense*), Mati kalai (*Phaseolus mungo*), Gram (*Cicer arietinum*), Sona mung (*Phaseolus radiatus*), Cow pea (*Vigna catiang*), Velvet beans (*Mucuna pruriens*).

A number of pure line types of Mati kalai (*Phaseolus mungo*) grown by the Economic Botanist to the Government of Bengal, besides certain well-defined local varieties, were tested. Not one was found to contain a trace of urease.

It might be here mentioned that during the course of this work a ground sample of Khulti kalai (*Dolichos biflora*) which is seen in the table to contain urease was tested two weeks later and gave no indication whatever of its

cc. N/10 HCl *required.*

Time in hours	Soya beans						Sword beans		Khulti kalai (*Dolichos biflora*)	Urena Lobata	Urena Lobata, another sample
	Yellow	Common	Chocolate	Spotted	Black	Rymbsa Ktang	U.S.A.	Dacca			
initial	0·4	0·5	0·5	0·8	0·5	0·5	1·2	0·6	0·2	0·2	0·4
0·5	1·8	2·2	2·0	2·9	1·5	1·4	8·7	5·9	2·7	1·9	1·7
1	3·6	3·5	3·0	4·2	3·0	2·7	13·0	15·4	3·2	4·0	3·9
1·5	4·6	4·4	4·6	5·6	4·4	3·7	15·7	16·7	3·7	5·4	5·2
2	5·4	5·4	5·0	6·7	5·2	4·1	15·9	16·7	4·2	—	6·8
2·5	—	—	—	—	—	—	—	—	—	—	7·9
3·5	—	—	—	—	—	—	—	—	—	9·1	
4	—	8·5	7·7	10·3	7·9	7·7	16·0				
5	—	9·7	8·9	12·2	9·4	8·5	—	—	6·2		
6	—	11·5	10·0	13·6	9·4	9·2	16·0	—	7·2		
7·5	—	—	—	—	—	—	—	—	—	10·4	
14	15·9	16·5	16·7	16·5	16·4	16·1	—	—	—	15·8	
15	—	—	—	—	—	—	—	—	—	15·8	
24	—	—	—	—	—	—	—	—	—	—	13·3
26	—	—	—	—	—	—	—	—	—	—	14·3
50	—	—	—	—	—	—	—	—	—	—	15·6

presence. Zemplèn [1912, p. 233] on the contrary found that powdered seed kept in a vacuum desiccator over sulphuric acid for six weeks was just as active as the fresh material. The powdered seed kept by me, however, was simply corked up in a test tube.

Summary.

(1) The urease activity of Sword bean (*Canavalia ensiformis*) is many times greater than that of any of the Soy bean varieties which were tested. This is the case with both American and Indian seed. The table shows that an extremely rapid production of ammonium carbonate takes place immediately on adding sword bean extract to urea solution.

(2) The urease activity of five of the six varieties of soya bean examined is practically identical. The sixth variety is distinctly more active.

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L. ON THE SO-CALLED "ACETONE-SOLUBLE PHOSPHATIDES."

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Though many points relating to the nature and distribution of the phosphatides still await solution, it has recently been shown that certain bodies which were supposed to be definite substances are really nothing more than lecithin contaminated by nitrogenous impurities [MacLean, 1913]. This observation has materially simplified the phosphatide problem, and it seems probable that further research will reduce the number of these bodies to a comparatively few well-defined substances. It is now definitely established that the only alcohol-soluble and acetone-insoluble phosphatide present in the majority of tissues is lecithin. The substance described as a di-amino-monophosphatide, and obtained from ether-extracted tissues by subsequent extraction with alcohol, is not a definite substance but a mixture of lecithin with certain basic bodies. Lecithin can be easily obtained from the mixture by emulsification with water and precipitation with acetone [MacLean, 1912]. The only tissue phosphatides identified with certainty are lecithin, kephalin, cuorin and sphingomyelin, and all these substances differ from ordinary neutral fat and from fatty acids in the fact that they are insoluble in acetone.

Under certain conditions lecithin is precipitated almost quantitatively from its ethereal solution by excess of acetone, and this property is constantly made use of in the separation of the phosphatides from accompanying fats and fatty derivatives.

While it is agreed that phosphatides in general are insoluble in acetone, certain observers have described another distinct class of these bodies—*acetone-soluble phosphatides.*

Thus Fränkel and Pari [1909] extracted ox-pancreas with acetone and from the acetone extract obtained a phosphatide which was isolated as the cadmium chloride combination. This compound, which they called vesalthin, had a nitrogen to phosphorus ratio of 1 : 1 and in many ways did not materially differ from the compound formed by lecithin with cadmium chloride. It had an empirical formula represented by $C_{32}H_{63}O_9NP \,.\, CdCl_2$ and contained

1·71 % N and 3·78 % P. N : P = 1 : 1.

Erlandsen [1907] obtained a somewhat similar substance from ox-heart; this substance he isolated as the platinum derivative. The dried heart tissue was extracted with ether, the ethereal extract concentrated to a syrup and excess of acetone added; on separating the precipitated lecithin the acetone solution still contained a phosphatide together with other acetone-soluble fatty bodies. On concentration of the acetone solution the phosphatide was not precipitated by the addition of more acetone but easily passed into solution. The residue obtained on evaporation of the acetone was extracted with a little alcohol and the phosphatide precipitated by the addition of an alcoholic solution of platinum chloride. The precipitate was washed with alcohol and dried in vacuo. The platinum chloride combination thus obtained had the empirical formula $(C_{33}H_{62}O_8NP)_2H_2PtCl_6$;

N = 1·81 %, P = 3·69 %; N : P = 1·09 : 1.

Now since lecithin has approximately the formula $C_{43}H_{80}O_9NP$ with 1·8% nitrogen and 4% phosphorus, it is obvious that the phosphatides of the above compounds of Fränkel and of Erlandsen contain a good deal more nitrogen than is present in lecithin. In fact the nitrogen percentage of the above *compounds* is almost exactly the same as that of *free* lecithin, while lecithin-cadmium-chloride contains about 1·4 % nitrogen and 3·90 % phosphorus.

Since it has been shown that lecithin very often contains impurities of a basic nature together with phosphorus compounds, it was possible that the above acetone-soluble phosphatides were nothing but impure lecithin which, owing to some physical or chemical change, had become soluble in acetone.

In order to test this, an ethereal extract of dried ox-hearts was made in the usual way. The greater part of the ether was evaporated, the lecithin precipitated by the addition of a large excess of acetone and the acetone filtrate evaporated to a thick syrup. This syrup contained large amounts of nitrogen and phosphorus indicating the probable presence of a phosphatide, but on the addition of more dry acetone no precipitate whatever was obtained.

This acetone was evaporated under reduced pressure and the syrup obtained allowed to flow slowly drop by drop into a large bulk of acetone in a tall cylinder. For each cc. of syrup about 200 cc. of acetone were taken. Immediately on the addition of the first few drops the liquid became hazy and then opalescent. After adding the calculated amount of syrup, the cylinder was shaken thoroughly, when a well-marked bulky flocculent precipitate separated out, leaving the yellowish tinged acetone quite clear. After standing overnight the supernatant fluid was separated, the acetone again evaporated and the syrup tested for nitrogen and phosphorus. As both were still present in considerable amount, the syrup was again added drop by drop to excess of acetone. Again a slight haziness appeared, followed by the formation of a small amount of flocculent precipitate. A third repetition of this process gave a very slight precipitate, but on the fourth attempt, the acetone to which the syrup was added remained perfectly clear, not a trace of precipitate being produced. On evaporation of the acetone the residue still gave distinct reactions for nitrogen and phosphorus. For purposes of description later on this residue will be called *syrup M*.

The different precipitates obtained above were collected and dissolved in a small amount of ether. On addition of acetone a well-marked precipitation at once took place and the substance behaved in all respects like lecithin. On analysis, however, the nitrogen content was found to be much too high, so the syrup was purified by emulsification with water and precipitation with acetone [MacLean, 1912]. A considerable amount of some basic impurity together with some phosphorus was separated off. The purified phosphatide was then dissolved in ether, precipitated with acetone and dried in vacuo over H_2SO_4. Analysis gave

$$N = 1{\cdot}81\ \%\ ;\quad P = 3{\cdot}96\ \%\ ;\quad N : P = 1{\cdot}01 : 1.$$

It was soluble in ether, alcohol, chloroform and benzene but quite insoluble in acetone. Its alcoholic solution was precipitated by cadmium chloride and by platinum chloride. On hydrolysis, choline was obtained. Its behaviour, in all respects like ordinary lecithin, left no doubt that this "acetone-soluble" substance was really lecithin.

An attempt was now made to investigate the substance present in the acetone syrup M mentioned above. When added drop by drop to pure dry acetone not the faintest haze appeared. Since it is well known that the presence of electrolytes often plays an important part in the precipitation of lecithin, it was thought that the addition of a trace of electrolyte to the

acetone might give a precipitate. Since the acetone used in these experiments was carefully dried over calcium chloride and distilled immediately before use, no electrolyte could be present. A trace of calcium chloride was now added to the acetone and the syrup run in drop by drop as before. Immediately a marked opalescence appeared, followed by a very distinct precipitate. This process was repeated as described above until no more precipitate could be obtained. The final acetone solution, on evaporation to a syrup, was found to be *free from nitrogen and contained only a very faint trace of phosphorus*. Thus the whole of the phosphatide was precipitated by acetone in the presence of a minute quantity of an electrolyte.

The different precipitates were mixed together, dissolved in ether, filtered and precipitated by acetone. The precipitate obtained was purified in the usual way with water and acetone, dissolved in ether, again precipitated with acetone and dried in vacuo. Analysis gave:

$$N = 1{\cdot}86\ \%\ ;\quad P = 3{\cdot}98\ \%\ ;\quad N : P = 1{\cdot}03 : 1.$$

In solubilities and other physical and chemical properties, it behaved exactly as ordinary lecithin. The whole of the phosphatide present in the original solution was therefore lecithin.

In another experiment, two ox-hearts were extracted with alcohol, the alcohol evaporated, the residue taken up with ether and precipitated with acetone as described. The acetone solution was allowed to stand in the ice-chest over night when a slight precipitate formed. This was filtered off, the acetone evaporated and the residue added drop by drop to excess of acetone containing *a trace of calcium chloride*. The proportions taken were 4 cc. syrup to 100 cc. acetone. A very marked flocculent precipitate was obtained. On separating the precipitate, evaporating the acetone and repeating the above process, another well-marked precipitate separated. On the other hand when a small amount of the syrup was added to pure acetone free from electrolytes the fluid remained quite clear.

The process was repeated six times, when it was found that practically no substance separated on the addition of the syrup to the acetone containing calcium chloride.

The syrup now obtained on the evaporation of the acetone contained 0·025 % phosphorus and 0·362 % nitrogen, *i.e.*, N : P = 11 : 1. The original syrup was divided now into parts:

A	*B*
Residual syrup which gave no precipitate when added to excess of acetone containing an electrolyte.	Precipitate obtained by adding syrup to acetone containing an electrolyte.

Syrup A. From the relation of the nitrogen to phosphorus it was obvious that only a small portion of the nitrogen, if any, was present in phosphatide form. On addition of an alcoholic solution of cadmium chloride no precipitate was obtained unless excess was added, when a slight opalescence occurred. It cleared up immediately on the addition of excess of alcohol. The same phenomenon occurred when platinum chloride in alcoholic solution was added.

When three drops of a 1 % alcoholic solution of lecithin were mixed with 5 cc. of the syrup, the addition of a few drops of cadmium chloride gave a distinct precipitate which did not disappear in excess of alcohol. Thus when a trace of lecithin was added the behaviour of the syrup was entirely different from that of the control, showing that the substance originally present was not lecithin. The reactions of the syrup with cadmium chloride and platinum chloride were exactly the same as those obtained with a basic water-soluble impurity which I had previously shown to be constantly associated with tissue lecithin [MacLean, 1913].

On extracting the syrup three times with water, the aqueous solution contained all but a trace of the nitrogen of the syrup, while no phosphorus could be detected. The part insoluble in water gave no precipitate whatever on the addition of cadmium chloride or platinum chloride in excess and was practically free from nitrogen. It is, therefore, clear that the nitrogen of the syrup was present in some water-soluble combination which contained no phosphorus or at most only a trace of phosphorus. This entirely excludes phosphatides.

Precipitate B obtained by adding syrup to acetone containing an electrolyte.

This substance was dissolved in ether and precipitated with acetone. It was then purified three times with acetone and water, and a water-insoluble residue obtained. The acetone-water solutions were mixed together and evaporated to a syrup. Substance *B* was thus divided into two parts :

(1) A portion insoluble in water.

(2) A water-soluble portion.

(1) *The water-insoluble portion.*

This constituted the greater part of the substance *B*. It was soluble in alcohol, ether, chloroform and benzene. From its ethereal solution it was precipitated almost quantitatively by acetone even in small amount,

It contained choline and its physical characteristics appeared identical with those of lecithin. On analysis it gave:

$$N = 1{\cdot}85\ \%\ ;\quad P = 4{\cdot}05\ \%\ ;\quad N : P = 1{\cdot}01 : 1.$$

From its analyses and reactions there can be no doubt that the substance was ordinary lecithin.

(2) *The water-soluble portion.*

This had all the properties of the water-soluble substance first described. It was insoluble in ether, soluble in alcohol containing a trace of moisture, but practically insoluble in water-free alcohol. On hydrolysis, no fatty acids were produced. Analysis gave:

$$N = 2{\cdot}05\ \%\ ;\quad P = 0{\cdot}43\ \%\ ;\quad N : P = 10 : 1.$$

A third experiment carried out with an old extract of heart on the lines indicated gave similar results. A substance was obtained from the acetone extract which had all the reactions of lecithin. Analysis gave:

$$N = 1{\cdot}89\ \%\ ;\quad P = 3{\cdot}94\ \%\ ;\quad N : P = 1{\cdot}05 : 1.$$

From these results it is clear that the ' acetone-soluble ' phosphatide described as obtained from ox-heart is really ordinary lecithin associated with a nitrogenous impurity. This lecithin is easily precipitated by acetone when certain electrolytes are present, but in the absence of these electrolytes the presence of fatty acids and fats renders it soluble in acetone. When separated, it behaves in all respects like ordinary lecithin both towards acetone and other reagents.

It is very probable that all phosphatides are insoluble in acetone, and that an application of the above methods to other tissues would result in eliminating from the literature certain substances which are supposed to be individual phosphatides, but which are really nothing more than impure lecithin.

Summary.

(1) The so-called acetone-soluble phosphatide of heart is impure lecithin. This substance can be easily separated from accompanying fats and fatty acids by means of acetone *containing a small amount of some electrolyte, such as calcium chloride.* In this condition it is associated with a basic impurity. When purified it has all the reactions of lecithin and is quite insoluble in acetone.

(2) The basic impurity is not a phosphatide; it is soluble in water and contains no fatty acids, while only a small and variable amount of phosphorus is present.

(3) It is probable that all acetone-soluble phosphatides described in the literature are merely lecithin contaminated with the nitrogenous impurity mentioned.

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LI. A NOTE ON THE BLACK PIGMENT IN THE SKIN OF AN AUSTRALIAN BLACK.

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(*Received August 25th, 1914.*)

The pigment occurring in the skin of black races is stated to absorb the violet and ultraviolet rays in the solar spectrum, and thus to act as a protection against the harmful effects of tropical sunlight. This absorption has only been demonstrated in the case of the whole skin [1913], and no references are to be found in the text books on Biochemistry to any work on the absorption spectrum of any of the melanin pigments which have been prepared from various sources.

The present note contains an account of the preparation of melanin, from the skin of an Australian Black who died in the local hospital, which was prepared in order to study the absorptive action on light of the isolated pigment.

The only instance recorded in the literature of the isolation of melanin from human skin is that of Abel and Davis [1896], who prepared a black pigment from the skin of a negro. The tissue was destroyed by heating either with 5–6 % potassium hydroxide, or with concentrated hydrochloric acid, and the pigment separated in the form of granules which contained the pigment and the pigment structure. The melanin was set free from these " pigment granules " by treatment for several days with hydrochloric acid. It was then extracted with a warm solution of potassium hydroxide in which it slowly dissolved, and finally precipitated by the addition of alcohol and ether. It was purified by repeatedly dissolving in warm alkali and reprecipitating. Abel and Davis found the composition of the " pigment granules " to be :

C = 52·83 %, H = 3·86 %, N = 14·01 %, S = 3·6 %, Fe = – %

and of the free pigment after treatment with alkali :

C = 53·56 %, H = 5·11 %, N = 15·47 %, S = 2·53 %.

In a paper on the melanin in black wool, Gortner [1910] stated that this pigment was readily destroyed by continued heating with a solution of caustic alkali. He recommended for the extraction the use of hot very dilute (0·2 per cent.) sodium hydroxide which dissolved out the pigment without decomposing it. In his opinion the heating with strong alkalies or acids, employed by Abel and Davis and by other workers in the extraction of melanins from other sources, was calculated to decompose a large proportion of the pigment.

In the present instance, however, it was found that a solution of alkali of the strength recommended by Gortner was too dilute to attack the skin, and in the preparation described below 5 per cent. potassium hydroxide was employed and the melanin dissolved out by heating on the water bath with this solution. To avoid continued heating of the dissolved melanin with the alkali, the material was treated with a successive number of small quantities of the solution, each portion being only allowed to act for a short time. The melanin when purified had the composition :

C = 60·12 %, H = 6·70 %, N = 11·89 %, S = — %, Fe = 0·21 %,

thus differing from that obtained by Abel and Davis. On combustion in oxygen a small quantity of ash was left, which contained iron. Abel and Davis record the presence of iron only in the "pigment granules."

A solution of the pigment both in 5 per cent. potassium hydroxide and in concentrated sulphuric acid was examined spectroscopically. In both cases the solutions absorbed all the rays in the violet, the blue and in part of the green to a wave length of about 515μ. Beyond this point there was slight blurring of the green and orange, whilst the red was practically unaffected.

Experimental.

A piece of skin from the back, 450 grams, was cut up as finely as possible and was warmed on the boiling water bath with 400 cc. of a 5 per cent. solution of potassium hydroxide. After about half an hour the solution was filtered through glass wool, and most of the liquid squeezed out of the residue by pressing with a pestle. The residue was then treated with a fresh lot of alkali in the same manner, and this process repeated until the material had been thoroughly macerated. The bulk of the material including the pigment went gradually into solution, and the filtrates on cooling deposited a good deal of fat which could be removed by filtering through glass wool. Each lot of extract as obtained, was at once cooled, most of the fat removed,

and the mixture acidified with hydrochloric acid. The pigment was thereby precipitated as a dark brown powder, and was gradually collected by filtering each of the mixtures through the same paper.

When as much as possible had been obtained, it was purified by repeatedly dissolving in warm 5 per cent. potassium hydroxide, filtering and precipitating with hydrochloric acid. The last traces of acid were removed by repeatedly suspending in distilled water and filtering. The brown powder was then dried on the filter paper by washing with alcohol and ether, and the paper folded and extracted in a Soxhlet apparatus, first with alcohol, and then with ether. Finally it was washed with pure carbon bisulphide, then with pure ether, and dried in a vacuum over sulphuric acid.

The product was a dark brown powder, insoluble in water. It was readily soluble in warm dilute potassium hydroxide forming a brown solution. It dissolved in cold concentrated ammonia and in cold concentrated sulphuric acid, being precipitated from the latter by dilution with water.

The substance contained carbon, nitrogen, sulphur and a trace of iron.

When dried it was found to take up moisture very rapidly, so that the samples taken for analysis were dried to constant weight in a vacuum over phosphoric anhydride at the temperature of boiling toluene. After combustion in oxygen a very small quantity (0·5 %) of a red coloured ash was left, in which the iron was determined colorimetrically with potassium thiocyanate, a standard iron solution being employed for comparison.

The nitrogen was determined by Kjeldahl, but unfortunately the quantity of material available was extremely small, so that the result must be accepted with some reservation.

Sufficient material was not obtained for a quantitative analysis of the sulphur. Analysis :

0·1169 g. ; 0·2577 g. CO_2 ; 0·0700 g. H_2O ; 0·25 mgm. Fe.
0·0387 g. ; 0·0046 g. N.
C = 60·12 % ; H = 6·70 % ; N = 11·89 % ; Fe = 0·21 %.

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LII. QUANTITATIVE ESTIMATION OF ASPARTIC AND GLUTAMINIC ACIDS IN THE PRODUCTS OF PROTEIN HYDROLYSIS.

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(*Received July 28th, 1914.*)

The figures obtained by the majority of workers when estimating glutaminic acid in caseinogen by separating the hydrochloride in the usual way, are uniformly about 11 per cent. In two cases, however, very much higher yields of the recrystallised hydrochloride have resulted. Thus Osborne and Guest [1911] found 15·55 per cent., and Foreman 15·9 per cent. (unpublished result). In these two cases the most favourable conditions for the separation were obtained, but could not be clearly defined. The accuracy of the results, however, still remained in doubt, and the method is unreliable.

The ease with which the glutaminic acid hydrochloride separates seems to vary with the protein under treatment and with the amount of glutaminic acid present. Plimmer [1912] states that separation of the hydrochloride "occurs in the case of caseinogen and certain vegetable proteins which contain from 10–40 per cent. of this amino-acid." If the protein contains under 10 per cent., then, as a rule, no hydrochloride will separate. Occasionally, however, a separation is obtained ; thus Hopkins and Savory [1911] working with Bence-Jones protein obtained 8·05 per cent.

When a protein contains so little glutaminic acid that separation as the hydrochloride before esterification is impossible, or any other difficulties are encountered, the estimation of this amino-acid must depend upon the working up of the higher boiling esters. The yield of glutaminic acid hydrochloride obtained in this way is always very poor when compared with that obtained by separation before esterification. An investigation of the unesterified portion will show that glutaminic and aspartic acids, probably

owing to their dibasic character, do not esterify so readily. From a quantitative standpoint, therefore, at any rate, the percentage of glutaminic acid obtained by working up the higher boiling esters appears to be valueless.

A direct method for the estimation of these two amino-acids is described below.

It seemed possible that a method might be based on some essential difference between the two types—dibasic and monobasic amino-acids. As it is well known that the calcium salts of non-nitrogenous dibasic organic acids have a much higher degree of insolubility in water or alcohol than the calcium salts of non-nitrogenous monobasic acids, it was thought that the same principle might apply in the case of the dibasic and monobasic amino-acids. Experiments were therefore made with a view to testing this matter.

Calcium chloride solution was added to a solution of the amino-acids obtained by the hydrolysis of caseinogen. No precipitate resulted, and on adding much alcohol only a small precipitate was obtained. When, however, another portion of the same solution was made alkaline with lime, a copious precipitate appeared on the addition of alcohol. The precipitate seemed to increase somewhat in quantity when the free ammonia was removed before the alcohol was added.

Solutions of all the monamino-acids found in proteins, with the exception of oxyproline, were then treated separately with lime and alcohol. Glutaminic and aspartic acids, cystine and tyrosine all gave calcium salts insoluble in alcohol. The calcium salts of the other monamino-acids, however, were found to be very soluble in alcohol, and the solutions all remained perfectly clear when the alcohol was added. Pyrrolidonecarboxylic acid has since been tried in the same way, and a copious precipitate was obtained.

Since these observations were made I have found that Abderhalden and Kautzsch [1910] have made the calcium salts of aspartic, glutaminic, and pyrrolidonecarboxylic acids separately from the pure substances in a similar way. They have not suggested, however, that any quantitative use could be made of these facts in reference to separations from hydrolytic products derived from proteins.

The precipitate obtained, by means of lime and alcohol, from the products of the hydrochloric acid hydrolysis of caseinogen, has been found to consist of the calcium compounds of the following substances:

(*a*) glutaminic and aspartic acids;

(*b*) a gummy substance very difficult to crystallise;

(*c*) a very small quantity of a pigmented substance precipitated from the cold aqueous solution by silver sulphate solution;

(*d*) a substance precipitated by phosphotungstic acid.

The calcium salt of tyrosine separates from the alcoholic filtrate on standing a long time.

The phosphotungstic precipitate, derived in the manner stated, from 20 g. caseinogen gave 0·57 g. dry substance after removing the phosphotungstic acid. An aqueous solution of this substance was neutralised with nitric acid and silver nitrate solution added. No precipitate resulted. On adding ammonia, however, a precipitate was obtained. This dissolved in excess. The further investigation of this substance is postponed.

Description of the Method.

20–40 g. of the protein are hydrolysed by boiling for 48 hours with three times the quantity of hydrochloric acid, and the liquid evaporated to a syrup at 45° and 15 mm., thus removing the large excess of hydrochloric acid.

Formation of the calcium salts and removal of the humins. The syrup is taken up in 200–400 cc. water, depending upon the quantity of protein taken. Pure calcium oxide is slaked, made into a cream with water, thoroughly cooled, and added to the liquid in sufficient quantity to ensure a good excess. As a rule, 0·5 g. calcium oxide to every gram of protein appears to be required. The flask is corked, and the cold liquid shaken for a few minutes and then filtered on a Buchner funnel, and the residue thoroughly washed with cold water. The insoluble part contains the humin bodies and the excess of lime.

Removal of excess of water and ammonia. The slightly coloured filtrate is evaporated at 40–45° and 15 mm., so that the remaining liquid will possess a volume corresponding to 3·5–4 cc. for each gram of protein taken. This operation also serves to remove all traces of ammonia which can be estimated if desired. As I have shown elsewhere [Foreman, 1914] glutaminic acid is converted into pyrrolidonecarboxylic acid to a considerable extent when its solution is boiled with excess of lime. No such change, however, occurs at 40–45°. The temperature is therefore not allowed to exceed this limit. The tube admitting air into the distilling flask should be fitted with a soda lime tube to prevent the entrance of carbon-dioxide.

Precipitation of the calcium salts insoluble in alcohol. Rectified spirit (97 per cent. alcohol) is now added to the solution in the distilling flask in small quantities at a time with vigorous shaking. The alcohol should be

added gradually, in order to prevent the precipitate forming a clot which would tend to carry down other substances. The first part of the precipitate is inclined to be sticky, and if the alcohol be gradually added with shaking, this part adheres to the sides of the flask as a thin uniform layer which can be afterwards easily washed. The addition of the alcohol is continued until further precipitation ceases. About a litre of alcohol is required as a rule.

The precipitate is now filtered on a Buchner funnel and the flask with its adhering layer as well as the contents of the funnel washed thoroughly with rectified spirit. Occasionally when the alcoholic washings enter the clear main filtrate, an opalescence appears on standing, and an insignificant amount of precipitate may separate. When this is filtered off further similar separations may be obtained in a like manner. These small precipitates contain no glutaminic or aspartic acids and may be neglected. The calcium salt of tyrosine separates slowly from such a mixture in this manner.

Quantitative removal of the calcium from the alcohol-insoluble calcium salts. The precipitate is taken up in about 300 cc. water and the calcium quantitatively removed by means of oxalic acid.

Removal of traces of chloride and a pigmented substance precipitated by cold silver sulphate solution. The calcium oxalate is filtered off and aqueous silver sulphate solution added to the cooled liquid until no further precipitate results. This removes a small amount of chloride and also a very small quantity of a peculiar pigmented substance, the precipitate presenting a flocculent appearance. If the liquid be heated on the water bath before filtering, the silver compound of the pigmented substance dissolves but the substance is precipitated by phosphotungstic acid, and is included in the precipitate obtained later on.

The slight excess of silver is now removed from the solution by means of sulphuretted hydrogen.

The slightly coloured filtrate from the silver sulphide is reduced to about half its bulk on the water bath to remove sulphuretted hydrogen.

Removal of substance precipitated by phosphotungstic acid. An aqueous solution of phosphotungstic acid (Merck's) is then added until the colour disappears from the solution, 5–10 g. being usually sufficient. The liquid at this stage will continue to yield insignificant traces of precipitate if more of the phosphotungstic acid solution be added, and about as much again is required in order to carry the precipitation to absolute completion. This further addition of phosphotungstic acid is unnecessary, however, as the following test shows.

A small test portion of the liquid is taken after the colour has disappeared, and made up so as to contain 5 per cent. sulphuric acid. To this is added a few drops of a 25 per cent. solution of phosphotungstic acid in 5 per cent. sulphuric acid. No precipitate results. By adding no more phosphotungstic acid after the disappearance of the colour, one avoids a much larger precipitate of barium phosphotungstate to be obtained on removing the excess, and possible loss of the amino-acids by occlusion is thus reduced to a minimum. The usual method of precipitation in 5 per cent. sulphuric acid was avoided, as in this case the presence of the acid is unnecessary, and loss by occlusion in a large barium sulphate precipitate, produced on its removal, is prevented.

Baryta water is now added to the solution at 45° until no further precipitate results. If the solution at this stage possesses a blue colour[1], the addition of baryta water should be continued until the colour completely disappears.

The small precipitate of barium phosphotungstate is filtered off, and the slight excess of baryta quantitatively removed by means of sulphuric acid.

Separation of the monamino-acids in the dry state. The small barium sulphate precipitate is filtered off, and the liquid evaporated to about 50 cc. on the water bath. It is then transferred to a weighed dish, containing a weighed stirring rod, and slowly evaporated to 20–25 cc. (corresponding to 40 g. protein taken originally) on a warm water bath. No trace of tyrosine appears. The amino-acids crystallise on cooling, and the semi-solid mass is stirred and placed in a vacuum desiccator over calcium chloride over night. By the next morning the amino-acids will have dried to hard gritty cakes. These can be broken up with the rod and placed for a further period in the vacuum desiccator, but as a rule the loss of weight in this second period is very small indeed.

Separation of the glutaminic and aspartic acids by extraction with cold glacial acetic acid in which these acids remain undissolved. The dry substance is then triturated with cold glacial acetic acid in the dish by means of a small glass pestle, and filtered on a small Buchner funnel. The glutaminic and aspartic acids remain undissolved. The solid is again placed in the dish and extracted in the same way with more cold glacial acetic acid. It is finally all transferred to the filter and washed two or three times with glacial acetic acid. The white solid, which comes off the filter paper quite cleanly, is transferred to a weighed dish, dried and weighed.

[1] A blue colouration appeared in the filtrate when using Kahlbaum's phosphotungstic acid. This did not occur when Merck's product was employed.

Treatment of the acetic acid extract. The extract yields a gum on removing the acetic acid. It is placed in a weighed porcelain dish and the acetic acid removed as completely as possible *in vacuo* at 40–45°.

The remaining traces of acetic acid can be removed in a vacuum desiccator over strong potash. Further drying may be carried out *in vacuo* over calcium chloride if considered necessary.

A further trace, usually under 0·1 g., may be obtained by extracting this dry residue a second time.

Estimation of pyrrolidonecarboxylic acid, present in the substance soluble in cold glacial acetic acid.

The moist gum is now weighed quickly and thoroughly mixed by stirring with a strong glass rod. The rod is then used to transfer a 0·2 g. portion to a weighing tube for an amino-nitrogen determination. Without delay a further 0·5 g. portion is weighed into a bulb of 4–5 cc. capacity, and about 2 cc. strong hydrochloric acid added. A long tube is then sealed to the stem of the bulb to act as a reflux condenser. The bulb is allowed to rest lightly on a sand bath, and the liquid boiled for about 12 hours. The liquid is then made up to 20 cc. with water, and the amino-nitrogen determined in a portion of this solution. The difference between the amino-nitrogen present before and after boiling with hydrochloric acid represents pyrrolidonecarboxylic acid. The total amount of this acid present in the moist gum can then be found, and its equivalent of glutaminic acid calculated. This is added to the weight of glutaminic acid separated as such, before calculating the percentage in the protein.

If confirmation be required, the remainder of the moist gum may be extracted with cold absolute alcohol in which pyrrolidonecarboxylic acid is soluble. The alcohol can then be evaporated as completely as possible from this extract in a weighed dish and the weight of this alcohol-soluble substance obtained. The amino-nitrogen can then be determined in portions weighed out, without delay, before and after boiling with strong hydrochloric acid in the manner already described.

Instead of weighing out portions of the gum it would have been easier to have made its aqueous solution up to a known volume. This procedure, however, was not adopted because of the difficulty of transferring the gum, derived from the evaporation of an aliquot portion, to the bulb for boiling with hydrochloric acid.

Estimation of the proportion of glutaminic acid to aspartic acid in the mixture of these two acids obtained.

The white substance, which was insoluble in cold glacial acetic acid, is then submitted to analysis. The proportion of glutaminic acid to aspartic acid is calculated on the carbon content of the mixture.

As the differences between the percentages of carbon in glutaminic and aspartic acids is as great as 4·73 the proportion can be determined with tolerable accuracy.

The purity of the mixture, and the proportions calculated from the carbon content can be checked by a nitrogen determination.

Separation of the two constituents of the mixture for further identification.

A known weight of the mixture is converted into the copper salts in plenty of water, and the filtered liquid allowed to cool. If crystals of copper aspartate do not separate after standing some time, the liquid is gently reduced in bulk until signs of crystallisation appear. After standing over night the light blue needles are filtered off, and an attempt may be made to obtain a further crop from the mother liquor in the same way. As a general rule, however, the first crop will contain practically all the aspartic acid. At any rate, this has been shown to be true when the aspartic acid is present to a greater extent in the mixture than the glutaminic acid. Occasionally, if allowed to stand too long, the characteristic small heavy dark blue prisms of copper glutaminate may appear mixed with the light blue needles. These crystals, however, can be separated quite easily from the needles by elutriation with cold water.

The filtrate from the copper aspartate is freed from copper by means of hydrogen sulphide and the liquid evaporated to dryness in a weighed dish. After taking the weight of the dry substance hydrochloric acid is added in the proportion of 1·8 to 2 cc. for each g. substance. The liquid is boiled for a short time, cooled and saturated with dry hydrochloric acid gas in the usual way. The glutaminic acid hydrochloride thus obtained can be weighed and analysed.

A maximum separation of glutaminic acid hydrochloride appears to result when the substance and the hydrochloric acid are taken in the proportion stated.

Should copper aspartate refuse to separate through being present in the mixture in too small a proportion, the glutaminic acid must be separated first as the hydrochloride. The filtrate from this is then evaporated to dryness and the copper aspartate may be obtained by working up this residue.

Results Obtained by Applying the Method to the Products of the Hydrolysis of Caseinogen.

The method as above described was applied to 40 g. "Hammarsten casein" containing 14·08 per cent. nitrogen and 9·3 per cent. moisture. Calculating on the basis that pure caseinogen contains 15·62 per cent. nitrogen, the 40 g. is equal to 36·05 g. of the pure substance. The hydrolysis was carried out by boiling with three times the quantity of hydrochloric acid (sp. gr. 1·16) for 48 hours. The following results were obtained:

Total weight of dry substance before extraction with the cold glacial acetic acid		13·48 g.
Weight of glutaminic and aspartic acids insoluble in the cold glacial acetic acid, dried to constant weight at 100°		
The first extraction	6·996 g.	
The second extraction	·025 g.	7·021 g.
Difference = weight of dry gum		6·46 g.

Analysis of the mixture of glutaminic and aspartic acids.

The white substance weighing 7·02 g. dried to constant weight at 100° gave the following results on analysis:

0·1684 g.; 0·2494 g. CO_2; 0·0902 g. H_2O.

0·1550 g.; 12·8 cc. nitrogen at 20° and 768 mm. by Dumas' method.

0·1634 g.; 27·0 cc. amino-nitrogen at 20° and 768 mm. by van Slyke's method.

The percentages calculated from these results are shown below side by side with the theoretical percentages present in glutaminic and aspartic acids:

	Calculated for glutaminic acid	Found	Calculated for aspartic acid
Carbon	40·82	40·39	36·09
Hydrogen	6·12	5·95	5·26
Nitrogen	9·52	9·57	10·52
Amino-nitrogen ..	9·52	9·56	10·52

The proportion of aspartic acid to glutaminic acid in the mixture is therefore shown as follows:

$$\frac{40{\cdot}82 - 40{\cdot}39}{40{\cdot}82 - 36{\cdot}09} = \frac{1}{11};$$

$$\frac{1}{11} \text{ of } 7{\cdot}02 = 0{\cdot}64 \text{ g. aspartic acid,}$$

$$\frac{10}{11} \text{ of } 7{\cdot}02 = 6{\cdot}38 \text{ g. glutaminic acid.}$$

Estimation of pyrrolidonecarboxylic acid in the gummy substance soluble in the cold glacial acetic acid.

Practically the whole of the 0·025 g. substance obtained by the second extraction came from the acetic washings of the 7 g. Through determining this fact two lots of gum were obtained :

A. from the main filtrate ;
B. from the washings.

These were treated separately, as it was afterwards found inconvenient to mix them.

When dried, as far as possible in the manner described, the two portions of the moist gum weighed as follows :

A. 6·29 g.
B. 2·48 g. } equal to 6·46 g. in the dry state.

The amino-nitrogen was then determined before and after boiling for 16 hours with hydrochloric acid, with the following results :

A. 0·161 g. gave 13·8 cc. amino-nitrogen at 20·8° and 768 mm. 0·4542 g. was boiled with 2·5 cc. strong hydrochloric acid for the time stated, and then made up to 20 cc. 10 cc. of this gave 27·1 cc. amino-nitrogen at 24·3° and 762 mm.

B. 0·2100 g. gave 19·2 cc. amino-nitrogen at 19° and 771 mm. 0·4705 g. was treated with hydrochloric acid in exactly the same way as *A*, and then made up to 20 cc. 8·6 cc. of this gave 22·9 cc. amino-nitrogen at 23° and 763 mm.

From these figures the following results were calculated :

	Weight of moist gum	Percentage of amino-nitrogen before boiling with HCl	Percentage after boiling with HCl	Difference
A.	6·29 g.	4·94	6·70	1·76
B.	2·48 „	5·24	6·41	1·17

Calculating the increase in amino-nitrogen into terms of glutaminic acid, it will be found that 1·163 g. is thus obtained from *A* and 0·305 g. from *B*, making a total of 1·468 g.

In order to prove that this increase in amino-nitrogen was due to the hydrolysis of pyrrolidonecarboxylic acid, the remaining gummy substance was extracted with cold absolute alcohol, which dissolves this acid, and the extract was boiled for 10 hours with hydrochloric acid after removing the alcohol. The brown solution, now containing glutaminic acid, was warmed

with animal charcoal and filtered. The filtrate was then evaporated to about 5 cc. and saturated with hydrochloric acid gas at 0°. The perfectly white glutaminic acid hydrochloride which separated, was then obtained in the dry state in the ordinary way, and weighed. After allowing for the portions of the original gummy substance previously removed 0·993 g. of the hydrochloride resulted.

As stated elsewhere, it is very probable that a small quantity of the pyrrolidonecarboxylic acid is decomposed when boiled with acid, thus explaining the brown colour which invariably appears. Other slight errors were introduced in the alcoholic extraction and the animal charcoal treatment. Taking these facts into consideration, the yield is consistent with that usually obtained by the hydrochloride method.

The glutaminic acid hydrochloride melted at 201–202°.

The following results were obtained on analysis:

0·1481 g.; 0·1593 g. CO_2; 0·0675 g. H_2O.

0·1529 g. gave by Dumas' method 9·1 cc. nitrogen at 18·5° and 753 mm.

0·1009 g.; 0·0091 g., equal to 9·02 per cent. of ash.

Allowing for the ash, the following figures were calculated:

	Found per cent.	Calculated for glutaminic acid hydrochloride per cent.
Carbon	32·25	32·69
Hydrogen	5·56	5·45
Nitrogen	7·52	7·62

It is difficult to account for the ash unless one assumes that it was derived from the phosphotungstic acid used early in the method.

The products of the hydrolysis of the 36·05 g. pure caseinogen therefore contained:

1·468 + 6·38 = 7·848 g. glutaminic acid,
0·64 „ aspartic acid.

The percentages in the protein work out as follows:

21·77 per cent. glutaminic acid,
1·77 „ „ aspartic acid.

Results of a previous trial with caseinogen.

For this trial, 20 g. "Hammarsten casein" equal to 18 g. of the pure substance were taken. The calcium salts were precipitated by alcohol in two fractions. Before adding the alcohol the volume was reduced at 40–45° and 15 mm. to about 100 cc. In the 40 g. trial, already described, the volume

was about 120 cc. before adding the alcohol, so that relatively more water was present when precipitating the calcium salts in this 20 g. trial.

The two fractions were obtained as follows. The alcohol was added in small quantities at a time with shaking until very little colour remained in the solution. The second fraction was obtained by completing the precipitation with more alcohol. For convenience these two lots of precipitate may be labelled *A* and *B*.

These two fractions *A* and *B* were worked up, in the way already described, omitting all precautions as to temperature. Finally the substance, instead of being dried in the desiccator before extracting with acetic acid, was heated in the dish on the water bath with occasional stirring until dry. The cakes were then crushed and dried to constant weight in the steam oven. The acetic acid was removed from the extracts on the water bath, and the gummy residues were afterwards dried to constant weight in the steam oven before analysis.

The following weights of dry substance resulted :

	Weight of dry substance before extraction with acetic acid	Weight of dry substance insoluble in acetic acid	Difference; weight of dry gum
Fraction *A*. ..	2·06 g.	1·129 g.	0·93 g.
„ *B*. ..	3·28 „	1·638 „	1·64 „
Total ..	5·34 „	2·767 „	2·57 „

Second extractions of the two gummy portions with cold glacial acetic acid yielded :

Fraction *A*. 0·081 g.
„ *B*. 0·077 „

The acetic acid was removed from these extracts on the water bath and the gums were dried in the steam oven. Portions were then taken, dried to constant weight, and analysed.

The following results were obtained :

	Fraction *A*; insoluble in acetic acid	Fraction *B*; insoluble in acetic acid	Fraction *A*. The dry gum soluble in acetic acid	Fraction *B*. The dry gum soluble in acetic acid
Carbon	40·08	40·51	45·45	46·04
Hydrogen	6·33	6·68	6·0	6·1
Amino-nitrogen ..	9·84	9·36	1·97	2·55
Kjeldahl nitrogen ..			11·26	11·31

Pyrrolidonecarboxylic acid contains :

Carbon	46·51 per cent.
Hydrogen	5·43 „ „
Nitrogen	10·85 „ „
Amino-nitrogen	nil

These two portions of gum contain smaller percentages of amino-nitrogen than was found in the gum from the 40 g. caseinogen trial. The total weight of dry substance before extraction with the acetic acid is also relatively less. Thus from the 20 g. caseinogen 5·34 g. were obtained and from the 40 g. caseinogen 13·48 g., giving a difference of 2·8 g. per 40 g. caseinogen. On calculating, it will be found that this 2·8 g. contains about 10 per cent. amino-nitrogen. By allowing less water to be present when precipitating the calcium salts with alcohol, 2·8 g. of some substance soluble in cold glacial acetic acid containing about 10 per cent. amino-nitrogen has therefore been obtained in addition. The identity of this constituent of the gum has not yet been established but the matter is receiving attention.

It will be noted that the analyses of the gums do not differ widely from the calculated figures for pyrrolidonecarboxylic acid. The total nitrogen is about 0·4 per cent. higher than the total nitrogen of this acid. If basic lead acetate solution be added to a portion of the aqueous solution of the gums a large precipitate, characteristic of pyrrolidonecarboxylic acid, results on adding plenty of alcohol. If this precipitate be filtered off and extracted with cold water, a coloured lead compound remains undissolved. On decomposing this with sulphuretted hydrogen a small quantity of brown substance (0·145 g. from 20 g. caseinogen) is obtained on evaporation. This body probably possesses a high nitrogen content, but has so far received no further attention.

In order to show more clearly that the increase in amino-nitrogen which is always obtained by boiling the gum with hydrochloric acid is due to the hydrolysis of pyrrolidonecarboxylic acid, the remaining gum was extracted with cold absolute alcohol, which dissolves pyrrolidonecarboxylic acid. The alcohol was removed from the extract, and the amino-nitrogen was determined before and after boiling with hydrochloric acid. It was argued that a much greater increase in the amino-nitrogen in this alcohol-soluble substance would give further satisfactory evidence on this point.

0·7808 g. of the dry gum was allowed to remain in contact with cold absolute alcohol for some time, in a desiccator to prevent absorption of moisture, stirring occasionally with a strong glass rod. The solution became slightly coloured, and the insoluble part gradually appeared in a powdery form. The liquid was filtered and the extraction completed with fresh cold alcohol. The alcohol was then completely removed in a vacuum desiccator over sulphuric acid, yielding 0·6267 of moist gum. Three portions of this were weighed out quickly, one for a moisture determination, and the other two

for amino-nitrogen determinations before and after boiling with strong hydrochloric acid.

0·1465 g. lost 0·0295 g. when dried at 100° till its weight was constant. The gum therefore contained 20·13 per cent. moisture, and the 0·6267 g. contained 0·5014 g. dry substance.

0·1420 g. of the moist gum gave by the nitrous acid method 4·25 cc. amino-nitrogen at 21° and 768 mm. The moist gum therefore contained 1·72 per cent. amino-nitrogen.

0·3261 g. was boiled with 1·6 cc. strong hydrochloric acid in a small bulb as before described, for 20 hours. The liquid was then made up to 20 cc. and the amino-nitrogen determined in 10 cc. of this solution. 24·2 cc. amino-nitrogen at 24·6° and 763 mm. were obtained. The percentage of amino-nitrogen calculated on the weight of moist gum taken was therefore 8·29.

By boiling with the acid the amino-nitrogen had therefore increased from 1·72 per cent. to 8·29 per cent.

Expressing this increase in terms of pyrrolidonecarboxylic acid hydrolysed by the acid, the 0·5014 g. of dry alcohol-soluble substance contained 0·3795 g. of this acid. As this 0·3795 g. was present in 0·7808 g. dry gum, 1·25 g. would be contained in the whole 2·57 g. dry gum obtained from the 18 g. pure caseinogen. This 1·25 g. corresponds to 1·42 g. glutaminic acid.

As the 0·5014 g. alcohol-soluble substance contains 0·3795 g. pyrrolidone carboxylic acid, the difference, 0·1219 g. contains the amino-nitrogen present before boiling with acid. It will be found by calculating, that this 0·1219 g. contained 8·8 per cent. amino-nitrogen. The figure should probably be nearer 10 per cent., as on boiling pure pyrrolidonecarboxylic acid with strong hydrochloric acid, 94 per cent. of change is generally accounted for, the remaining 6 per cent. probably decomposing to produce something giving the brown colour which always appears.

The portion of the 0·7808 g. gum insoluble in the alcohol amounting to 0·2704 g. and equal to 4·9 per cent. of the caseinogen should contain no amino-nitrogen. It gave a dark blue copper salt insoluble in alcohol which showed no tendency to crystallise. The investigation of this substance is proceeding.

The total weight of glutaminic and aspartic acids thus accounted for in this 20 g. caseinogen trial is shown as follows :

	Weight in g.	Percentage of the protein
Separated by means of the glacial acetic acid ..	2·925	16·22
From the pyrrolidonecarboxylic acid	1·42	7·88
	4·345	24·1

The total percentage of glutaminic and aspartic acids in the protein obtained in the 40 g. caseinogen trial was 23·54. The results obtained in the two trials therefore practically correspond.

In order to compare the two results the figures are shown side by side, as follows :

	Per cent. separated as glutaminic and aspartic acids	Per cent. glutaminic acid, estimated from the pyrrolidonecarboxylic acid content of the gum	Total per cent.
Trial *A* .. 20 g. caseinogen	16·22	7·88	24·1
Trial *B* .. 40 g. caseinogen	19·47	4·07	23·54[1]

It will be noted that in trial *A*, the amount of pyrrolidonecarboxylic acid present in the gum was greater than in trial *B*. The amount of glutaminic and aspartic acids separated as such, however, is correspondingly less. When carrying out trial *A*, however, no temperature precautions in the final stages were taken, as already explained. In another paper it will be shown that glutaminic acid changes into *l*-pyrrolidonecarboxylic acid when its aqueous solution is boiled. The change also occurs, but to a much smaller extent, at the temperature obtained in a liquid contained in an open dish on the boiling water bath. It has also been shown that even strong acids like hydrochloric and sulphuric do not completely inhibit the change until they are present to the extent of 3 per cent. and 8 per cent. respectively. These facts were not determined at the time trial *B* was carried out, the only information available at that time being that a change took place when an aqueous solution of glutaminic acid was heated with lime. The temperature precautions taken in the final stages of trial *B* were instinctive. The inference is obvious that these precautions account for the higher yield of glutaminic and the corresponding lower yield of pyrrolidonecarboxylic acid.

When carrying out trial *B* the liquid from time to time was frequently evaporated, and sometimes heated in an open dish or in a flask on the water bath, which will account for the conversion of glutaminic acid into pyrrolidonecarboxylic acid to the extent of 4·07 per cent. of the protein. As it has also been shown in another place that *l*-pyrrolidonecarboxylic acid is hydrolysed to glutaminic acid by boiling for 4 hours with strong hydrochloric acid, it is very difficult to conceive of the pyrrolidonecarboxylic acid required by this 4·07 per cent. glutaminic acid existing in the original hydrolytic liquid obtained by boiling the caseinogen for 48 hours with strong hydrochloric acid. Its formation from glutaminic acid, afterwards, appears to need no further discussion.

[1] I consider 23·54 the more accurate figure.

The Method applied to the Soluble Protein of the Swede Turnip.

This work was carried out by G. Williams, who has given me permission to refer to his results, which are awaiting publication. He was unable to obtain any glutaminic acid as the hydrochloride before esterification. In working through the ester process he obtained 2·75 per cent. aspartic and 0·26 per cent. glutaminic acid.

Using the new method he obtained 6·98 per cent. aspartic acid and 3·18 per cent. glutaminic acid, without working up the gummy substance containing pyrrolidonecarboxylic acid which it has been shown can be formed from glutaminic acid during the operations. The percentage of glutaminic acid will be increased when this gum has been worked up.

The glutaminic and aspartic acid mixture insoluble in the cold glacial acetic acid, gave the following analysis shown side by side with the calculated percentages in the pure ingredients:

	Calculated for aspartic acid per cent.	Found in the mixture per cent.	Calculated for glutaminic acid
Carbon	36·09	37·44	40·81
Hydrogen ..	5·27	5·67	6·12
Nitrogen ..	10·52	10·18	9·52

The method already described for separating the two constituents of the mixture was afterwards applied and practically all the aspartic acid was obtained as copper aspartate and nearly all the glutaminic acid partly as copper glutaminate, separated by elutriation, and the remainder as the hydrochloride from the mother liquor from the copper salts. These products all gave very satisfactory analyses.

Experiments to show that the Calcium Salts of Glutaminic and Aspartic Acids can be Precipitated Quantitatively by Means of Alcohol.

1 g. pure glutaminic acid and 1 g. pure aspartic acid were dissolved separately in about 20 cc. water, and these solutions were treated in exactly the same way. Pure calcium oxide previously made into the form of a cream with water, and cooled, was added until present in excess. The liquids were then reduced to 4 cc. at 40–45° and 15 mm. and rectified spirit added until precipitation ceased. The precipitates were filtered off and thoroughly

washed with alcohol. The filtrate and washings in each case were combined and the nitrogen content of the whole liquid determined by Kjeldahl's method. The same amount of N/10 acid, viz. 0·15 cc., was neutralised by the ammonia in each case. 99·78 per cent. of the glutaminic acid and 99·8 per cent. of the aspartic acid therefore had been precipitated under these conditions.

In a previous experiment, using 1 g. glutaminic acid, the volume of the aqueous solution of calcium glutaminate was 30–40 cc. before adding the alcohol; 83 per cent. was precipitated, determined in the way described. The amount of water present before adding the alcohol is therefore a factor that decides the yield.

It has been shown that practically the same percentages of glutaminic and aspartic acids were obtained in the 20 g. and 40 g. caseinogen trials. In the 20 g. trial, however, the volume of the aqueous solution of the calcium salts before adding the alcohol was in the proportion of 4 to 5 cc. for each gram of caseinogen originally taken, whilst in the 40 g. trial it was 3 to 3·5 cc. It therefore appears that the yield of glutaminic and aspartic acids is not affected if these limits be observed when separating them from proteins by this method. When the smaller quantity of water is present, however, more amino-nitrogen is present in the glacial acetic acid extract.

Additional evidence for the quantitative character of the precipitation of the calcium salts of the two dibasic acids was obtained by working up the alcoholic filtrate from the 40 g. caseinogen trial. The alcohol was removed, and the remaining liquid reduced to a smaller bulk than for the first precipitation. Alcohol was again added and a very small amount of precipitate, which took a long time to settle, resulted. This was filtered off, and two further small quantities obtained in succession by repeating this whole operation. These three precipitates were combined, and worked up in the same way as the main precipitate. No traces of glutaminic and aspartic acids, however, were found. The aqueous solution obtained at the finish which should have contained these acids if they had been present was neutral to litmus even when highly concentrated, and the small quantity insoluble in cold glacial acetic acid was found to consist of tyrosine. It is therefore claimed that all the glutaminic and aspartic acids were present in the main precipitate.

Summary.

1. The calcium salts of glutaminic and aspartic acids are precipitated quantitatively from their aqueous solutions by means of alcohol, provided that these solutions are sufficiently concentrated.

2. These calcium salts are also precipitated quantitatively in the same manner, from a solution containing the calcium salts of the amino-acids resulting from the acid hydrolysis of proteins.

3. Glutaminic and aspartic acids are practically insoluble in cold glacial acetic acid, and this fact is made use of in separating them from the other products obtained.

Pyrrolidonecarboxylic acid was present in the acetic acid extract.

4. Evidence is given here, as well as in another paper [1914], to show that glutaminic acid is converted into pyrrolidonecarboxylic acid to some extent during the operations, through exposing the solutions from time to time to temperatures in the neighbourhood of 80–90°.

This pyrrolidonecarboxylic acid can be reconverted into glutaminic acid by boiling with hydrochloric acid, and the degree of change from ring nitrogen to the amino-form enables an estimation to be made.

5. Other unidentified substances probably of great importance are obtained by the method.

6. The application of the method to two proteins has given substantially greater yields of glutaminic and aspartic acids than obtained by the older methods, and it is believed that the results are quantitative.

The method applies equally to proteins which contain small percentages of either of the two amino-acids, and only a small quantity of protein is needed.

Addendum.

The foregoing description applies to the method as it has been used up to the present. Subsequent work, published in another paper [1914], has shown that at such temperatures as the solutions attained when heated on the water bath from time to time during the operations, glutaminic acid is transformed to some extent into pyrrolidonecarboxylic acid. Evidence has already been given to show that the pyrrolidonecarboxylic acid found when applying the method to caseinogen was formed in this way. A further trial is therefore in progress wherein the temperature at any stage of the

operations will not be allowed to exceed 45°. There can be very little doubt that these extra precautions will result in the isolation of the whole of the glutaminic acid as such.

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LIII. THE TRANSFORMATION OF GLUTAMINIC ACID INTO *l*-PYRROLIDONECARBOXYLIC ACID IN AQUEOUS SOLUTION.

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In another paper [1914] an account will be found of a new method for the estimation of aspartic and glutaminic acids in the hydrolytic products of proteins, depending upon the insolubility of their calcium salts in alcohol. In studying the influence of lime upon glutaminic acid in aqueous solution at higher temperatures it was found that the amino-nitrogen content had been considerably reduced. The experiments described below were carried out in order to investigate this matter. The study of the behaviour of the new product when treated in aqueous solution with lime and alcohol was also an object of the experiments.

The glutaminic acid hydrochloride used in this and the other experiments described in this paper was a recrystallised specimen obtained from a caseinogen hydrolysis. Its purity is shown by the following analysis:

	Found per cent.	Calculated per cent.
Carbon	32·61	32·69
Hydrogen	5·80	5·45
Amino-nitrogen ..	7·74	7·63

25 g. glutaminic acid hydrochloride were dissolved in 700 cc. water, 28 g. of pure calcium oxide (about three times the equivalent) were added, and the mixture left in the autoclave for 2·5—3 hours at 140—145°.

The excess of calcium oxide was filtered off, and the liquid was reduced to about 100 cc. at 45° and 15 mm. Rectified spirit (97 % alcohol) was then

added in small quantities at a time with shaking, until no further precipitate resulted. The precipitate was filtered off and washed with rectified spirit, and by extraction with cold water was separated into two fractions, one of which was difficultly soluble and the other easily soluble. These two fractions and the alcoholic filtrate were treated separately as follows :

Fraction *A*, the difficultly soluble portion ;
„ *B*, the easily soluble portion ;
„ *C*, the alcoholic filtrate.

The calcium was quantitatively removed from fractions *B* and *C* by means of oxalic acid, the alcohol having previously been evaporated from fraction *C*. The calcium was precipitated from fraction *A* as the sulphate by digesting with a slight excess of normal sulphuric acid.

Treatment of Fractions A *and* B. The solutions obtained from these two fractions were then treated separately as follows :

Very small amounts of hydrochloric acid were removed by means of silver sulphate solution. The excess of silver sulphate was decomposed with sulphuretted hydrogen, and the filtrates were freed from sulphuretted hydrogen by evaporation to half bulk on the water bath. The sulphuric acid was then quantitatively removed by means of baryta.

Separation of a substance soluble in cold glacial acetic acid. The filtrates from the barium sulphate were then evaporated to dryness, and dried in the water oven. The residues were extracted with cold glacial acetic acid by triturating in the evaporating dishes with a pestle. The insoluble portions were filtered off by suction and washed on the filter with cold glacial acetic acid. The acetic acid was evaporated slowly from the filtrates leaving gummy residues which gradually crystallised on standing a long time over potash. These, as well as the insoluble portions, were dried and weighed.

The following weights of dried substances were obtained :

	Insoluble in cold glacial acetic acid	Soluble in cold glacial acetic acid
Fraction *A*	6·76 g.	0·86 g.
„ *B*	1·8 g.	4·1 g.

The 6·76 g. portion of Fraction *A* when crystallised from water gave characteristic crystals of glutaminic acid, which melted at 213–214°. The 1·8 g. portion of Fraction *B* also gave the correct melting-point for glutaminic acid.

Treatment of the 4·1 *g. portion of Fraction* B.

The amino-nitrogen content of this acetic acid soluble portion was estimated by the nitrous acid method.

0·1405 g. gave 2·15 cc. nitrogen at 17° and 747 mm. equal to 0·87 per cent. amino-nitrogen.

This portion was again extracted with a small quantity of glacial acetic acid, filtered by suction, and washed on the filter with glacial acetic acid, the washings, however, were kept separate. The insoluble part when dried weighed 0·61 g.

The acetic acid was slowly evaporated from this main filtrate by allowing the dish to remain on a warm water bath. When reduced to a small bulk the very slightly coloured gummy liquid was placed in a desiccator over strong potash solution. Large crystals separated on standing a long time, the whole of the gummy liquid eventually crystallising completely, the crystals all having the same appearance. These crystals were stirred with about 1 cc. cold glacial acetic acid, filtered by suction, and washed with a mere trace of acetic acid. The larger crystals were left on the filter in a colourless state. The object of this separation was to obtain a specimen completely devoid of light brown colour so that its specific rotatory power could be determined with accuracy. The weight of colourless crystals obtained was 0·47 g. A portion taken from this specimen commenced to soften at 142° and completely melted without colouration at 158°.

The filtrate from the colourless specimen again crystallised completely when treated in the manner already described, and portions were taken from this for analysis. The weights given below were constant at 100°.

The following results were obtained:

0·1449 g.; 0·2520 g. CO_2; 0·0748 g. H_2O; C = 47·43; H = 5·73 per cent.

0·1385 g. gave, by Dumas' method, 13·5 cc. nitrogen at 17° C. and 740 mm.; N = 11·00 per cent.

0·3023 dissolved in 24·76 g. glacial acetic acid gave 0·34° depression of the freezing-point by the cryoscopic method, giving a molecular weight of 140.

The colourless specimen was used for the following determinations:

0·3965 g. was dissolved in water and made up to 20 cc. at 20°. The amount of rotation obtained in a 200 mm. tube was − 0·45°: $[\alpha]_D^{20^\circ} = -11{\cdot}35^\circ$.

The amino-nitrogen was determined in 7·05 cc. of the solution used for the rotation, equal to 0·1398 g. substance: 0·2 cc. nitrogen was obtained by the nitrous acid method.

5 cc. of the same solution, equal to 0·0991 g. substance required 1·5 cc. N/2 soda for neutralisation to delicate litmus paper. The calculated equivalent of N/2 soda is 1·53 cc. The carboxylic group was therefore exercising its full acidity.

On comparing the figures given in the following table, it will be seen that the substance was pyrrolidonecarboxylic acid. The calculated figures for pure glutaminic acid from which the substance was made are also shown side by side.

	Calculated for glutaminic acid per cent.	Found per cent.	Calculated for pyrrolidonecarboxylic acid per cent.
Carbon	40·83	47·43	46·51
Hydrogen	6·12	5·73	5·43
Nitrogen	9·52	11·00	10·85
Amino-nitrogen ..	9·52	traces	*nil*

The specific rotatory power −11·35° agrees very closely with that found by Abderhalden and Kautzsch [1910], who give figures for *l*-pyrrolidonecarboxylic acid ranging from −10·8° to − 11·52°.

The substance was soluble in alcohol, strongly acid to litmus, and its melting-point agrees with that found by Menozzi and Appiani [1892] and also by Abderhalden and Kautzsch [1910] for *l*-pyrrolidonecarboxylic acid. The carbon is 0·9 per cent. too high, but all the other facts appeared to be so conclusive that a repetition of the combustion was thought to be unnecessary.

Treatment of Fraction C.

After filtering off the calcium oxalate, the hydrochloric acid was removed by means of silver sulphate solution. Excess of silver and sulphuric acid were then removed in the manner previously described. The liquid was evaporated to dryness, and the residue was dried and found to weigh 3 g. This weight would have been undoubtedly less if more water had been removed previously to precipitating the calcium salts with alcohol. This 3 g. was not extracted with cold glacial acetic, as a determination of its amino-nitrogen content appeared to give all the information required.

0·1315 g. gave by the nitrous acid method 4·8 cc. nitrogen at 15·5° and 761 mm. equal to 2·13 per cent. amino-nitrogen.

Assuming this substance to consist of a mixture of glutaminic acid and pyrrolidonecarboxylic acid, and calculating the amino-nitrogen as present

in glutaminic acid, the 3 g. contains 0·67 g. glutaminic acid and 2·33 g. pyrrolidonecarboxylic acid.

The weights of the two substances separated at the different stages are given in the following table:

	Insoluble in cold, glacial acetic acid	Soluble in cold, glacial acetic acid
Fraction *A* ..	6·76 g.	0·86 g.
„ *B* ..	2·41 „	3·49 „
„ *C* ..	0·67 „	2·33 „
Total	9·84 „	6·68 „

The 6·68 g. pyrrolidonecarboxylic acid corresponds to 7·61 g. glutaminic acid. 43—44 per cent. of the glutaminic acid originally taken had therefore been converted into pyrrolidonecarboxylic acid.

Transformation of glutaminic acid into l*-pyrrolidonecarboxylic acid in aqueous solution in the presence of excess of calcium hydroxide, at* 100°.

1 g. portions of glutaminic acid hydrochloride were mixed with 1·2 g. pure calcium oxide and 28 cc. water, *i.e.* in the same proportions as in the previous experiment. These mixtures were boiled in small flasks fitted with reflux condensers, on a sand bath, for different lengths of time. When boiling, the temperature recorded on a thermometer with its bulb immersed in the liquid was 100°. The liquids were cooled, and sufficient acetic acid added to dissolve the excess of calcium oxide. The solutions were then made up to 50 cc., and the amino-nitrogen determined in 10 cc. portions by the nitrous acid method.

A further similar mixture of the substances in the same proportion was kept at 40—45° for 8 hours, and the amino-nitrogen determined in the same way.

The results obtained are shown in the following table:

Weight of substance taken	Temperature	Time	Per cent. amino-nitrogen	Percentage transformed
0·9925 g.	100°	2·5 hrs.	6·45	16·66
0·9955 „	„	5 hrs.	6·17	20·29
0·9933 „	„	7·5—8 hrs.	5·91	23·64
0·9820 „	40—45°	8 hrs.	7·70	*nil*

The amino-nitrogen content of the glutaminic acid hydrochloride originally taken was 7·74 per cent. and the percentages shown had therefore been transformed.

The experiment was repeated using 0·16 g. of a specimen of free glutaminic acid (M.P. 213–214°), the other ingredients being in the same proportion,

viz., 0·24 g. calcium oxide and 5·6 cc. water. After 5 hours boiling, the amino-nitrogen present in the solution was determined. There resulted 20·7 cc. at 19° and 763 mm. equal to 7·47 per cent. amino-nitrogen. Pure glutaminic acid would have contained 9·52 per cent.; 21·53 per cent. of the glutaminic acid had therefore been transformed.

In order to show that the same substance, viz., pyrrolidonecarboxylic acid, had been formed, the solutions left over from the amino-nitrogen determinations were combined. The calcium was removed by means of oxalic acid, and the hydrochloric acid by silver sulphate solution. The excess of silver and sulphuric acid were removed by means of sulphuretted hydrogen and baryta. The liquid was evaporated to dryness, the residue dried, and extracted with cold glacial acetic acid. The small amount of gummy liquid obtained by removing the acetic acid from the extract, crystallised very slowly when allowed to stand over potash. The crystals obtained were exactly similar in appearance to those isolated in the previous experiment. The whole of the preparation was thoroughly dried in a vacuum desiccator over calcium chloride, and the amino-nitrogen determined in a portion of the dry substance by the nitrous acid method. The following result was obtained:

0·1865 g. gave 3·45 cc. nitrogen at 16° and 766 mm., equal to 1·09 per cent. amino-nitrogen.

Effect of other bases.

It was thought, at this stage, that baryta would have the same effect as lime. On carrying out a similar experiment substituting for the lime its equivalent of baryta, however, all the glutaminic acid remained unchanged.

Weight of glutaminic acid hydrochloride taken	Temperature	Time	Per cent. amino-nitrogen
0·9940 g.	100°	5 hrs.	7·63

The effect of substituting other bases for the lime was then ascertained. A pure specimen of the free glutaminic acid was used for this purpose, the amino-acid, water and base being in exactly the same proportion, *i.e.* 0·34 g. to 4 equivalents of the base made up to 12 cc. with water. These mixtures were boiled on a sandbath for 5 hours, under a reflux condenser and made up to 20 cc. with water. The amino-nitrogen was then determined in an aliquot portion. The following results were obtained:

Base	cc. taken from the 20 cc.	Nitrogen obtained, cc.	Temperature and pressure	Amino-nitrogen per cent.	Per cent. changed
$Sr(OH)_2$ ..	9·9	27·85	20° 760 mm.	9·47	*nil*
NaOH ..	9·75	27·7	21° 760 „	9·51	„
NH_4OH ..	10·0	37·1	20·5° 760 „	10·4	„

According to van Slyke [1912] 38·9 per cent. of the nitrogen of the ammonia is liberated by nitrous acid in 5·5 minutes. Applying this correction for the ammonia found present in the solution after the five hours boiling the figure 10·4 was calculated. Although this result is only approximate it suffices to show that no change occurs in the presence of ammonia.

The outstanding difference between calcium hydroxide and the hydroxides of the other alkaline earths is one of solubility in water.

Thus calcium hydroxide is soluble only to the extent of 0·08 per cent. at 100°, while the other bases are very soluble at 100°. Of the three extra equivalents of lime in the 12 cc. solution only about 9 milligrams would be soluble. In the case of the baryta and strontia, however, all the excess would be dissolved. Lactam formation from a salt of glutaminic acid not hydrolytically dissociated appeared to be unlikely. It was therefore concluded that the excess of baryta, strontia and soda were preventing hydrolytic dissociation of the salts. In the case of the lime there was insufficient in solution to do this. The calcium salt of pyrrolidonecarboxylic acid also present would be dissociated, and an equilibrium obtained after 21 per cent. of the glutaminic acid had been transformed.

For this view to be tenable some of the glutaminic acid should be changed, if only one equivalent of baryta were used instead of four. The following experiment was therefore carried out to test this point.

A mixture of 0·17 g. glutaminic acid, 0·375 g. barium hydroxide (one equivalent) and 6 cc. water was boiled for 5 hours under a reflux condenser, and the whole of the solution used for the amino-nitrogen determination with the following results :

Nitrogen	Per cent. Amino-N.	Per cent. changed
24·3 cc. at 20·2° and 768·5 mm.	8·26	13·24

Transformation in aqueous solution.

Having thus established that hydrolysis of the salts of glutaminic acid, involving the presence of the free acid in the equilibrium mixture, appeared to be a condition essential to the formation of the lactam from those salts, there was no apparent reason why the change should not occur simply in aqueous solution, with no base present. This contention was supported by evidence obtained previously when making a preparation of the free acid from its hydrochloride. On this occasion 10 g. of the hydrochloride equal to 8 g. of the free acid were dissolved in water and to this solution heated in a flask on the water bath hot silver sulphate solution was added

until in slight excess. The excess was removed by sulphuretted hydrogen and the filtrate evaporated to half its bulk, on the water bath. Sufficient baryta was then added to the hot liquid to combine quantitatively with the sulphuric acid. After allowing to stand on the water bath until the barium sulphate could be easily filtered, the barium sulphate was removed, and the liquid evaporated on the water bath to crystallisation. The following fractions were obtained:

Crop 1	white	4·29 g.	M.P. 213—214°
„ 2	„	1·49 „	„ 198—199°
Mother liquor evap. to dryness and dried	light brown colour	1·55 „	Softening commenced at about 130°, all melted by 151°

The 10 g. glutaminic acid hydrochloride was taken from a very good specimen, the analysis of which is given on page 481.

The carbon, hydrogen and amino-nitrogen were then determined in the mother liquor portion of 1·55 g., with the following results:

0·1353 g.; 0·2225 g. CO_2; 0·0707 g. H_2O.

0·1580 g.; 7·4 cc. nitrogen at 19° and 764 mm. by the nitrous acid method.

	Calculated for glutaminic acid	Found	Calculated for pyrrolidone-carboxylic acid
Carbon	40·83	44·85	46·51
Hydrogen	6·12	5·81	5·43
Amino-nitrogen ..	9·52	2·71	*nil*

The 1·55 g. evidently consisted of a mixture of glutaminic acid and pyrrolidonecarboxylic acid, as a calculation based on the carbon content showed 29·2 per cent. of glutaminic acid present in the mixture, and on the amino-nitrogen content, 28·5 per cent.

It has already been shown that the carboxylic group of pyrrolidonecarboxylic acid exercises its full acidity when titrated with N/2 soda using delicate litmus paper. Osborne has shown that one of the carboxylic groups of glutaminic acid is neutralised by soda to delicate litmus paper. The amount of soda required to neutralise equal weights of these two acids should therefore be about the same.

The titration of a portion of the 1·55 g. gave a result which agreed with these facts.

As the glutaminic acid hydrochloride originally taken for this experiment gave very satisfactory figures on analysis, the conclusion arrived at was that this 1·1 g. pyrrolidonecarboxylic acid had been produced from glutaminic acid during the repeated heatings on the water bath when in aqueous solution.

As all the facts pointed to the change occurring in aqueous solution the following experiments were carried out.

Two portions of 0·34 g. taken from different specimens of glutaminic acid both melting at the correct temperature were each dissolved in 12 cc. water and boiled on a sand bath for 5 hours under a reflux condenser. The solutions were made up to 20 cc. and the amino-nitrogen determined in 10 cc. by the nitrous acid method.

The following results were obtained :

	Nitrogen cc.	Temperature and pressure	Amino-nitrogen per cent.	Per cent. changed
Specimen *A* ..	13·0	20° & 763 mm.	4·39	53·89
„ *B* ..	13·3	22·2° & 762 „	4·44	53·37

The amount of rotation was determined in a 200 mm. tube in the solution from specimen *B* before using it for the amino-nitrogen determination.

A reading of $-0·01°$ was obtained, the liquid being practically inactive, $[\alpha]_D^{20°} = -0·3°$.

The specific rotatory power of the original glutaminic acid Specimen *B* was also determined and found to be $[\alpha]_D^{20°} = +11·03°$.

The specific rotatory power of *l*-pyrrolidonecarboxylic acid previously found was $-11·35°$. Abderhalden [1909] gives $+10·5°$ for glutaminic acid in aqueous solution.

It is therefore evident that somewhere about half the glutaminic acid is converted into *l*-pyrrolidonecarboxylic acid by boiling the aqueous solution for 5 hours.

The next experiment was designed for the purpose of finding the effect of evaporating solutions containing glutaminic acid on the water bath. With this object in view 0·34 g. was dissolved in 150 cc. water and evaporated to dryness on the water bath. The time taken was 2·5 hours and the temperature of the liquid ranged from 81 to 83°.

When dry, the substance was dissolved in a little normal soda solution and made up to 20 cc. The amino-nitrogen was then determined in 10 cc. with the following results :

cc. Nitrogen	Temperature and pressure	Amino-nitrogen per cent.	Per cent. changed
27·2	18·6° & 761 mm.	9·23	3·1

The influence of temperature is therefore considerable. This gives an explanation for the fact that only 1·1 g. pyrrolidonecarboxylic acid appeared as the result of the repeated evaporations on the water bath, carried out in

the course of working up the 10 g. glutaminic acid hydrochloride already described.

The effect of acids upon the transformation.

Experiments were then carried out to show the effect of acids upon the transformation. 0·17 g. portions and the required amounts of the various acids were in each case made up to 6 cc. with water and the solutions boiled on the sand bath for 5 hours under reflux condensers. The amino-nitrogen was then determined in the whole of each solution. The results are given in the following table:

Acid	Ratio of the acid to glutaminic acid in equivalents	Percentage of acid in the solution	Amino-nitrogen obtained cc.	Temperature and pressure	Amino-nitrogen; percentage of original substance	Per cent. changed
Acetic ..	4	4·6	10·5	19° & 760 mm.	3·55	62·7
Hydrochloric	2	1·38	22·3	22 & 768	7·51	21·12
Sulphuric ..	4	3·72	21·85	19·8 & 768	7·44	21·85
„ ..	2	1·86	16·9	18·5 & 771·5	5·82	38·87
„ ..	8	7·44	25·85	20·5 & 768·5	8·78	7·77

In calculating the number of equivalents in Column 2 of this table, glutaminic acid is considered as a mono-acid base, having regard to the function of its amino-group.

It will be noted that the inhibiting effect upon the lactam formation appears to correspond with the strength of the acid employed. Thus two equivalents of sulphuric acid have the same effect as one equivalent of hydrochloric acid. In the case of the acetic acid the liquid was boiled for 6 hours instead of 5 by mistake. At any rate, however, it may be said that weak acids such as acetic acid have no inhibiting effect. As glutaminic and aspartic acids occur together in proteins, the influence of aspartic acid was tested: 0·17 g. glutaminic and 0·17 g. aspartic acid were together dissolved in 12 cc. water and boiled for 5 hours as before. The amino-nitrogen estimation showed that 53·57 per cent. of change had taken place. It therefore appears that aspartic acid exercises no influence, as the same change was produced in water alone. It is, however, noteworthy that twice as much water *i.e.*, 12 cc. instead of 6 cc., was used for the treatment of this 0·17 g. glutaminic acid.

The figures obtained for the sulphuric acid show that as the amount of acid present in the solution increases, the inhibiting effect also increases, and it may be gathered that when the solution contains about 8 per cent. sulphuric acid, no change at all would occur. It appears reasonable to assume that the same result would be obtained with 3 per cent. hydrochloric acid present.

Reconversion of ring-nitrogen to the amino-form by means of strong hydrochloric acid.

In order to ascertain whether the reverse change, *i.e.*, *l*-pyrrolidonecarboxylic acid into glutaminic acid, occurs on using stronger solutions of a mineral acid, the following experiment was carried out:

0·3285 g. of *l*-pyrrolidonecarboxylic acid was boiled for 4 hours with three times the quantity of strong hydrochloric acid. The liquid was then made up to 20 cc. and the amino-nitrogen determined in 10 cc.

Nitrogen obtained, cc.	Temperature and pressure	Amino-nitrogen percentage of original substance	Per cent. changed
28·6	14·3° C. & 760 mm.	10·24	94·4

The original *l*-pyrrolidonecarboxylic acid contained practically no amino-nitrogen (0·14 g. gave 0·2 cc.). It is therefore obvious that strong hydrochloric acid hydrolyses it to glutaminic acid.

A further experiment, using 5 to 6 times the quantity of hydrochloric acid and boiling for 8 hours, gave a change of 92·2 per cent. of the pyrrolidone carboxylic acid into glutaminic acid. The time required for the complete change has not yet been determined.

The solutions became brown in colour as the boiling continued, and it therefore appears possible that traces of the pyrrolidonecarboxylic acid are decomposed. This colouration appears very frequently when dealing with solutions of this substance.

Abderhalden and Kautzsch [1910] have also shown that pyrrolidonecarboxylic acid is converted into glutaminic acid by heating with hydrochloric acid. They succeeded in obtaining glutaminic acid hydrochloride from the resulting solution.

Secondary formation of pyrrolidonecarboxylic acid in solutions of the products of the hydrolysis of proteins.

As *l*-pyrrolidonecarboxylic acid is hydrolysed to glutaminic acid by boiling with three times the quantity of hydrochloric acid for 4 hours, it is very difficult to conceive of its presence in the liquid arising from a 48 hours' hydrolysis of a protein with strong hydrochloric acid. Up to the present, no experiments similar to those described have been carried out with the inactive pyrrolidonecarboxylic acid, but it is a reasonable assumption that this also will be hydrolysed by strong hydrochloric acid. It has been shown in this paper how pyrrolidonecarboxylic acid may be formed

from glutaminic acid in aqueous solutions and even in solutions containing appreciable percentages of the mineral acids, as well as when solutions presumably containing a hydrolysed salt of glutaminic acid are heated. There are therefore several ways open for the secondary formation of pyrrolidonecarboxylic acid during the treatment of the products of protein hydrolysis. From these considerations it would therefore appear that *l*-pyrrolidonecarboxylic acid is a secondary substance when obtained from the products of the hydrolysis of proteins by hydrochloric acid.

The fact that an increased yield of glutaminic acid hydrochloride generally results when any solution of amino-acids containing glutaminic acid is boiled with strong hydrochloric acid before saturating with hydrochloric acid gas is very probably also accounted for by a re-transformation of pyrrolidonecarboxylic into glutaminic acid.

That even prolonged boiling with hydrochloric acid leaves glutaminic acid quite untouched, is shown by the following experiment:

0·2972 g. pure glutaminic acid was boiled for 48 hours with three times the quantity of hydrochloric acid in a small bulb to which a long tube condenser was sealed. The solution was made up to 20 cc. and the amino nitrogen determined in 9·65 cc. equal to 0·1434 g. glutaminic acid:

Nitrogen obtained, cc.	Temperature and pressure	Amino-nitrogen per cent.	Per cent. changed
23·6	19·5° C. & 761 mm.	9·49	*nil*

Summary.

1. Glutaminic acid is converted into *l*-pyrrolidonecarboxylic acid, to a large extent when its aqueous solution is boiled for a length of time.

2. The change also occurs, but to a much smaller extent, at 81—83°, the temperature of a liquid evaporated in an open dish on the water bath.

3. The same change occurs on boiling an aqueous solution of a salt of glutaminic acid, but to a smaller extent than in an aqueous solution of the amino-acid.

No change takes place when excess of the base is present, probably because hydrolysis of the salt is prevented.

4. Very weak acids have no inhibiting effect upon the lactam formation. The mineral acids, however, do inhibit the change to an extent proportional to their strength and to the amount present. About 8 per cent. sulphuric

acid and about 3 per cent. hydrochloric acid present in the solution of glutaminic acid inhibit the change altogether.

5. The reverse change, viz., of *l*-pyrrolidonecarboxylic to glutaminic acid occurs on boiling with strong hydrochloric acid.

6. Glutaminic acid is unaffected by boiling with strong hydrochloric acid.

7. Pyrrolidonecarboxylic acid may be formed from glutaminic acid in many of the operations involving evaporation or heating when in aqueous solution, during the working up and separation of the products of protein hydrolysis. When found amongst these products, there appears to be no doubt as to its secondary nature.

8. *l*-Pyrrolidonecarboxylic acid is precipitated as the calcium salt by adding excess of alcohol to its aqueous solution previously made alkaline with calcium oxide; when present with glutaminic acid the two calcium compounds are precipitated together.

9. These two substances are also precipitated by adding basic lead acetate solution, and then excess of alcohol, to their aqueous solutions.

10. Glutaminic and *l*-pyrrolidonecarboxylic acids have been obtained in a state of purity by means of two successive extractions of their mixture with cold glacial acetic acid.

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LIV. THE ACTIVATION OF TRYPSINOGEN.

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In a previous paper I showed [1901, 2] that if minced pancreas were kept at room temperature with water or saline solution in presence of chloroform, or with dilute alcohol, the extracting liquid at first contained little or no free trypsin and rennin, but that after a variable time, it might be 5, 12, or 19 days, it suddenly began to develop activity, and in the course of the next few days reached its maximum tryptic and rennetic power. This clearly pointed to an auto-catalytic reaction, and I supposed trypsin itself to be the activator. Small quantities of it, liberated spontaneously from the trypsinogen, would in such a case activate the extract with rapidly accelerating velocity. In seeming proof of such a hypothesis I found that small quantities of active extracts containing trypsin had a very marked activating power upon inactive extracts, whilst a repetition of these experiments with pancreatic juice gave a similar result [1902, 1913]. Mellanby and Woolley [1912] showed that extracts of intestinal mucous membrane containing enterokinase, when added to pancreatic juice, liberated the trypsin at a rate which was slow at first, but which increased with rapidly accelerating velocity to a maximum as the end of the reaction was approached. This result appeared to me to confirm my own experiments, and to indicate that the later and rapid portion of the activation process is due chiefly to the catalytic action of the trypsin liberated in the earlier stages by the enterokinase. For reasons to be adduced later on, I find this hypothesis to be erroneous, and my fresh experimental data point to the following explanation of this vexed problem: *The trypsin liberated by the enterokinase gradually sets free an enzyme, termed deuterase* (to indicate that it acts secondarily to enterokinase) *from a precursor which is present in pancreatic juice and extracts, and this deuterase is mainly responsible for the later stages of the activation process.* Hence in all my previous

experiments in which I have attributed activating powers to free *trypsin*, I now conclude that they are really due to *deuterase liberated by the trypsin from a pro-deuterase.*

Method of Experiment.

The method of estimation of trypsin adopted has already been explained in former papers [1901, 1; 1913]. It depends on Robert's metacasein reaction, suitably corrected, and as a rule takes 3 to 5 minutes to perform, or exceptionally from 2 to 60 minutes. As previously pointed out, this reaction is a test for the rennin rather than the trypsin, but under most conditions the two enzymes run parallel in activity, and so a measure of one is likewise a measure of the other. My earlier experiments were made with a fibrin digestion method which took 20 or 30 minutes for completion, and though it gave useful results it is clearly unsuitable for estimating rapid activation processes.

In all the experiments described below, unless special mention is made to the contrary, both the extracts and pancreatic juice were brought to a fivefold dilution before activation. To the extracts Na_2CO_3 was added up to 0·05% (*i.e.* a centinormal solution), whilst each sample of pancreatic juice used was titrated against N/10 HCl, using methyl orange as indicator, and its alkalinity after dilution reduced to one of 0·05 % Na_2CO_3 by the addition of suitable amounts of N/10 HCl.

The enterokinase was prepared by scraping off the mucous membrane from the upper half of the small intestine of the cat or monkey, grinding it with a little sand, and mixing thoroughly with two to six times its weight of chloroform water. The extract was filtered off 20 to 40 hours later, and kept with chloroform. All the extracts and pancreatic juice were kept in a refrigerator at a temperature of 1° to 5° when not in use.

The Activation of Pancreatic Juice by Enterokinase.

In the few experiments recorded previously on the time relations of the activation of pancreatic juice by enterokinase, I pointed out that the latter part of the activation showed approximately the same velocity whatever the quantity of enterokinase employed. This velocity is so great that its determination is necessarily somewhat inexact, but the plan adopted in the present experiments was to estimate the time taken over the *final half* of the activation process. It was possible to make two or three trypsin estima-

tions during the most rapid stage, as well as other estimations before and after it. These results were plotted out, and the straight line joining the estimations made during the most rapid stage continued till it reached the point of maximum tryptic activity. That is to say, for the purpose of calculating the velocity of the final half of activation it was assumed that there was no slowing down of the activation process as it tended towards completion. Doubtless some slowing does exist, but it is so slight that no good experimental evidence of it could be obtained.

In Fig. 1 is shown the effect of activating pancreatic juice with cat's enterokinase. The 10 % of extract used took 39 minutes to activate at 37°,

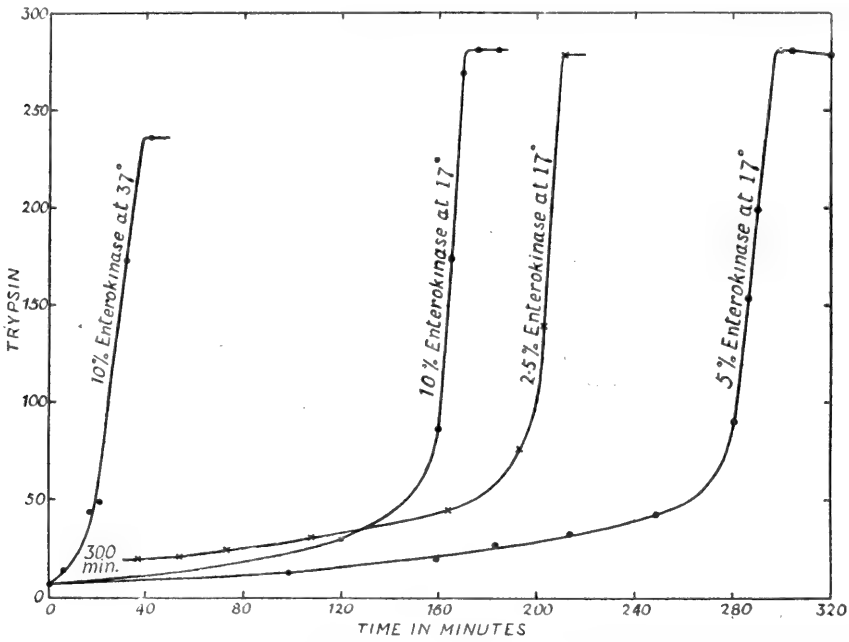

Fig. 1.

but 172 minutes at 17°. These values give a temperature coefficient of 2·10 for each rise of 10° between the limits mentioned, or a similar value to those obtained in most other enzyme actions. Nevertheless temperature does not by any means affect every portion of the curves in the same manner. The last half of the activation process apparently took 12 min. at 37°, but only 8 min. at 17°. The larger value obtained at the higher temperature is doubtless an experimental error due to the difficulty of drawing the slope of the curves correctly when only a small number of experimental results are available, but all the results agree in showing that the latter half of the

activation process is comparatively little affected by temperature. In the other two curves shown in the figure, which indicate the activation produced by 5 % and 2·5 % of enterokinase at 17°, the times taken for the last half of the process were 11 and 8 minutes respectively, or the average time taken at 17° is 9 minutes. In an experiment at 27° the time taken was 13 minutes. Experiments made at 37° with other samples of juice gave the following times for the last half of activation : (*a*) with 10 % of E., 7 min. ; with 5 %, 7 min. ; (*b*) with 6 % of E., 5 min. ; with 3 %, 6 min. ; (*c*) with 10 % of E., 9 min. ; (*d*) with 10 % of E., 5 min. (average of five experiments). Hence the average value obtained at 37° works out at 7 min., whilst the average at 17° and 27° is 10 min. In my previous paper I stated that the time taken for the most rapid doubling of tryptic activity was about 4 min., but if the results there quoted are re-calculated in accordance with the method now used, the time taken over the final half of activation works out at about 5 min.

As will be pointed out again later on, the small influence of temperature upon the final stages of activation of pancreatic juice by enterokinase is due to their being induced for the most part by deuterase, the activating power of which is almost unaffected by temperature.

It will be noted that the time taken for the last half of activation is independent of the quantity of enterokinase used. On the other hand the time taken over the whole process is greatly influenced by it, though not quite in inverse proportion. Thus in the experimental results above recorded activation by 10 % of E. took 172 min., by 5 % took 296 min., and by 2·5 % took 512 min., or in the ratio of 1 : 1·7 : 3·0, instead of 1 : 2 : 4. Another sample of juice was activated at 37° by 10 % of E. in 30 min., and by 5 % of E. in 50 min. Another sample was activated by 10 % of E. in 69 min., and by 4 % of E. in 137 min. On the other hand a fourth sample was activated by 6 % of E. in 32 min., and by 3 % of E. in 83 min., but this exception cannot be held to counterbalance the other three results. Mellanby and Woolley [1913, p. 356] quote one experiment in which the activation time was almost exactly in inverse proportion to the quantity of enterokinase, but in another experiment [1912, p. 375] it was less affected than in any of the results above quoted.

It is of interest to compare the minimum and maximum velocities of activation effected by enterokinase at the beginning and end of its action. For instance, in the above recorded experiment when 2·5 % of E. acted on pancreatic juice at 17°, the tryptic value increased from 7 units to 20 units

in the first 337 minutes, or at the rate of 1 unit in 26 minutes, and as already mentioned it finally doubled itself in 8 min. This increase works out at the rate of 1 unit in 0·057 min., or is 456 times more rapid than the initial rate. Probably it is in reality quite 1000 times more rapid than the real initial rate, as there can be no doubt that the velocity of activation towards the end of the 337 minutes interval was considerably greater than at the beginning. In the experiment with 5 % of E. the velocity observed during the 0 to 99 min. interval was 210 times slower than the final rate observed. With smaller quantities of E. than 2·5 % the difference between the initial and final rates would be greater still, and differences of such an order are seen with most samples of pancreatic juice. Even when kept in a refrigerator they activate themselves spontaneously in about five days. This is presumably due to the presence of minute quantities of enterokinase, which for the first two or three days liberate scarcely appreciable quantities of trypsin, but which finally lead to the familiar rapid activation. Mellanby and Woolley find that extracts of every tissue in the body accelerate the activation of pancreatic juice, and so presumably contain small quantities of enterokinase. The activation of pancreatic extracts observed by me in my original experiments must likewise have been due to the presence of variable traces of enterokinase.

The extreme slowness of the initial rate of activation induced by small quantities of enterokinase, coupled with the fact that the final rate is practically independent of the amount present, clearly points to the existence of a dual reaction in the process. It is not possible to explain such a result by assuming that a co-enzyme is liberated during the course of activation which augments the action of the enterokinase, for in such a case the final velocity would be influenced by the amount of enterokinase present.

The Activation of Pancreatic Extracts by Enterokinase.

For most purposes it is much more convenient to use pancreatic extracts than pancreatic juice, as they can be more readily obtained, and are much more constant in their properties [Vernon, 1901, 2, p. 316]. If fresh sheep's or pig's pancreas be minced up and mixed with four times its weight of glycerin, the extract filtered off one to three weeks later contains large quantities of trypsinogen and only traces of trypsin, and does not activate itself for months if kept in a refrigerator. If, on the other hand, the minced gland be mixed with three parts of glycerin and one of water, the extract begins

to activate itself in about three weeks, and attains its maximum tryptic power in about 5 or 6 weeks. This tryptic power it retains with very slight diminution for many months. If the minced gland be extracted with a mixture of equal parts of glycerin and water it affords an active extract in a fortnight or less, for the larger the proportion of water present in the extracting liquid the quicker the traces of enterokinase in the gland substance exert their action.

The pancreas of animals other than the pig and sheep may yield different results on extraction, partly because the amount of enterokinase contained

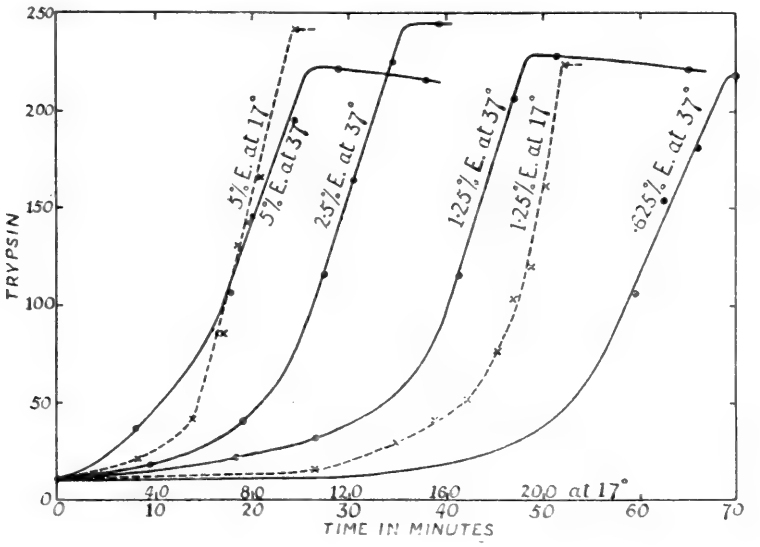

Fig. 2.

in them is variable, and because they may contain an anti-body. For instance, a concentrated glycerin extract of monkey's pancreas was found on two occasions to activate spontaneously in a fortnight. On the other hand a 75 % glycerin extract of ox-pancreas did not activate at all in three weeks.

Inactive glycerin extracts of sheep's pancreas are activated by enterokinase in a very similar manner to pancreatic juice, as can be seen from the curves given in Fig. 2. The times required for complete activation at 37° by 5, 2·5, 1·25 and 0·625 % of E. were 26, 36, 49 and 70 minutes respectively, or were very far from being in inverse proportion to the quantity of enterokinase. The times taken for the last half of activation were 8, 8, 8, and 10 minutes respectively, or were almost independent of the quantity of enterokinase. The results obtained at 17° are represented as dotted line

curves. The time scale on which the values are plotted is four times smaller than that for the values at 37°, and so it can be seen at a glance that the rate of activation at 17° by 5 % and 1·25 % of E. was about four times slower than in the corresponding experiments at 37°. The actual temperature coefficients work out at 1·93 and 2·07 respectively. It will be noted that the slope of the final portion of the dotted line curves is considerably steeper than that of the other curves, and in fact the times taken for the last half of activation by 5 and 1·25 % of E. were 24 and 17 minutes respectively, or two to three times longer than in the corresponding experiments at 37° instead of four times longer. The more marked influence of temperature

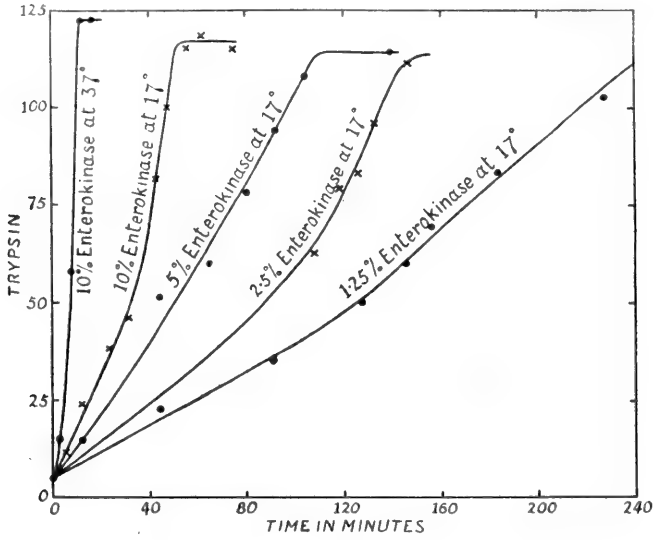

Fig. 3.

on the final stages of activation than in the experiments with pancreatic juice probably indicates that the deuterase factor is relatively less responsible for them.

Extracts of pig's pancreas are not activated by enterokinase in nearly such a typical manner as those of sheep's pancreas. As can be seen from Fig. 3, they much more nearly approach straight lines, or show comparatively little acceleration of velocity in the latter part of the activation. That is to say, the activation is throughout induced very largely by enterokinase, and deuterase is much less important than in the activation of sheep's pancreas extracts. Still it probably accounts for over half of the total activation. This is shown by determining the time taken over the last half of activation, and calculating it as a percentage on the total activation time. The per-

centage times observed are given in the table. They were obtained with various enterokinase preparations, two of which were much more active than the others, and so the percentage amounts of enterokinase given in the first column only hold for about two-thirds of the data. For the remaining third they represent a fivefold diluted solution.

Enterokinase added	Percentage time taken over last half of activation at 37°	17°	1°	Mean
10 %	37, 31	50, 37, 28	22, 20	32
5	—	43, 28	21	31
2·5	42, 32, 28	30, 30	25	31
1·25	34, 22, 22	44	—	30
0·625	21	—	—	21
Mean	30 % at 37°	36 % at 17°	22 % at 1°	

It will be seen that the relative time taken for the last half of activation was about 31 % of the whole time, or was about two-fifths of that required for the first half of activation. Hence even if deuterase were not responsible for any of this first half, it must have induced the larger part of the second half of activation. It will be noted that with a single exception the percentage time is almost constant whatever the amount of enterokinase used for activation. That is to say, with diminishing quantities of enterokinase the slope of the last half of the activation curves changed in almost the same proportion as the time taken for the whole of activation. This points to the final stages of activation being chiefly induced by enterokinase, but evidence indicating that it is largely due to deuterase is afforded by classifying the results according to temperature. In addition to experiments at 37° and 17°, a number were made in which the activating mixture was kept in a vessel surrounded by pounded ice and water, or at a temperature of 0–1°. From the bottom line of the table it will be seen that on an average in these experiments the last half of activation took only 22 % of the total time, as against 33 % taken in the experiments at 17° and 37°. From these data it can be calculated that deuterase must have been responsible for nearly twice as much of the last half of activation at 1° as at 17° and 37°. This result is in accordance with the conclusion that the action of deuterase is almost unaffected by temperature. The considerable difference in the form of the activation curves at 1° and 17° is well shown by comparing Figs. 4 and 3. These results were obtained with extracts of the same pig's pancreas, but the sample used for the experiments shown in Fig. 4 had remained longer in contact with the gland substance before filtration, and so contained much more trypsinogen.

It will be seen from the curves in Figs. 3 and 4 that the time of total activation of pig's pancreas extracts does not vary in inverse proportion to the quantity of enterokinase. Thus the activation times at 17°, with 10, 5, 2·5 and 1·25 % of E. were 52, 109, 148 and 248 min. respectively, whilst at 1°, with 10, 5, and 2·5 % of E. they were 143, 200 and 282 min. respectively. In some observations at 37°, with 10, 2·5 and 1·25 % of E. they were 14, 45 and 65 min. respectively. Experiments with other extracts of pig's pancreas gave similar results. Six sets of experiments made at 37° and 17° with 10 to 1·25 % of E. gave temperature coefficients of 2·07, 1·97, 1·93, 1·93, 1·86 and 1·81, or 1·93 on an average, whilst two sets of experiments at 17° and 1° gave temperature coefficients (likewise for a 10° interval) of 2·24 and 1·94.

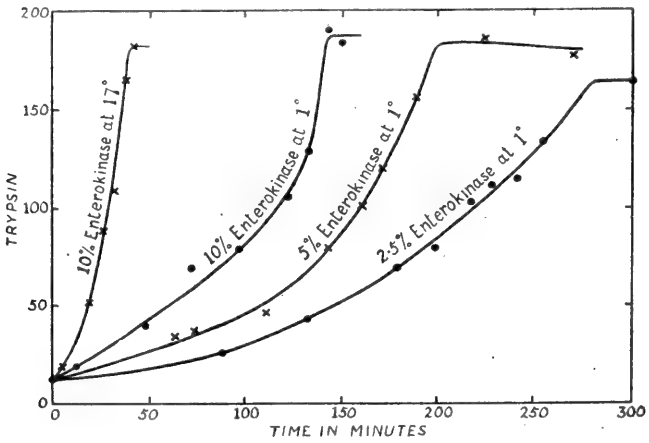

Fig. 4.

Extracts of ox-pancreas gave activation curves similar to those obtained with pig's pancreas. 5 % of E. took 32 minutes to activate at 37° and 108 min. at 17° (temp. coefficient 1·84), whilst the last half of activation took 28 and 31 % respectively on the total activation time.

The difference in the character of the activation curves obtained with extracts of sheep's and of pig's pancreas probably depends on a number of factors. As will be shown later on, various extracts not only contain different proportions of pro-deuterase and of an anti-body, but the rate of liberation of deuterase by trypsin and the action of deuterase on trypsinogen is influenced by various conditions. For instance, products of proteolytic activity retard the formation, or the action, of deuterase much more than the action of enterokinase. In Fig. 5 is shown the effect of adding 0·2 to 3·5 % of Witte

peptone to sheep's pancreas extract which was then activated by 5 % of E. at 37°. It will be seen that with increasing quantities of peptone the rate of the last part of activation, as compared with that of the first part, was more and more diminished. The actual percentage time of the last half of activation on the total time when 0, 0·2, 0·6, 2·0 and 3·5 % of Witte peptone was present was 16, 29, 27, 31 and 46 % respectively. Probably the peptone acts chiefly by retarding the action of the trypsin on pro-deuterase, for it is well known to retard its action on proteins. For instance, I found [1904, 2, p. 350] that 2 % of Witte peptone reduced the digestive action of trypsin on fibrin by 12 %.

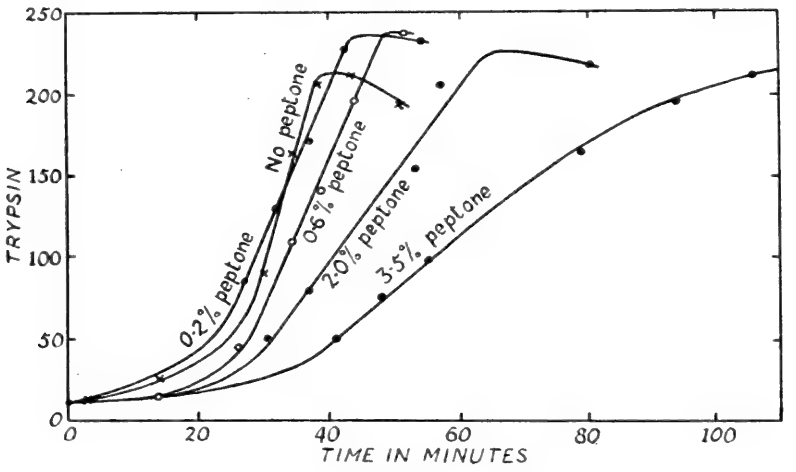

Fig. 5.

Under other conditions the action of enterokinase is much more retarded than that of deuterase. For instance, 5 % of it took 157 minutes to activate a glycerin extract of sheep's pancreas which was diluted with half its volume of water but without the addition of Na_2CO_3, as against 44 minutes when it was diluted to 5 volumes of 0·05 % Na_2CO_3. The last half of activation, however, took only 35 min. as against 15 min., or 22 % of the total activation time as against 34 %. Again, a pig's pancreas extract diluted 50 % with water only, took 203 min. as against the normal 56 min., but took only 12 % of the total time over the last half of activation instead of 28 %. The same extract, diluted 50 % with Na_2CO_3 up to a concentration of 0·08 %, took 139 min. for activation, but only 12 min., or 9 % on the total activation time, over the last half of activation. Presumably this great retardation of the enterokinase is due chiefly to the glycerin itself. Arguing on the

supposition that the gland substance which was extracted by four times its weight of glycerin contained 75 % of water, these activating solutions must have contained about 56 % of glycerin.

The Activation of Trypsinogen by Deuterase.

In previous experiments made with pancreatic juice and extracts I found [1902] that the addition of active pancreatic extracts caused a more rapid activation than the addition of intestinal extracts containing enterokinase. This result I have fully confirmed in my recent experiments.

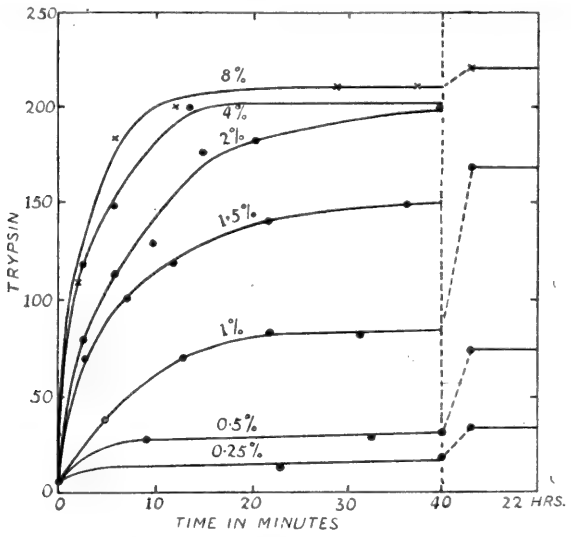

Fig. 6.

In Fig. 6 is shown the effect of adding 0·25 to 8 % of an active glycerin extract of pig's pancreas to an inactive extract at 17°. It will be seen that 8 or 4 % of extract activated almost completely in about 16 minutes, whilst 2 % activated in about 40 minutes. The form of the activation curve is entirely different from that obtained with enterokinase. Activation begins very rapidly and gradually slows down to zero as the available mass of trypsinogen diminishes, or deuterase appears to act logarithmically like most other enzymes. However, its action does not as a rule obey the logarithmic law, for it will be noted that the activation slows down and nearly ceases before the whole of the trypsinogen is converted. This is especially well seen when 1 % or less of deuterase extract was employed. In these experiments the activation almost stopped in the first 10 or 20 minutes, and then continued

with such extreme slowness that even 22 hours later, as can be seen from the data on the extreme right of the figure, a large portion of the trypsinogen was still unconverted. As will be shown later, this very slow and continuous conversion must be due to the action of fresh deuterase gradually liberated by the trypsin from the pro-deuterase contained in the inactive extract.

It should be stated that in these experiments the tryptic value of the active extract added was always deducted from the tryptic value observed. Also it was found that the deuterase continues to activate the trypsinogen of the extract to some extent after the sample has been added to the diluted milk for estimation, though not so rapidly as in the original solution. Hence in plotting out the curves *half* of the time taken for each estimation—which was generally 3 or 4 minutes—was added to the actual time from start of experiment to beginning of the estimation. In the enterokinase experiments no such correction was made, as the enterokinase appeared to cease activating on mixing with the milk.

A large number of activation experiments have been made with different substrates and different deuterase extracts, and they show that no single law of action will suffice to explain them. From evidence which will be adduced later it seems probable that both the trypsinogen and the deuterase in pancreatic extracts exist in various colloidal states, the condition of which can be readily changed, and which greatly influence their interaction. For instance, in Fig. 10 (see below) is recorded another set of experiments made with the same pancreatic extracts as were used in the experiments just described, but three months previously. It will be seen that the activation curves obtained with 0·7 and 1·2 % of deuterase approach the logarithmic type much more closely than those in Fig. 6. In still another experiment with the same inactive glycerin extract of pig's pancreas, but with a different deuterase extract (obtained by extracting pig's pancreas with two parts of N/10 HCl; slightly over-neutralising 17 hours later with solid Na_2CO_3, and filtering off), [*cf.*, Mellanby and Woolley, 1914, 1], the activation curve was definitely logarithmic in type. It is impossible to determine the total amount of trypsinogen present in an extract with great accuracy, for in the first place the method of trypsin estimation employed is only moderately accurate, and it does not give quite the same results from day to day as the samples of milk vary somewhat in quality. This glycerin extract had on previous occasions developed a maximum tryptic value of 236, 229, 219, 212 and 211, when kept at 17° with deuterase, but in the present experiments developed a value of only 200. Taking this to represent the effective maxi-

mum, and deducting from it the 7 units of free trypsin present in the extract before activation, it will be seen from the table that the results obtained at 17° and 37° roughly follow the logarithmic law for uni-molecular reactions, viz. :

$$K = \frac{1}{t} \log \frac{1}{1-x}.$$

The most exceptional values were those obtained in the two initial estimations. They are probably due to the correction made for the activation which continues during the trypsin estimation being too great.

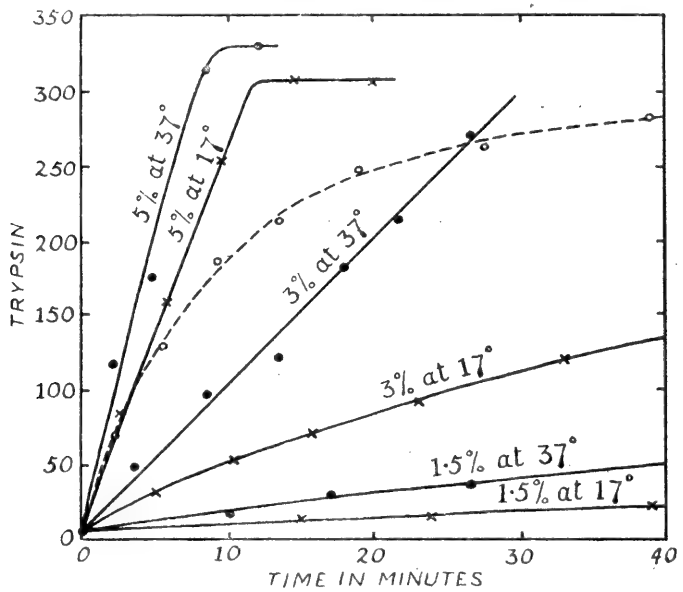

Fig. 7.

At 17° C.			At 37° C.		
Trypsin liberated (x)	Time (t)	K	Trypsin liberated (x)	Time (t)	K
46	3·5 min.	0·034	48	3·25 min.	0·038
69	8·4	0·023	69	8·0	0·024
95	13·0	0·023	101	15·5	0·021
109	18·0	0·020	114	23·75	0·016
127	23·5	0·020	155	35·5	0·020
170	69·0	0·014	182	42·75	0·029

In another series of experiments, made with extracts of sheep's pancreas, the rate of activation as a rule followed the linear law. In Fig. 7 is shown the effect of adding active glycerin extract of sheep's pancreas to inactive extract, and it will be seen that the activation curves are almost straight lines,

and continue so nearly to their completion. Yet these very same extracts, when tested some four months later, gave activation curves more or less midway between the linear and logarithmic types. Also, the inactive extract, at the same time that it was giving linear curves when activated by the active extract of sheep's pancreas, under some circumstances gave nearly logarithmic curves when activated by the HCl extract of pig's pancreas above mentioned. It should be stated that this extract had begun to activate spontaneously immediately it was prepared, but since at the time it was required for this experiment it had not quite completed its activation, a trace of enterokinase (0·1 %) was added to it and it was kept 24 hours before use. The action of 10 % of this mixture is shown in the dotted line curve of Fig. 7. Similar curves of a logarithmic type were obtained on activating with 6 and 4 % of the mixture, but 3, 2, 1·5 and 1 % of it gave absolutely linear activation curves. In their case the available mass of substrate must have been in excess of the maximum upon which the enzyme could act at any moment.

Five days after the HCl extract had activated itself completely its action on inactive sheep's extract was again tested, and 10 % of it, acting at 37° and at 17°, yielded curves almost identical in form and position with those shown on the extreme left of Fig. 7. The changed action seemed to depend partly on the lack of a trace of enterokinase, for after keeping some of the completely activated HCl extract for 24 hours with 0·1 % of E., it again yielded a curve approaching the logarithmic type. On the other hand some of the HCl extract likewise gave logarithmic instead of linear curves when diluted and kept five days with 0·05 % Na_2CO_3. Also, the inactive extract of sheep's pancreas under certain conditions, to be mentioned later, yielded curves very similar to those shown in Fig. 6, whilst it yielded curves about midway between the logarithmic and linear types when activated by active extract of pig's pancreas.

A glycerin extract of ox-pancreas, acted on by an active glycerin extract of monkey's pancreas, gave nearly linear curves (*cf.* Fig. 16).

These very conflicting results suggest that a change in the colloidal condition of enzyme and substrate, and in their manner of interaction, is effected very easily. More striking proof of the existence of different kinds of trypsinogen in pancreatic extracts came to light in quite another way. It was found that the maximum tryptic value induced on addition of enterokinase to inactive extract was always less than when it was kept with active extract (*i.e.* deuterase). The difference in the two maxima

attained varied considerably even in the same extract at different times. For instance, a glycerin extract of pig's pancreas was filtered off from the gland substance after 23 to 26 days' extraction, and was then found to develop a maximum tryptic value of 132 on addition of enterokinase. Four days later the maximum was 160 ; twelve days later it was 187, but three months later it averaged 215 in various experiments. On keeping this same extract with various deuterase extracts it developed an average tryptic value of 221, hence after the three months' interval practically all of its trypsinogen was in the same form. On the other hand a glycerin extract of sheep's pancreas, even after it had been kept three months, developed a maximum tryptic value of only 252, 236, and 244 in various experiments on addition of

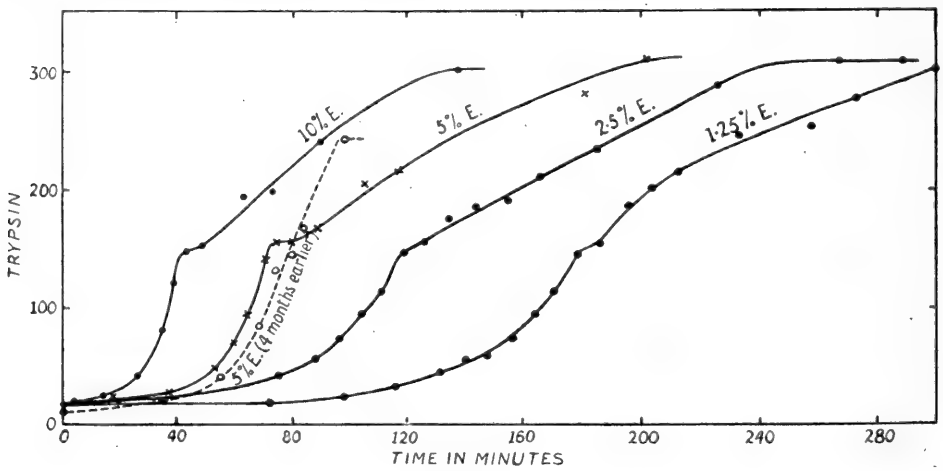

Fig. 8.

enterokinase, but one of 334, 313 and 312 when deuterase was added. That is to say, on an average only 76 % of the trypsinogen was converted into trypsin on the addition of enterokinase. Of course the extract did attain the same maximum after addition of enterokinase as after the addition of deuterase if it were given sufficient time, and if the destruction of the liberated trypsin were avoided by carrying out the experiment at 17° or 1°, but this only occurred slowly and was quite distinct from the rapid activation to a nearly constant level which is so typical of the action of deuterase.

After the glycerin extract just mentioned had been kept another four months in cold storage, it was found that nearly *half* of its trypsinogen had passed into the relatively stable form. This is strikingly proved by the series of experiments recorded in Fig. 8, in which it was acted on by 10 to

1·25 % of enterokinase at 17°. With 10 % of enterokinase the typical activation was completed in 41 minutes, the last half of activation taking only 15 % of the total time. Then the activation of the second half of the trypsinogen proceeded at a rate about seven times slower than the previous maximum rate, and it took about 97 minutes for its completion. The action of 5 % of E. was equally striking, but with 2·25 % and 1·25 % of E. the change in the slope of the curve after activation of the first half of the trypsinogen was not so marked. It will be noted that within the limits of experimental error the slope of the last half of the activation curves is almost the same whatever the amount of enterokinase present, *i.e.*, it is a deuterase effect.

The dotted line curve shows the action of 5 % of E. (another preparation) on this same extract of sheep's pancreas four months previously. It is one of the results already recorded in Fig. 2. Unfortunately the rate of activation in these earlier experiments at 17° was not investigated after the supposed maximum had been reached. It had been investigated in the corresponding experiments at 37°, and was then found to fall off rapidly. The reason is that the trypsin is so unstable at 37° that its destruction rate is greater than its activation rate, when such activation occurs at the slow speed shown by the latter half of the curves in Fig. 8.

The Activation of Pancreatic Juice by Deuterase.

Pancreatic juice is activated by deuterase in the same way as pancreatic extracts. In Fig. 9 (p. 510) is shown the effect of 1 to 3 % of a glycerin extract of pig's pancreas at 37°, and of 3 % at 17°. The curves are rather irregular in form, owing largely to the fact that the final stages are complicated by the activating effect of fresh deuterase liberated from the pro-deuterase present in the juice. The difference in the rate of activation induced by 3 % and by 2 % of deuterase at 37° is so remarkable that it might be thought to be due to some experimental error: but a repetition of the experiment a day later gave almost the same result. The two sets of values obtained with 3 % of deuterase are shown in the figure.

Experiments on the activation of pancreatic juice by the deuterase of activated juice will be described in a subsequent section.

The Temperature Coefficient of Deuterase.

As already mentioned, the activation of trypsinogen by deuterase is only slightly affected by temperature. A striking proof of this statement

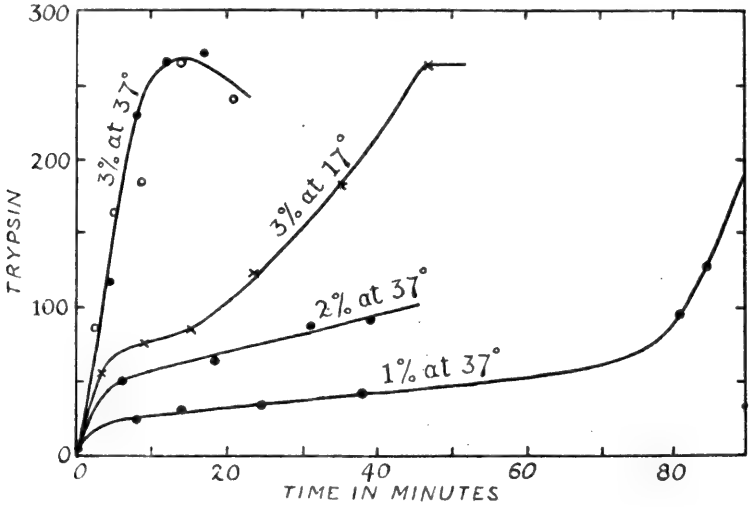

Fig. 9.

is afforded by the table showing the relationship between time and trypsin liberated which was given a few pages back. The average velocity coefficient, K, was 0·0223 at 17°, and 0·0247 at 37°, or nearly the same. A number of

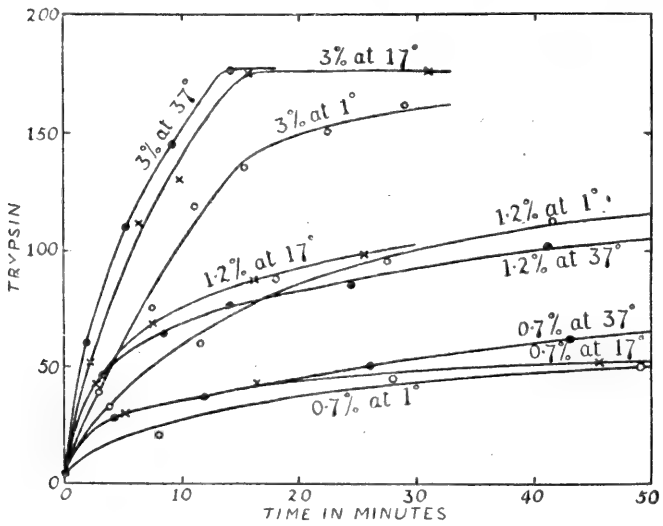

Fig. 10

other experiments in which an active glycerin extract of pig's pancreas was allowed to activate an inactive glycerin extract of pig's pancreas gave almost the same result. One series is shown in Fig. 10, and here we see that though 3 % and 0·7 % of deuterase activated rather more quickly at 37° than at 17°, 1·2 % activated more quickly at 17° than at 37°, or the average of the three sets of experiments indicates that the temperature change was practically without influence. At 1°, however, there is a distinct retardation in the rate of activation, especially in the initial stages. Later on there is an acceleration, and in the experiments with 1·2 and 0·7 % of deuterase we see that 30 minutes after the start the activation was proceeding at about the same rate at 1°, 17°, and 37°. This acceleration is due in part to the greater stability of deuterase at the low temperature, but there must be some other factor concerned, as deuterase is only very slowly destroyed at 17°.

As can be seen from the curves given in Fig. 7, the action of deuterase on extract of sheep's pancreas is considerably affected by temperature; but the actual temperature coefficient is not nearly so high as might be imagined at first sight. It is true that the rate of activation by 3 % of deuterase at 37° was nearly three times greater than at 17°, and by 1·5 % of deuterase, three and a half times greater, but it can be calculated from the curves that 3 % of deuterase at 37° activated the trypsinogen nine times more rapidly than did 1·5 %, and at 17°, fourteen times more rapidly. Hence in order to arrive at the real temperature coefficient, the relative amounts of deuterase required to induce the same rate of activation at 37° and at 17° must be calculated. It is found that 2·5 % of deuterase at 37° would activate at the same rate as 3·5 % at 17°, or that the temperature coefficient for this extract is 1·18.

It is a more accurate method to determine by direct experiment the relative amounts of deuterase required to activate at the same rate at 37° and 17°, and this has been done in two instances. It was found that 2 % of an active glycerin extract of pig's pancreas at 37° activated glycerin extract of ox pancreas at almost exactly the same rate as 3 % at 17°, or that the temperature coefficient was 1·22. Again, 2·5 % of active glycerin extract of sheep's pancreas at 37° activated pancreatic juice at the same rate as 3·0 % at 17°, or gave a temperature coefficient of 1·09.

The temperature coefficients for the activation of trypsinogen by enterokinase and deuterase are collected in the table for the sake of contrast. Only the data obtained between the temperature limits of 17° and 37° are included.

		Temp. coefficient (10°)	
Enterokinase activating	Pancreatic juice	2·10	2·00
	Extract of sheep's pancreas (2·07, 1·93)	2·00	
	„ „ pig's pancreas (2·07, 1·97, 1·93, 1·93, 1·86, 1·81)	1·93	
	„ „ ox pancreas	1·96	
Deuterase activating	Pancreatic juice	1·09	1·12
	Extract of sheep's pancreas	1·18	
	„ „ pig's „	1·00	
	„ „ ox „	1·22	

A large number of experiments were made in which the data were insufficient to give an accurate temperature coefficient, so in order to compare

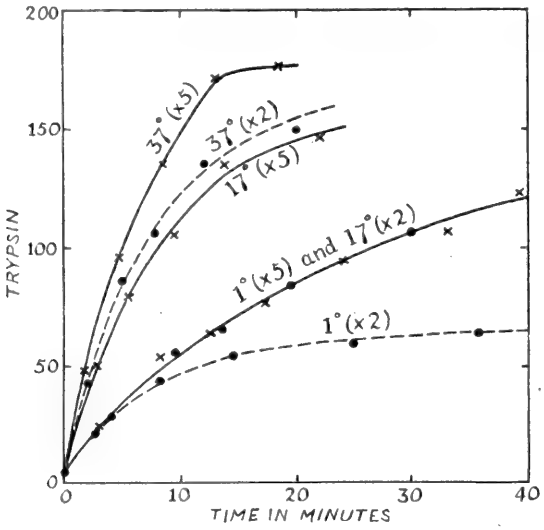

Fig. 11.

the temperature effects the method adopted was to read off from the plotted curves the times taken for liberation of 50 % of the total amount of trypsin set free in 30 or 40 minutes at 37°, and then find the time taken for the liberation of the same amount of trypsin at 17°. The time of liberation of 50 % of trypsin is taken for comparison rather than that of the completion of activation, as it can be estimated more accurately.

The differences of temperature effect observed with extracts of pig's pancreas and of sheep's pancreas appear to depend on the quality of both enzyme and substrate. Taking first the experiments made with glycerin extracts, it was found that when pig's pancreas deuterase activated sheep's pancreas trypsinogen, it took 1·4 times longer at 17° than at 37°, instead of 1·5 to 3·1 times longer as in the corresponding experiments when sheep's

deuterase activated sheep's trypsinogen. In the converse experiment when sheep's deuterase activated pig's trypsinogen it took, in various experiments, 1·2, 1·3 and 2·3 times longer at 17° than at 37°, whereas we have seen that pig's deuterase activated pig's trypsinogen as quickly at the lower temperature as at the higher. Hence the temperature effect depends partly on enzyme, but probably the physical condition of the substrate is the most important factor, and it can readily be modified so as to influence the temperature effect enormously. This is well shown by the two series of curves reproduced in Fig. 11. In one experiment, the results of which are represented by continuous line curves, some of the same glycerin extract of pig's pancreas, which on fivefold dilution with 0·05 % Na_2CO_3 was activated as quickly at 17° as at 37°, was diluted fivefold with water only. It was activated by 5 % of active pig's pancreas extract, and we see that its activation was distinctly slower at 17° than at 37°, and very much slower at 1°. In the other experiment the same extract was diluted with an equal volume of water only, and then it took four times longer to activate at 17° than at 37°, and much longer still at 1°. The curve obtained at 17° happens to be identical in position with the 1° curve of the more diluted extract.

Experiments with the active HCl extract of pig's pancreas previously mentioned gave similar results. When allowed to activate glycerin extract of pig's pancreas it took 1·6 times longer at the lower temperature, and when activating sheep's extract it took, in various experiments made at different times with different amounts of enzyme, 2·2, 2·7, 2·8, 3·8 and 6·4 times longer at the lower temperature.

The Rate of Liberation of Deuterase.

We have seen that the addition of small quantities of active pancreatic extracts to inactive extracts rapidly activates the trypsinogen, but if a sample of this freshly activated extract be added to more inactive extract it is found to possess little or no activating power. For instance, in Fig. 12 we see the effect of adding 3 % of active extract of pig's pancreas to inactive extract at 17°. Activation was almost complete in 30 minutes, and a minute after this time (marked by an arrow in the figure) a sample of the mixture containing 3 % of the extract was added to more inactive extract at 17°, and we see from the lowest curve in the figure that it had extremely little activating power. Another sample of the mixture taken 48 minutes after had distinctly more activating power: one taken 115 minutes after much more activating

power still, whilst a sample taken 175 minutes after had almost as much activating power as the original pancreatic extract. It is concluded, therefore, that a freshly activated extract contains scarcely any deuterase, but that this enzyme is gradually liberated from the pro-deuterase present in the extract by the action of the free trypsin. In proof of this hypothesis, it is found that the rate of development of activating power is dependent on temperature. On repeating the experiment at 1°, it was found that 3 % of the active extract activated the inactive extract to about the same point as that indicated in Fig. 11 in 90 minutes. At times ranging from 145 to 696 minutes later, 3 % samples of it were added to inactive extract at 17°,

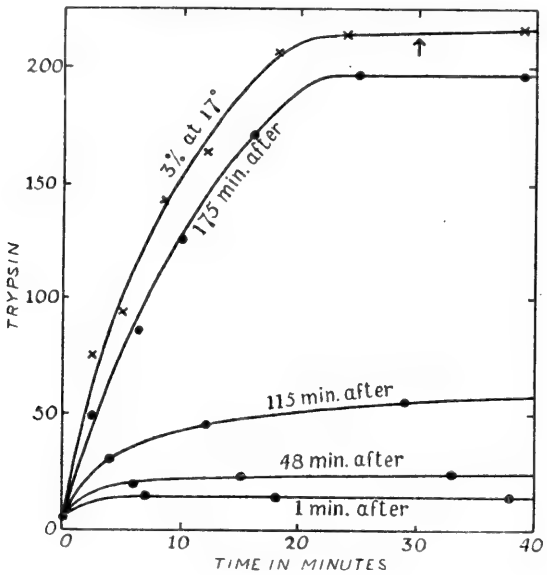

Fig. 12.

and it was found that the activation curves were of the same character as those shown in Fig. 11, except that the rate of generation of activating power in the mixture—which was kept throughout at 1°—was about four times slower than before. This is shown by the curves given in Fig. 13, which indicate the percentage of trypsin liberated in 40 minutes on addition of 3 % samples of the regenerating mixtures to inactive extract. When the results recorded in Fig. 12 are plotted out in this manner, it is seen that the rate of development of activating power gets more and more rapid from start to finish. The results obtained at 1° are plotted out on a time scale reduced fourfold, and we see that the curve obtained is of similar form and position to that obtained at 17° ; *i.e.* that the rate of development of activating

power in freshly activated extracts is four times slower at 1° than at 17°. At 37° it is probably about four times quicker than at 17°, but the results are unsatisfactory owing to the extreme instability of the liberated deuterase at this temperature. Thus an experiment was made in which 3 % of active extract was added to inactive extract at 37°, and it activated it almost completely in 24 minutes. At times ranging from 5 to 139 minutes later 3 % samples of this mixture—kept throughout at 37°—were added to inactive extract at 17°, and the percentages of trypsin liberated in 40 minutes are shown in Fig. 13. We see that though the activating power of the mixture

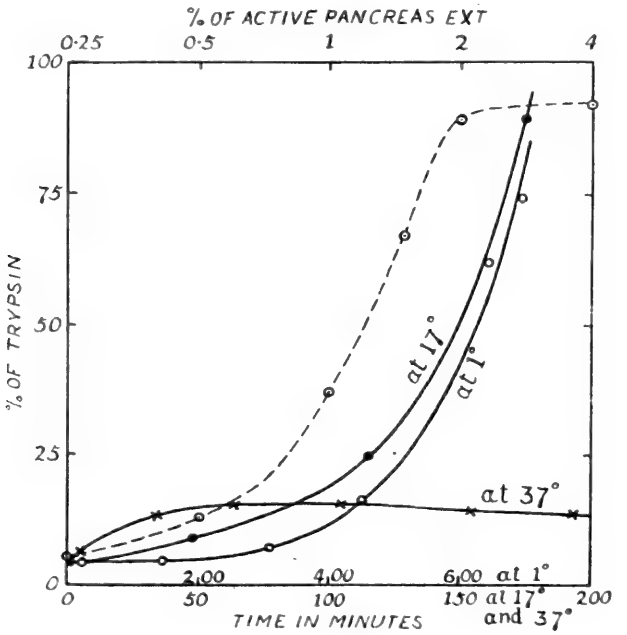

Fig. 13.

at first developed much more rapidly than that kept at 17°, it quickly slowed down, and after 105 minutes began to deteriorate.

The rapidly accelerating rate of formation of deuterase in freshly activated extracts is a mysterious and unexpected phenomenon. It is true that within certain limits the amount of trypsin liberated in an inactive extract on the addition of active extracts increases in greater proportion than corresponds to the extract added. For instance, the curves in Fig. 6 indicate that the addition of 0·25, 0·5, 1 and 2 % of active extract to inactive extract caused the liberation, in 40 minutes, of 12, 28, 79 and 193 units of trypsin respectively, or in the proportion of 1, 2·3, 6·6 and 16·1, instead of 1, 2, 4

and 8: but the rate of acceleration of deuterase formation is much more rapid than this. It occurs in geometrical progression, as is shown by comparing the curves of deuterase formation in Fig. 13 with the dotted line curve. This curve indicates the percentage of trypsin liberated by 0·25 to 4 % of active extract (*cf.* Fig. 6), but plotted out on the logarithmic scale given at the top of the figure. The curve is very similar in form to those indicating the rate of formation of deuterase at 17° and 37°, and hence it follows that this likewise occurs in geometrical progression.

In contradistinction to this geometrical increase in the rate of deuterase liberation is the fact that on addition of small quantities of deuterase to inactive extracts a certain amount of trypsinogen is activated almost at once, but then the activation slows down and continues so slowly as to be incomplete even 24 hours later (*cf.* Fig. 6). One would have thought that the trypsin liberated by the deuterase added would at once begin to liberate more deuterase from the pro-deuterase present, and that in consequence the activation would soon begin to accelerate again as it does in the presence of enterokinase. So far from that, the results recorded in Fig. 6 indicate that 0·5 % of deuterase, though it liberated 26 units of trypsin in 40 minutes, liberated only an extra 42 units in 22 hours. Again, 1 % of deuterase liberated 78 units in 40 minutes and an extra 90 units in 22 hours, though at the end of this time 52 units of trypsin still remained in the zymogen form. Experiments with sheep's pancreas extracts showed that the activation induced by deuterase continued much more steadily than that in pig's pancreas extracts, but even in them no acceleration was observed. The explanation of this result is unknown, but it seems to be dependent on conditions, such as the presence of anti-bodies, which do not obtain in pancreatic juice. As already pointed out, the results recorded in Fig. 9 clearly indicate that the addition of deuterase to pancreatic juice causes not only a rapid initial activation of a portion of the trypsinogen, but after an interval of slow activation this is succeeded by a second acceleration which must be due to the liberation of fresh deuterase. The results obtained in another experiment at 17° are recorded in Fig. 14 (see below), and two of the curves there reproduced show this secondary acceleration. In a similar experiment carried out at 37° the secondary acceleration appeared in every case.

It is evident that if time be given for the deuterase in freshly activated extracts to be liberated, the capacity for activation can be transmitted from extract to extract indefinitely. For instance, 5 % of an active extract of pig's pancreas was used to activate inactive extract of pig's pancreas,

and 4·5 hours later 5 % of this mixture was added to more inactive extract. 15 hours later 5 % of *this* mixture was used to activate still more inactive extract, and 5·5 hours later 5 % of this last mixture was added to still another sample of inactive extract. All the activation curves in the series were practically the same in form.

Experiments with glycerin extracts of sheep's pancreas gave results similar to those obtained with extracts of pig's pancreas. For instance, 5 % of active sheep's extract was added to inactive extract at 17°, and 36 minutes later the addition of 5 % of this mixture to more inactive sheep's

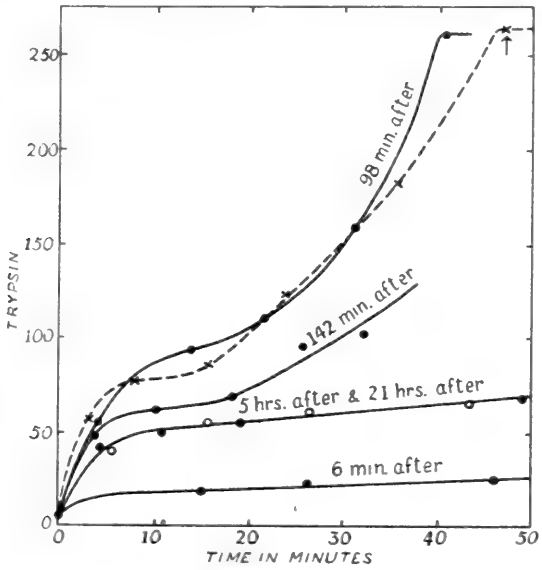

Fig. 14.

extract at 17° liberated 8 units of trypsin ; 51 minutes later it liberated 13 units ; 150 minutes later 24 units, but 343 minutes later, 165 units. Hence there was a rapid acceleration in the rate of deuterase formation in the latter part of the experiment, as in the previous series of experiments. The activation curves were somewhat similar in form to those shown in Fig. 12, and were not linear like the sheep's pancreas curves shown in Fig. 7.

Pancreatic juice yielded similar results to pancreatic extracts. In an experiment at 17°, 3 % of active extract of pig's pancreas activated the juice completely in 47 minutes, and 6 minutes later 5 % of the active juice was added to inactive juice at 17°, but as can be seen in Fig. 14 its activating power was but small. When it was tested again 98 minutes after it had probably reached its maximum activating power, for when tested 142 minutes

after it was distinctly less active. When tested 5 hours and again 21 hours after it had less activating power still. Hence the curves shown in the figure seem to indicate that the activating power of freshly activated pancreatic juice reaches its maximum two to four times more quickly than in pancreatic extracts, and that the deuterase formed is so unstable as to undergo rapid destruction even at 17°. In a similar experiment at 37° 3 % of active extract activated the juice completely in 13 minutes, and the immediate addition of 5 % of this active juice to inactive juice at 37° had as much activating effect as that shown in the lowest curve in Fig. 14. A sample taken 25 minutes later had nearly as much effect as that shown in the " 142 minutes after " curve of Fig. 14, whilst a sample taken 104 minutes after had slightly less effect than that shown in the lowest curve of Fig. 14. Another experiment at 37° showed that the freshly activated juice had more activating power 14 minutes after it had completed activation than either immediately after or 40 minutes after. Hence it is evident that at 37° the deuterase of freshly activated juice is extraordinarily unstable. It is quickly liberated, but within a very few minutes most of it is destroyed.

A Comparison between the Activating and Tryptic Powers of Extracts.

The experimental results thus far described are explicable on the hypothesis which I put forward in previous papers to the effect that trypsin itself is a powerful activator of trypsinogen. I found that fresh pancreatic extracts, rich in trypsin, had relatively much greater activating power than extracts which had deteriorated to some extent in tryptic power as the result of being kept. Also I found that the trypsin (or rennin) in freshly prepared extracts is so unstable that even when kept with water only (and no Na_2CO_3) at 38° about 50 % of it is destroyed in an hour. That in kept extracts is much more stable, and may be destroyed at the rate of only 12 % per hour [Vernon, 1901, 1, p. 196]. Hence I concluded that trypsin exists in various states of stability, and that only unstable trypsin has much activating power. It was suggested to me by Prof. W. M. Bayliss that I had no good evidence that the activating substance was this unstable trypsin, and further experiments supported his contention, for I found that in various freshly prepared active pancreatic extracts there was no relationship between activating power, trypsin content and stability of trypsin. It is quite true that if any extract deteriorates as the result of being kept, its activating power diminishes

much more rapidly than its tryptic power, but this is because the deuterase it contains is much more unstable than the trypsin.

Anything more than a rough comparison between the activating powers of extracts is useless, in that such comparisons do not hold at all closely at different concentrations. A comparison of the activation curves obtained with pig's pancreas extracts and sheep's pancreas extracts which are shown in Figs. 6 and 7 proves this. In experiments made at 37° it was found that 3 % of active sheep's extract activated inactive sheep's extract at the same rate as 1·25 % of active pig's extract, or that its activating power was 42 % as great. A similar experiment at 17° showed it to be 40 % as great. In other experiments made at different times upon inactive pig's extract it was found that 6, 4·5 and 3 % of active sheep's extract activated at the same rate as 2·0, 1·5 and 0·65 % of active pig's extract, or was 33, 33 and 22 % as active in the respective experiments. On an average, therefore, the sheep's extract had only 34 % of the activating power of the pig's extract, in spite of the fact that its tryptic power was almost exactly the same. The HCl extract of pig's pancreas, though it had three-fourths the tryptic power of the glycerin extract, had only a sixth its activating power, whilst a watery extract of pig's pancreas (made by extracting for 42 hours with 2 parts of chloroform water, filtering off and keeping with chloroform) had about half the tryptic power of the glycerin extract, but only a thirtieth the activating power. These experiments with pig's pancreas extracts confirm those formerly made by me [1901, 2, p. 295], in which I found that 0·9 % NaCl extracts of the pancreas of various animals had much less activating power, relative to their trypsin content, than their glycerin extracts.

The most striking evidence of the lack of correspondence between activating power and tryptic power was obtained with the other two extracts recorded

Pancreatic Extract	Relative activating power	Mean	Trypsin	% Trypsin destroyed in 1 hour at 37°
Glycerin extract of pig		100	294	12
„ „ „ monkey	83, 62, 52	66	83	16
„ „ „ sheep	42, 40, 33, 33, 22	34	293	65
HCl „ „ pig		17	218	17
H_2O „ „ pig		3·5	144	—
Glycerin „ „ ox		1	183	78

in the table. A glycerin extract of monkey's pancreas was found to have only about a fourth as much tryptic power as the glycerin extract of pig's pancreas, but two-thirds its activating power. This relative richness of

the monkey's gland in pro-deuterase may help to explain the fact that both the concentrated glycerin extracts made with the pancreas of healthy Macaque monkeys, which had been killed instantaneously, activated themselves spontaneously within a fortnight of their preparation.

In contradistinction to monkey's pancreas extract, a 75 % glycerin extract of ox-pancreas, though it had nearly two-thirds the tryptic power of the pig's extract, had no activating power at all in most experiments. As there was a slight indication of it in one or two instances, its actual power is recorded in the table as 1 % on that of pig's extract. As will be shown in the next section, the extract did contain some deuterase, but its activating powers were masked by the presence of an anti-body.

The stability of the trypsin in pancreatic extracts was tested by diluting them fivefold with water and Na_2CO_3 to 0·05 %, and keeping for an hour (or two hours) at 37°. Under such conditions no less than 78 % of the trypsin of the ox-pancreas extract was destroyed, and 65 % of that in the sheep's extract. The other extracts were much more stable, and the glycerin extract

Extract	% trypsin destroyed in 1 hr. at 37°	Mean
Glycerin extract of ox pancreas	82, 76, 76	78 %
,, ,, ,, sheep's ,,	71, 70, 70, 69, 64, 59, 52	65
HCl ,, ,, pig's ,,	21, 19, 17, 12	17
Glycerin ,, ,, pig's ,,	27, 26, 23, 23, 22, 21, 21, 14 (two hours)	(22)
,, ,, ,, monkey's pancreas	17, 16	16

of pig's pancreas had only 22 % of its trypsin destroyed in *two* hours. The stability of the trypsin probably depends on its colloidal state, and so varies according to the manner in which an extract of a gland is prepared, for my earlier experiments with pig's pancreas extracts gave a very different result from the present ones. When tested by a fibrin digestion method it was found that the trypsin of a glycerin extract was destroyed at the average rate of 69 % per hour by 0·4 % Na_2CO_3 at 38°, and at the rate of 39, 32, 24 and 23 % per hour by water only (in this case tested by the metacasein method). That is to say, it was destroyed about three times more rapidly than in the present experiments, if we assume that 0·05 % Na_2CO_3 is rather more injurious than water only. A glycerin extract of sheep's pancreas was destroyed at the average rate of 80 % per hour by 0·4 % Na_2CO_3, and at the rate of 66, 60, 53 and 39 % per hour by water only, or was about as unstable as in the present experiments.

The great instability of deuterase as compared with trypsin is proved by several experiments described in the previous section, in which the activating power of juice kept at 17° and 37° was found to deteriorate rapidly,

though the tryptic power was almost unaffected at 17°, and only moderately affected at 37°. Also I showed previously [1913, p. 335] that pancreatic juice activated at 37° has much less activating power on inactive juice than if it is activated at 17°. Deuterase retains its activity almost undiminished for months in glycerin extracts, but is very unstable in aqueous extracts. This is indicated by the small activating power of the HCl and aqueous extracts in the present experiments, and of the 0·9 % NaCl extracts described in my original experiments. Possibly the trypsin itself attacks it. Arguing from the great retardation to the action of deuterase exerted by Witte peptone, it seems probable that pro-deuterase is a protein substance which is first converted into deuterase, and then destroyed, by the digestive action of trypsin.

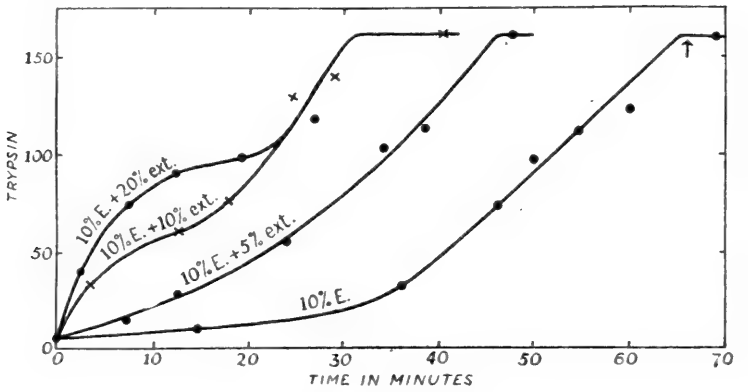

Fig. 15.

The Augmentor Action of Intestinal Extract on Deuterase.

It was stated in an earlier section that when ox-pancreas extract is acted on by enterokinase, it shows the typical acceleration in the velocity of the last half of activation. On an average this latter half took 29 % of the total activation time, so the acceleration was not marked, but the mere fact that it exists at all points to the liberation of deuterase in the extract, or is in apparent contradiction to the result described above. Further experiment showed clearly that there is a formation of deuterase in the course of activation. The addition of 10 % of E. to the extract at 17° gave the curve shown in Fig. 15. Forty-one minutes after the point in this curve marked with an arrow had been reached, 20 % of the activated mixture (containing 2 % of E.) was added to more inactive ox-extract together with 8 % of fresh E. That is to say, the inactive extract was now being activated by 10 % of E., together

with any deuterase which may have been present in 20 % of the previously activated mixture. The curve obtained shows the rapid initial velocity of activation followed by a gradual slowing which is the typical deuterase effect. This is probably followed by a second acceleration of activation, though by an oversight sufficient experimental data were not obtained to prove it, but in another experiment made with 10 % of E. + 10 % of the activated extract 138 minutes after activation, this second quickening of acceleration is shown. Presumably it is dependent on the enterokinase present. In another experiment made 76 minutes after activation, with 10 % of E. + 5 % of the activated mixture, the activation is considerably accelerated, but at a steady rate throughout.

This experiment leaves no doubt that a moderate amount of deuterase must have been liberated from pro-deuterase in the extract of ox-pancreas during the course of its activation by enterokinase, but in such a case how is one to explain the apparent absence of deuterase as shown in the experiments of the type described in the last section ? The subject is a difficult and complex one, and I did not attempt to solve it in detail, but the experiment to be described points to the existence of an anti-body which neutralises the deuterase unless its action is augmented by the presence of enterokinase. Some of the extract of ox-pancreas was activated at 17° by the addition of 5 % of active extract of pig's pancreas, and 8, 56, 128 and 174 minutes after activation was practically complete 5 % samples of this activated extract were added to more inactive extract, together with 5 % of E. In every case activation occurred more rapidly than in the control experiment in which E. alone was added. With the 128 minutes sample it was completed in 81 minutes instead of the 109 minutes taken in the control experiment, and with the other samples in 84 to 90 minutes. In the absence of E. the samples had no activating effect at all.

The augmentor action of intestinal extract upon deuterase was proved by a number of experiments. For instance, Fig. 16 shows the effect upon inactive extract of ox-pancreas at 37° of (*a*) 1·25 % of deuterase (active glycerin extract of monkey's pancreas); (*b*) 1·25 % of deuterase which had been diluted tenfold and kept with 10 % of E. at 17° for 2 minutes before mixing with the inactive ox extract; (*c*) 1·25 % of deuterase kept with the E. for 62 minutes before mixing with the inactive extract. We see that in this last experiment activation occurred considerably quicker than in the first or even in the second experiment. In fact it was almost doubled in rate, as can be seen on comparing the curve with the control

curve obtained with 2·5 % of active extract. Hence it is evident that the trace of E. in some way augments the action of the deuterase, and that to get the best result it is necessary for the deuterase and E. to be mixed for some little time before they are added to the inactive extract. In another experiment, with different extracts, it was found that when the deuterase and E. were mixed for 32 minutes before, they had just the same activating effect as when they were mixed 69 minutes before, activation being in each case completed in 18 minutes instead of 24 minutes, the time taken when they were mixed only 1 minute before. If the time for which they are mixed be too prolonged the activating effect is diminished rather than increased,

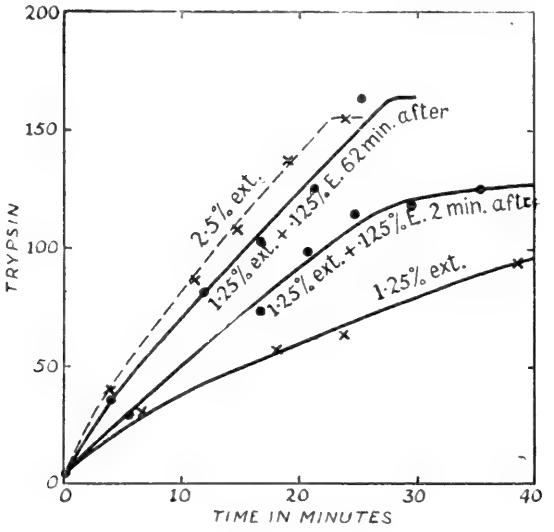

Fig. 16.

as the enzymes appear to destroy one another. Thus in one experiment it was found that if they were mixed 148 minutes before adding to the inactive extract there was no augmentation of activation at all, whilst in experiments in which they had been mixed for 24 hours there was a considerable diminution of activation.

It might be thought that the increased rate of activation was due directly to the E. but this is not so, for the actual amount added in the experiment recorded in Fig. 16, viz., 0·125 %, had by itself no appreciable activating effect in the time for which the experiment lasted. In another experiment the proportion of E. added was large enough to produce a considerable amount of activation. An extract of sheep's pancreas was activated at 37° by (*a*) 2 % of deuterase (active extract of pig's pancreas); (*b*) 0·25 % of E.

(a very active preparation); (*c*) 2 % of deuterase + 0·25 % of E., mixed together in presence of 0·05 % Na_2CO_3 and kept at 37° for 1·5 minutes before adding to the inactive extract. This mixture liberated 43, 104, 164 and 191 units of trypsin after 10, 15, 20 and 25 minutes respectively, whilst the sum of the amounts liberated by the deuterase and the E. after the same time intervals amounted only to 22, 43, 77 and 145 units.

An experiment in which enough E. was taken to activate a glycerin extract of pig's pancreas completely in 60 minutes at 17° is recorded in Fig. 17. This activation is compared with that produced by (*a*) 1 % of deuterase (active extract of pig's pancreas); (*b*) samples of 1 % of deuterase + 0·5 %

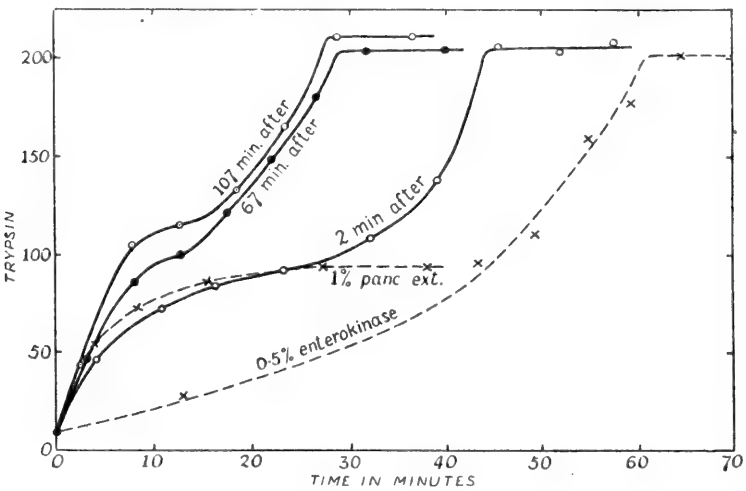

Fig. 17.

of E. mixed together at 17° in presence of 5 volumes of 0·05 % Na_2CO_3, and added to the inactive extract, 2, 67 and 107 minutes later. The curve obtained with the 2 minutes mixture shows a less rapid initial rate of activation than that produced by 1 % of deuterase alone, and much less than the sum of the effects produced by 1 % of deuterase + 0·5 % of E. The 107 minutes mixture, however, yielded an activation curve the earlier part of which would about correspond to the sum of the deuterase and E. curves, and the later part of which represents a distinct augmentation on such effects. The retardation produced when deuterase and E. solutions are mixed in certain proportions and added immediately to inactive extract appears to be greater the larger the proportion of E. used, for in another experiment at 17°, upon inactive sheep's extract, the addition of a mixture of 5 % of deuterase (active sheep's extract) + 10 % of E. (an old and rather weak preparation) caused

activation in 95 minutes when added immediately after mixing, though the 10 % of E., when acting alone, caused activation in 58 minutes. The mixed deuterase + E., when tested 62 minutes after mixing, took 77 minutes to activate ; 116 minutes after mixing, 62 minutes to activate ; 187 minutes after, 36 minutes, and 229 minutes after, 32 minutes. The 5 % of deuterase, when acting alone, activated three-fourths of the trypsinogen in the first 33 minutes, and then almost stopped, but even the activation induced by the 229 minutes' mixture was only equal to the sum of the activation rates induced by the deuterase alone and the E. alone. Hence in this experiment the large quantity of E. added, or some anti-body contained in the preparation, exerted a retarding action throughout.

General Conclusions.

It is desirable to discuss briefly some of the objections which may be levelled against my hypothesis, and to show in what respects it can be reconciled with previous observations. In the first place it may be said that it is inherently improbable that two different enzymes should be required to bring about one and the same effect. In reply to this I would point out that several different agents are capable of liberating secretin from prosecretin. In addition to acids the liberation is effected by soaps, alcohol, 0·6 % NaCl and other substances [Matsuo, 1913]. These liberating substances cannot all act in the same way ; neither can pepsin and trypsin attack proteins in the same way, and so there is no real difficulty in the fact that enterokinase and deuterase attack trypsinogen in different ways. The extremely low temperature coefficient observed for deuterase, viz., 1·12, is unique, but comparatively low coefficients have been observed with other enzymes. Kastle and Loevenhart [1900] obtained one of 1·3 for the action of esterase on ethyl butyrate. Tammann [1892], Kjeldahl [1881], and O'Sullivan and Tompson [1890] independently obtained the same coefficient, viz., 1·4, for the action of invertase on cane sugar, whilst Senter [1903] obtained a coefficient of 1·5 for the action of catalase on hydrogen peroxide. Most other enzymes investigated, including enterokinase, have been found to give coefficients of 2·0 to 2·6 [for literature see Euler, 1910], but Bayliss [1904] observed one of 5·3 for the action of trypsin on caseinogen, and as I [1904, 1, p. 364] observed one of 2·2 to 1·8 for the action of trypsin on Witte peptone one must conclude firstly that different enzymes have very different coefficients from one another, and secondly that even with the same

enzyme the coefficient varies with the nature of the substrate. In this connection it will be remembered that deuterase gave coefficients varying from 1·0 to 1·2 with different substrates.

Bayliss [1911] points out that the temperature coefficient of adsorption is, so far as investigated up to the present, a low one. He himself found that the rate at which equilibrium is attained in the adsorption of Congo red by paper is accelerated 1·36 times for each rise of 10° between 10° and 50°. Presumably the still lower coefficient obtained with deuterase is likewise compatible with the view that its action is an adsorption process, and involves no chemical change such as is associated with the action of enterokinase. The low coefficient of deuterase cannot be taken to indicate that it is not a true enzyme, for we have seen that it acts catalytically according to the logarithmic or the linear law like other enzymes. Moreover it is an extremely unstable body.

As another objection to my hypothesis it may be said: What is the need of a dual mechanism of activation? A convincing answer is supplied by the experiments of Terroine [1910] and of Mellanby and Woolley [1914, 2], who have shown that pancreatic steapsin is extraordinarily unstable in the presence of free trypsin. If enterokinase is added to pancreatic juice, the steapsin persists only so long as the quantities of trypsin present are minute. Hence, as Mellanby and Woolley point out, it is essential that the digestion of fat in the small intestine should take place in the absence of much free trypsin. This condition is observed in the upper part of the small intestine provided that there is not much enterokinase present, for in such a case the rapid liberation of trypsin from the trypsinogen of the juice may be delayed for many minutes.

An objection may be raised to the fact that enterokinase apparently has two distinct actions. We have seen that it not only liberates trypsin from trypsinogen, but it heightens the activity of deuterase on trypsinogen. This fact naturally suggests that deuterase is merely a co-enzyme of enterokinase, but arguments precluding this view have been adduced in a previous section. It may be found that these two properties of enterokinase are in some way related to one another, though at present we cannot say how. Again, it is possible that enterokinase acts by neutralising an anti-body to deuterase which is present to a greater or less extent in all extracts. Moreover, the augmentor effect on deuterase may not be due to enterokinase at all, but to some other unknown substance present in intestinal extracts.

The nature of the change involving the conversion of trypsinogen into trypsin is unknown. Mellanby and Woolley suggest that enterokinase is a proteolytic ferment which activates trypsinogen by digesting it, and splitting off free trypsin from a protein moiety with which it is firmly combined. Some of my earlier experiments [Vernon, 1903] show this hypothesis to be very improbable. I determined the proportions of trypsinogen precipitated from a glycerin extract by the addition of various strengths of alcohol, and on repeating the experiment a few days later when the trypsinogen had been converted into trypsinogen I found the precipitability of this trypsin to be almost exactly the same. It might be argued that the alcohol really precipitated protein from the extract, which absorbed and carried down trypsinogen or trypsin with it, but in reply I would point out that I found the precipitability of another enzyme in the extract, viz. amylopsin, to be quite different from that of the trypsin. Hence it is highly improbable that a protein + trypsin body would be adsorbed exactly to the same extent as trypsin alone. In all probability the conversion of trypsinogen into trypsin is a transformation involving no change in the size of the molecule, and this can be induced either by a mechanism which involves a chemical process at some stage, or by a purely adsorption process, *i.e.*, by enterokinase or by deuterase.

If the results described in this paper be compared with those given previously [Vernon, 1913], it might be thought that they are to some extent mutually contradictory. Thus I showed that inactive juice could readily be activated by the addition of activated juice, whether this contained enterokinase or not, but the activation curves which I gave do not, except in one experiment, show the initial rapid activation which is typical of the action of deuterase. The explanation is that I did not as a rule happen to catch my samples of activated juice at a time when they still retained much of their unstable deuterase, and that in consequence the curves given in Figs. 2 and 4, and the curve on the right of Fig. 3 of my previous paper, are comparable to the lowest curve in Fig. 9 of the present paper, whilst the curve on the left of Fig. 3 of my previous paper is comparable to that on the extreme left of Fig. 9 of the present paper. Again, Mellanby and Woolley [1913, p. 355] quote an experiment in which they activated juice by enterokinase, and every few minutes during the course of activation they took 10 % samples of it and added them to inactive juice. On plotting out their results it can be calculated that the sample taken when the primary activation was just completed had nearly twice the activating power of the sample

taken when it was just begun, and yet they do not admit that this result affords proof of the existence of any activating agent other than enterokinase. As is indicated by the results described in this paper, they would have found that their activated juice had a much greater activating power if they had tried it a few minutes after it had completed its activation, and still better, if they had carried out the experiment at 17° instead of 37°.

Summary.

1. The experiments described in this paper lead to the conclusion that in the activation of trypsinogen by enterokinase, the trypsin liberated in the earlier stages by the direct action of the enterokinase gradually sets free an enzyme (termed *deuterase*, to indicate that it acts secondarily to enterokinase) from a precursor, and that this deuterase is mainly responsible for the later stages of the activation process.

2. In support of this hypothesis it is found that in the activation of pancreatic juice by enterokinase, the rate of activation during the last half of the process may be 1000 times more rapid than the initial rate, and it is independent of the quantity of enterokinase used. The total activation time varies with the temperature (temp. coefficient for 10° = 2·00), but the time taken over the last half of activation is only slightly influenced by temperature. This is because it is chiefly dependent on deuterase, the temperature coefficient of which ranges from 1·00 to 1·22. Enterokinase and deuterase are also distinguished by the fact that the former is relatively much more retarded in its action by glycerin, and the latter, by Witte peptone.

3. The addition of active pancreatic extract or juice to inactive extract or juice causes activation which is rapid at first, but which quickly slows down almost to zero. This is due to its containing dueterase, which acts according to a logarithmic or linear law like other enzymes. The freshly activated extract or juice contains very little deuterase, but it is gradually liberated from pro-deuterase in the course of the next few minutes (at 37°), or next hour or two (at 17°). The rate of its liberation increases in geometrical progression.

4. There is no relationship between the amounts of deuterase and trypsin present in pancreatic extracts prepared from various animals. Glycerin extract of monkey's pancreas contained only a fourth as much trypsin as glycerin extract of pig's pancreas, but two-thirds as much deuterase. Some experiments seemed to show that extract of ox pancreas contained no deuterase

at all, but this was because its presence was masked by an anti-body. Deuterase is extremely unstable, except in presence of glycerin, and therefore aqueous pancreatic extracts have relatively little activating power, whilst freshly activated pancreatic juice was observed to lose most of its activating power in two hours at 37°. Probably the deuterase is destroyed by the trypsin.

5. The stability of the trypsin in different extracts is extremely variable, and bears no relationship to activating power. That in ox pancreas extract was destroyed at the rate of 78 % per hour by 0·05 % Na_2CO_3 at 37°, whilst that in pig's pancreas extract was destroyed at 12 % per hour. Trypsinogen also exists in different degrees of stability, and in one extract half of it was found to be activated by enterokinase seven times more slowly than the other half.

6. The activating power of deuterase may be nearly doubled by the addition of small quantities of intestinal extract which themselves have no appreciable activating power. Nevertheless deuterase is not a co-enzyme of enterokinase.

I wish to express my thanks to Dr G. von Anrep and Dr C. Lovatt Evans for their kindness in providing me with samples of pancreatic juice.

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LV. THE INFLUENCE OF EXCESSIVE WATER INGESTION ON PROTEIN METABOLISM.

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A number of observations have been made upon the effects of increased ingestion of water on the output of nitrogen. As the various results obtained by previous workers are referred to later, it will suffice here to state that there is almost unanimous agreement that if water be freely exhibited to an animal in nitrogenous equilibrium there accompanies the consequent diuresis an increased excretion of nitrogen. As to whether this increased nitrogenous excretion signifies a mere mechanical flushing out of waste products or an accelerated catabolism of protein, opinion has been sharply divided. Fowler and Hawk [1910] undertook an investigation on the subject and extended the field of observation to include creatine and creatinine. They found regarding these, that the addition of three litres of water per day produced a decrease in the output of creatinine and led to the appearance in the urine of considerable amounts of creatine.

The primary object of the present research was to investigate the accuracy of these findings. In view of the fact, however, that most of the earlier workers conducted their experiments on animals and limited their observations either to the total nitrogen or the urea, it was thought that further information might be obtained by making a more complete urinary analysis in the human subject and by varying the protein intake in the different experiments.

Method.

The subjects of the experiments, normal healthy males, were given a fixed creatine-free diet for several days, until the daily excretion of the various nitrogenous constituents of the urine had become approximately constant.

Thereupon, in addition to the diet, a given quantity of water was drunk each day for three days, whereafter, the diet was continued for several days more without the extra water. Some of the water was taken with the meals and the rest in the intervals between. The protein content of the food was varied in the different experiments from 27 to 319 grams per day. The urine was collected in 24 hour samples, the collection being completed at 9 a.m. before either food or water had been taken for the day.

In one of the experiments, No. IV, the water taken during the preliminary period was fixed in amount. The daily requirements however, as indicated by the inclination of the subject to drink, were not uniform. In all the others therefore, to ensure that the conditions on the pre-water days would be as natural as possible, the amount taken was not restricted. It was considered that this procedure would give a more perfectly normal period for comparison with the period of excessive consumption, and that the volume of the urine would indicate the average daily intake, with sufficient accuracy for the purposes of the research.

The methods of analysis adopted were: total nitrogen—Kjeldahl: ammonia—Folin: urea Exp. II, III and IV,—Folin, I and V,—the urease method described by Plimmer and Skelton [1914]: amino acids—Sörensen's formalin titration method: creatine and creatinine—Folin.

Protocols.

Experiment I. Very low protein diet.

Diet. In grams, apple 100, banana 150, potato 400, butter 75, sugar 75, cocoa 10, bread 200, containing approximately protein 27 g., fat 70 g., carbohydrate 325 g.

Subject aet. 33; weight at beginning of exp. 67·8, at end 65·8 kilos.

The diet was continued for a preliminary period of eight days. On the 9th, 10th and 11th days three litres extra of water were drunk. Owing to the rapid loss of weight of the subject the experiment was stopped on the 12th day.

The results of the first six days are omitted, the only points of interest being a continuous loss of weight, an excretion of nitrogen in excess of the intake and a uniform decrease of creatinine from 1·367 to 1·320 grams per day.

TABLE I.

Day of exper.	Weight kilos	Urine c.c.	Tot. N. grs.	Urea N. grs.	NH_3 N. grs.	Amino Acid N. grs.	Creatinine grs.	Creatine grs.	Per cent. of Tot. N. as urea	Remarks
7	65·97	412	6·300	4·151	0·259	0·113	1·320	—	65·9	Fixed diet
8	65·97	405	6·300	4·157	0·185	0·143	1·328	—	65·9	„
9	65·80	2830	8·610	6·629	0·231	0·163	1·330	—	76·9	3 lit. H_2O extra
10	66·02	3545	6·325	4·534	0·266	0·161	1·315	—	71·6	„
11	65·80	3602	6·125	4·388	0·280	0·108	1·313	—	71·6	„

Experiment II. Moderately low protein diet.

Diet. In grams, oatmeal 100, bread 300, butter 80, apple 70, cocoa 10, sugar 100, milk 300 c.c., containing approximately protein 48 g., fat 93 g., carbohydrate 346.

Subject aet. 22; weight at beginning of exp. 57·8, at end 57·7 kilos.

The diet was continued for a preliminary period of seven days. On the 8th, 9th and 10th days three litres extra of water were drunk. On the 11th, 12th and 13th days the diet was continued without extra water. The first five days are omitted from the table, as they show no points of interest. The first post-water day is also omitted, as the analysis showed the day's collection to have been contaminated.

TABLE II.

Day	Body wt. kilos.	Urine c.c.	Tot. N. grs.	Urea N. grs.	NH_3 N. grs.	Amino Acid N. grs.	Creatinine grs.	Creatine grs.	Per cent. of Tot N. as urea	Remarks
6	57·7	600	9·07	6·10	0·342	0·1834	1·350	—	67·3	Fixed diet
7	57·7	670	8·77	5·95	0·347	0·1788	1·421	—	67·8	„
8	58·4	2980	9·83	6·90	0·459	0·3633	1·360	—	70·2	3 lit. H_2O extra
9	58·2	3105	8·96	6·12	0·465	0·1827	1·360	—	68·3	„
10	58·2	3400	9·24	6·41	0·459	0·1970	1·377	—	69·4	„
12	57·7	880	9·38	6·44	0·336	0·1970	1·343	—	67·6	Fixed diet
13	57·7	870	9·46	6·59	0·403	0·1480	1·350	—	69·7	„

Experiment III. Moderately high protein diet.

Diet. In grams, sugar 100, "plasmon" 60, cheese 200, dried skimmed milk 50, bread 400, cocoa 20, butter 50, apple 100, containing approximately protein 160 g., fat 73 g., carbohydrate 359 g.

Subject same as Exp. II, aet. 22; weight at beginning of exp. 57·2, at end 57·4 kilos.

On the 5th, 6th and 7th days three litres extra of water were drunk. On the third post-water day the diet was stopped. The table gives the results from the third day.

TABLE III.

Day	Body wt. kilos	Urine c.c.	Tot. N. grs.	Urea N. grs.	NH_3 N. grs.	Amino Acid N. grs.	Creatinine grs.	Creatine	Per cent. of Tot. N. as urea	Remarks
3	57·2	1220	17·53	13·98	0·468	0·1274	1·377	—	79·7	Fixed diet
4	57·3	1040	17·47	14·03	0·392	0·1060	1·381	—	80·3	,,
5	58·8	3450	19·04	14·54	0·609	0·1000	1·332	—	76·4	3 lit. H_2O extra
6	58·8	3895	19·06	15·87	0·566	0·1081	1·405	—	83·3	,,
7	58·9	2730	18·72	14·99	0·549	0·1172	1·343	—	80·1	,,
8	57·6	1350	19·49	16·26	0·426	0·2744	1·400	—	83·4	Fixed diet
9	57·4	1220	20·12	16·67	0·440	0·0792	1·340	—	82·9	,,

Experiment IV. Very high protein diet.

Diet. In grams, dried skimmed milk 600, cheese 300, bread 300, water 3 litres containing approximately protein 319 g., fat 153 g., carbohydrate 179 g.

Subject aet. 21; weight at end of exp. 57 kilos.

In this experiment the diet was continued for 15 days. On the 5th and on the 9th, 10th and 11th days 9·6 litres of water were drunk in addition to the water used in preparing the food.

TABLE IV.

Day	Urine c.c.	Tot. N. grs.	Urea N. grs.	NH_3 N. grs.	Amino Acid N. grs.	Creatinine grs.	Creatine grs.	Per cent. of Tot. N. as urea	Remarks
1	1930	39·82	33·59	0·250	0·241	1·660	—	84·4	Fixed diet
2	1980	40·24	33·83	0·241	0·249	1·480	—	84·1	,,
3	2180	42·19	33·90	0·246	0·264	1·563	—	80·4	,,
4	2260	43·88	35·03	0·206	0·324	1·554	—	79·8	,,
5	7400	40·19	36·41	0·477	0·103	1·533	—	90·6	9·6 litres H_2O
6	2150	34·60	31·71	0·202	0·348	1·588	—	91·4	Fixed diet
7	2110	38·19	35·36	0·283	0·267	1·650	—	92·6	,,
8	2040	40·22	36·58	0·299	0·398	1·604	—	91·0	,,
9	8370	40·19	37·05	0·576	0·268	1·586	—	92·2	9·6 litres H_2O
10	8500	40·57	36·68	0·563	0·436	1·459	—	90·4	,,
11	8070	39·24	36·12	0·499	0·387	1·547	—	92·1	,,
12	2440	34·16	31·28	0·252	0·348	1·687	—	91·6	Fixed diet
13	2150	36·34	33·46	0·333	0·279	1·605	—	92·1	,,
14	2160	36·89	33·96	0·345	0·285	1·619	—	92·1	,,
15	2300	40·40	37·18	0·361	0·363	1·642	—	92·0	,,

Experiment V.

Diet creatine-free, containing protein 110, fat 67, carbohydrate 325 g.

In this experiment nitrogenous equilibrium had not been attained before the increased water intake. Its results are omitted in discussing the effects of water on the excretion of total nitrogen.

TABLE V.

Urine c.c.	Tot. N. grs.	Urea N. grs.	NH_3 N. grs.	Amino Acid N. grs.	Creatinine grs.	Creatine grs.	Per cent. of Tot. N. grs.	Remarks
1298	10·885	8·458	0·322	0·243	1·330	—	77·7	Fixed diet
898	12·810	9·940	0·280	0·261	1·340	0·02	77·6	„
3741	13·583	10·962	0·378	0·257	1·340	0·02	80·7	3 litres H_2O extra
4020	13·02	10·596	0·406	0·229	1·385	—	81·4	

Results.

Total Nitrogen. In all except Exp. IV the increased ingestion of water causes a distinct rise in the excretion of total nitrogen. There is, however, in the degree of the increase a lack of uniformity, which corresponds to the divergent results obtained by different workers. Thus Forster [1878] found an increase of 90 %, Heilner [1906] 40 %, Voit [1860] 25 %, Mayer [1880] 9 %, Gruber [1901] 7 %, Fränkel [1877] 6–12 %, and Salkowski and Munk [1877] about 3 %. Dubelir [1891], Seegen [1871] and Straub [1899] found no distinct increase. Heilner [1906] suggests that the results vary with the amount of food. Voit and Forster worked with fasting dogs, while Dubelir and others used dogs on an ample diet of flesh. In the present series of experiments the greatest increase was obtained in Exp. I, where the protein was abnormally low, whereas in Exp. IV with the high protein intake there was no increase. In Table VI the whole series is compared.

TABLE VI.

No. of exper.	Protein in diet. approx. g.	Excretion of Tot. N. on last pre-water day, g.	Excretion of Tot. N. on first water day, g.	Increase in Tot. N. g.	Increase per cent.
1	27	6·30	8·6	2·3	36·5
2	48	8·77	9·83	1·06	12·1
3	160	17·47	19·04	1·57	8·9
4	319	40·22	40·19	*nil*	*nil*

It is thus seen that the percentage increase of total nitrogen excreted on excessive water ingestion tends to vary inversely with the amount of protein fed.

In Exp. IV there is evidence of a marked retention of nitrogen on the cessation of the excessive water intake. After the single water-drinking day the amount excreted fell from 40·19 to 34·69 grams. After the three-day period of increased water intake it fell from 39·24 to 34·16, and only returned to its former level on the fourth day.

There would appear to be two distinct factors involved, one, whose action is immediate and whose influence is most manifest in protein deficiency, tending to cause a flushing out of nitrogenous end products, and another, whose influence is more prolonged, tending to cause a retention of nitrogen.

Ammonia. There is in every instance a distinct rise in the ammonia output which coincides exactly with the increased water intake. This is in keeping with the results of Fowler and Hawk [1910], who argued that the water stimulated gastric secretion and the increased ammonia represented the amount necessary to neutralise the excess of HCl thereby produced. If this argument were well founded the increase in ammonia output should be more or less parallel with the increase in water drunk. As appears in Table VII, there is no evidence of such parallelism.

The increase of water passing through the system must accelerate the flow of blood through the liver and of lymph through the tissues. In the process of deaminisation, as the NH_2 moiety is detached it would tend to be carried off by the accelerated flow of blood and lymph and excreted as ammonia instead of being converted to urea. If this supposition be correct, the increase in ammonia would be in some degree proportional to the extent of deaminisation taking place in the tissues, which in turn would be influenced by the amount of protein in the food. The following table shows that the increase in ammonia more or less corresponds to the amount of food protein being catabolised.

TABLE VII.

No. of exper.	Protein in diet, g.	NH_3N. Aver. excret. on non-water days, g.	NH_3N. Aver. excret. on water days, g.	Increase g.	Percentage increase
1	27	0·222	0·259	0·037	16·6
2	48	0·345	0·461	0·116	33·6
5	110	0·301	0·392	0·091	30·2
3	160	0·430	0·575	0·145	33·7
4	319	0·273	0·529	0·256	93·8

The increase in ammonia evidently represents a mere flushing-out process, and the amount of the increase depends upon the amount of ammonia being liberated in the liver and other tissues.

Urea. The rise in the urea output is most marked. Both in absolute amount and in percentage of total nitrogen excreted as urea, the increase is maintained throughout the water and the post-water periods. Thus, in Exp. I. on the third water day the total nitrogen falls from 6·3 grams on the pre-water days to 6·125, while the urea rises from 4·154 to 4·388. In Exp. II

the average urea output of the post-water days is 6·52 as against 6·03 on the pre-days. In Exp. III the figures are: pre-water 14 g., post-water 16·46 g. In Exp. IV, on the last day of the records, i.e. the fourth day after the period of increased water intake, the total nitrogen output is 40 g. as compared with an average of 41·53 g. in the preliminary period, while the urea, instead of decreasing with the total nitrogen, rises from 34·09 to 37·184 g. The prolongation of the increased excretion of urea beyond the period of increased diuresis is evidence that the increase cannot be accounted for by a mere mechanical flushing out of urea accumulated in the tissues.

Table VIII shows the percentage of total nitrogen excreted as urea in the preliminary period and in the period following the beginning of the increased consumption of water including both the water and the post-water days.

TABLE VIII.

	Average daily excretion in preliminary period			Average daily excretion on water and post-water days			
No. of exper.	Tot. N. g.	Urea g.	Per cent. of Tot. N. as urea	Tot. N. g.	Urea g.	Per cent. of Tot. N. as urea	Increase per cent.
1	6·3	4·154	65·9	7·02	5·184	73·8	7·9
2	8·92	6·025	67·5	9·374	6·492	69·3	1·8
3	17·50	14·005	80·0	19·286	15·666	81·2	1·2
	41·532	34·09	82·8	38·27	35·07	91·6	8·8
5	11·848	9·199	77·6	13·30	10·779	81·0	3·4

It will be seen that the greatest increase in the excretion of urea occurs in Exp. I, where there is a protein deficiency, and in Exp. IV, where the protein intake is excessive.

Amino acids. In Exps. I and II on the low protein diets there is an immediate rise in the excretion of amino acids on the first day of increased water ingestion. In Exp. III with a moderately high protein intake there is a slight drop, and in Exp. IV, where the protein intake is higher, the drop is marked. Table IX compares the series.

TABLE IX.

No. of exper.	Protein intake, g.	Amino acid N. Average in prelim. period	Amino acid N. First water day	Difference
1	26	0·128	0·163	+·035
2	48	0·181	0·363	+·182
5	110	0·255	0·257	+·002
3	160	0·117	0·100	−·017
4	319	0·270	0·103	−·168

While there is an absence of uniformity in the results, they are such as suggest the condition, that with a deficiency of protein in the food the stimulated diuresis due to increased water intake causes an initial washing out of the amino acids, while with an excess of protein the increased water intake causes an initial retention of these. As these results are not maintained, they would appear to be due to the sudden alteration in the amount of fluid passing through the system.

Faecal nitrogen. Only in Exp. I was the collection and analysis of faeces made. The daily evacuation was fairly regular, taking place in the forenoon. The first day's collection was discarded, as they belonged more properly to the preceding period. Those of the first water day were regarded as belonging to the preliminary period and those of the first post-water day as belonging to the water period. The results obtained were :

Preliminary period		Increased water period	
Dried weight	Total nitrogen	Dried weight	Total nitrogen
19·42 g.	0·927 g.	12·9 g.	0·542 g.

These results are in agreement with those of Fowler and Hawk [1910]. Too much importance should not be attached to the results of a single experiment, but even keeping in view the fact that the faecal nitrogen is not all derived from food residues, the results seem to suggest that the increased consumption of water is productive of a more complete utilisation of the ingested protein.

Creatine and creatinine. In Exps. II, III and IV there is on the water days an apparent decrease in the excretion of creatinine. On these days, however, the bulk of the urine was enormously increased and the concentration of the creatinine correspondingly decreased. It was necessary, therefore, to depart from the routine mode of analysis on the water days. In some instances 30 c.c. of urine were taken for analysis instead of 10 c.c., and in other instances, after development of the colour, dilution was carried only to 250 c.c. instead of to 500 c.c. Both of these deviations, which are recommended by Folin [1904] in such cases, in his original description of the colorimetric method are productive of error, showing a reduction in the amount of creatinine found which is quite fictitious. In Exps. I and V the urine from the beginning was diluted to 5,000 c.c. and the dilution and method of analysis rigidly adhered to throughout the experiment. In these, on the water days there appears no decrease in the amount of creatinine excreted. It may be concluded, therefore, that the decrease found in the earlier experiments should be ascribed to faulty methods of analysis. On no occasion was the increased

consumption of water followed by the appearance of creatine in the urine. The influence of excessive water ingestion on the excretion of creatine and creatinine has already been dealt with [Burns and Orr, 1914] and need not be further discussed here.

Discussion of Results.

The source of the increased excretion of nitrogen on excessive consumption of water has been much discussed. V. Noorden [1907], after a review of the literature, says: "It all turns on the flushing of nitrogenous end products out of the system." He bases his conclusion largely on the work of Neumann [1899], who found that, as the consumption of water rose, the urinary nitrogen rose, the rise being most marked on the first day and rapidly disappearing, and that on a return to a normal consumption of water there occurred a retention of nitrogen. The initial increased output and subsequent retention have been here verified, but the compared results obtained on the varying protein intakes do not support the view that the increase represents a mere flushing out of end products. If that view were correct, the greatest increase would appear where there existed the greatest amount of end products, which one would expect to be during the period of highest protein intake. We have seen however that it is just in this case that no increase takes place, a result which is in agreement with the findings of those who worked with dogs on a heavy flesh diet, and that, on the contrary, the highest increase is found in Exp. I, where the protein was abnormally low, viz. 27 grams per day, the diet consisting mostly of carbohydrate and fat, in which case one would expect the minimum accumulation of nitrogenous end products liable to be flushed out. While it is not to be doubted that there occurs a flushing-out process, to which the increase of ammonia appears entirely due, the whole of the influence exerted by the water cannot be ascribed to this mechanical cause. The results obtained on the varying protein intakes, together with the fact that the increased percentage of urea continues on the post-water days, after the mechanical flushing-out process has ceased, indicate that the water has a direct influence on the protein metabolism.

Voit [1860] believed that the influence of the water is to produce an increased protein catabolism, a view supported by Forster [1878] and others. In conditions of protein deficiency as in fasting dogs with which Voit worked, such a conclusion appears reasonable. In Exp. IV, however, where the protein of the food is excessive in amount, there appears on the addition of

the extra water no increase in the urinary nitrogen, but there occurs a most marked rise both in the absolute amount of urea and in the percentage of total nitrogen excreted as urea, a result obtained in all the experiments. While it is doubtful, therefore, whether in conditions of protein sufficiency excessive consumption of water causes an increase in the amount of protein catabolised, as indicated by the urinary nitrogen, there appears to result in every case a stimulation of the catabolic processes, leading to a more complete disintegration of the protein molecule and the production of those end products whose immediate destination is excretion.

The subsequent marked retention of nitrogen in Exp. IV is in agreement with the results obtained by Neumann [1899] and Fowler and Hawk [1910]. In view of the fact that the retention takes place on the highest protein diet (319 grams per day), when there would be a surplus of circulating protein and non-protein nitrogenous material within the economy, it is improbable that the amount retained, viz. about 8 grams after the one day's water drinking, and in addition to that about 14 grams after the three day's period, would represent an addition to either of these bodies. It is more probable that the retained nitrogen represents an increase in tissue protein. If this supposition be regarded as well founded, we would be able to conclude that the influence of the water is to stimulate both the analytic and the synthetic phases of protein metabolism.

An observation made on the body temperature in Exp. I lends support to this view. During the preliminary period the average temperature was 97·1° F., and during the water days the average was 97·6° F. Heilner [1907] observed an increased production of heat in rabbits on increased water ingestion.

Summary.

The excessive ingestion of water produces :

1. An increased excretion of urinary nitrogen which is most marked on a low protein diet.

2. A retention of nitrogen on the return to normal consumption of water in the case of excessive protein intake.

3. An increase in the percentage of total nitrogen excreted as urea.

4. A marked increase in the excretion of ammonia.

5. No excretion of creatine and no decrease in the excretion of creatinine.

6. A decrease in the faecal nitrogen which is interpreted as indicating a more complete utilisation of the food protein.

It is suggested that the results indicate that the influence of the increased water consumption is to accelerate both the catabolic and the anabolic phases of protein metabolism.

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LVI. THE DETERMINATION OF THE COMPOSITION OF THE DIFFERENT PROTEINS OF OX AND HORSE SERUM BY THE METHOD OF VAN SLYKE.

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Recent investigations have shown that serum albumin and serum globulin exhibit well marked differences in chemical composition. Abderhalden [1903, 1905] working with the proteins of horse serum found that albumin yielded no glycocoll on hydrolysis, while globulin gave 3·5 % of this amino acid. Haussmann [1899] and Gümbel [1904] studied the albumin of horse serum by Haussmann's nitrogen distribution method and Gibson [1912] using the same method determined the nitrogen distribution in the pseudo-globulin from horse serum. The results obtained by these three authors show that albumin contains a greater proportion of basic or diamino nitrogen than globulin. Bywaters and Tasker [1913] determined the sulphur and carbohydrate content of the albumin and globulin of horse serum. They found that albumin contains about 2 % of sulphur and 0·25 % of carbohydrate, while globulin yields less sulphur (1·17 %) and more carbohydrate (3·23 %). They also demonstrated the presence of phosphorus in globulin but could find none in albumin.

The globulin occurring in serum is of two kinds, viz. euglobulin and pseudo-globulin. The physical properties and solubilities of these two substances are very different, and recently evidence has been brought forward to show that they differ also in chemical composition. Hardy [1905] was the first to show that globulin contained phosphorus. He was unable to prepare a phosphorus-free globulin by extraction with alcohol and ether and concluded that the phosphorus of globulin is not due to entangled lecithin. Hardy also

succeeded in preparing an osazone from the insoluble phosphorus-containing globulin but failed to do so from the soluble phosphorus-free globulin. Haslam [1912, 1913] prepared pseudo-globulin free from phosphorus and showed that euglobulin contains about 0·1 % of phosphorus, about half of which he was able to extract by means of alcohol and ether. Hardy and Haslam worked with the proteins of ox serum.

In the experiments described in this paper the different proteins of ox and horse serum have been prepared and their chemical composition has been determined by the method of protein analysis recently introduced by van Slyke [1911]. This method has the advantages of yielding approximately quantitative results and requiring the use of relatively small quantities of material, and indicates the nature of the nitrogenous products resulting from complete acid hydrolysis. The whole proteins of the serum, the albumin, the total globulin, the pseudo-globulin and the euglobulin have been prepared in as pure a condition as possible. Each protein was finally obtained in the dry condition and weighed quantities of the air-dried preparations were analysed. Duplicate analyses were carried out in all cases in which sufficient material was available. Details of the preparation of the various proteins and the figures obtained in the analyses of the same are recorded at the end of the paper.

Results.

The proteins of ox serum were studied first. Unfortunately, the amount of pure protein available was not sufficient in any case for duplicate analyses to be carried out, and in one case (viz. the euglobulin prepared from ox serum by one-third saturation with ammonium sulphate) only 2·5 g. of material were used. After hydrolysis the solution employed for analysis in this instance contained 235 milligrams of nitrogen: of this, only 52 milligrams were present as diamino nitrogen and the determination of the four diamino acids was carried out on this small quantity. On account of the small amount of material used, less confidence is felt in the result of this particular analysis than in the others. It has been found by experience that the analysis of proteins by van Slyke's method is conveniently carried out on solutions containing about 400 milligrams of nitrogen; in the case of the serum proteins such solutions contain about 100 milligrams of diamino nitrogen. In the case of the proteins of horse serum larger quantities of material were prepared and in three cases enough material was available for duplicate analyses to be carried out.

The results of the analysis of the different proteins of ox and horse serum are summarised in the following tables:

TABLE I.

Summary of the analyses of the different proteins of ox serum, in percentages of the total nitrogen.

	Whole Protein		Albumin		Total Globulin		Pseudo-Globulin		Euglobulin ($(NH_4)_2SO_4$ method)		Euglobulin (Panum's method)	
Ammonia N ..	7·05		5·8		7·7		7·5		9·3		8·0	
Melanin N ..	1·60		1·1		2·0		1·9		2·0		2·5	
Cystine N ..	2·75		3·5		2·0		1·9		2·0		1·4	
Arginine N ..	11·05	31·75	10·4	36·9	10·9	28·2	10·8	27·1	11·6	26·6	11·7	28·7
Histidine N ..	4·40		6·7		6·3		4·8		3·8		6·5	
Lysine N ..	13·55		16·3		9·0		9·6		9·2		9·1	
Amino N of the Filtrate	56·65	58·80	54·2	56·5	59·8	62·0	61·7	63·3	57·9	60·7	58·0	59·4
Non-amino N of the Filtrate	2·15		2·3		2·2		1·6		2·8		1·4	
Sum	99·20		100·3		99·9		99·8		98·6		98·6	

TABLE II.

Summary of the analyses of the different proteins of horse serum, in percentages of the total nitrogen.

	Whole Protein		Albumin		Total Globulin		Pseudo-Globulin		Euglobulin ($(NH_4)_2SO_4$ method)		Euglobulin (Panum's method)	
Ammonia N ..	7·25		6·65		7·95		7·7		7·90		8·0	
Melanin N ..	1·60		0·95		2·30		2·2		2·25		2·3	
Cystine N ..	2·10		3·10		1·65		1·7		1·65		1·8	
Arginine N ..	9·50	30·00	9·90	34·75	8·00	25·25	8·9	26·2	8·25	25·30	9·4	26·5
Histidine N ..	5·60		5·85		4·80		5·8		5·45		5·2	
Lysine N ..	12·80		15·90		10·80		9·8		9·95		10·1	
Amino N of the Filtrate	59·95	61·95	56·55	58·15	62·65	64·80	61·3	64·2	62·20	64·35	61·0	62·8
Non-amino N of the Filtrate	2·00		1·60		2·15		2·9		2·15		1·8	
Sum	100·80		100·50		100·30		100·3		99·80		99·6	

Discussion of Results.

From a consideration of the figures given in the two preceding tables it is evident that the composition of serum albumin is very different from that of any of the globulins. Albumin yields on hydrolysis a greater proportion of diamino acids than the globulins, a result which is in agreement with those obtained by Haussmann [1899], Gümbel [1904] and Gibson [1912], who used the nitrogen distribution method devised by Haussmann. Further, albumin

yields a greater proportion of cystine than globulin. In the case of ox serum albumin 3·5 % of the total nitrogen occurs as cystine, while the average for the four globulins is 1·8 %. The corresponding figures for the albumin and globulin from horse serum are 3·1 % and 1·7 %. The results for cystine are in agreement with those of Abderhalden [1903, 1905], who obtained a greater amount of cystine from albumin than from globulin, and of Bywaters and Tasker [1913], who found that the sulphur content of albumin is greater than that of globulin. Estimations of the lysine, arginine, and histidine content of the serum proteins have not hitherto been carried out. From the figures given above for these amino acids it is clear that in the case of both sera examined the albumin differs from the globulins in containing a much larger amount of lysine. It is also to be observed that albumin yields a smaller amount of ammonia and melanin on hydrolysis than the globulins.

Other experimental evidence regarding the lysine content of albumin and globulin has been obtained. Van Slyke and Birchard [1914] determined the free amino nitrogen of a number of proteins and showed that on treatment with nitrous acid in van Slyke's apparatus for half an hour the volume of nitrogen gas evolved corresponded to one half the lysine nitrogen of the protein. Similar experiments have been carried out with the albumin and globulin prepared from ox serum. It was found that albumin yielded about twice as much nitrogen as globulin on treatment with nitrous acid and that in each case the nitrogen obtained was almost exactly one half the lysine nitrogen of the respective proteins. Full details of these experiments will be published shortly.

The marked difference in composition of the proteins of the serum is of interest when considered with reference to the question of the conversion of albumin into globulin, a change which according to Moll [1904, 1906] occurs when serum is heated for an hour at 60° after the addition of a little dilute alkali. Gibson [1912] and Bywaters and Tasker [1913] brought forward evidence which showed that albumin solutions treated in the manner described by Moll yielded a protein which is not identical with naturally occurring globulin and Abderhalden [1903, 1904] showed that globulin yields glycocoll on hydrolysis while albumin does not. The figures given in the above tables show other important differences in the chemical composition of albumin and the globulins of serum, particularly a striking difference in the relative amounts of lysine. When the whole of the evidence dealing with the chemical nature of the serum proteins is considered it is not easy to understand the mechanism of the process by which such simple treatment

of one protein can lead to the formation of another possessing such a widely different chemical composition.

The figures obtained in the analyses of the different globulins indicate that these substances are very similar in chemical composition. Euglobulin was prepared by two methods: (1) by the method of Panum [1851], and (2) from the previously prepared and purified "total globulin[1]" by one-third saturation with ammonium sulphate. A portion of this "total globulin" was set aside for analysis and the remainder was separated into pseudo-globulin and euglobulin by repeated salting out with ammonium sulphate. Analysis of these different globulin preparations by the method of van Slyke failed to reveal any notable differences in chemical composition. The agreement is particularly close in the case of the globulins prepared from horse serum. In the case of the ox serum globulins, the euglobulin prepared by one-third saturation with ammonium sulphate shows a slightly higher ammonia and lower histidine nitrogen value than the other three, but in other respects the agreement is fairly close. As pointed out above, the analytical errors in this particular estimation are probably greater than in the other cases as the analysis was carried out on a much smaller quantity of material. Larger quantities of this protein were prepared from horse serum and the figures obtained on analysis are almost identical with those obtained for the pseudo-globulin and the "total globulin."

The compositions of the different globulins are of interest when considered in connection with the recent work of Chick [1914]. She suggests that the relationship between euglobulin and pseudo-globulin is a very close one, and that under certain conditions pseudo-globulin may undergo a process of "denaturation" whereby a substance is formed the properties of which are very similar to those of euglobulin. She suggests that this artificial euglobulin may be a mechanical complex resulting from the interaction and mutual precipitation of two colloidal systems (viz. pseudo-globulin solution and lipoid emulsion) and that the euglobulin of serum may be a protein-lipoid complex of similar origin. The evidence brought forward in this paper regarding the composition of these two proteins lends support to the idea that euglobulin may be related to pseudo-globulin in some such way as Chick suggests. The conversion of pseudo-globulin into euglobulin is a process which, on chemical grounds, would be more easily understood and more capable of explanation than the conversion of albumin into globulin.

[1] The term "total globulin" refers to the protein precipitated from the diluted serum by half saturation with ammonium sulphate. It consists of pseudo-globulin and euglobulin.

Experimental Details.

Preparation of the different serum proteins.

Euglobulin was prepared by the method of Panum [1851], and also by means of ammonium sulphate. Albumin, total globulin, and pseudo-globulin were prepared by salting out with ammonium sulphate. The albumin and globulin were first separated by half saturation with ammonium sulphate. The serum was diluted with four volumes of distilled water and an equal volume of saturated ammonium sulphate solution added. The precipitated globulin was centrifuged off and the solution containing the albumin was saturated with powdered ammonium sulphate. The albumin was filtered off, dissolved in distilled water, and the albumin solution brought to a volume equal to that of the original *diluted* serum. An equal volume of saturated ammonium sulphate solution was added, the precipitated globulin was filtered off, and the solution containing the albumin was again saturated with powdered ammonium sulphate. The albumin was dissolved and reprecipitated five times in all. The albumin solution was always brought to the same volume as that of the original diluted serum before the addition of the saturated ammonium sulphate solution.

The total globulin of the serum was dissolved and reprecipitated five times in the case of ox serum and six times in the case of horse serum. The globulin after each precipitation was dissolved in distilled water and the solution was diluted until the volume was the same as that of the original diluted serum. A portion of the total globulin was then set aside for analysis and the remainder was separated into euglobulin and pseudo-globulin by Haslam's method [1913]. Each globulin was dissolved and reprecipitated five times in the case of ox serum and six times in the case of horse serum globulin.

The different proteins prepared as described above were finally dissolved in distilled water and dialysed against running tap water until free from ammonium sulphate. The aqueous solutions of the different proteins were evaporated to dryness in a modified form of the apparatus described by Martin [1896], a dropping funnel, the exit tube of which was drawn out to a fine capillary, being substituted for the Berkefeldt filter. The bath containing the two drying bottles was maintained at a temperature of 40° and the larger bottle which acts as a receiver was immersed in ice. The dried proteins obtained in this way were reduced to a fine powder and analysed.

Euglobulin was also prepared by the method of Panum [1851]. The serum was diluted ten times with distilled water and normal acetic acid added in sufficient amount to precipitate the euglobulin. The amount of acetic acid necessary for complete precipitation was previously determined by tests on small quantities of the diluted serum. After standing overnight the clear supernatant fluid was decanted and the precipitated euglobulin centrifuged. It was purified by being dissolved in a minimum and measured quantity of decinormal sodium hydrate and reprecipitated by the addition of an exact equivalent of decinormal acetic acid. This process was repeated four times and the euglobulin was finally washed twice in the centrifuge tubes with distilled water.

A sample of the original serum was dried in Martin's apparatus.

Methods of Analysis.

In carrying out the analyses of the different serum proteins the experimental conditions were kept as constant as possible. From four to five grams of material were boiled with 80 cc. of 20 % hydrochloric acid and the rate of hydrolysis was followed by testing samples of the fluid at intervals in van Slyke's apparatus. The hydrolysis was continued for 20 to 22 hours.

After hydrolysis of the protein the excess of hydrochloric acid was removed *in vacuo* and the hydrolysed material washed into a 250 cc. flask. Duplicate quantities of 20 cc. of the solution were used for the determination of the total nitrogen, and 200 cc. of the solution were used in all cases for analysis. The sum of the ammonia nitrogen, melanin nitrogen, total nitrogen of the filtrate, and the total nitrogen of the bases (the last-named being determined in two parts) should be the same as the amount originally present, as determined separately in the 20 cc. portions. The experience gained in the study of these serum proteins shows that with the exercise of ordinary care throughout the course of the analysis it is not difficult to recover the whole of the nitrogen.

In separating the diamino acids from the monamino acids the same amount of phosphotungstic acid (15 g.) was used, and the precipitation was carried out at a volume of 200 cc. in all cases. After the addition of the phosphotungstic acid the mixture was heated on the water bath until most of the precipitate had redissolved, and after cooling to room temperature the vessel was allowed to stand for 48 hours in the cold room. The precipitate of the bases was washed with an ice-cold solution of phosphotungstic acid in hydrochloric acid, 120 to 140 cc. of solution being used.

TABLE III.

Details of observations.

Material	Quantity taken g.	Total N: cc. N/10 acid per 20 cc.	Total N: g. N per 200 cc.	Ammonia: cc. N/10 acid per 200 cc.	Ammonia: g. N per 200 cc.	Melanin: cc. N/10 acid per 200 cc.	Melanin: g. N per 200 cc.	Cystine: g. $BaSO_4$ per 40 cc.	Cystine: g. N per 200 cc.	Arginine: cc. N/10 acid per 100 cc.	Arginine: g. N per 200 cc.
A. Ox serum											
1. (*a*) Whole protein	4	24·2 24·1	0·3381	16·9	0·0237	3·8	0·0053	0·0240	0·0072	6·2	0·0347
(*b*) Whole protein	4	24·5 24·6	0·3437	17·6	0·0246	4·0	0·0056	0·0209	0·0063	6·1	0·0342
2. Albumin	4	27·9 28·0	0·3913	16·2	0·0277	3·3	0·0046	0·0380	0·0114	6·7	0·0375
3. Total globulin	4	26·5 26·4	0·3703	20·4	0·0286	5·5	0·0077	0·0170	0·0051	6·7	0·0375
4. Pseudo-globulin	4	27·2 27·2	0·3808	20·5	0·0287	5·2	0·0073	0·0160	0·0048	6·8	0·0381
5. Euglobulin $(NH_4)_2SO_4$ method	2·6	16·8 16·8	0·2352	15·6	0·0218	3·5	0·0049	0·0075	0·0022	4·3	0·0241
6. Euglobulin Panum's method	4	28·05 28·50	0·3958	22·7	0·0318	7·0	0·0098	0·0102	0·0031	7·7	0·0431
B. Horse serum											
7. (*a*) Whole protein	5·04	33·0 32·9	0·4613	23·7	0·0332	5·6	0·0078	0·0245	0·0073	7·3	0·0409
(*b*) Whole protein	4·95	33·0 33·2	0·4634	24·3	0·0340	5·0	0·0070	0·0241	0·0072	7·3	0·0409
8. (*a*) Albumin	4	29·5 29·5	0·4130	19·8	0·0277	3·1	0·0043	0·0341	0·0102	6·85	0·0384
(*b*) Albumin	4	29·4 29·7	0·4137	19·5	0·0273	2·9	0·0041	0·0345	0·0103	6·7	0·0375
9. (*a*) Total globulin	4	28·4 28·2	0·3962	22·6	0·0316	6·4	0·0090	0·0170	0·0051	5·2	0·0291
(*b*) Total globulin	4	29·2 29·4	0·4102	23·1	0·0323	6·9	0·0097	0·0115	0·0034	5·2	0·0291
10. Pseudo-globulin	5	35·7 35·6	0·4991	27·5	0·0385	7·8	0·0109	0·0203	0·0061	7·4	0·0414
11. (*a*) Euglobulin $(NH_4)_2SO_4$ method	4·59	32·3 32·3	0·4522	26·1	0·0365	7·3	0·0102	0·0176	0·0053	6·3	0·0353
(*b*) Euglobulin $(NH_4)_2SO_4$ method	4·62	32·8 32·9	0·4599	25·7	0·0360	7·6	0·0106	0·0162	0·0048	6·0	0·0336
12. Euglobulin Panum's method	4·5	29·5 29·5	0·4130	23·6	0·0330	6·9	0·0097	0·0159	0·0048	6·4	0·0358

TABLE III.

Details of observations.

Total N of Bases			Amino N of Bases		Amino N of Filtrate		Total N of Filtrate		Total N recovered g. per 200 cc.
cc. N/10 acid per 100 cc.	cc. N/10 acid per 100 cc. + arginine titration	g. N per 200 cc.	cc. N per 40 cc.	g. N per 200 cc.	cc. N per 200/15 cc.	g. N per 200 cc.	cc. N/10 acid per 200/6 cc.	g. N per 200 cc.	
29·0	35·2	0·0985	22·1; 16°; 768 mm.	0·0648	22·6, 22·7; 17°; 760 mm.	0·1962	24·6, 24·7	0·2071	0·3346
28·9	35·0	0·0980	22·5; 18°; 765 mm.	0·0651	22·7, 22·9; 16°; 768	0·2005	25·45, 25·50	0·2140	0·3422
41·4	48·1	0·1347	31·0; 16°; 774 mm.	0·0916	18·2, 18·4; 15°; 774[1]	0·2172	20·6, 20·7	0·2313	0·3933
27·4	34·1	0·0955	18·7; 17°; 763 mm.	0·0542	26·3; 19°; 762	0·2265	28·6, 28·4	0·2394	0·3712
26·7	33·5	0·0938	19·2; 18°; 764 mm.	0·0555	28·0, 27·8; 19°; 762	0·2400	29·9, 29·9	0·2512	0·3810
14·5	18·8	0·0526	10·6; 15°; 764 mm.	0·0310	16·1, 16·0, 16·2; 15°; 764	0·1415	18·3, 18·1	0·1529	0·2322
29·4	37·1	0·1039	19·5; 16°; 764 mm.	0·0568	20·0, 20·3; 16°; 764[1]	0·2349	22·0, 21·8	0·2453	0·3908
39·2	46·5	0·1302	29·5; 22°; 770 mm.	0·0843	32·4, 32·6; 20°; 773	0·2820	35·2, 35·2	0·2957	0·4669
38·5	45·8	0·1282	29·2; 21°; 765 mm.	0·0832	32·9, 33·0; 21°; 768	0·2829	35·5, 35·4	0·2978	0·4670
41·4	48·25	0·1351	31·6; 17°; 766·5 mm.	0·0184	27·3, 27·3; 17°; 766	0·2385	30·0, 29·9	0·2516	0·4188
40·5	47·2	0·1321	31·3; 17°; 766·5 mm.	0·0182	27·5, 27·6; 18°; 766	0·2394	29·7, 29·7	0·2495	0·4130
28·0	33·2	0·0930	20·8; 20°; 770 mm.	0·0600	28·8, 28·9; 19°; 773	0·2517	31·7, 31·7	0·2663	0·3999
27·5	32·7	0·0916	20·9; 20°; 764 mm.	0·0598	30·5, 30·4; 19°; 770	0·2640	32·9, 33·0	0·2768	0·4104
35·9	43·3	0·1212	25·8; 21°; 764 mm.	0·0734	36·7, 36·7; 22°; 763	0·3114	39·4, 39·4	0·3309	0·5015
30·8	37·1	0·1039	22·8; 18°; 752 mm.	0·0648	33·6, 33·4; 18°; 752	0·2856	35·8, 36·0	0·3015	0·4521
32·4	38·4	0·1075	23·4; 20°; 762 mm.	0·0667	34·2, 34·4; 18°; 752	0·2925	36·4, 36·5	0·3062	0·4603
29·1	35·5	0·0994	21·9; 24°; 757 mm.	0·0608	31·1, 31·1; 25°; 755	0·2572	32·0, 32·2	0·2696	0·4117

[1] Solution brought to 200 cc.

One of the more difficult of the estimations in the process is the determination of the total nitrogen of the bases, which is carried out on the same solution used for the determination of the arginine. In these analyses the practice was adopted of continuing the digestion for some time after the solution becomes clear, gently moving the fluid round the sides of the flask from time to time in order to wash down into the acid any particles which may have crept on to the upper parts of the digestion flask. The figures for the histidine nitrogen show the widest variation in duplicate analyses. This value is not determined directly but is calculated from the results of three other determinations, viz. the arginine nitrogen, the amino nitrogen of the bases, and the total nitrogen of the bases. As van Slyke points out these three values can all be determined accurately, and the duplicate analyses for the histidine should not vary by more than one per cent. of the total nitrogen.

The following estimations were carried out on the solution of hydrolysed protein :

(1) Total nitrogen was estimated in 20 cc. of the original solution.

(2) Ammonia nitrogen and melanin nitrogen were estimated in 200 cc. of the original solution.

(3) After removal of the ammonia and melanin the separation with phosphotungstic acid was carried out.

(4) The filtrate from the phosphotungstic acid precipitate of the bases was made to 150 cc. and the following estimations made :

(*a*) Total nitrogen of the filtrate in 25 cc.

(*b*) Amino nitrogen of the filtrate in 10 cc.

(5) The solution of the bases was made to 50 cc. and the following estimations made :

(*a*) Cystine nitrogen in 10 cc.

(*b*) Amino nitrogen in 10 cc.

(*c*) Arginine nitrogen and total nitrogen of the bases in the same sample of 25 cc.

The detailed figures obtained in the analysis of the different serum proteins are given in the tables. Table III (pp. 548, 549) contains a record of the actual observations, the volumes given in the headings referring in every case to the amount of the original solution corresponding to the volume actually employed as explained above. In Table IV the results are expressed as percentages of the total nitrogen.

TABLE IV.

Results of analyses expressed in percentages of total nitrogen.

Material	Ammonia N	Melanin N	Cystine N	Arginine N	Histidine N	Lysine N	Amino N of Filtrate	Non-Amino N of Filtrate	Total N recovered
A. Ox serum									
1. (*a*) Whole protein	7·0	1·6	2·1	10·2	3·4	13·3	58·0	3·2	98·8
Corrected for solubility of bases			2·9	11·2	4·5	13·4	56·5	1·8	
(*b*) Whole protein	7·1	1·6	1·8	9·9	3·2	13·5	58·3	3·9	99·3
Corrected			2·6	10·9	4·3	13·7	56·8	2·5	
2. Albumin	5·8	1·1	2·9	9·6	5·7	16·2	55·5	3·6	100·4
Corrected			3·5	10·4	6·7	16·3	54·2	2·3	
3. Total globulin	7·7	2·0	1·4	10·1	5·3	8·9	61·1	3·4	99·9
Corrected			2·0	10·9	6·3	9·0	59·8	2·2	
4. Pseudo-globulin	7·5	1·9	1·2	10·0	3·9	9·5	63·0	3·0	100·0
Corrected			1·9	10·8	4·8	9·6	61·7	1·6	
5. Euglobulin $(NH_4)_2SO_4$ method	9·3	2·0	0·9	10·2	2·2	9·0	60·1	4·9	98·6
Corrected			2·0	11·6	3·8	9·2	57·9	2·8	
6. Euglobulin Panum's method	8·0	2·5	0·8	10·9	5·6	9·0	59·3	2·6	98·7
Corrected			1·4	11·7	6·5	9·1	58·0	1·4	
B. Horse serum									
7. (*a*) Whole protein	7·2	1·7	1·6	8·8	4·9	12·8	61·1	3·0	101·1
Corrected			2·1	9·5	5·8	12·9	60·0	1·9	
(*b*) Whole protein	7·3	1·5	1·5	8·8	4·6	12·6	61·0	3·2	100·5
Corrected			2·1	9·5	5·4	12·7	59·9	2·1	
8. (*a*) Albumin	6·7	1·0	2·4	9·3	5·1	15·7	57·7	3·1	101·0
Corrected			3·1	10·0	6·1	16·0	56·5	2·0	
(*b*) Albumin	6·6	0·9	2·5	9·0	4·6	15·7	57·8	2·4	99·5
Corrected			3·1	9·8	5·6	15·8	56·6	1·2	
9. (*a*) Total globulin	8·0	2·3	1·3	7·3	4·1	10·6	63·5	3·7	100·8
Corrected			1·9	8·1	5·1	10·7	62·2	2·4	
(*b*) Total globulin	7·9	2·3	0·8	7·1	3·6	10·7	64·3	3·1	99·8
Corrected			1·4	7·9	4·5	10·9	63·1	1·9	
10. Pseudo-globulin	7·7	2·2	1·2	8·3	5·0	9·7	62·4	3·9	100·4
Corrected			1·7	8·9	5·8	9·8	61·3	2·9	
11. (*a*) Euglobulin $(NH_4)_2SO_4$ method	8·0	2·2	1·1	7·8	4·2	9·8	63·1	3·5	99·7
Corrected			1·7	8·5	5·0	9·9	62·0	2·4	
(*b*) Euglobulin $(NH_4)_2SO_4$ method	7·8	2·3	1·1	7·3	5·1	9·9	63·6	3·0	100·1
Corrected			1·6	8·0	5·9	10·0	62·4	1·9	
12. Euglobulin Panum's method	8·0	2·3	1·1	8·7	4·3	10·0	62·3	3·0	99·7
Corrected			1·8	9·4	5·2	10·1	61·0	1·8	

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LVII. THE GELATINISATION OF PECTIN IN SOLUTIONS OF THE ALKALIES AND THE ALKALINE EARTHS.

By DOROTHY HAYNES.

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Pectin substances have been known since the year 1825, when Bracannot[1] extracted from the roots and stems of a number of plants a substance to which he gave the name of pectic acid; about the same time Payen extracted a similar substance from the root of *Ailanthus glandulosa*. This discovery was followed up by other investigators, and Frémy's researches [1848] placed beyond doubt the existence in plant tissues of a class of substances distinct from the celluloses, though strongly resembling them. Frémy shewed that the parent substance, to which he gave the name of pectose, exists together with cellulose in the cell walls of green fruits, as well as in other parts of plants, and passes into solution during the process of ripening; it may also be brought into solution by boiling with acids. To this soluble variety he gave the name of pectin. Pectin undergoes further changes in boiling, and Frémy recognised two distinct varieties, parapectin, which is precipitated by neutral lead acetate, and metapectin, which is produced by boiling pectin or parapectin with acids, and is precipitated by barium chloride. Frémy also discovered the coagulating action of the enzyme pectase. These results have been confirmed and extended by later observers, especially by Mangin [1892, 1893], and a series of pectin substances has been recognised, differing in solubility, coagulable under different conditions, and marked by increasing acidity.

The action of alkalies on pectin was regarded by Frémy and his successors as resulting in the formation of an acid substance to which the name of pectic acid has been given; this being insoluble is precipitated by acids from alkaline solutions of pectin. The insoluble compounds of the alkaline earths

[1] For a résumé of early researches on pectin, see *Science Progress*, **6**, 344.

were looked upon as salts of pectic acid, and the action of pectase has been ascribed to the formation of calcium pectate. These conclusions of Frémy have been accepted by later observers ; nevertheless the existence of pectic acid, and in particular its action in determining gelatinisation, must be regarded as in the highest degree uncertain. The work of recent years on adsorption compounds has taught us that change of physical properties and state of aggregation frequently occurs without any definite change of chemical composition. Gelatinisation and loss of solubility cannot be regarded as evidence in themselves of chemical change. All that is definitely known of pectin in this connection may be summarised as follows :

(1) Pectin boiled with water and acids becomes increasingly acid in reaction.

(2) It is coagulated reversibly by the alkalies, irreversibly by the alkaline earths ; the action of acids on this coagulum leaves in both cases an insoluble residue.

(3) Certain salts, e.g. calcium chloride, in very concentrated solution, bring about a coagulation which is reversible.

(4) Coagulation by pectase is dependent upon the presence of calcium salts [Bertrand and Mallevre, 1894].

Although this last fact can be simply explained by the supposition that pectic acid is produced and is precipitated in the form of its calcium salt, this is quite uncertain, and the identification of this salt with the product of the reaction of pectin with calcium hydroxide is entirely unsupported by evidence.

The chemical composition of pectin has not at present been determined with any certainty. Analyses vary considerably but indicate a composition very near to, if not exactly, that of a carbohydrate ; from the fact that pectin substances give furfural on distillation with hydrochloric acid and mucic acid when treated with nitric acid, the presence of galactan and pentosan groups in the complex molecule has been deduced. The analyses indicate that there may be a slight excess of oxygen in the molecule above the carbohydrate percentage, and Tollens [1914] has suggested that this may be explained by the existence of a few carboxyl groups in the molecule ; if these are condensed with hydroxyl in the neutral pectin their progressive hydrolysis would account for the increasing acidity of pectin solutions when these are hydrolysed. In conformity with this hypothesis Tollens suggests a formula $6\,(C_{10}H_{18}O_9) + O$ or $5\,(C_{10}H_{18}O_9) + C_{10}H_{18}O_{10}$.

The object of the experiments described below was the study of the mechanism of gelatinisation and its dependence upon chemical change in the case of pectin solutions gelatinised by the action of the alkalies and alkaline earths.

Experimental.

The pectin used in the following experiments was prepared principally from limes; a little lemon pectin was also used. The fruit was cut up and boiled with water for several hours until quite soft; the liquid was then filtered through muslin and the residue further extracted with boiling water, until the greater part of the pectin had been removed. The liquid was evaporated to a small volume, alcohol was added to precipitate the pectin, and the jelly thus produced was drained through muslin, redissolved in water, and reprecipitated by alcohol many times. No attempt was made to remove calcium salts. The solutions were finally boiled until all alcohol was driven off. With the specimens prepared later solutions were made from the dry powder obtained by washing the pectin jelly with alcohol until most of the water was removed, drying at 100° and powdering. The solutions were distinctly acid and highly viscous, they could be kept indefinitely in the presence of chloroform; a slight sediment was formed on standing apparently due to the presence of mechanically contained impurities. Pectin prepared in this way is readily precipitated by neutral lead acetate, and therefore consists largely, if not entirely, of the variety distinguished by Frémy and Mangin as parapectin; barium chloride gives no precipitate but concentrated solutions form a jelly on standing.

The following are particulars of the different pectin solutions used:

(*a*) Prepared from limes; concentration (determined by evaporating 5 cc. at 100°) 0·48 g. in 100 cc.

(*b*) Prepared from limes; concentration 0·25 g. (dry powder) in 100 cc.; ash 2·72 per cent.

Acidity 10 cc. = 0·5 cc. N/25.

(*c*) Prepared from lemons; concentration approx. 0·25 g. (dry powder in 100 cc.); determined by evaporating 5 cc. at 100° = 0·31 g. in 100 cc.

Acidity 10 cc. = 0·3 cc. N/25.

The weight of residue left by evaporating solution (*b*) to dryness was not determined; the concentrations of (*b*) and (*c*) were nearly the same, (*b*) being slightly the more concentrated.

Measurements of the Rate of Precipitation of Pectin by the Alkalies and Alkaline Earths.

If solutions of the alkaline earths are added to a solution of pectin containing 1 per cent. or even less, a stiff jelly is produced; concentrated solutions of potassium hydroxide produce a similar effect. If the pectin solution is very dilute, a gelatinous precipitate is formed, resembling in appearance a small precipitate of aluminium hydroxide; if the alkali is also sufficiently dilute, the rate of precipitation becomes measurable. In very dilute solutions, if the mixture is allowed to stand without shaking, a very attenuated jelly is formed; this is very unstable; a slight movement causes rifts to form in the jelly, and precipitation takes place almost instantaneously. From this it appears that the first action of barium hydroxide or other alkali is the formation of a continuous jelly, which being mechanically unstable is readily broken up in dilute solution; with a larger excess of hydroxide the mechanical instability is so great that precipitation takes place immediately. That a jelly is formed in the first place is confirmed by the more or less sudden change which takes place in the appearance of the mixture in all cases previous to precipitation. With potassium hydroxide the action is very similar, but very much higher concentrations are necessary for gelatinisation and precipitation, and the precipitate forms less sharply; the rate of precipitation is consequently less easily measured.

The following method was employed for the measurement of the rates of precipitation: the solution of alkali or of alkaline earth was measured into a small flask and diluted to a volume such that with the addition of pectin solution it measured 50 cc. During the experiment the temperature was kept constant (usually at 15°) by immersing the flask in a glass trough filled with water. To ensure immediate precipitation the flask was constantly shaken. The time was measured from the moment at which the addition of pectin solution was made to the moment of precipitation; it was found possible to measure to within a quarter of a minute, and the results obtained usually agreed to within half a minute or less. The pectin solution used for all rate measurements was the solution (*a*) unless otherwise mentioned.

Precipitation by the hydroxides of the alkaline earth metals.

It was found that in all cases the rate of precipitation was nearly proportional to the square of the concentration; in the following tables the product time $\times$ (concentration)^2 is given for comparison.

In the diagram (Fig. 1) the reciprocals of the times are plotted against the squares of the concentrations.

The effect of change of temperature has not been measured, but the rate increases very rapidly with a small increase of temperature.

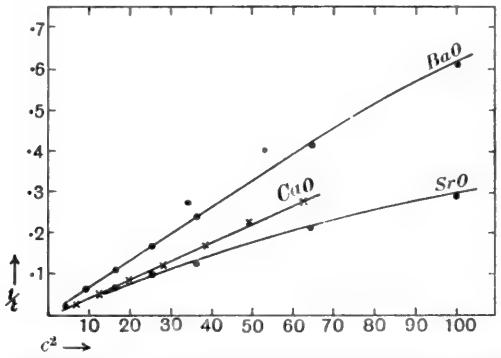

Fig. 1. Rate of precipitation of pectin by the alkaline earths (Tables II B, III and IV).

TABLE I.

Rate of precipitation of pectin by barium hydroxide.

Conc. of BaO_2H_2 mg. equiv. per litre	A. 2 cc pectin in 50 cc.		B. 1 cc. pectin in 50 cc.		C. 0·5 cc. pectin in 50 cc.	
	t (mins.)	c^2t	t (mins.)	c^2t	t (mins.)	c^2t
c	1 measurement		1 measurement		2 measurements	
2	35	140	30	120	34	136
3	—	—	—	—	16[1]	144
4	8·5	136	9	144	9·25	148
6	4	144	4	144	5	180
8	2·5	160	3	192	3	192
10	1·5	150	2	200	2	200

[1] 1 measurement.

Another series gave the following results :

TABLE II.

Rate of precipitation of pectin by barium hydroxide.

Conc. of BaO_2H_2 mg. equiv. per litre	A. 3 cc. pectin in 50 cc.		B. 2 cc. pectin in 50 cc.	
	t (mins.)	c^2t	t (mins.)	c^2t
c	1 measurement		2 measurements	
2	47·5	190	41·9	167
3	17[1]	153	16·25	146
4	9·25	148	9·1	146
5	—	—	6·0	150
6	4	144	4·1	148
8	2·5	160	2·4	152
10	1·75	175	1·6	162

[1] 2 measurements.

The series in Table II is the more accurate; in the most dilute pectin solutions the precipitate does not form very sharply.

TABLE III.

Rate of precipitation of pectin by strontium hydroxide.

Conc. of SrO_2H_2 mg. equiv. per litre c	2 cc. pectin in 50 cc. t (mins.)	c^2t
	2 measurements	
4	17·25	276
5	10·0	250
6	7·6	274
8	4·6	296
10	3·4	337

TABLE IV.

Rate of precipitation of pectin by calcium hydroxide.

Conc. of CaO_2H_2 mg. equiv. per litre c	A. 2 cc. pectin in 50 cc. t (mins.)	c^2t	B. 1 cc. pectin in 50 cc. t (mins.)	c^2t
	2 measurements		2 measurements	
2·6	37·4	253	30·9	209
3·5	19·25	236	16·9	206
4·4	11·5	223	11·0	213
5·3	8·1	228	7·9	222
6·2	6·0	231	6·5	250
7·0	4·5	220	4·75	233
7·9	3·6	226	3·9	242

A direct comparison was made of the rates of precipitation by barium, strontium, and calcium hydroxides in equivalent quantities. The following results were obtained:

TABLE V.

mg. equiv. per litre c	3 cc. of pectin solution (c) in 50 cc. t (mins.) Ba		Sr		Ca	
4	14		18		17	
3·8	6·5		9	9·75	7·5	8
9·9	2·5	2·5	3·75	4	3·25	3·25

In comparing the foregoing results, it must be remembered that the time measured is that necessary for precipitation, and not that which is required for the formation of the jelly. As soon as the jelly forms absorption of hydroxide takes place, and this causes the framework of the jelly to stiffen and contract, so that spontaneous precipitation takes place at all but very

small concentrations; at these concentrations precipitation can be brought about by shaking, but it is probably necessary in this case also for a certain amount of absorption to take place in the jelly before it can be precipitated. This amount will obviously vary with the character of the jelly, and is probably less for barium, which gelatinises very readily, than for strontium or calcium. The rate of absorption will depend upon the concentration of the hydroxide and the absorption capacity of the jelly; the latter factor is markedly greater for calcium than for the other hydroxides.

The abnormality of the strontium curve in position and character is probably due to the influence of the absorption factor in precipitation, which

TABLE VI.

Rate of precipitation of pectin solutions of varying concentrations by barium hydroxide (conc. 4 mg. equiv. per litre).

Conc. of pectin cc. solution (*a*) in 50 cc.	*t* (mins.) I.	II.
1	16·5	15·5
2	13	—
3	10·5	10·75
4	10	—
5	9·5	9·5
10	9·5	8·5
15	9	—
20	9	—
25	9	—
30	—	9·5
40	10	—
50	—	10·5

becomes unimportant for barium on account of the insolubility of the jelly, and for calcium on account of the rapidity with which absorption takes place. The effect will be that a quantity will be added to the time required for gelatinisation, which will decrease as the concentration increases, but will increase relatively to the total time of precipitation, thus causing the precipitation constant to increase. A slight increase of the precipitation constant is to be observed in the barium curves also. This increase in the precipitation constant cannot be ascribed to changes in the degree of dissociation, even where, as for barium hydroxide, the change would be of a corresponding order of magnitude in the two cases, for the conductivity curve shews that at the concentrations employed barium hydroxide dissociates according to the equation

$$BaOH^{\cdot} = Ba^{\cdot\cdot} + OH',$$

no non-ionised hydroxide being present, and it will be evident from the results and conclusions to be given below that the formation of $BaOH^{\cdot}$ ions is unlikely to affect the rate of precipitation. These remarks anticipate results and conclusions which will be given in detail at a later stage.

The effect of varying the quantity of pectin is shewn in Tables VI and VII.

TABLE VII.

Rate of precipitation of pectin solutions of varying concentrations by strontium hydroxide (conc. 5 mg. equiv. per litre).

Conc. of pectin cc. solution (a) in 50 cc.	t (mins.)
1	10·25
3	10·25
4	10·50
5	11·75

These tables shew that for very dilute solutions the rate of precipitation increases with increase in the quantity of pectin present; for larger concentrations an opposite effect is produced and the rate of precipitation gradually decreases.

The effect of salts on precipitation.

Potassium chloride added to a solution of barium hydroxide retards the precipitation of pectin; this is to be expected as double decomposition will take place to some extent.

TABLE VIII.

Rate of precipitation of pectin by barium hydroxide in the presence of potassium chloride.

2 cc. of pectin solution in 50 cc.
Conc. of BaO_2H_2 4 mg. equiv. per litre.

Conc. of KCl mg. mols. per litre	t (mins.)
0	9·1
10	10·75
20	11·5

The effect of salts having a common ion is to increase the rate of precipitation; it has been possible to measure the accelerating effects for both ions, since potassium hydroxide in dilute solution has itself no precipitating action on pectin.

TABLE IX.

Rate of precipitation of pectin by barium hydroxide in the presence of salts having a common ion.

A. 2 cc. of pectin solution in 50 cc.
Conc. of BaO_2H_2 4 mg. equiv. per litre.

	t (mins.)		
	$BaCl_2$		KOH
Conc. mg. equiv. per litre	I. (1 measurement)	H. (2 measurements)	(1 measurement)
2	7	7·25	6·25
4	6	5·75	4·75
6	5	4·75	3·5
8	4	4·25	3·25
10	3·5	3·75	3·00

B. 2 cc. of pectin solution in 50 cc.
Conc. of BaO_2H_2 2 mg. equiv. per litre.

	t (mins.)	
Conc. mg. equiv. per litre	$BaCl_2$ (2 measurements)	KOH (1 measurement)
2	21·4	17·5
4	16·4	11·75
6	13·4	10
8	11·75	8
10	9·5	—
12	9·25	—
20	—	3·25

C. 0·5 cc. of pectin solution in 50 cc.
Conc. of BaO_2H_2 4 mg. mols. per litre.

	t (mins.)	
Conc. mg. equiv. per litre	$BaCl_2$ (1 measurement)	KOH (1 measurement)
2	8·5	7
4	7	5·5
6	6·5	5
8	5·5	4·5
10	5·5	3·5
20	4·5	—

The corresponding measurements for calcium chloride were found to be partially invalidated by impurities in the calcium chloride and are therefore omitted ; the results were however similar. Tables of the effect of strontium chloride and nitrate on strontium hydroxide and of potassium hydroxide on calcium hydroxide are given.

TABLE X.

Rate of precipitation of pectin by strontium hydroxide in the presence of strontium salts.

2 cc. of pectin solution in 50 cc.
Conc. of SrO_2H_2 4 mg. equiv. per litre.

Conc. (mg. equiv. per litre) $Sr(NO_3)_2$ and $SrCl_2$	t (mins.)	
	$Sr(NO_3)_2$ (3 measurements)	$SrCl_2$ (1 measurement)
2	10·75	12
4	8·6	10
6	7·6	9·25
8	6·9[1]	—
10	6·25	7·75
14	5·25	6·25
20	4·65	5·25

[1] 2 measurements.

TABLE XI.

Rate of precipitation of pectin by calcium hydroxide in the presence of potassium hydroxide.

2 cc. of pectin solution in 50 cc.
Conc. of CaO_2H_2 3·5 mg. equiv. per litre.

Conc. of KOH (mg. equiv. per litre)	t (mins.) (2 measurements)
2	13·1
4	9·9
6	8·75
8	7·1
10	6.4
12	6·0
20	4·5

It will be observed from these tables that the rate of precipitation is accelerated both by potassium hydroxide and by the salts of the alkaline earth metals; the effect of the latter is less than that of potassium hydroxide. It is of course possible that the anion exerts an inhibitory effect; but this is an unlikely supposition since, as is shewn later, the effect of potassium salts on the rate of precipitation by potassium hydroxide is nearly identical, and since chloride ions are not appreciably absorbed by the pectin jelly. The absorption of other anions has not been determined, but there is no reason to suppose the chloride ion in any way exceptional. A large part of the difference in the rates for barium chloride and potassium hydroxide is certainly due to the incomplete dissociation of the former; the uncertainty

as to the dissociation of divalent salts makes it difficult to say whether the whole difference can thus be accounted for, but the similarity of the two curves (see Fig. 2) makes this very probable; it is possible however that a small part of the discrepancy may be due to absorption effects. The conductivity curves for strontium chloride and nitrate are very uncertain at great dilutions; they however indicate that the dissociation of strontium chloride falls off with considerably greater rapidity than that of the nitrate as concentration increases, and this difference probably suffices to account for the small differences in the rates measured.

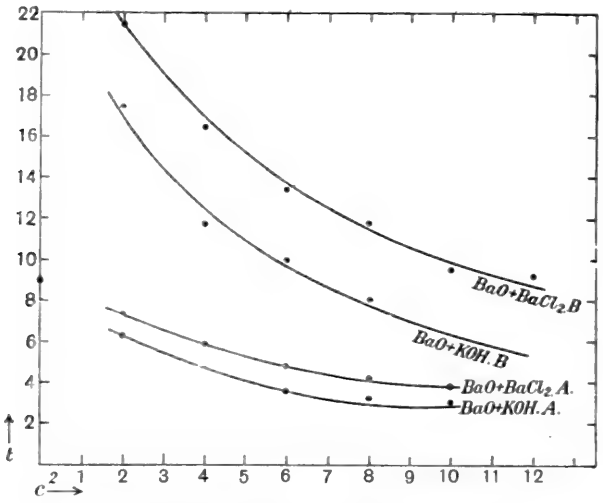

Fig. 2. Rate of precipitation of pectin by barium hydroxide in the presence of barium chloride and potassium hydroxide (Table IX A, B).

Since the rate of gelatinisation of pectin is proportional to the square of the concentration of the hydroxide added, and is accelerated by the addition of either ion, it is concluded that the rate of gelatinisation is proportional to the product of the concentrations of alkaline earth metal and hydroxyl; the following table gives the value of this product for equal times in the case of:

(1) barium hydroxide (C_1),

(2) barium hydroxide and barium chloride (C_2),

(3) barium hydroxide and potassium hydroxide (C_3),

assuming complete dissociation; the values of the concentrations for equal times are obtained by interpolation from the results in Table II B and Table IX A.

TABLE XII.

Value of the product $Ba \times OH$ for equal values of t.

t	BaO_2H_2 C_1[2]	$BaCl_2$ +4 mg. equiv. BaO_2H_2 per litre 4 (C_2+4)	KOH +4 mg. equiv. BaO_2H_2 per litre 4 (C_3+4)
3	52	—	56
4	36	46	35
5	29	37	30
6	23	30	20
7	18	21	—

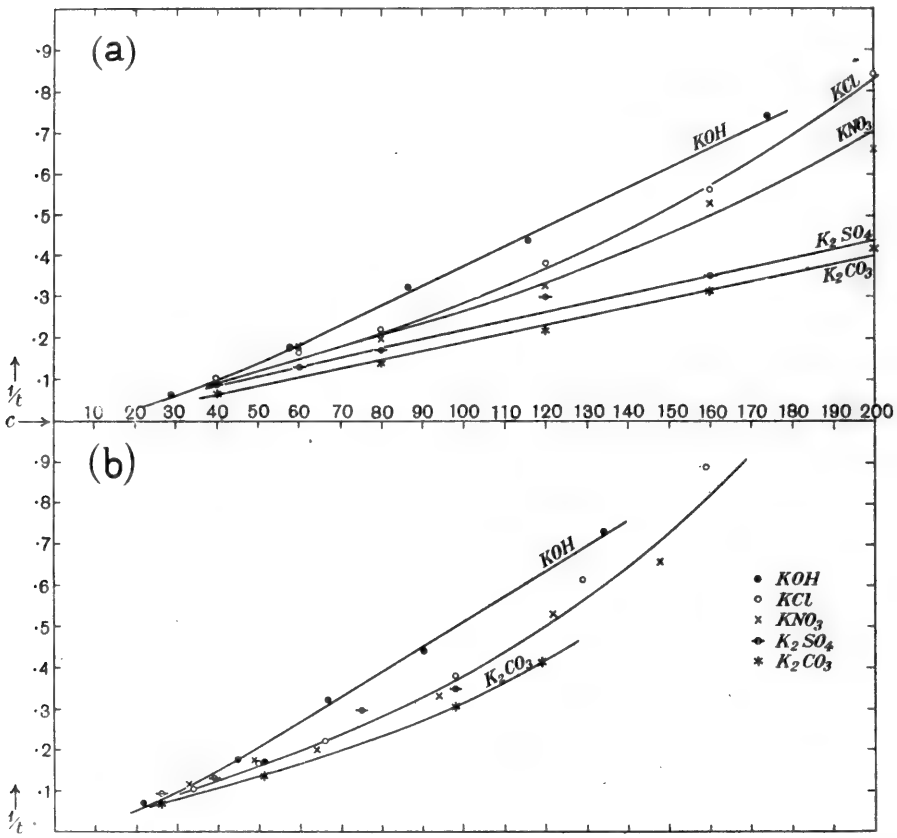

Fig. 3. Rate of precipitation of pectin by potassium hydroxide and by mixtures of potassium hydroxide and potassium salts.

(*a*) c = conc. of potassium salts in the presence of 290 mg. equiv. per litre of potassium hydroxide.

(*b*) c = conc. of potassium ions above 244 mg. equiv. per litre.

As will be seen the values for additions of potassium hydroxide correspond very closely with those for barium hydroxide alone; for barium chloride the percentage difference decreases as the concentration decreases.

Precipitation by Potassium Hydroxide.

TABLE XIII.

Rate of precipitation of pectin by potassium hydroxide.

2 cc. of pectin solution in 50 cc.		
Conc. (c) mg. equiv. per litre	t (mins.) 2 measurements	$(C - 310)\,t$
319	15·15	186
348	5·75	218
377	3·1	209
406	2·25	216
464	1·4	212

The values of $1/t$ plotted against the concentration (Fig. 3 (a)) give a straight line cutting the x axis at the concentration 310; the values of the constant $(C - 310)\,t$ are given in the table.

The following table shews the effect on the rate of precipitation of additions of various potassium salts to potassium hydroxide.

TABLE XIV.

Rate of precipitation of pectin by potassium hydroxide in the presence of potassium salts.

2 cc. of pectin solution in 50 cc.
Conc. of KOH 290 mg. equiv. per litre.

Conc. mg. equiv. per litre	KCl t (mins.) 2 measurements	KNO_3 t (mins.) 2 measurements	K_2CO_3 t (mins.) 2 measurements	K_2SO_4 t (mins.) 2 measurements
40	9·5	9·0[1]	14	10·75
60	6·0	5·75	—	7·50
80	4·5	5·0	7·25	6·0
120	2·6	3·0	4·50	3·4
160	1·6	1·9	3·25	2·9
200	1·1	1·75	2·4	2·4

[1] 1 measurement.

Fig. 3 (a) shews these results graphically, the reciprocals of the values of t being plotted against the concentrations; the curves thus obtained only diverge slightly from a straight line, except in the case of potassium chloride.

If however the values of $\frac{1}{t}$ are plotted against the concentrations of the potassium ion, a different result is obtained; the curves for KCl, KNO_3 and K_2SO_4 fall together at the lower concentrations, though they diverge slightly as the concentration increases, and the curves for KCl and KNO_3 for which

the measurements extend further than those for the salts of the dibasic acids shew marked curvatures towards the y axis. For the sake of clearness the mean curve for the three salts is given in the figure (Fig. 3 (*b*)); the curve for potassium carbonate is shewn separately. The discussion of these results is deferred until later.

The calculation of the ionic concentrations was made on the assumption that for salts of monobasic acids $C_3 = C_2 (C_1 + C_2)$ and of dibasic acids $C_3 = C_2 (C_1 + C_2)^2$, where

C_1 = concentration of KOH,

C_2 = concentration of salt,

C_3 = that concentration at which the salt in pure solution is equally dissociated.

If the square root or cube root of the product of the ions be taken to represent the effective ionic concentration, we have

$$C_3^2 \alpha_3^2 = C_2 (C_1 + C_2) \alpha_1 \alpha_2$$

and

$$C_3^3 \alpha_3^3 = C_2 (C_1 + C_2)^2 \alpha_1^2 \alpha_2,$$

where α_1 represents the mean dissociation of the mixture of salt and potassium hydroxide; for salts of monobasic acids $\alpha_1 \alpha_2 = \alpha_3^2$ approximately; the formula will therefore enable the degree of dissociation of the salt in the mixture to be calculated with fair accuracy; for salts of dibasic acids $\alpha_1^2 \alpha_2 > \alpha_3^3$, the value found for C_3 will therefore be too small and the degree of dissociation too high; for small concentrations however the percentage error is not very great.

The following table gives the data for the calculation of the degree of dissociation of the various salts between the concentrations 40 and 200 mg. equiv. per litre in the presence of potassium hydroxide at a concentration of 290 mg. equiv. per litre.

TABLE XV.

Salt	Concentration (c)	C_3	λ	λ_∞	α
KCl	40	115	1040	1240	84
	200	313	985	—	79·5
KNO_3	40	115	970	1180	82
	200	313	875	—	74
K_2CO_3	40	163	850	1300	65·5
	200	363	775	—	59·5
K_2SO_4	40	163	855	1310	65·5
	200	363	780	—	59·5
KOH	290	290	1905	2270	84
	319	319	1895	—	83·5
	464	464	1853	—	81·5

The concentrations of dissociated salt (C) corresponding to the concentrations in Tables XIII and XIV and the product of these concentrations with α are therefore as follows:

TABLE XVI.

A. KOH.

C	αc
290	244
319	266
348	289
377	311
406	334
464	378

B. *Salts.*

	KCl	KNO_3	K_2CO_3	K_2SO_4
C	αC	αC	αC	αC
40	34	33	26	26
60	50	49	39	39
80	66	64	51	51
120	98	94	75	75
160	129	122	98	98
200	159	148	119	119

The decrease in the dissociation of KOH with increasing salt concentrations has been disregarded.

The following table shews the action of potassium chloride on varying quantities of potassium hydroxide:

TABLE XVII.

5 cc. of pectin solution (c) (from lemons) at 20°.

Conc. mg. equiv. KOH per litre	Conc. mg. equiv. KCl per litre	t (mins.) 1 measurement
686	0	1·5
343	200	2·5
171	300	5·25
103	340	14

Absorption of the Alkaline Earths by Gelatinised Pectin.

The process of gelatinisation is accompanied by absorption; in dilute solution the precipitate can be easily filtered off, and it was possible therefore to determine by titration the quantity of hydroxide that had been absorbed. Since the change of volume on gelatinisation is inappreciable, the following method was adopted for the estimation of the amount of hydroxide absorbed.

A mixture of alkaline earth, pectin and water was made in a flask of known volume and was allowed to stand for a definite time, usually half an hour.

It was then filtered, the first part of the filtrate was rejected and a part of the remainder, usually one-half of the original volume, was titrated with standard acid.

To avoid absorption of carbon dioxide the filtering funnel was fixed in a bell jar, through which passed a funnel provided with a stopcock; a little potash was placed inside the bell jar, and all communications to the outer air were closed with soda lime tubes. After standing, the contents of the flask were emptied rapidly into the stoppered funnel; the first part of the filtrate was then allowed to run through, after which the whole apparatus was removed, so that the filter drained into a dry flask. To ensure constant temperature the whole apparatus, together with the water and solutions, used for the mixture, was kept in a zinc cupboard, maintained at a constant temperature by means of a thermoregulator. A variation of 5° did not affect the results appreciably.

Absorption takes place rapidly, and, except in the case of the most dilute solutions, apparent equilibrium is established in less than half an hour; the following measurements shew the variation in the rate of absorption in very dilute solution.

TABLE XVIII.

Rate of absorption of barium hydroxide in dilute solution by gelatinised pectin.

Volume 100 cc. 10 cc. of pectin solution (*C*).

Final conc. of BaO_2H_2 100 cc. = cc. N/25	Absorption		
	0·5 hr.	1 hr.	2 hrs.
5	1·7	2·0	2·3
	1·8	2·1	2·2
	1·7	—	—
11	2·1	2·2	2·3
	2·1	—	—
38	2·8	—	2·6
	2·6	—	—

It will be noticed that, except at extreme dilution, equilibrium was established within the half hour; the variations for the concentrations 11, though these probably indicate a slight increase, are within the errors of experiment. This however was not true equilibrium, as a slow absorption continued to take place even after several days; this slow absorption may be due to chemical action, but is more probably caused by slow diffusion of hydroxide into the interior of the jelly. A similar effect was observed by Morawitz [1910] in his experiments on the absorption of salts by charcoal,

and was ascribed by him to diffusion. In the case of calcium, which is much more strongly absorbed than either barium or strontium, the increased absorption is appreciable after 4·5–5 hrs. The following measurements for calcium and strontium are given for comparison.

TABLE XIX.

Absorption after 4·5–5 hrs.

Vol. 100 cc. 10 cc. of pectin solution (*C*).

A. *Strontium Hydroxide.*

Final conc. 100 cc. = cc. N/25	Absorbed after 0·5 hr.	Absorbed after nearly 5 hrs.
11	2·1	2·2
36	2·9	2·9
90	3·6	3·8

B. *Calcium Hydroxide.*

	after 0·5 hr.	after 4·5 hrs.
8	2·5	2·7
	2·5	—
	2·5	—
27–29	3·1	3·5
	3·2	after 24 hrs.
		4·8

In these experiments the absorption was determined by titrating with N/25 HCl and phenolphthalein in the cold ; the alkaline earth solution was added from a pipette, which was washed out into the flask ; water and pectin were then added, and the flask was filled to the mark and thoroughly shaken. In all the absorption experiments the water used was boiled to free it from carbon dioxide. The order in which the mixture was made—whether it was diluted before or after the addition of pectin—was found to be without effect. The concentration of hydroxide was determined for each measurement by a blank titration in which pectin was absent. The mixture was allowed to stand half an hour, and the amount of absorption was then measured by the difference in the value given by the titration of 50 cc. in the two cases, the mixture having been filtered as described above. The absorption at this point was assumed to have attained a definite state, probably by saturation of the outer layer of the jelly-forming substance ; that this assumption is justified is confirmed by the regularity of the absorption curves obtained. Differences between two titrations giving the amount absorbed usually agreed within less than 0·1 cc. ; the greatest variations amounted to 0·15. These are doubled in the table which expresses the absorption for the total volume.

TABLE XX.

Absorption of the alkaline earths by gelatinised pectin.

Vol. 100 cc. 10 cc. pectin solution (*C*). *T* 20°–25°.

BaO_2H_2 2 measurements		SrO_2H_2 1 measurement		CaO_2H_2 2 measurements	
Final conc. 100 cc. = cc. N/25	Absorbed cc. N/25	Final conc. 100 cc. = cc. N/25	Absorbed cc. N/25	Final conc. 100 cc. = cc. N/25	Absorbed cc. N/25
11	2·1	11	2·1	8	2·5
38	2·7	36	2·9	30	3·15
66	3·0	—	—	51	4·05
94	3·2	90	3·6	73	4·55

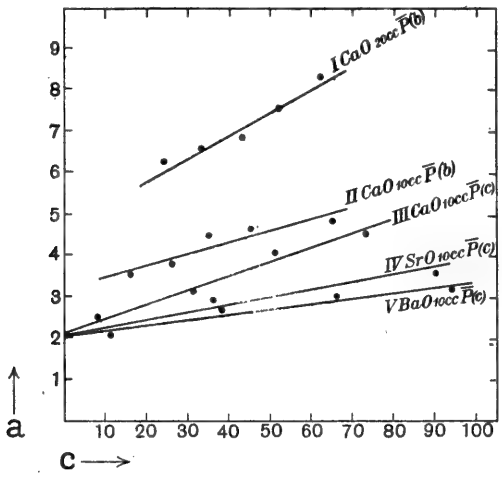

Fig. 4. Absorption of alkaline earths by pectin (Tables XX, XXI).

It will be seen (Fig. 4) that these points lie on converging straight lines which nearly meet on the y axis; if Table XVIII be referred to it will also be noticed that, even at very great dilutions, the amount of barium hydroxide absorbed coincides with the corresponding line, if sufficient time be allowed for equilibrium to be established. Strontium and calcium do not gelatinise pectin so readily, and in consequence absorption cannot be determined at equally great dilutions; this is especially the case for strontium.

It was unfortunately impossible to extend these results as material was lost during an interruption of the work, and the concluding experiments were made with a very limited quantity of pectin; some earlier measurements are however given, made with a different specimen of pectin, which shew the effect on the absorption of increasing the concentration of the pectin. The measurements are not quite so accurate as the preceding, the method

employed being slightly different ; the concentration of the calcium hydroxide was in this case determined by titration of the original solution, and a measured volume of this solution was added from a pipette. This method had two defects, which were avoided by the method described above ; the amount of carbon dioxide absorbed during the mixing process was unchecked, and the delivery of calcium hydroxide solution from the pipettes was not quite regular, this being due to a tendency of the solution to behave towards glass as to a greasy surface, in consequence of which drops were liable to adhere to the pipette. The flasks and pipettes used were carefully calibrated. In a given series the greatest differences between two measurements of the absorption was 0·3 c.c.

TABLE XXI.

Absorption of calcium hydroxide by gelatinised pectin.

Vol. 100 cc. T 18°–20°.

A. 20 cc. of pectin solution (*b*).

Final conc. 100 cc. = cc. N/25	Absorbed cc. N/25 Series I (2 measurements)	Series II (2 measurements)	Mean
24	6·3	6·15	6·25
33	(7·3)	6·55	6·55
43	6·9	6·8	6·85
52	7·45	7·65	7·55
62	7·65	7·95	7·8

B. 10 cc. of pectin solution (*b*).

Final conc. 100 cc. = cc. N/25	Absorbed cc. N/25 (2 measurements)
16	3·55
26	3·8
35	4·5
45	4·65
65	4·85

The mixture was left for half an hour as in the preceding measurements, and equilibrium was tested by measuring the absorption after 1 hour at low and high concentrations. The corresponding curves are shewn in Fig. 4 I, II.

Absorption in the presence of calcium or barium chloride.

A fairly concentrated solution of calcium chloride was necessary to increase the absorption by a measurable amount ; the following table shews the increase in absorption for different concentrations of calcium hydroxide ; 30 cc. or 70 cc. of approximately N/25 calcium hydroxide were mixed with 20 cc. of pectin and varying quantities of calcium chloride, and the volume was made up to 200 cc.

TABLE XXII.

Absorption of calcium hydroxide by gelatinised pectin in the presence of calcium chloride.

Vol. 200 cc. 20 cc. of pectin solution (*b*). *T* 18°–20°.

A. 70 cc. CaO_2H_2 added: final conc. 200 cc. = 62–66 cc. N/25.

	Absorption			
cc. N/25 $CaCl_2$	(1)	(2)	(3)	Mean
0	7·25	7·25	7·25[1]	7·25
10	7·7	7·7	7·45	7·62
50	8·4	8·25	8·35	8·33
B. 30 cc. CaO_2H_2 added: final conc. 200 cc. = 23–26 cc. N/25.				
0	5·8	5·8	5·85[1]	5·82
10	(6·9)	6·15	—	6·15
50	6·7	6·7	—	6·7

[1] After standing 1 hour.

In order to determine whether or not chloride ions were absorbed, 10 cc. of the filtrate from the mixtures A and B of the preceding table, each containing 50 cc. $CaCl_2$ in 200 cc., were neutralised and titrated with N/10 $AgNO_3$, potassium bichromate being used as indicator; 5 cc. of the original $CaCl_2$ solution required 23·95 $AgNO_3$. The equivalent quantities of the solutions to which pectin had been added required:

A. 23·8 cc.

B. 24 cc.

It appears therefore that pectin does not absorb chloride ions.

TABLE XXIII.

Absorption of barium hydroxide by gelatinised pectin in the presence of barium chloride.

Vol. 100 cc. 20 cc. of pectin solution (*b*). *T* 17°–18°.

A. Final conc. of BaO_2H_2: 100 cc. = 67–69 cc. N/25.

	Absorbed		
cc. N/2 $BaCl_2$	(1)	(2)	Mean
0	6·6	6·5	6·55
10	8·2	7·9	8·05
30	8·0	—	8·0
B. Final conc. of BaO_2H_2: 100 cc. = 9–10 cc. N/25.			
5	5·0	4·7	4·85
40	5·1	4·8	4·95

Table XXIII shews the absorption of barium hydroxide in the

presence of varying quantities of barium chloride; the concentration was determined for each measurement by a blank test in the absence of pectin; titrations were made by adding 20 cc. of N/2 HCl to the mixture where necessary, and titrating with N/25 HCl in the presence of phenolphthalein. In this case the solutions were boiled to eliminate carbon dioxide, but this was found to be without effect upon the result.

Theoretical.

It has been shewn that the rate of precipitation of pectin by the alkaline earths is very nearly proportional to the product of the concentrations of hydroxyl and metallic ion, and that this product may, with great probability, be taken as a measure of the rate of gelatinisation; the theoretical implications of these results remain to be considered.

(1) The fact that both anions and kations are concerned in the precipitation excludes the possibility that the alkali acts by a process of diffusion into the suspended pectin complex, since in this case the rate of diffusion and consequently of precipitation also, would be dependent upon the rate of diffusion of the more slowly moving ion; i.e. the rate of precipitation would be determined in general by the metallic ion alone.

(2) It is also improbable that precipitation is due to surface adsorption. This usually takes place with great rapidity and it is quite unlikely that the process of adsorption would be prolonged through a considerable interval, as in this case; moreover when adsorption occasions precipitation this has been found to be generally due to the adsorption of an ion having a charge opposite to that of the adsorbent [see especially Morawitz, 1910]. Since both positive and negative ions are concerned in the precipitation of pectin, this cannot be due to any change of potential brought about by adsorption.

(3) A third possibility is that the rate of gelatinisation is a measure of the rate of chemical change.

It may be seen from the measurements of absorption that the amount of alkaline earth which combines with pectin is small, therefore the concentrations of alkaline earth may be taken as constant throughout the time t, and we have on this assumption

$$\frac{dx}{dt} = RC_1C_2(a-x)^p,$$

where a is the initial quantity of pectin present; x is the amount transformed in time t; C_1, C_2 the concentrations of metallic ion and hydroxyl.

Putting $p = 1$ we have

$$RC_1C_2 = \frac{1}{t} \log \frac{a}{a-x}.$$

Now $\frac{a}{a-x}$ is constant for all the measurements of a series since it must be presumed that gelatinisation occurs when a definite quantity of pectin has undergone change.

Hence $$C_1C_2t = K.$$

It is therefore possible to express the rate of gelatinisation of pectin as determined by the velocity of a chemical change in which pectin reacts with barium, strontium or calcium and hydroxyl ions ; the reaction equation will probably be of the form

$$\overline{\text{P}}. \cdot \text{OH} + \text{BaO}_2\text{H}_2 = \overline{\text{P}}. \cdot \text{OBaOH} + \text{H}_2\text{O}.$$

It may be observed in Tables I–IV that the value of the constant c^2t is usually too high for the first measurement of the series, and that the error is greater, the greater the quantity of pectin present ; this may be accounted for not only by the slight acidity of the pectin but also by the fact that when the value of c is very small, an appreciable error is made in taking c as constant throughout the time t.

It has been shewn that the rate of precipitation of pectin by potassium hydroxide is conditioned by the concentration of potassium ions ; it is to be remarked, however, that gelatinisation does not take place below a definite and fairly high concentration. Since above this concentration the rate of gelatinisation increases proportionately to the increase of concentration, it may be assumed that hydrolysis takes place, and that the reaction may be represented by the equation

$$\overline{\text{P}}. \cdot \text{OH} + \text{KOH} \rightleftarrows \overline{\text{P}}. \cdot \text{OK} + \text{H}_2\text{O}$$

or

$$\overline{\text{P}}. \cdot \text{OH} + \text{K}^{\cdot} + \text{OH}' \rightleftarrows \overline{\text{P}}. \cdot \text{OK} + \text{H}_2\text{O}$$

which takes place in both senses ; the pectin cannot be itself dissociated since the reaction takes place slowly, and therefore could only take place between ions if it were dependent upon a process of diffusion, which has been shewn to be extremely improbable. The equation shews that the rate of reaction for a given concentration of hydroxyl will be determined by the concentrations of the potassium ions.

The hypothesis of chemical change affords therefore a partial explanation of the character of the curves shewn in Fig. 3. These curves indicate, however, that while for small additions of salt the rate of precipitation is nearly pro-

portional to the increase of the potassium ions, for larger additions the rate of precipitation increases more rapidly, the effect being ultimately greater than that produced by an equal quantity of potassium hydroxide ; moreover the specific character of the salt begins to assert itself. This appears to point to a molecular reaction between salt and pectin, and this reaction as is to be expected is more evident in the case of the salts of monobasic than in that of those of dibasic acids. The curve for potassium carbonate suggests that the error in the calculation of the degree of dissociation is preceptibly greater in this case than in that of potassium sulphate, which is probable since the ordinary solution of potassium carbonate contains hydrolysed salt, whereas in the present case hydrolysis is prevented by the excess of potassium hydroxide.

The hypothesis of a reaction between salt molecules and pectin is supported by the general analogy between the reactions described here and those of the sugar group. The constitution of the so-called saccharates and the analogous glucose compounds with the alkaline earths is doubtful, but formulae of the type assumed here for the pectin compounds, in which CaOH or its analogues replace a hydrogen atom, e.g. $C_{12}H_{21}(CaOH)O_{11}$, $C_6H_{11}(CaOH)O_6$, are not improbable, and have been strongly supported by some investigators [see especially Lippmann, 1904, p. 1701]. Compounds with the alkalies are similarly formed, e.g. $C_{12}H_{21}KO_{11}$ [Tollens, 1914, p. 400]. The sugars are also known to form molecular compounds with sodium chloride and many other salts [Tollens, 1914, p. 404 ; Lippmann, 1904, p. 1323].

In the ordinary notation the equation

$$\frac{dx}{dt} = KC_1C_2(a - x)$$

represents the reaction of one molecule of pectin with e.g. the ions Ba and OH. Now the molecule of pectin is certainly large, and is made up, at least to a very great extent, of condensed carbohydrate groups ; these groups will be largely independent of one another, and therefore it is probable that for the purposes of this equation they may be regarded as independent molecules. The equation will therefore represent the rate of reaction of a carbohydrate group with the ions Ba and OH, rather than that of the whole complex molecule.

The rate of reaction of pectin with barium hydroxide (Table VI) increases with the concentration of the pectin while this is very small ; at higher concentrations the rate becomes stationary, and it finally decreases. There is probably more than one cause for this behaviour. The fact that the rate

of reaction decreases much more rapidly for strontium than for barium suggests that absorption plays an increasing part, and that for the reason given on page 558, gelatinisation and precipitation become less and less coincident as the concentration of pectin increases. But besides this other factors may be brought into play ; unchanged pectin probably acts to impede gelatinisation, and there is good reason to believe that the degree of dispersion of pectin decreases with the concentration, so that the active mass will increase less rapidly than the concentration ; the importance of this latter factor in dilute solution is however entirely problematical.

Turning now to the absorption compounds which gelatinised pectin forms with the alkaline earths, it must be remarked that the term " absorption compound " has been used in its most general sense to denote a compound of continuously varying composition. Reference to the curves in Fig. 4 shews that the absorption curves are straight lines cutting the y axis at varying points. The simplest explanation of a straight line absorption curve is that of solid solution. Surface adsorption is precluded both by the character of the curve and by the slowness with which the condition of equilibrium is reached at small concentrations, which again favours the hypothesis of a solid solution. If surface adsorption occurs at all it must be to a very small extent.

If V be the volume of the jelly ; A the amount of alkaline earth absorbed ; N the partition coefficient of alkaline earth between jelly and solution, we have for a solid solution at concentration C

$$\frac{CV + A}{CV} = N, \qquad \text{i.e. } A = CV\,(N - 1),$$

which gives a straight line when V is constant.

It has been shewn that a true equilibrium is not reached under the conditions of the experiments, and that absorption continues for days, probably owing to a slow process of diffusion. This has been taken to mean that the apparent equilibrium is due to saturation at the surface ; V will consequently be a function of the surface area. Now $V\,(n - 1) = \tan\theta$ if θ is the angle the absorption curve makes with the axis of x, and it will be seen by comparing curves I and II Fig. 4 that $2\tan\theta_2$ is greater than $\tan\theta_1$; accordingly $\frac{V_2}{2}$ is greater than V_1, i.e. the specific surface increases as the concentration of the pectin decreases.

The absence of surface adsorption is important, since in this case the point at which the straight line cuts the y axis will give the composition of

the compound of alkaline earth and pectin. This is confirmed by the convergence of the curves for calcium, strontium and barium (Fig. 4 III, IV, V) which approximately meet at a point on the y axis. These curves give the absorption for 10 cc. of pectin solution (c) in 100 cc.; the solutions were therefore very dilute, and the convergence to a point is to be attributed to this fact. At higher concentrations it is most unlikely that this occurs, and some experimental evidence was obtained on this point, but it needs further investigation.

Taking 1·9 (2·2 − 0·3 for the acidity) as combining with 10 cc. of pectin solution containing approximately 2·5 grms. of dry pectin per litre, we have 330 as the weight of pectin combining with the equivalent of alkaline earth. This figure is certainly too high since the specimen of pectin was not pure, and no allowance was made for the weight of ash. It may be compared with the formula $6(C_{10}H_{18}O_9) + O$ suggested by Tollens. The weight of the $C_{10}H_{18}O_9$ group is 282.

It is well known that the alkaline earths are easily soluble in sugar solution, and cane sugar has been shewn to form a series of solid solutions with lime [Cameron and Patten 1911], so that in this case again the resemblance of pectin to the members of the sugar group is preserved. It has been shewn that calcium chloride as such is not absorbed by pectin; it merely serves to increase the absorption of the calcium hydroxide, and a similar effect is obtained with barium chloride and barium hydroxide. This may be a salting out process, due to decreased dissociation; but it seems more probable, at any rate in the case of calcium, that the chloride increases the solubility of the hydroxide in pectin as it does in water [Cameron and Bell 1907], but to a greater extent, thus increasing the value of the partition coefficient n.

Theory of gelatinisation of pectin.

References to the theory of gelatinisation have been hitherto avoided, and it has been assumed that alterations of physical or chemical properties may suffice to bring this process about, without reference to the mechanism of the change. This now remains to be considered, and it will be necessary, as a preliminary, to discuss shortly the current theories of gelatinisation [see the historical introduction, Bachmann 1912].

Until very recently jellies have been almost universally supposed to consist of two phases separated by a process of desolution during the solidification of the original sol. According to this theory where desolution results in

gelatinisation the one phase separates in droplets, which remain suspended in the continuous phase, and afterwards cohere, and in most cases solidify, forming the framework of the gel. Hardy [1900] observed this process for mixtures of alcohol, water and gelatin, and he found it possible, by suitably varying the conditions of experiment, to obtain droplets of almost ultra-microscopic size.

Hardy's work gave fresh confirmation to the widely accepted honeycomb theory of gel structure due to Bütschli's classical researches on the structure of gelatin [1898], a theory which was also supported by Quincke's theoretical investigations, and by the authority of van Bemmelen, who used it for the interpretation of his later work ; it was however adversely criticised in some quarters ; notably by Pauli [1902] who regarded the structure observed by Bütschli in coagulated gelatin as a secondary structure due to the coagulation, and not as preexisting in the uncoagulated jelly. To establish this point he investigated the action of the coagulating agent, and arrived at the conclusion that essential differences exist between the processes of gelatinisation and coagulation, a conclusion very important for the theory of these processes. He shewed that the one process could be accelerated by means which caused retardation in the other ; that e.g. grape sugar added to gelatin accelerates gelatinisation and raises the temperature of solidification, while it hinders precipitation ; and that the independence of the two processes was also proved by the fact that coagulation could take place as well in solid as in liquid gelatin. He also pointed out certain discrepancies between the results of Bütschli's theory and experiment, and he concludes that the " species of coagulation " worked by chromic acid, alcohol, etc. in solidified gelatin must be regarded as a true coagulation, essentially different from the preceding process of solidification, that the honeycomb structure observed in the coagulation is secondary, and that those appearances in uncoagulated gelatin which Bütschli regarded as due to this structure must be looked upon rather as the result of imperfect homogeneity.

Pauli's conclusions did not meet with general acceptance, and the theory of Bütschli held its ground until the introduction of ultra-microscopic methods of research allowed the application of a conclusive test. By this means Bachmann [1912] has shewn that gelatin jellies possess a far more complicated structure than the theory of Bütschli would allow, being built up of microns, sub-microns, and probably still finer elements. Bachmann points out also that the jellies prepared by Hardy are of a very different character from those of gelatin, being soft, white and opaque, rather than clear and stiff. Such

difference in character may well denote an altogether different method of formation.

The continuity of properties which has so often been observed between sol and gel has led some investigators to regard gels as sols of greatly increased vicosity, but vicosity alone cannot account for their properties. Hatschek [1911] has pointed out that gels differ from highly viscous substances, such as pitch, in their inability to flow, as do these, however slowly. Any theory of gels must take account of this rigidity, as also of the heterogeneity of structure which they exhibit, which is usually of the same order as that of the sol from which they are derived.

Bütschli's theory of the structure of gels replaced an older theory due to Nägeli; this "micellar theory" contemplated the gel as consisting of molecular aggregations or *micelle* of varying degrees of complexity, separated by films of water and attracted together more or less strongly according to the thickness of the intervening film. The breakdown of Bütschli's theory has brought about a return to more or less similar conceptions [cf. Zsigmondy 1912, p. 72]. These afford a fairly satisfactory explanation of the coagulating effect of change of potential on inorganic sols, but are less successful where organic sols are concerned, the gelatinisation of which takes place at much smaller concentrations, and appears to be largely independent of electrical conditions, and there is some tendency at present to regard gelatinisation in these as due to the properties which organic sols share with true solutions, rather than to those which they possess in common with the suspensoids, i.e. as a process akin to crystallisation, rather than as a process of aggregation due to a change in the conditions of suspended particles. It must be recognised however that our present ignorance of the nature of aggregation processes makes this a somewhat provisional distinction. Procter [1911] regards the sol of gelatin in this light, and considers its gelatinisation to be due to the formation of a molecular network, while the work of Pauli [1913] on protein solutions has shewn the relationship of the aggregation processes which take place in these to the phenomena of crystallisation.

It has been shewn that the rate of gelatinisation of pectin solutions may be regarded as determined by rate of chemical combination; this at once suggests a crystallisation process. It is of course to be expected that chemical change in suspended particles might cause aggregation and precipitation, but it is difficult to conceive that the rate of aggregation would be directly proportional to the rate of chemical change. Those aggregation processes, the rates of which have been studied, have been shewn to depend upon the

adsorption of oppositely charged ions [cf. Freundlich and Ishizaka, 1913: Paine, 1912], and in these cases the rate of precipitation shews no simple relation to that of adsorption; also where aggregates separated by films of water are concerned, rate of diffusion must determine rate of chemical action, and moreover, in the present case, the extreme dilution at which pectin gelatinises in alkaline solution makes any but a molecular hypothesis difficult of acceptance. Therefore here also the "crystallisation" hypothesis affords the simplest explanation of the facts.

During the last few years von Weimarn's [1911] work has thrown much light on the theory of gelatinisation, and since his theory is the most complete expression of the "crystallisation" theory, an attempt will be made to outline this in order to compare with it the results obtained for pectin jellies.

Von Weimarn regards inorganic jellies as concentrated highly disperse suspensoid solutions. He has himself prepared jellies from barium sulphate and other inorganic substances, and he has shewn that in general any substance under suitable conditions may be prepared in this form, the requisite conditions being to combine a sufficiently high degree of dispersion with a sufficiently high concentration. His theory of crystallisation, which is on the lines of the preceding theories of Lehmann and Tammann but permits of wider application, shews the relation of these jellies to substances in ordinary crystalline form. He regards crystallisation as the result of two independent processes (1) the formation of molecular aggregates by sudden concentration, and (2) the growth of centres by diffusion, either of molecules or of small aggregates. The first process gives rise to a suspensoid solution, and from this crystals are formed by the second process of diffusion. The rate of the first process, which von Weimarn takes as proportional to the relative supersaturation (i.e. the ratio of the quantity of substance capable of separating out to the solubility), determines the degree of dispersion; where this is great the degree of dispersion will be high. Now if the second process is impeded or inhibited, stable suspensoid solutions will arise, and this will evidently take place when the degree of dispersion is so high that the greater part of the substance present separates during the first process in the form of small molecular aggregates, for these will neither diffuse rapidly nor combine readily with one another, since their vector properties will be small. A stable suspensoid solution is therefore formed, when a substance separates from a solution whose relative supersaturation is very high; the greater the solubility the greater the relative supersaturation, and the higher the degree

of dispersion necessary for stability ; concentrated suspensoid solutions will take the form of a jelly.

As a test of his theory von Weimarn prepared a number of disperse solutions by the admixture of solutions containing substances which mutually precipitate one another ; e.g. barium sulphate was obtained at different degrees of dispersion by mixing solutions of barium chloride and magnesium sulphate at various concentrations. At the highest concentrations jellies of cellular structure were produced, since the reaction took place at the surface where the two solutions were in contact, and by maintaining the same degree of relative supersaturation with a lesser solubility (by alteration of the solvent) a jelly of network structure could be obtained.

In the application of this theory to organic sols, von Weimarn points out that between true solutions and disperse solutions an absolute continuity of properties exists, and that " the properties peculiar to the solutions of simple substances decrease in intensity as the molecular complexity increases." These complex solutions gelatinise readily because their molecules diffuse with difficulty, and with increase of concentration form groups practically incapable of overcoming the internal friction of the solvent, while cohesion is weak since the energy of the molecule is exhausted in its complicated internal structure. They form in fact disperse solutions, in which at small concentrations diffusion and vectorial cohesion are as much suppressed as in the highly concentrated disperse solutions of inorganic substances, and with a similar result. A lattice work is thus formed, the particles of which are bound together by vectorial forces, and which may therefore be entitled crystalline, but which will be soft and weak, and will be penetrated by the solvent, and greatly deformed [von Weimarn 1912].

The size and nature of the particles of which pectin solutions are formed have not been determined, but there is some reason to believe that the degree of dispersion decreases with increase of concentration, since the difficulty of filtration increases ; only very dilute solutions can be filtered through hardened filter paper without separation of the dissolved pectin. The characteristics are those of an emulsoid, and the solutions may very probably consist of a suspension of aqueous pectin in a dilute pectin solution. In this case the reaction with the alkalies and the alkaline earths will take place in the solution and at the surface of the particles. As the reaction proceeds a supersaturated solution will be formed ; this, as has been shewn, will solidify to a jelly at any concentration at which a molecular network can be produced. At very small concentrations where suspended pectin must be almost entirely

absent, the solution will remain supersaturated until practically the whole of the pectin present has been transformed, for the presence of a foreign substance will greatly impede both diffusion and cohesion, and will thus hinder the formation of a continuous network. As concentration increases the relative supersaturation previous to gelatinisation will increase, while the fraction of pectin transformed will decrease ; the amount separating out will thus increase with the concentration, but in a smaller ratio. These considerations afford an explanation of the positions of the points at which the curves in Fig. 4 cut the y axis, as also of the rate measurements in Table VI.

Summary.

(1) The rate of gelatinisation of pectin is not determined by the rate of diffusion nor by the rate of surface adsorption of the alkali or alkaline earths producing gelatinisation.

(2) It can be expressed as a velocity equation determined by the concentration of the reacting substances, when the initial concentration of pectin is constant.

(3) It is therefore regarded as determined by the rate of a chemical reaction—the substitution of H by BaOH, K etc.

(4) That a reaction of this kind takes place is supported by the fact that the constitution deduced for compounds of the alkalies and alkaline earths is strikingly analogous to that of the corresponding compounds of the mono- and di-saccharides. Similar molecular compounds with salts are probably also formed.

(5) Gelatinised pectin forms solid solutions with the alkaline earths which may be compared with the solid solutions of lime and cane sugar.

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LVIII. THE ACTION OF POISONS ON REDUCTASE AND ATTEMPTS TO ISOLATE THIS ENZYME.

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Although it has been shown [Harris, 1910] that tissue-reductase is comparatively insoluble, the following attempts at its extraction have been made; they led not to its being isolated but to a study of the action of poisons on it.

Extracts or solutions of reductase were made thus: 1. A liver extract; the livers of four pigeons, before the animal heat had left them, were crushed in the juice-press, ground up with sand in a mortar in the presence of 200 cc. of 0·75 per cent. sodium chloride solution and the mixture allowed to remain under toluene for 24 hours. 2. The pectoral muscles of two pigeons, still warm, were crushed in presence of glycerol and allowed to remain under this liquid for 24 hours. 3. The pectoral muscles of two other pigeons were crushed with glycerol and 0·75 per cent. sodium chloride solution and the mass allowed to stand for 24 hours. There were, then, three extracts or solutions of reductase; a saline extract of liver, a pure glycerol extract of muscle, and a glycerol and saline extract of muscle.

The presence of reductase in the extract was detected by determining whether it effected the reduction of the two-banded oxyhaemoglobin to the one-banded haemoglobin. In the reduction experiments, one part of extract was mixed with two parts of a blood solution prepared by diluting 10 cc. of freshly defibrinated cat's blood to 250 cc. with water. The reduction was carried out at 40°.

The progress of the reduction of oxyhaemoglobin to the fully reduced pigment was followed spectroscopically, a small direct vision spectroscope being employed. The replacing of the two bands of oxyhaemoglobin by the

one band of fully reduced haemoglobin gives an end-point which is not affected by any personal factor. But the disappearance of the two bands is from a very early period accompanied by the appearance of a fainter band between them in the intralinear green, so that at a certain stage of the reduction the single band of the reduced haemoglobin is flanked by the remains of the two bands of the oxyhaemoglobin. The pigment was never considered fully reduced until the remains of these margins of the single band had completely disappeared. In this way it was possible to obtain an end-point of considerable definiteness. It was evident that owing to the opacity of the mixture of extract and diluted blood in even a narrow test tube, the spectrum of the oxyhaemoglobin could not be seen sufficiently distinctly to be certain of the end-point. It was found that glass vessels made by drawing out short lengths of glass tubing (6 mm. diam.) to a conical end answered the purpose very well, for if we were in doubt as to the persistence of the two bands, when the spectroscope was directed across the tube itself, it was only necessary to look through the conical part of the tube where the mixture was sufficiently transparent to obtain a distinct spectrum. Complete reduction was considered to have taken place at the first moment the two bands had vanished, and when they were no longer distinguishable as ribbed margins to the single band.

It was previously ascertained that neither the glycerol, the saline solution nor a mixture of these effected the reduction of oxyhaemoglobin at 40°.

A mixture of the glycerol and saline extract of the pigeon's muscle with the blood solution had its oxyhaemoglobin completely reduced in two minutes in one experiment, and in three in another. Indeed the small quantity of residual blood in this extract of muscle was found to be in the reduced condition. The pure glycerol extract was incapable of effecting reduction. The saline extract of the liver completely reduced the oxyhaemoglobin in 16 minutes. There was evidence, therefore, that a certain amount of reductase passed into solution in a menstruum of glycerol and normal saline both from disintegrated liver and from muscle, but that evidently it did not pass into solution in the pure glycerol.

Efforts were next made to precipitate the reductase from these extracts or solutions. A quantity of the saline glycerol solution of hepatic reductase was thrown into an excess of absolute alcohol, and the precipitate allowed to stand for some time. The alcohol was then decanted, the precipitate washed with water and dried in a current of air at room temperature. This precipitate was shaken with glycerol and normal saline and some of the

resulting cloudy liquid mixed with dilute blood in a conical observation tube. No appreciable reduction could be detected at 40° even at the end of an hour. The filtrate from the alcohol precipitate had no reducing power. The toxicity of alcohol towards reductase was thus once more demonstrated, it having been previously found [Harris, 1911] that alcohol destroyed the power of reductase to reduce soluble Prussian blue. The glycerol and saline extract of the pectoral muscles was saturated with ammonium sulphate, but it was found that neither the precipitate itself nor the filtrate from it was able to effect any appreciable reduction of oxyhaemoglobin. In other words, ammonium sulphate inactivates or poisons reductase.

The poisonous influence of many substances towards enzymes has long been recognised, and has been the subject of considerable investigation. For example, the poisonous action of a number of substances on the catalase of the blood has been investigated by Senter [1903]. Indeed, many of the enzymic poisons are the same substances which inhibit the action of certain inorganic catalysts, as has been shown by the investigations of Bredig and his pupils during the last few years. The effect of different poisons on inorganic catalysts and enzymes is illustrated in the accompanying table, in which is shown the concentration of different poisons that is necessary entirely to destroy the catalytic action of colloidal platinum [Bredig and v. Berneck, 1899] and of catalase [Senter, 1903] on hydrogen peroxide.

TABLE I.

Poison	Colloidal platinum	Catalase
H_2S	1: 300000 molar	1: 1000000 molar
HCN	1: 20000000 „	1: 1000000 „
$HgCl_2$	1: 2000000 „	1: 2000000 „
$Hg(CN)_2$	1: 200000 „	1: 300 „
I in KI	1: 5000000 „	1: 50000 „
$NH_2(OH)HCl$	1: 25000 „	1: 80000 „
Aniline	1: 5000 „	1: 40000 „
As_2O_3	1: 50 „	1: 2000 „
CO	very poisonous	no paralysis
HCl	1: 3000 molar	1: 100000 molar
NH_4Cl	1: 2000 „	1: 1000 „
HNO_3	no paralysis	1: 250000 „

As our previous work has shown [Harris and Creighton, 1912] that the liver and other organs contain a reducing enzyme, reductase, and that attempts to isolate this enzyme have indicated that its activity is greatly reduced by contact with foreign substances, it seemed desirable to investigate the action of small quantities of a number of substances towards it. For

this purpose the reductase contained in cat's liver-juice was employed. The action of the foreign substances on the reductase was measured by determining the difference between the time required for the reduction of oxyhaemoglobin to the one-banded condition, by pure liver-juice, and by liver-juice containing some of the foreign substance with which it had been in contact for a definite time. The progress of the reduction was followed spectroscopically. In the experiments, a mixture of 1 cc. of fresh liver-juice and 1 cc. of an aqueous solution of the poison was allowed to remain in contact for ten minutes, at the end of which time 2 cc. of the blood solution (dilution 1 : 25) were added and the mixture introduced into observing tubes which were then placed in a thermostat at 40°. The spectrum of these mixtures was examined every half-minute. The times required for the reduction at 40° by liver-juice which contained foreign substances were compared with the time required for the reduction, at the same temperature, of a *normal* mixture containing 1 cc. of liver-juice, 1 cc. of distilled water and 2 cc. of the blood solution. Only such substances were employed as were found to produce no change in the spectrum of oxyhaemoglobin. It was not possible, therefore, to use a number of desirable substances such as copper sulphate, iodine, acids, alkalies, etc. The results of the experiments are given in Table II. The times given in this table represent the means of several experiments which seldom differed by more than a few per cent. and which, in most cases, were identical.

TABLE II.

Ten minutes required for reduction of normal mixture.

Poison	Time required for the reduction of mixtures containing 1 cc. of liver-juice, 2 cc. of blood solution and 1 cc. of an aqueous solution of the poison having a concentration of	
	0·01 molar	0·1 molar
As_2O_3	33 minutes	
KCN	30 „	34 minutes
$HgCl_2$	17 „	blood destroyed
Na_3AsO_3	17 „	„ „
$AuCl_3$	15 „	16 minutes
OsO_4	13 „	19 „
$MnCl_2$	11 „	25 „
NH_4Br	11 „	13 „
HCHO	10 „	48 „
NH_4Cl	9 „	9 „

The solution of As_2O_3 employed was somewhat more dilute than 0·01 molar.

In Table II the different substances are arranged in the order of their poisoning action with respect to the 0·01 molar concentration. The order is quite different with respect to the higher concentration. At the higher concentration some of the substances are relatively much more poisonous than at the lower. Thus formaldehyde, which exhibits no toxic action in the smaller concentration, is the most powerful poison in the list when its concentration is increased to 0·1 molar.

The poisonous action of the foregoing foreign substances is of physiological interest. A new method of studying the action of poisons on one phase of tissue-respiration has been used. It is possible that it may be found a convenient one where injection of poison into the entire animal is not desirable. One substance only of those examined exerted no retarding action on reductase, namely, ammonium chloride. This is in accordance with what is known of ammonium chloride therapeutically; it is a mild expectorant and a ciliary excitant and is not a toxic substance at all in the ordinary meaning of the word. It may be said that the more poisonous a substance is, the longer is the time required for reductase to effect complete reduction in the presence of the weaker concentration of the substance. Judged by this, neither ammonium bromide nor manganese chloride is a poison, and this, at least as regards the former salt, is quite in agreement with the experience of therapeutics. Ammonium bromide, except in exceedingly high doses, is not a poison. It is, however, exceedingly interesting that mercuric chloride and potassium cyanide, for instance, substances well known to be most energetic in destroying the activity of the inorganic ferment colloidal platinum and of catalase should be among the most toxic of the substances which were examined. In concentrations so dilute as 0·01 molar they interfered with the activity of reductase, causing the reduction to be lengthened one and a half times in one case and three times in the other. Quite distinctly toxic, even in such dilution, is sodium arsenite. Not far behind it come gold chloride and osmic acid, the latter a well-known protoplasmic poison. It need be a matter of no surprise that eight of the ten substances examined, more or less at random, should be toxic for reductase. None of these substances is a constituent of the normal cell-lymph in which reductase must act. The slightest trace of any of these salts or acids is foreign to the environment of living cells, and as such cannot but be highly injurious to them. Nevertheless reductase is found within limits to be fairly resistant, for concentrations of these poisons ten times as high as 0·1 molar did not entirely prevent it exerting its reducing action. In the case of formaldehyde

the time taken to reduce at the higher concentration, 0·1 molar, was more than quadrupled, being prolonged to 48 minutes.

That dilute formaldehyde should not exert a poisonous action on tissues is in keeping with what is believed to be its rôle in the anabolic processes carried out by vegetable protoplasm. Formaldehyde has been proved to be the first organic substance produced in the synthesis of carbohydrate in the plant, under the influence of sunlight on chlorophyll. This function of formaldehyde is incompatible with its being in high dilutions a protoplasmic or enzymic poison.

The fact that the poisons studied behave towards some substance in fresh tissue exactly as they behave towards catalysts in general, is an additional indication that that substance in tissue-juice is itself catalytic.

The physiological significance of the relative insolubility of reductase is undoubtedly this, that its sphere of action is *within* the cell. Inside the cell but separated from the oxygen-containing lymph by only the more or less permeable membrane or wall of the cell, the reducing enzyme extracts oxygen from the circumambient lymph. Reductase could not perform this particular service in tissue-respiration if it could dissociate from cell proteins and become dissolved in the lymph and blood. It would then circulate and perform a rôle wholly different from that which it evidently does perform at the surface of, but still inside, the living cells. Reductase is not a secretion, it is an *endo*-enzyme.

Summary.

1. It has been found that the reductase contained in pigeon's liver and muscle is soluble to some extent in normal saline, and in a mixture of normal saline and glycerol but not in pure glycerol. All attempts to isolate the active enzyme have failed.

2. The poisonous action of ten substances, chosen more or less at random, towards reductase has been investigated.

3. In conclusion, the action of the poisons on reductase and the insolubility of the latter have been discussed from the physiological standpoint.

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LIX. ON THE RESPIRATORY EXCHANGE IN FRESH-WATER FISH. Part II: ON BROWN TROUT.

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The influence of diminution of oxygen content of water on fish life has been investigated by many observers, notably by Duncan and Hoppe-Seyler [1893], Chopin and Nikitin [see Kupzis, 1901] and König and Hünnemeier [1901]. The results generally indicate that fish—of very different varieties, including trout—die when the oxygen content of the water reaches 1–0·4 cc. per litre.

In the method employed by most observers the fish were placed in a closed vessel, and the gradual diminution of the amount of dissolved oxygen was brought about by the breathing of the animals themselves. When asphyxia took place samples of the water were withdrawn and analysed.

The subject has been more recently dealt with by Hans Winterstein [1908], who made use of roach (*Leuciscus erthyrophthalmus*). In his experiments the tension of the dissolved oxygen was reduced by passing through the water a continuous stream of nitrogen, and his method was not very dissimilar from that adopted in the experiments to be described in this paper.

Winterstein found that in the case of roach an oxygen content of 0·7 cc. per litre, or a tension of 2·2 % of an atmosphere, was compatible with life, but when the oxygen was reduced to 0·4–0·5 cc. per litre, *i.e.* a tension of 1·3–1·5 % of an atmosphere, asphyxia took place.

In Part I of this series of papers [1914], we showed that trout use more oxygen per kilo. per hour than some other varieties of fresh-water fish, and further that within the limits of temperature of our climate the oxygen absorbed was a function of temperature. Between 10° and 20° C. the oxygen absorption per kilo. per hour was approximately doubled. As the volume

of water that must be pumped through the gills is inversely as the oxygen content of the water, it seemed likely that the asphyxial tension would be greater at higher temperatures than at lower, and this difference would be dependent on the facts that at the higher temperatures the fish require more oxygen, and that under such conditions more work has to be performed to obtain the oxygen. It seemed of interest therefore to investigate the effects of diminishing tension of oxygen in the case of trout, and to determine the least quantity of oxygen per litre compatible with life at various temperatures.

The method adopted was a modification of that described in our earlier paper. The animals were placed in an hermetically closed vessel containing known volumes of water and air. The air was displaced by a *continuous*

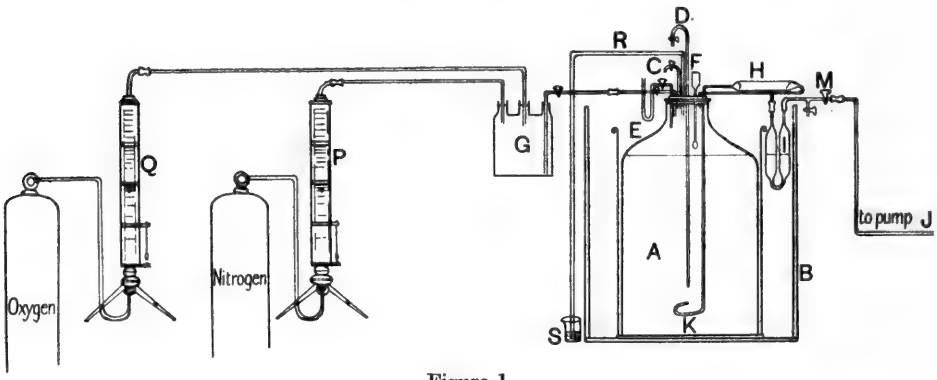

Figure 1.

current of nitrogen, or a mixture of nitrogen and oxygen of known composition. The gas above the water was then pumped through the water continuously. If the gas above the water consisted of nitrogen, the quantity of oxygen dissolved in the water was gradually reduced. Samples of water were withdrawn when the fish entered into the asphyxial condition, or at any desired point prior to this, and analysed. If the gas above consisted of any definite mixture of oxygen and nitrogen, the oxygen in the water was gradually reduced until equilibrium was attained between the gas above the water and that in solution, and the fish could then be observed under any desired oxygen tension.

Apparatus employed.

The apparatus used was that fully described in Part I with some modifications. The essential parts of the apparatus with the modifications introduced

are shown in Figure 1, but the pumping mechanism is omitted, as it was exactly the same as in the earlier experiments.

In these experiments a much larger volume of water was used—about 45 litres—and the gas space above the water was consequently only 2–3 litres in capacity. Only one fish of about eight inches in length and approximately 90–100 grams in weight was used in each experiment. The reason for using only one animal and a relatively very large volume of water was to prevent any appreciable increase in the carbon dioxide pressure during the experiment. The oxygen reservoir in the old apparatus was replaced by a small mixing bottle *G*, of about 0·5 litre capacity, connected through two gas measuring instruments *P* and *Q* with cylinders containing respectively compressed oxygen and nitrogen. In this way pure nitrogen or any desired mixture of oxygen and nitrogen could be delivered continuously into the gas space above the water in *A*.

The excess of gas escaped through the tube *R* which was just closed by a water seal *S*. The meters *P* and *Q* were "Rotameters" made by the Deutsche Rotawerke Co. of Aachen. The "Rotameter" consists of a glass tube slightly increasing in diameter from below upwards and divided in a "litres-per-hour" scale. The gas on entering from the lower opening sets in rapid rotation, about a vertical axis, a cylindrical float on the side surface of which a spiral groove is cut. The float rotates freely and visibly in the gas stream without coming in contact with the walls of the tube, and rises in the tube to a height corresponding to the volume of the gas passing through. The tube is marked with an empirically graduated scale giving the litres per hour. The oxygen meter was graduated to read from 0 to 50 litres per hour, and the nitrogen meter from 0 to 100 litres. Analyses of the mixed gases showed that the instruments gave reasonably accurate results—quite as accurate as would be obtained by the use of any of the ordinary forms of volume meters—and they were much more convenient. In performing an experiment the bottle *A* was filled to the desired point with tap water and left in the thermostat *B* to attain the temperature required. A weighed fish was then introduced; the air in the gas space was displaced by nitrogen or any desired mixture of oxygen and nitrogen, and a current of this gas passed through at a known rate. The gas in the space above the water was then pumped through and through the water by means of the pumping mechanism *I*, *H*, and spray *K* at a rate of about 100 cc. per few seconds. The rate could be varied by adjustment of the pumping mechanism. In this way the composition of the gas dissolved in the water was

gradually changed to that corresponding to the partial pressure of the gases in the space above. This was a slow process, owing to the large bulk of water employed, and, even when pure nitrogen was passed through the gas space, it took several hours, the exact time depending on the rate and efficiency of spraying, to get rid of the oxygen in the water. Measured samples of water were withdrawn by displacement of mercury in the manner described in our former paper, at desired periods. The gases were pumped out of the samples by means of the pump described by Buckmaster and Gardner [1910, 1912] and analysed in the usual way. An example will make clear the mode of calculation. A fish weighing 80 g. was placed in the apparatus at 1 p.m., and the current of nitrogen started at 65 litres per hour. The temperature of the water was 6° C. The pump was now started and at 4.40 p.m. the fish turned over on his back in an asphyxial condition. A sample of water was now withdrawn, measuring 53·5 cc. This sample yielded to the pump gas which after standing over potash to get rid of carbon dioxide measured 1·83 cm. at 14·15° and constant volume (48·65 cc.). After treatment with alkaline pyrogallate it measured 1·76 cm. at 14·10°. The water therefore contained 0·79 cc. of oxygen and 20·02 cc. nitrogen, measured at 0° and 760 mm., per litre. Taking Winkler's value 0·04181 as the absorption coefficient of oxygen at 6° (Landolt, Börnstein, Meyerhoffer's tables) the partial pressure of the oxygen is $\frac{0{\cdot}79}{10 \times 0{\cdot}04181} = 1{\cdot}89$ per cent. of an atmosphere.

The results, we believe, are accurate. They do not depend, as in the experiments described in Part I, on analytical differences before and after a period of time, and are not therefore affected by the sources of error there described.

Experimental results.

It was easy, of course, to determine the gas content of the water at the point at which the animal entered into the asphyxial condition, but it was very difficult to find out at what point prior to this the animal began to suffer inconvenience.

Observations on the behaviour of trout under diminishing oxygen pressure showed that between 10° and 25° the animals exhibited strikingly similar behaviour. With fully saturated water the animals moved about continually in leisurely fashion, breathing in a normal manner. As the oxygen diminished the respiratory movements gradually increased in rapidity and force, and acquired a dyspnoeic character, as has been described by Baglioni [1908]

and other observers. At the beginning the animals spent the greater part of their time moving about gently. As the oxygen gradually diminished, they became more active, rushing about and jumping into the gas space above the water. These periods of activity alternated with periods of rest at the bottom with the dyspnoeic respiration noted. Gradually the periods of rest became longer relatively to the periods of activity, and ultimately the fish remained for the bulk of the time at the bottom with occasional short rushes to the surface. As the oxygen approached the asphyxial tension they remained all the time at the bottom and ultimately quietly rolled over on their backs. In this condition they died in the course of three or four minutes, but if at this stage oxygen was sprayed through the water they recovered in the course of 5 to 10 minutes, and afterwards appeared to suffer no apparent ill effects. The asphyxial condition evidently takes place within quite small limits of oxygen tension. At or below this they die, but slightly above they recover. Their behaviour can, we think, be explained by the increased volume of water the animals have to pump through their gills to get the necessary oxygen, as the tension of the gas diminishes below a certain value, necessitating the expenditure of a considerable amount of energy.

In quantitative experiments samples of water were withdrawn when the animals turned over on their backs and were in an asphyxial condition. Before reaching this point it was difficult to decide exactly when the animal began to suffer inconvenience, but an arbitrary point was selected, viz. when the animal's periods of rest at the bottom became well established, and the alternating periods of activity were quite short. The point arbitrarily selected was when the periods of rest were about three minutes, the active periods occupying only a few seconds.

At temperatures below 6° or thereabouts the animals behaved rather differently. At these low temperatures the animals, at the beginning, remained quietly at the bottom, with only occasional excursions round the bottle and to the surface. This continued for a considerable period and until the oxygen tension was very considerably reduced. No marked dyspnoeic respiration was noticed. As the oxygen tension further diminished the fish became very active, and ultimately spent most of their time rushing round near the surface, and often jumping and thrusting their noses out into the gas above the water. Asphyxiation generally took place during a period of activity and the fish fell over on their backs and gradually sank. Death took place rapidly, but again if oxygen was sprayed through recovery took

place in a very few minutes. At these low temperatures samples were taken at the asphyxial point only.

The results of a number of experiments are given in Table I.

TABLE I.

		Arbitrary initial pt.		Asphyxial point		Normal	
Weight of fish	Temp. of exp. °C.	c.c. of oxygen at 0° and 760 in water per litre	Partial pressure in % of 1 atm.	c.c. of oxygen at 0°, 760 in water per litre	Partial pressure % of 1 atm.	c.c. of oxygen 0°, 760 in water per litre	Partial pressure in % of 1 atm.
90 g.	6·4°	—	—	0·79	1·89	8·6	
90 „	9·5°–10°	3·75	9·86	0·81	2·19	8·0	
80 „	18°	3·19	9·9	1·49	4·62	6·61	
80 „	17°	5·05	15·4	1·37	4·17	6·75	app. 21 %
90 „	24°	3·55	12·33	1·97	6·83	5·89	
88 „	25°	3·18	11·23	2·4	8·49	5·78	
1	2	3	4	5	6	7	8

In a number of other experiments fish were kept for the greater part of a day at tensions which analysis showed were between the arbitrary points given in column 3 of the above table and the asphyxial points without any apparent subsequent ill effects, though judging by their dyspnoeic breathing the animals were in a state of distress. How long the animals could have existed in such a condition we did not attempt to determine. Winterstein kept roach under like conditions at a tension above the asphyxial point for about a week, but we did not push our experiments with trout to such lengths. Experience showed us that trout do not take kindly to being kept in a confined space for long periods, and to keep them healthy a very considerable space per fish is necessary, and it was felt that if conclusive results were to be obtained by keeping fish in the above apparatus for long periods a very large number of experiments would be necessary, and the fish would have to be observed after returning to the aquarium for long periods further. Our trout accommodation did not permit of this.

Discussion of results.

A glance at columns 5 and 6 of Table I will show that the oxygen content of the water when asphyxiation took place was much higher than was found by Winterstein in the case of roach. The oxygen content at 6°–10° was of much the same order as that at which he was able to keep roach alive for a week.

In our earlier paper we showed that the oxygen consumption of trout in fully saturated water increases with increase in temperature—within the ordinary limits of temperature it doubles itself for a rise of 10°,—and consequently the fish would have to pump a much larger volume of water through their gills at a higher temperature than at a lower to obtain the necessary amount of oxygen. Hence as pointed out at the beginning of this paper we should expect the oxygen content at the asphyxial point to be greater at higher temperatures than at lower. This conclusion is fully borne out by the figures in columns 5 and 6. The rate of increase with temperature of the figures in column 5 appears to be somewhat greater than the rate of increase in oxygen consumption under normal conditions of fully saturated water. This we think can be well explained by the increased work done in respiration under low tension of oxygen at higher temperatures. The figures in column 3 at the arbitrary point selected do not appear to show much variation, but we know nothing of the other factors governing them.

From a consideration of our own work and that of other observers there can be no doubt that trout can exist at tensions of oxygen considerably lower than normal, but judging by the type of respiration and the general behaviour of the animal, the trout under conditions between those represented in columns 3 and 5 were in a condition of distress which even in their natural environment would have a very deleterious effect. Such conditions of oxygen content might of course be readily produced by pollution of the natural water.

We take this opportunity of expressing our thanks to the Government Grants Committee of the Royal Society for aid in carrying out this work.

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LX. THE CHEMICAL INVESTIGATION OF THE PHOSPHOTUNGSTATE PRECIPITATE FROM RICE-POLISHINGS.

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The chemistry of rice-polishings has attracted many workers ever since it was shown that there occurred in the polishings a substance, apparently of a relatively simple chemical constitution, which had a powerful curative effect when administered in cases of beri-beri, or experimental beri-beri, produced by a diet of polished rice.

Practically all the communications which have appeared on the subject have, however, concerned themselves chiefly with that particular fraction of the polishings which contained the curative substance and have to a greater or less extent neglected other fractions which were obtained.

The objects of this investigation were, primarily, to repeat the attempt to isolate the curative substance in a pure condition, and, at the same time, to carry out as exhaustive an examination of the other simple nitrogenous substances contained in the phosphotungstic acid precipitate as possible, in the hope that some new substance might be isolated which could be regarded as a degradation product of the unstable curative substance.

We will not give a detailed account of the findings of previous workers, but will refer to and discuss them in the course of the description of our work.

Experimental.

For the purpose of this investigation 620 kilograms of rice-polishings[1] were rendered practically free from fatty substances by petroleum ether extraction, after which they were well extracted three times with alcohol in

[1] This fractionation was begun in 1913 [Funk, 1913, 1], but only the silver nitrate-baryta fraction was thus investigated.

a large extractor. The combined alcoholic extracts, which possessed a deep golden colour, were then freed from alcohol under diminished pressure at 30°, and yielded a thick, dark brown syrup.

This residue was now extracted with 10 per cent. sulphuric acid and the extract diluted with an equal volume of water. On the addition of a 50 per cent. solution of phosphotungstic acid a heavy precipitate was thrown down which was filtered off, washed with 5 per cent. sulphuric acid, and dried.

The dry phosphotungstates, which were to be investigated, were now decomposed by excess of baryta, and, after removal of the excess of baryta, the filtrate was neutralised with hydrochloric acid and concentrated to a thick syrup at low temperature and under diminished pressure.

Extraction of this syrup with alcohol removed a certain amount of inorganic salts, which remained behind insoluble and which for the most part consisted of potassium salts, and the alcoholic extract, after diluting with an equal volume of water, was precipitated by alcoholic mercuric chloride solution.

The heavy white sublimate fraction was filtered off, and, after removal of the excess of mercury from the filtrate and evaporation in vacuo, the purines present were precipitated together with silver chloride by the addition of 20 % silver nitrate in dilute nitric acid solution.

The combined precipitate of silver purine compounds and silver chloride was removed by filtration, and to the filtrate sufficient excess of silver nitrate solution was added to ensure that all compounds present which were capable of forming silver compounds had done so, when they were precipitated as such by the addition of an excess of baryta.

The filtrate from the silver nitrate-baryta fraction was now freed from excess of silver by means of hydrochloric acid, and from excess of baryta by sulphuric acid and the remaining basic substances were precipitated by the addition of phosphotungstic acid.

Four fractions were thus obtained:

1. Sublimate fraction;
2. Purine fraction;
3. Silver nitrate-baryta fraction;
4. Residual phosphotungstic acid fraction;

and these were investigated separately.

1. Investigation of the Sublimate Fraction.

This fraction, which weighed, when dry, 1505 g., was decomposed in aqueous suspension by means of sulphuretted hydrogen. The filtrate from the mercury sulphide possessed a bright blue colour which, however, rapidly faded on standing. It was decided to investigate this filtrate by two different methods, and accordingly it was divided into two equal portions.

Method I.

This half of the filtrate, in which the basic substances were present in the form of their hydrochlorides, was treated with silver nitrate and silver nitrate and baryta so as to obtain a purine fraction, a histidine fraction, and an arginine fraction. The final filtrate from these separations being freed from excess of baryta and silver was reprecipitated by phosphotungstic acid.

The four sub-fractions of the sublimate fraction were then investigated individually.

Purine sub-fraction of the sublimate fraction. This weighed 220 g. and consisted for the most part of silver chloride. It was ground up with water and the silver removed by sulphuretted hydrogen.

The filtrate from the silver sulphide, on concentration in vacuo at 30° C. to dryness, yielded a small, dark-coloured semi-crystalline residue. On recrystallisation from dilute alcohol, a very small amount of a slightly dirty white, granular substance was obtained. This compound on heating darkened at 220°, but did not show a melting point below 290°. Micro-analysis was attempted, using the method of Pregl, but gave variable figures, and it was found that the substance was not sufficiently pure for an elementary analysis to be made.

It was attempted to make a derivative which could be sufficiently purified to analyse, but the only one which could be isolated with readiness was the picrate, of which, however, we did not obtain sufficient to carry out an analysis.

The substance was a purine, gave a positive reaction with the xanthine test and the diazobenzenesulphonic acid test, a negative result with Wheeler and Johnson's reaction, and a doubtful positive reaction with Capranica's test for guanine.

The picrate was rather insoluble in cold water but more readily soluble in hot, insoluble in alcohol and showed no melting point below 290°.

From the bulk of the evidence we considered that we had isolated guanine, but in the absence of analytical data we could not be certain of this point.

The filtrate from the guanine picrate yielded about a milligram of a crystalline picrate melting without further purification at 145°, but owing to the extremely small amount at our disposal we were unable to make any further examination of this substance.

No other substances were isolated from this fraction.

Histidine sub-fraction of the sublimate fraction. A small fraction which weighed 9 g. It was decomposed in the usual manner in aqueous suspension by means of sulphuretted hydrogen, and the filtrate from the silver sulphide on concentration in vacuo at 30° yielded a very small amount of a residue which was obviously very impure.

This residue was taken up in 200 cc. of water and precipitated by alcoholic mercuric chloride. The white mercuric chloride salt was decomposed, and yielded on concentration of the filtrate from the mercury sulphide a small residue from which a crystalline platinum chloride salt was prepared.

The platinum double salt, after one recrystallisation from dilute alcohol, crystallised in fine needles, melted at 239–240°, and gave on analysis, after drying at 100° C. in vacuo, the figures:

7·643 mgms.; 2·439 mgms. platinum; 31·91 % Pt.
Calculated for choline platinichloride; 31·73 % Pt.

No substance could be isolated from this fraction other than this small amount of choline which was probably in this fraction as an impurity.

Arginine sub-fraction of the sublimate fraction. This fraction, which only weighed 0·4 g., did not give us a sufficient residue on decomposition to enable us to identify or isolate anything from it, other than a small trace of inorganic salts.

Phosphotungstic acid sub-fraction of the sublimate fraction. This final sub-fraction was decomposed by means of lead acetate and the filtrate from the lead phosphotungstate freed from lead, acidified with hydrochloric acid and concentrated at low temperature and under diminished pressure to a thick syrup. Extraction of this syrup with absolute alcohol left a small amount of insoluble inorganic salts, and the alcoholic extract was then precipitated with alcoholic mercury chloride.

The heavy sublimate salt was recrystallised twice from water and then decomposed. The filtrate from the mercury sulphide was concentrated as far as possible and the thick syrup thus obtained allowed to stand in an

evacuated desiccator over potash and calcium chloride. After some weeks there was obtained a crop of fine needle-shaped crystals which were extremely hygroscopic and closely resembled choline hydrochloride in appearance. A platinum double chloride was prepared, and, after one recrystallisation from dilute alcohol, melted at 240°.

Analysis (substance dried at 100° in vacuo).

22·155 mgms.; 7·023 mgms. platinum; 31·71 % Pt.
Calculated for choline platinichloride; 31·73 % Pt.

A fine crystalline picrate was also obtained, which on recrystallisation from water separated in fine clusters of golden yellow prisms which melted sharply at 240°.

The presence of comparatively large amounts of choline in rice-polishings has been noticed by many previous workers [Funk, 1911; Suzuki, Shimamura and Odake, 1912; etc.].

The mother liquors from the recrystallisation of the choline mercury chloride were decomposed by means of sulphuretted hydrogen and the filtrate from the mercury sulphide concentrated at low pressure and temperature to a thick syrup. Extraction of this syrup with cold alcohol yielded a substance which was rather insoluble in alcohol and which was not inorganic in nature. It was removed by filtration and after washing with alcohol examined. Without further purification it melted at 242° and gave a fine double salt with platinic chloride.

This salt, after one recrystallisation from dilute alcohol, melted at 242°.

Analysis (substance dried at 100° in vacuo).

6·412 mgms.; 1·869 mgms. platinum; 30·43 % Pt.
6·927 mgms.; 2·110 mgms. platinum; 30·46 % Pt.
Calculated for betaine platinichloride (mp. 242°); 30·28 % Pt.

The occurrence of the well-known and widely distributed plant base betaine, although not previously observed in rice-polishings, did not surprise us, and we subsequently obtained some interesting results on this point which will be described later.

The alcoholic solution after separation of the betaine hydrochloride was fractionated with platinum chloride but nothing further than small quantities of choline and betaine was isolated.

Method II. (*Sublimate Fraction.*)

The other half of the filtrate from the decomposition of the sublimate precipitate was acidified with sulphuric acid until it contained 5 % of the acid and was then precipitated with phosphotungstic acid. The precipitated phosphotungstates were then treated by a method which we are now using to a considerable extent in our investigations, namely extraction with acetone[1].

It has already been shown that the phosphotungstates vary a great deal in their solubilities in acetone or dilute acetone and advantage has been taken to some extent of this fact by Wechsler [1911]. So far as we know, however, there is no published record of any attempt to employ the different solubilities of the phosphotungstates as a means of separation.

We are, at the present time, making an extensive examination of these solubilities in the hope that by this means a new and useful process may be added to biochemical methods. Preliminary results, a few of which are contained in this communication, lead us to believe that the method may, when fully worked out, be of considerable value, but we cannot pass a verdict until we have completed our investigation of the subject.

In this particular case the phosphotungstates were ground up in a mortar in the cold with acetone until the extract ceased to possess a brown colour, when the insoluble residue was removed by filtration and washed with acetone. We find that extraction in the warm is undesirable, as it generally results in the formation of an extremely sticky mass which is very difficult to deal with, and we have always found that a far better separation is effected when the extraction is carried out at room temperature.

By this means, we obtained a deep brown solution of acetone-soluble phosphotungstates and a small residue insoluble in acetone.

Acetone-soluble phosphotungstates from the sublimate fraction. The deep brown solution was decomposed by shaking well with an excess of a saturated aqueous solution of lead acetate. After standing, the precipitated lead phosphotungstate was filtered off and the filtrate freed from excess of lead by means of sulphuretted hydrogen. The clear solution thus obtained was rendered free from sulphuretted hydrogen and fractionated, as in Method I, by means of silver nitrate, and silver nitrate and baryta into a purine fraction, a histidine fraction, an arginine fraction, and then, after removal of the excess of baryta and silver, into a final phosphotungstic acid fraction.

[1] This method was used for the first time in the fractionation of yeast and cod-liver oil by Funk, shortly to be published.

The purine fraction on decomposition yielded the same purine that we had previously encountered and which we believe was guanine, but here, again, insufficient was obtained to render the identification absolute.

Nothing definite could be isolated from the histidine and arginine fractions, which were very small.

The final phosphotungstic acid precipitate yielded on decomposition practically pure choline, and no betaine could be recognised or isolated from this fraction.

Acetone-insoluble phosphotungstates from the sublimate fraction. This residue, weighing 40 grams, was decomposed by lead acetate, the filtrate freed from excess of lead by sulphuretted hydrogen, acidified with hydrochloric acid and evaporated to a thick syrup under diminished pressure at 30°.

As no crystalline derivative could be isolated directly from this syrup, a sublimate salt was made and recrystallised from water. On decomposition it yielded a hydrochloride, which was soluble in alcohol and melted sharply at 263°.

A platinum double salt was prepared, which, after one recrystallisation from dilute alcohol, melted at 253° and after drying at 100° C. for two hours in vacuo gave the following figures on analysis:

14·896 mgms.; 4·478 mgms. platinum; 30·07 % Pt.
10·826 mgms.; 3·245 mgms. platinum; 29·98 % Pt.
Calculated for nicotinic acid platinichloride (mp. 253°); 29·72 % Pt.

The mother liquor from the mercurichloride precipitation on decomposition yielded a platinichloride which was readily identified as betaine platinichloride.

Apparently, in the investigation of this fraction by utilising the separation of the phosphotungstates by means of acetone, into an acetone-soluble and an acetone-insoluble portion, we have been able to separate the choline practically quantitatively from the betaine and nicotinic acid, thereby rendering the identification of the small quantity of the latter possible by removing the choline into another fraction.

We have therefore found the Sublimate Fraction to contain mainly choline together with small quantities of betaine, nicotinic acid and probably traces of guanine. It is of interest to note that nicotinic acid has been isolated from this fraction, which it has already been shown contains a small amount of the curative substance. The parallel occurrence of nicotinic acid

and the curative substance has already given rise to the suggestion made by one of us [Funk, 1913, 2] that nicotinic acid may be a degradation product of the curative substance. This point will, however, be referred to again later.

2. Investigation of the Purine Fraction.

This fraction, weighing 47 g., was decomposed by means of sulphuretted hydrogen and the clear filtrate from the silver sulphide concentrated under diminished pressure at 30°. After concentration to half bulk, the excess of nitric acid in the solution was quantitatively removed by means of nitron and after removal of the nitron nitrate the concentration continued to dryness. During the concentration two crops of a sandy solid were obtained which were removed by filtration. This substance, which was impure, weighed 0·3 g. and was recrystallised from water, in which it is very insoluble. Thus purified, it showed all the reactions of adenine, but did not give very concordant figures on analysis by the micro-combustion method of Pregl.

The picrate closely corresponded with adenine picrate.

Analysis (dried at 100° C. in vacuo for two hours).

2·740 mgms.; 0·717 cc. N. at 21° C. and 770 mm.—30·62 % N.
Calculated for adenine picrate .. .. .. 30·77 % N.

From the tarry residue left after the separation of the adenine and further concentration of the mother liquor, nothing very definite could be isolated except a very small quantity of a crystalline picrate melting, without further purification, at 280°. The amount, however, was far too small to admit of any examination.

The purine fraction consists mainly of adenine together with possible traces of other purines.

3. Investigation of the Silver Nitrate-Baryta Fraction.

This fraction, which, as originally shown by Funk, contains practically the whole of the curative substance, has already been very exhaustively investigated and the results of the investigation published elsewhere [Funk, 1913, 2]. In that case 2·5 g. of a highly active substance were obtained, which after one recrystallisation from dilute alcohol gave a white substance crystallising in small needles which melted at 233°.

This substance did not give the Millon test nor the uric acid or phenol

tests, it was sparingly soluble in water and on analysis, using the micro-combustion method of Pregl, the following figures were obtained:

	C.	H.	N.
(1)	58·80 %	3·93 %	10·58 %
(2)	58·89 %	3·89 %	10·64 %

From these figures the formula $C_{26}H_{20}O_9N_4$ was arrived at to represent this substance.

Calculated figures for $C_{26}H_{20}O_9N_4$:

C.	H.	N.
58·62 %	3·79 %	10·53 %

The substance did not yield all its nitrogen by Kjeldahl's method.

From the mother liquor of this substance another crop of crystals was obtained which were somewhat less coloured and apparently more soluble in water.

After recrystallisation from dilute alcohol, this substance melted at 234° and no depression of the melting point was observed when melted together with a sample of synthetic nicotinic acid.

Analysis gave the following figures:

	C.	H.	N.
(1)	58·37 %	3·93 %	11·11 %
(2)	58·45 %	4·06 %	10·97 %

Calculation for nicotinic acid:

C.	H.	N.
58·30 %	4·08 %	11·34 %

The identity of the product from rice-polishings was further established by the melting point and analysis of the picrate.

Barger [1914] has remarked that the substance described with melting point 233° to which the provisional formula $C_{26}H_{20}O_9N_4$ had been given, showed a very close resemblance to nicotinic acid both as regards its melting point and its elementary composition, and he suggested that the possibility was not excluded that this body might in reality be merely nicotinic acid contaminated with a small quantity of a highly active substance rich in carbon.

It must be remembered that the substance $C_{26}H_{20}O_9N_4$ was not described as being curative, and, in fact, it was found that it has no greater curative action than nicotinic acid. With regard to the suggestion that the compound $C_{26}H_{20}O_9N_4$ is identical with nicotinic acid, our work goes to show that it is quite correct.

Both substances crystallise in the same form and the crystals melt at 232–234°. A mixed melting point determination gave the figure 232°.

The two picrates were prepared and both presented the same crystalline appearance. They both melted at 215°, and a mixed sample also melted at 215°. Their copper salts were also prepared and were identical in appearance.

Analysis of copper salt of $C_{26}H_{20}O_9N_4$ dried at 100° C. in vacuo:

(1) 19·100 mgms.; 4·180 mgms. CuO; 20·13 % Cu.
(2) 19·060 mgms.; 4·93 mgms. CuO; 20·65 % Cu.
Calculated for the copper salt of nicotinic acid; 20·67 % Cu.

The identity of the substance previously described as $C_{26}H_{20}O_9N_4$ with nicotinic acid could now be doubted no longer, and a careful examination of the mother liquid of these two crops of crystals did not result in the isolation of any other substance than nicotinic acid. All trace of the curative substance had disappeared, and a fraction, which originally had shown very marked curative properties, now consisted of nothing other than nicotinic acid, which possesses very slight action.

All attempts which we have made to isolate the elusive curative substance from this fraction, in which it originally occurs, have failed, and we can only hope that one or other of the new methods which we are testing at the present time, will give a process by which it can be isolated before it has time to become inactive.

4. Investigation of the Phosphotungstic Acid Fraction.

This fraction, weighing 195 g., was treated by grinding in a mortar with acetone, as already described, until the acetone extract ceases to be coloured.

The two fractions thus obtained were then investigated separately.

Acetone-insoluble sub-fraction of fraction 4. This weighed 25 g. and was decomposed by means of lead acetate. The filtrate, after being freed from lead, was acidified with hydrochloric acid and concentrated at low temperature and pressure to a syrup. As no definite crystalline substance could be obtained from this syrup, alcoholic mercuric chloride was added, and the precipitated mercury chloride salt removed by filtration.

On decomposition the mercurichloride yielded a small amount of a rather hygroscopic hydrochloride from which a well-defined platinichloride was prepared.

After one recrystallisation from dilute alcohol the platinum salt melted at 232°.

Analysis (dried at 100° C. in vacuo).

(1) 11·670 mgms.; 4·024 mgms. platinum; 34·48 % Pt.
(2) 7·155 mgms.; 2·463 mgms. platinum; 34·42 % Pt.

After another recrystallisation from dilute alcohol it melted at 234°.

(3) 4·235 mgms.; 1·453 mgms. platinum; 34·39 % Pt.

The small quantity of this salt which we obtained prevented us from making any further examination of it, and we have so far been unable to identify it.

The filtrate from the platinum double salt, together with the mother liquors from its recrystallisation, were decomposed and attempts made to prepare a gold salt from the residue, but without success.

The filtrate from the mercuric chloride precipitation was decomposed and concentrated at low temperature and pressure to dryness. A very small residue of impure crystalline hydrochlorides was obtained which, however, contained inorganic substances. Alcohol extraction removed the organic portion, which yielded a small quantity of a platinichloride melting with decomposition at 280°, but the quantity obtained was far too small to admit of purification or examination.

Acetone-soluble sub-fraction of fraction 4. This weighed 175 g. and was decomposed in the usual manner by means of lead acetate. The filtrate, after freeing from lead, was concentrated at low temperature and pressure to a thick syrup. Nothing crystallised from this residue on standing, so saturated aqueous picric acid was added. A small amount of a brownish yellow semi-crystalline picrate separated out and was removed by filtration. This picrate was very insoluble in cold water and very little more soluble in hot. It was recrystallised from water and separated as a golden brown powder. It darkened on heating at 200° but showed no melting point below 289°. Determinations of its content of picric acid were made by the nitron method on a sample dried at 100° in vacuo for two hours. The figures obtained were:

(1) 0·0749 g.; 0·1388 g. nitron picrate; 78·45 % picric acid.
(2) 0·0395 g.; 0·0732 g. nitron picrate; 78·43 % picric acid.

Portions of the picrate and of the mother liquid from its recrystallisation were decomposed with ether and hydrochloric acid, and yielded upon

concentration to dryness a very small residue of the base in the form of its hydrochloride.

It was itself insufficiently pure to examine, and attempts to prepare a platinichloride, a gold salt or a picrolonate were all unsuccessful.

Guanidine picrate melts at 315°, exhibits similar solubilities to the compound we isolated from rice, and contains 79·10 % of picric acid. It is possible that we were here dealing with guanidine picrate, but we were unable to be sure of this point because of the very small quantity which was at our disposal.

The filtrate from the precipitation of this picrate was rendered free from picric acid by means of acidification with hydrochloric acid and shaking out with ether, and was then concentrated at 37° under diminished pressure. During the concentration a crystalline substance separated out and was filtered off, after which the concentration was continued to dryness.

The crystalline substance, which had separated out during the concentration, was recrystallised from dilute alcohol and separated out from that solvent in the form of small needles. Without further purification it melted sharply at 235°.

The isolation of this substance was of considerable interest to us, as we have encountered it several times in the investigation of rice-polishings. If the total phosphotungstate precipitate from rice-polishings is decomposed by means of lead acetate, and the filtrate, after freeing from lead and acidifying with hydrochloric acid, concentrated to dryness, a semi-crystalline residue of hydrochlorides is obtained. If now, this residue be extracted and recrystallised from dilute alcohol, the same crystalline substance melting at 235° is obtained.

On several occasions we have isolated this substance, and, after having collected some few grams, we decided to investigate it. It was recrystallised once again from alcohol and then melted sharply at 234°.

It was noticed that on drying, the crystals often—although not in every case—developed a fine blue colour. Some crystals apparently developed the colour to a far greater extent than others, and we found that the production of the colour was due to a photochemical action. The colour disappears when the crystals are dissolved, and when they separate out again they are perfectly white, so long as they are covered by the solvent. When they are removed from the solution and placed in the light they quickly develop the colouration again, and we have found them to develop this colouration after as many as six recrystallisations.

We have not yet satisfied ourselves as to the cause of the colouration, but believe it is in some way the result of the action of light upon minute traces of tungsten compounds which have been persistently retained from the phosphotungstic acid decomposition. This however was never observed in other fractionations.

The substance which we had now obtained in an apparently pure condition was analysed by micro-combustion. Concordant figures could not, however, be obtained on duplicate analyses, despite further purification of the substance by recrystallisation from dilute alcohol. The substance did not give all its nitrogen when estimated by Kjeldahl, and in many ways resembled nicotinic acid hydrochloride, which, however, as prepared from nicotinic acid, always melts at 263°.

A well-defined platinum double salt was prepared, which after one recrystallisation from dilute alcohol melted sharply at 253°, the theoretical melting point for the platinichloride of nicotinic acid, and no depression of the melting point was observed when a mixed sample of the two platinichlorides was melted.

Analysis (dried at 100° C. for two hours in vacuo).

(1) 7·746 mgms.; 2·318 mgms. platinum; 29·86 %.
(2) 10·479 mgms.; 3·136 mgms. platinum; 29·93 %.
Calculated for platinichloride of nicotinic acid; 29·72 %.

The free base was liberated from the hydrochloride by means of silver acetate, and from the product thus obtained pure nicotinic acid was isolated, which was in every respect identical with the synthetic acid.

We were unable to explain the difference in the melting points of what we imagined were two distinct modifications of the hydrochloride of nicotinic acid, and could not account for the inconstant figures obtained on analysis of the hydrochloride melting at 235°, until an attempt to recrystallise the latter compound from absolute alcohol provided us with a clue, which, after investigation, resulted in the clearing up of the whole matter.

It was found that the compound melting at 235° was not all equally soluble in absolute alcohol and that a portion of it was very insoluble in cold alcohol.

This less soluble portion was fractionated with cold alcohol, and eventually a white crystalline substance was obtained which was very insoluble in cold alcohol and which after one recrystallisation from dilute alcohol melted sharply at 245°. A platinichloride was prepared and formed a fine,

orange-coloured, needle-shaped, crystalline compound which after one recrystallisation from dilute alcohol melted at 242°.

Analysis. 13·133 mgms.; 3·998 mgms. platinum; 30·44 % Pt.
Calculated for betaine platinichloride (mp. 242°); 30·28 % Pt.

It also gave a picrate which melted at 182° and was identical in every respect with betaine picrate.

We then realised that in the compound melting at 235° we were dealing with some complex, probably of the nature of mixed crystals, of nicotinic acid hydrochloride and betaine hydrochloride.

We therefore decided to effect a separation of the two constituents, and after several attempts found that this could be quantitatively carried out by utilising the insolubility of the copper salt of nicotinic acid.

The hydrochloride was dissolved in water and was warmed on the water bath at 60° for several hours with excess of a saturated solution of copper acetate until no further deposition of any copper salt occurred. The precipitated copper salt and the clear blue mother liquor were then separated, and decomposed individually by means of sulphuretted hydrogen.

The pale green, amorphous copper salt on decomposition and concentration of the filtrate from the copper sulphide yielded a crystalline hydrochloride, which after one crystallisation from dilute alcohol was shown to be identical with nicotinic acid hydrochloride. It melted at 264°, its picrate at 214°, and none of these derivatives showed any depression in its melting point when melted with the corresponding derivative of nicotinic acid.

The bright blue-green filtrate from the insoluble copper salt of nicotinic acid was decomposed in a similar manner, and yielded a hydrochloride, very insoluble in cold alcohol, which after a single recrystallisation from alcohol melted at 245° and was identical with betaine hydrochloride. Its picrate melted at 181°, and no depression of this melting point was observed on melting it together with a sample of betaine picrate. Its gold salt melted at 209° but on recrystallisation from 1 % hydrochloric acid, the other form melting at 247° crystallised out.

Analysis. 11·884 mgms. gold salt; 5·119 mgms. gold; 43·09 % Au.
Calculated for betaine aurichloride (mp. 247°); 43·11 % Au.

We also tried other methods of separating the two constituents of this complex, but found no method so useful as that which we have described in which we used copper acetate.

Alcohol fractionation removed the betaine hydrochloride gradually, but

we were not able to prepare a pure specimen of nicotinic acid by this process. Fractionation of the picrate of the complex also gave us fairly pure betaine picrate in the less soluble fractions, but we were unable to obtain nicotinic acid picrate free from betaine picrate.

It was of interest to us to try the curative action of the complex hydrochloride, and accordingly doses were administered by intra-muscular injection into pigeons suffering from experimental beri-beri, but we did not observe any arrest of the symptoms or of the onset of death. Betaine salts were likewise examined and found to be entirely without curative action.

The residue left after concentration of the mother liquors, from which the complex hydrochloride melting at 235° had separated out, was then examined, but only yielded a mercurichloride. This was decomposed and found to consist of choline as identified by its platinichloride, melting at 242°.

Analysis (dried at 100° C. in vacuo).

7·643 mgms.; 2·439 mgms. platinum; 31·91 % Pt.

Required for choline platinichloride; 31·73 % Pt.

The filtrate from this small quantity of choline mercurichloride on decomposition yielded a small residue which gave a platinichloride. After a single recrystallisation it melted at 237°.

Analysis (dried at 100° C. for two hours in vacuo).

(1) 8·418 mgms.; 2·906 mgms. platinum; 34·57 % Pt.

(2) 15·092 mgms.; 5·199 mgms. platinum; 34·45 % Pt.

This platinichloride seemed, despite its somewhat higher melting point, identical with that which had been isolated in the acetone-insoluble subfraction of this fraction. Here again we obtained insufficient to render much further examination possible. A small quantity was decomposed and the free hydrochloride prepared. No gold salt, picrate or picrolonate could be obtained, and consequently we have been unable to prepare any other derivative by means of which it might be identified.

There is the bare possibility that it might be the platinichloride of choline contaminated with traces of potassium platinichloride, which it is known are persistently retained by the choline salt, but it did not give the periodide test for choline.

No other substance could be isolated from this fraction, from which we have separated betaine, choline, nicotinic acid, a picrate that resembles guanidine picrate, and the platinichloride of a base which we have been unable to identify.

We may now summarise our findings, and it is here that a convenient point is reached at which to discuss some of the results of previous workers.

From an exhaustive fractionation of the phosphotungstic acid precipitate from the alcoholic extract of fat-free rice-polishings, we have isolated comparatively large amounts of choline, nicotinic acid and betaine, together with small amounts of the purines, guanine and adenine and traces of a substance which may be possibly guanidine.

Choline and nicotinic acid have been found by the majority of the previous workers, because they occur in larger amounts than any of the other constituents. We have, whilst carrying out this work, paid particular attention to the published work of Suzuki, Shimamura and Odake [1912] in the hopes that we might be able to find some corroboration of their results. We have, however, with a few exceptions, been entirely unable to do so. By decomposition of the phosphotungstic acid precipitate from rice-polishings with baryta and subsequent concentration of the filtrate, they obtained a product which was highly curative in action and which they designated "Rohoryzanin I."

In spite of the fact that it is quite obvious that "Rohoryzanin I" is a complex mixture of basic substances, they boiled it with 3 % hydrochloric acid for two hours and then looked on the products they isolated from the fluid as hydrolytic products of Rohoryzanin.

From this hydrolysis they obtained two acids, which they termed α and β acids, choline, nicotinic acid, and glucose. Thus they said that from 100 parts of Rohoryzanin there were obtained on hydrolysis 10 parts of the α and β acids, 30 parts of choline and nicotinic acid and 23 parts of glucose.

The two acids were obtained by allowing the hydrolysed solution of Rohoryzanin to cool, when golden brown coloured crystals separated out. These were separated, washed with a small quantity of cold water, and recrystallised from alcohol, when two different products, separated by their different solubilities in alcohol, were obtained, and from the elementary analyses the formulae $C_{10}H_8O_4N$ and $C_{18}H_{16}O_9N_2$ were given to the two acids.

Funk (1913, 1) tried to prepare these two bodies, following exactly the method described by Suzuki and his co-workers, but has never yet succeeded in obtaining them. Whenever the product which these authors termed "Rohoryzanin" was hydrolysed a clear solution was obtained which did not deposit any crystalline substance whatever on cooling. From this solution on concentration we have now separated the hydrochlorides of nicotinic acid and betaine by their different solubility in alcohol, but we can hardly believe

that these were the substances described asthe acids $C_{10}H_8O_4N$ and $C_{18}H_{16}O_9N_2$. Unfortunately, beyond giving a few solubilities and reactions the authors do not give much information about these two products.

The Japanese workers, from their descriptions of "Rohoryzanin," were apparently under the delusion that they were dealing with a single substance and give several reactions for it. They even claim that a characteristic reaction for "Rohoryzanin" is the *p*.-diazobenzenesulphonic acid test.

It was found that the substance described as "Rohoryzanin" does give the "diazo" reaction, but we believe that the purine content of this crude mixture of bases is quite sufficient to account for a positive reaction being obtained.

Suzuki, Shimamura and Odake also describe nicotinic acid, choline and glucose amongst what they term "die Spaltungsprodukte des Rohoryzanins," obtained by hydrolysis with 3 % hydrochloric acid.

With regard to nicotinic acid, we ourselves lean towards the belief that its occurrence in rice-polishings has some relationship to the occurrence of the curative substance, and that it may possibly even be a degradation product of the active body, but in the case of choline we firmly believe that it exists as such, and in comparatively large amounts, in the polishings.

The workers, whose work we are criticising so fully, purified their "Rohoryzanin I" by means of precipitation with tannin, and thereby obtained another product which they called "Rohoryzanin II," and which was three times more active than the original substance. The yield, however, was very small.

From a concentrated aqueous solution of "Rohoryzanin II," they obtained on addition of picric acid a precipitate of a golden-brown crystalline picrate. They separated it and found that it contained as an impurity nicotinic acid picrate, due to "Rohoryzanin II" containing impurities of nicotinic acid.

They succeeded in preparing this picrate, "Oryzanin picrate," free from nicotinic acid picrate and further purified it by recrystallisation from acetone.

Thus prepared, it was described as a golden-brown picrate crystallising in microscopic needles. It is insoluble in ether and petroleum ether, rather insoluble in water, more soluble in hot water, alcohol or ether. The yield was very poor, and very little detail is given beyond the few solubilities given above. It was highly active when administered to pigeons suffering from experimental beri-beri and was regarded by the discoverers as the curative substance of rice-polishings in the form of its picrate.

We have attempted to prepare this substance but have not yet succeeded

in doing so. Among the substances which we isolated from rice-polishings was one picrate which corresponded in many respects with the picrate described by the Japanese authors. This picrate, already described in a previous portion of the paper, we believe to have been guanidine picrate. Whether this is so or not we are not sure, but we certainly know that it possesses no curative action whatever.

Not one of the picrates which we isolated demonstrated any such action, and we have been quite unable to confirm the isolation of the curative substance in the form of its picrate as described by Suzuki and his collaborators.

Summary.

1. We have carried out an exhaustive fractionation of the phosphotungstic acid precipitate from an alcoholic extract of rice-polishings.

2. We have once again confirmed the presence of considerable amounts of choline and nicotinic acid in the polishings, and have also detected the presence of betaine, adenine, guanine and possibly guanidine. Small amounts of other substances were isolated but in quantities too small to admit of identification.

3. We have repeated the work of the Japanese authors Suzuki, Shimamura and Odake, who claimed to have isolated the curative substance in the form of its picrate, but have been unable to confirm this finding, as well as some other portions of their work.

4. We have ourselves failed to isolate the curative substance which occurs in rice-polishings. It is apparently decomposed during the fractionation and all trace of it is lost.

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LXI. A COMPARISON BETWEEN THE MOLECULAR WEIGHTS OF PROTAGON AND OF THE PHOSPHATIDE AND CEREBROSIDES OBTAINABLE FROM IT.

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(*Received Nov. 6th, 1914.*)

Introduction.

The following observations were carried out with the object of obtaining fresh and conclusive information concerning the question whether protagon is a mixture of phosphatides and cerebrosides or whether it contains these substances in chemical combination. All authors are agreed that it is possible to prepare from protagon two cerebrosides, namely cerebrin and homocerebrin (phrenosin and kerasin of Thudichum), and a phosphatide, sphingomyelin. But while according to Thudichum and his followers protagon is simply a mixture of these three substances, together perhaps with a number of other simpler substances, the followers of Gamgee maintain that protagon contains these substances in chemical combination which is easily broken up with the liberation of the constituent phosphatide and cerebrosides, just as haemoglobin is easily broken up into globin and haematin.

Hitherto all attempts to decide this question have been made by studying the behaviour of protagon on the one hand and of cerebrin, homocerebrin and sphingomyelin and mixtures of the three on the other hand towards certain solvents such as alcohol, chloroform, pyridine and others. But this line of argument has not only failed to bring about an agreement but has actually led to an impasse.

The observations of one group of authors are incompatible with those of another. Thus Thudichum and Rosenheim and Tebb state that protagon has

an indefinite and variable melting point, contains relatively large amounts of potassium, and completely alters its composition on recrystallisation from large volumes of alcohol; in other words, its behaviour is that of a mixture of a phosphatide and cerebrosides. On the other hand, on subjecting these statements to re-examination Gamgee, Roscoe, Baumstark, Ruppel, and Cramer find that protagon has a definite melting point, is free from potassium and retains its composition on recrystallisation from large and small volumes of alcohol; in other words its behaviour, according to these authors, is not that of a mixture of a phosphatide and cerebrosides. There is therefore hardly any common ground on which a discussion as to the nature of the substance in question is possible along these lines. Indeed the only conclusion an impartial observer can draw, is that the substance investigated by Thudichum and his followers is not identical with the one studied by Gamgee and his followers. Nor are these contradictions to be found only between the statements of those who attack and those who defend the existence of protagon as a definite compound. The observations of Posner and Gies that protagon retains its composition when dissolved in warm alcohol and cooled immediately, however large the volume of alcohol, is diametrically opposed to the statements of Rosenheim and Tebb. No explanation has ever been offered by the latter observers to account for this contradiction, and it is difficult to understand how these authors can quote each other in support of their views.

A new and more decisive line of argument is necessary in order to settle this question. It is offered in this paper and is as follows. The chemical composition of cerebrin and homocerebrin is fairly well known owing mainly to the work of Thudichum, Kossel and Freytag, Thierfelder and Levene and Jacobs. Thierfelder [Kitagawa and Thierfelder, 1906] from his observations on the products of acid hydrolysis of cerebron, which is either identical with or closely allied to cerebrin, has calculated the formula $C_{48}H_{93}O_9N$ which has also been accepted by Levene and Jacobs [1912] as the result of their analysis. The molecular weight of cerebron according to this formula would therefore be 827. In the case of the closely allied homocerebrin the molecular weight has been determined directly by means of the elevation of the boiling point by Kossel and Freytag and was found to lie between 945 and 1027. In the case of sphingomyelin our knowledge depends mainly on the statement of Thudichum, who from a study of the products of hydrolysis gives to it the formula $C_{52}H_{104}O_9N_2P$. The molecular weight of sphingomyelin would be, accordingly, 931.

The three substances which can be obtained from protagon therefore have molecular weights of such an order of magnitude that, if they follow Raoult's law, a definite elevation of the boiling point should be noticeable in solutions of moderate concentrations (3 %–5 %) of these substances, especially if one uses a solvent with a relatively high constant such as chloroform. One gram of a substance with the molecular weight 1000 would, in a 4 % solution in chloroform, elevate the boiling point by about 0·10°. If therefore protagon were a mixture of these substances it should, with the concentrations given, produce a distinct elevation of the boiling point of chloroform.

On the other hand, if protagon contained these substances in chemical combination it would have a molecular weight at least approximating to 3000. It may, of course, be considerably higher; in fact Cramer calculated from the amount of sulphur in protagon a molecular weight of 5778. If therefore protagon were a combination and not a mixture of cerebrosides and phosphatides the boiling point of a 3 % to 5 % chloroform solution of protagon should show either no elevation at all of the boiling point of chloroform or only a very slight one compared with that produced by cerebrin, homocerebrin, sphingomyelin or mixtures of these substances.

Observations on the boiling point of chloroform solutions of these substances by means of Beckmann's method will therefore give a final and decisive answer to the question whether protagon is a chemical combination or a mixture of phosphatides and cerebrosides. And since with substances of high molecular weights the determination of the molecular weights can only be approximate, it may be pointed out that this answer is not dependent on the exact numerical evaluation of the molecular weights nor upon slight quantitative differences between them, but upon differences of such an order of magnitude as to be qualitative.

Experimental.

The observations were made by Beckmann's method with an apparatus having an electrical heating device. The amount of chloroform used was measured in every case by volume, not by weight, and amounted to 25 cc. The chloroform was "chloroform from chloral" except in one case when "chloroform from acetone" was used. In almost every case a reading of the boiling point of the pure solvent was taken both before and after the experiment in order to be able to correct for changes due to variations in

barometric pressure. The boiling point of a solution was taken as the point where three successive readings at intervals of five minutes gave constant results.

Protagon was prepared from ox brain by the method of Wilson and Cramer: after completely exhausting the fresh minced brain tissue by repeated extraction with cold acetone and cold ether at room temperature the residue was extracted rapidly with boiling alcohol. Two alcohol extracts only were made. The white precipitate, obtained on cooling the alcoholic extract, was twice recrystallised. No more than ten ox brains were worked up at any one time. The process was repeated with new quantities of fresh ox brains until about 12 g. of dry protagon had been obtained in the form of a snow-white pulverulent powder. It is not advisable to work with large quantities of brain tissue.

Part of the protagon obtained in this way was used for the preparation of cerebrin and homocerebrin, by boiling it with baryta water. This method, which was used originally by Parcus and by Kossel and Freytag, has been re-introduced again recently by Lorrain Smith and Mair [1910] and by Loening and Thierfelder [1911]. Protagon is made into a fine emulsion with saturated baryta water, heated under a reflux condenser in a vigorously boiling water bath for one hour and the mixture filtered. The residue, after boiling with a mixture of alcohol and acetone, is repeatedly extracted with boiling acetone from which on cooling a mixture of cerebrin and homocerebrin separates out. The mixture thus obtained was recrystallised twice from boiling acetone and was obtained eventually in the form of a white powder more granular in appearance than protagon.

Since it was the object of these observations to compare the behaviour of protagon with that of a mixture of the cerebrosides which can be prepared from it, the separation of the two cerebrosides was not carried out. The observations made with protagon, and with the mixture of cerebrin and homocerebrin respectively, are recorded in Tables I and II.

These observations show clearly that the size of the protagon molecule is very much larger than that of the molecules of cerebrin and homocerebrin. Indeed the protagon molecule is so large that even in concentration exceeding 5% it fails to produce an elevation of the boiling point of chloroform. Now it is possible to calculate from the amount of galactose liberated from protagon, cerebrin, and homocerebrin respectively that *one gram* of protagon contains 0·6 to 0·7 gram of cerebrosides and 0·4 to 0·5 gram of sphingomyelin. Even if one assumed that sphingomyelin had a very much larger molecule

than the data of Thudichum would indicate, or if one assumed that for some reason or other it did not follow Raoult's law, perhaps by aggregating in solution into a colloidal form,—even then the elevation of the boiling point produced by *one gram* of protagon ought to be at least as great as that produced by 0·6 to 0·7 gram of the cerebrosides obtained from it. As a matter of fact, however, 0·7 gram of a mixture of cerebrin and homocerebrin

TABLE I.

Boiling point of chloroform solutions of protagon.

No. of experiment	Volume of chloroform in cc.	Weight of substance in solution	Boiling point	e	Barometer
		—	59·62		
I.	25	0·6664	59·62	0	734
		—	59·62		
		—	59·92		
II.	25	0·9730	59·92	0	742
		—	59·92		
		—	60·50		
III.[1]	25	0·9430	60·45	−0·05	not read
		1·5920	60·45	−0·05	
		—	60·50		

[1] In observation No. III chloroform from acetone was used.

TABLE II.

Boiling point of a chloroform solution of a mixture of cerebrin and homocerebrin.

No. of experiment	Volume of chloroform in cc.	Weight of substance in solution	Boiling point	e	Barometer
		—	59·50		
		0·7305	59·53	+0·03	
IV.	25	1·5140	59·63	+0·13	728
		—	59·50		

is sufficient to produce a distinct elevation of the boiling point of 25 cc. of chloroform, while even double the quantity of protagon is incapable of producing that effect. It follows, then, that the observations recorded in Tables I and II are incompatible with the view that protagon is a mixture of sphingomyelin, cerebrin and homocerebrin.

It will be noted that the elevation of the boiling point produced by cerebrin and homocerebrin increases with increasing concentration out of proportion to the amounts of substance added. This irregularity has no

bearing on the problem under discussion but it is of some general interest since a similar phenomenon has been observed in the case of other substances with large molecules, for instance in the case of colloidal solutions of silicic acid, tungstic acid and molybdic acid. It will also be noted in one case (Experiment No. III) that the boiling point of a chloroform solution of protagon instead of remaining constant showed a slight diminution, which, however, was not increased on adding more protagon. Since in this case a less pure sample of chloroform ("chloroform from acetone") was used, the irregularity may probably be referred to this fact.

Although a chloroform solution of protagon shows no elevation of the boiling point, yet the presence of protagon does not hinder the boiling point of chloroform being affected by the addition of another substance. This is shown by the fact that the addition of a weighed quantity of naphthalene to 25 cc. of a chloroform solution containing 0·6664 gram of protagon produced an increase of the boiling point commensurate with its molecular weight, 128. Thus the addition of 0·4240 g. naphthalene gave an elevation of the boiling point $e = 0{\cdot}37°$, molecular weight calculated, 119. The further addition of 0·4306 g. naphthalene produced a further elevation, $e = 0{\cdot}37°$, molecular weight calculated, 121.

In order to compare the molecular size of sphingomyelin with that of cerebrin, homocerebrin, and protagon, sphingomyelin was prepared by heating crude protagon with pyridine to 50° for 20 minutes. The precipitate which falls out on cooling the pyridine is removed by filtration. After washing with cold alcohol and drying in vacuo it had a phosphorus percentage of 2·3 %; it represents according to Rosenheim and Tebb [1910] sphingomyelin with a slight admixture of cerebrosides. The pyridine filtrate from sphingomyelin when poured into an excess of acetone gives a precipitate consisting of cerebrosides with a slight admixture of sphingomyelin. The sphingomyelin obtained in this way was not freed from its slight admixture of cerebrosides, since the object of these observations is to obtain comparative values for protagon on the one hand, and the substances or mixtures of the substances which can be obtained from it, on the other.

The observations made with sphingomyelin prepared in this way are given in Table III. It will be seen that sphingomyelin produces a distinct elevation of the boiling point of chloroform, thus indicating that the size of the molecule of sphingomyelin is of about the same order of magnitude as that of cerebrin and homocerebrin and very much smaller than that of protagon. The observations with sphingomyelin, therefore, confirm the

conclusions arrived at from the observations with cerebrin and homocerebrin.

The preparation of sphingomyelin and the cerebrosides from protagon by heating the latter with pyridine made it possible to compare protagon with a mixture of sphingomyelin and cerebrosides in approximately the

TABLE III.

Boiling point of chloroform solution of sphingomyelin (with slight admixture of cerebrosides).

No. of experiment	Volume of chloroform in cc.	Weight of substance in solution	Boiling point	ε	Barometer
		—	60·56		
V.	25	0·6842	60·60	+0·04	754
		—	60·35		
VI.	25	1·0948	60·42	+0·07	752

TABLE IV.

Boiling point of a chloroform solution of a mixture of sphingomyelin, cerebrin and homocerebrin.

No. of experiment	Volume of chloroform in cc.	Weight of substance in solution	Boiling point	ε	Barometer
		—	60·35		752
		1·0948 (sphingomyelin)	60·42	+0·07	
VII.	25	+ 0·7210 (cerebrosides)	60·45	+ +0·03	
		+ 0·7196 (cerebrosides)	60·47	+ +0·02	
		—	60·25		749

Note that in this table the changes in the elevation of the boiling point produced by the successive addition of sphingomyelin and cerebrosides are given separately with reference to each single addition and not collectively. For a detailed discussion see text.

same proportions in which they can be obtained from it. For that purpose the chloroform solution of sphingomyelin obtained in Experiment No. VI, Table III, was used. After having obtained a constant value for the boiling point of this solution the cerebrosides obtained by the pyridine treatment described above were added. The results are given in Table IV. There was a rapid fall of barometric pressure on the day on which this observation was carried out. Experiment No. V, Table III, was made on the forenoon of the

same day, Experiment No. VI, Table III, and the observations recorded in Table IV, on the afternoon of that day. It will be seen that the barometer fell from 754 mm. at 9 a.m. to 749 mm. at 3 p.m. There is, of course, a corresponding fall of the boiling point of the pure solvent. Since in the observations recorded in Table IV the boiling points of three different solutions were determined, one hour and a half elapsed between the beginning and the end of these observations, and even during that time a distinct fall in boiling point of the pure solvent was noticed.

Although the barometric variations would tend to diminish any elevation in the boiling point produced by the addition of cerebrosides, there is nevertheless clear evidence of a rise in the boiling point of the chloroform solutions of sphingomyelin on every addition of cerebrosides. Taking the last observations it will be seen that a mixture of about 1·1 gram sphingomyelin and 1·4 gram cerebrosides having a phosphorus percentage of 1 % corresponding to that of protagon produced a total rise of 0·22°. Of this rise 0·07 is due to the sphingomyelin present, and consequently a rise of 0·15 is due to the cerebrosides. This is approximately the same value as that obtained before in the observation recorded in Table II. In other words, the effect produced by a mixture of sphingomyelin, cerebrin and homocerebrin is simply the additive effect produced by the substances constituting the mixture. If protagon were a loose molecular compound of phosphatides and cerebrosides which could be reconstituted by simply mixing these substances together as has been suggested by some writers, the addition of cerebrosides to the solution of sphingomyelin should diminish the elevation of the boiling point produced by sphingomyelin. As a matter of fact the opposite takes place. The observations show then again that protagon is not a mixture of sphingomyelin, cerebrin and homocerebrin, but that it must contain these substances in chemical combination. They also show that it is not possible to reconstitute protagon by mixing together in certain proportions the cerebrosides and phosphatides which can be prepared from it.

Discussion.

The observations recorded in this paper show that the molecule of protagon is very much larger than that of cerebrin, homocerebrin or sphingomyelin. It follows therefore that protagon is not a mixture of these substances but holds them in chemical combination. It is also shown that the behaviour of a mixture of cerebrin, homocerebrin and sphingomyelin is

different from that of protagon and that it is not possible to reconstitute protagon by simply mixing together these simpler substances in certain proportions. This again shows that protagon is not a mixture of phosphatide and cerebrosides. It also shows that protagon is not a loose molecular compound of these substances which is formed when these substances occur together in solution.

While the observations recorded in this paper establish the fact that protagon is a chemical combination and not a mixture of cerebrosides and phosphatides, they do not answer the entirely different and much less important question whether protagon is only one such combination of phosphatides and cerebrosides or a mixture of several such combinations.

Although the essential difference between these two questions is obvious, it is necessary to emphasise it, because they have been confused in the past. Those who held with Gamgee that protagon is not a mixture of phosphatides and cerebrosides have been represented by their opponents as maintaining that protagon is not a mixture; they have been criticised for not bringing forward any evidence to prove that protagon is a single substance, and from their inability to do so it has been argued, not that protagon is a mixture, which might be logical, but that protagon is a mixture of phosphatides and cerebrosides, which is quite illogical. In this way the entire protagon question has become obscured and distorted. It is obvious that the fact that protagon is a chemical combination of cerebrosides and phosphatides does not exclude the possibility that protagon consists of a mixture of several such combinations. This possibility has in fact been pointed out by Kossel and Freytag and by Cramer. The question whether any complex substance isolated from tissues is a single substance or a mixture of homologous substances is as difficult to decide in the case of protagon as in the case of haemoglobin or any other protein. Even in the case of many phosphatides and cerebrosides it is not yet settled. Fortunately no considerations of great theoretical or practical importance depend on the question whether protagon is a single substance or a mixture of several protagons. But considerations of quite a different order of importance are involved in the question whether protagon is a mixture or a chemical combination of cerebrosides and phosphatides. This, however, does not appear to be clearly understood if one may judge from the statements frequently made in the literature, so that a brief indication of the significance of this question is called for.

If the views of Thudichum were correct, phosphatides and cerebrosides

must be considered to be the most complex forms of lipoids present in tissues. Many text-books actually represent the chemistry of lipoids in this way. Now phosphatides are lipoids containing phosphorus but no galactose; cerebrosides are substances containing galactose but no phosphorus. Any substance isolated from tissues which contains both phosphorus and galactose is, from this point of view, considered to be a mixture of phosphatides and cerebrosides and is, therefore, subjected to methods of purification, no matter how drastic, until substances either free from phosphorus or free from galactose are obtained. The substances obtained after such treatment are then considered to exist preformed in the tissues.

We find accordingly substances obtained by boiling brain with baryta water unhesitatingly accepted as preformed constituents of nervous tissue. But since it has been shown in this paper that it is possible to isolate from nervous tissue a substance having a larger molecule than either phosphatides or cerebrosides, and since this substance contains both galactose and phosphorus in its molecule, it is necessary to recognise the existence of a group of lipoids, more complex than either phosphatides or cerebrosides. Since in the case of protagon, which is the best known representative of this group, cerebrosides preponderate in the building up of the molecule, and since protagon in its physical properties resembles cerebrosides and is unlike the typical phosphatides, the term "phosphorised cerebrosides" or briefly "phospho-cerebrosides" as proposed by Cramer [1911] seems most convenient.

The observations recorded in this paper also furnish an answer to the much debated question whether protagon can be decomposed by the application of solvents such as alcohol and pyridine. As pointed out in the introduction, up to the present time the protagon controversy has centred entirely round this question. It has been shown that by the long repeated application of warm alcohol it is possible to prepare from protagon cerebrosides and phosphatides. The fact itself is not disputed; what is in dispute is the interpretation which is to be placed upon it. Thudichum and his followers deny that this process takes place under conditions involving the decomposition of protagon and they describe this process as a "fractional crystallisation." But their statements in regard to this point are always made dogmatically and no evidence is offered in support of them. Gamgee and his followers claim that protagon is easily decomposed by alcohol and Wilson and Cramer have shown that the conditions which make a separation of protagon into phosphatides and cerebrosides possible also produce a distinct change in the physical constants of protagon. More recently Rosenheim and Tebb have

used pyridine as a means of preparing the cerebrosides and sphingomyelin from protagon. Rosenheim [1913] describes the method as a separation by cold pyridine although the process involves a preliminary heating to 40° and claims again that pyridine is an inert solvent and that the process excludes the possibility of decomposition. Again no evidence is offered in support of this claim and it is clear that, if the possibility of a decomposition of protagon by alcohol is admitted, as it is now admitted by Rosenheim, a solvent like pyridine which has a strongly alkaline reaction is even more likely to bring about such a change.

The method of separation by pyridine has been used and described in this paper. The molecular weights of the substances obtained by this method have been examined both when isolated and when mixed together again in the proportions in which they were obtained from protagon. Since protagon has a very much larger molecule than the substances or mixtures of substances which can be obtained from it by the use of pyridine or alcohol, it is evident that the methods used for the preparation of cerebrosides and sphingomyelin from protagon involve a decomposition of protagon.

Summary.

Observations have been made by Beckmann's method on the elevation of the boiling point of chloroform produced by the addition of protagon on the one hand, and of the phosphatide (sphingomyelin) and the cerebrosides (cerebrin and homocerebrin) obtainable from protagon, on the other hand. The observations show that protagon has a very much larger molecule than the phosphatide and cerebrosides mentioned, either when separated or when mixed together. It follows that protagon is neither a mixture of these substances, nor a loose molecular compound formed when sphingomyelin, cerebrin and homocerebrin occur together in solution. The observations prove that protagon is a chemical combination of cerebrosides and a phosphorus-containing lipoid (sphingomyelin). It represents a group of lipoids more complex than either phosphatides or cerebrosides which is most suitably classified by the term "phospho-cerebrosides."

While these observations prove that protagon is a "phospho-cerebroside," *i.e.* a chemical combination of a phosphatide and cerebrosides and not a mixture of these substances, they afford no evidence with regard to the entirely different problem whether protagon is a simple "phospho-cerebroside" or a mixture of several "phospho-cerebrosides."

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References to the older literature are not given in this paper, as a complete reference to the literature on protagon and cerebrosides up to 1910 is given in the chapter by Cramer on Protagon, Cerebrosides and allied substances in the *Biochem. Handlexicon*.

LXII. NOTES ON REGULATOR MIXTURES, RECENT INDICATORS, ETC. II.

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(*Received Nov. 10th, 1914.*)

In preparing the second edition of this chart I have taken the opportunity, in these notes, of attaching to it recent additions to the data which it is intended to present.

The first chart [1910] owed its origin almost entirely to a paper by Sörensen [1909, 2]: the accompanying matter was explanatory, and gave in addition a technique for applying the indicator method of Friedenthal and Salm [1907] to coloured fluids, without the use of neutral dyes.

On this occasion I have been able to avail myself of a later monograph by Sörensen [1912] and also one by Michaelis [1914]. In these are collected an invaluable store of information on the subject of indicators, solutions of standard reaction, the electrometric measurement of hydrogen ion concentration, and the properties of amphoteric electrolytes which form the foundations of so much biochemical work. In addition I have included the results of some determinations in these laboratories which are here published for the first time; and data from the general literature which are not to be found in either of the sources mentioned.

INDICATORS.

Confusion arises in descriptions of indicators and their relative practical utility because they are regarded by different authors from one or other of two essentially different points of view.

In earlier books, *e.g.* Cohn [1899] and Glaser [1901] and even in many contemporary papers indicators are described as "good" for certain titrations when their colour change occurs at a point where the reacting

substances are in chemically equivalent proportions. It is natural, therefore, that phenolphthalein should be described as "no good with ammonia," but admirable for titrating acetic acid with caustic soda.

There is, however, an alternative standard whereby an indicator may be judged, *i.e.* the fidelity with which it, by its tint, represents the [H] of a solution into which it is introduced.

From this standpoint these notes have been written and an indicator will be the more highly valued the less its accuracy is impaired by the presence of protein, neutral salts or other condition of practical expediency. It may be remarked in passing that phenolphthalein works perfectly well as a quantitative indicator of reaction over its sensitive range even in solutions containing ammonia. The following experiment may be cited:

20 cc. of Sörensen borate solution (NaH_2BO_3) containing phenolphthalein gave against the 0·10 N calomel electrode, with saturated KCl as connecting fluid, at 13°, a hydrogen E.M.F. 0·8630 volt ($P_H^+ = 9{\cdot}26$).

20 cc. 1 per cent. ammonium chloride solution containing the same amount of phenolphthalein +0·95 cc. N NaOH gave a liquid having the same tint. Its hydrogen E.M.F. was 0·8620 volt at 14° ($P_H^+ = 9{\cdot}21$).

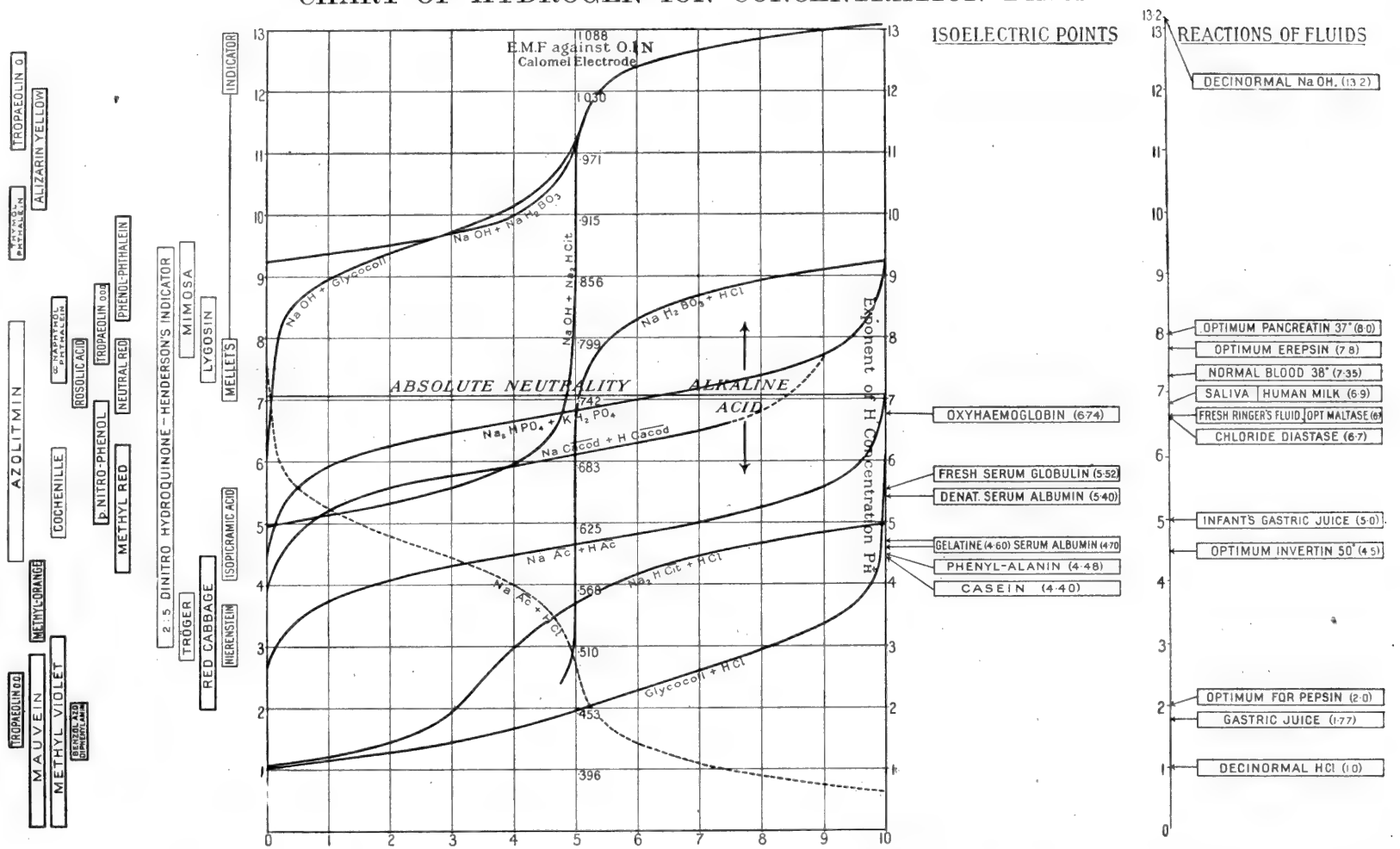
As an extreme example of those dyes which may give misleading and useless results when used as *indicators of reaction* may be mentioned *alizarin-sulphonic acid* which is even more accurate than methyl orange, when used to titrate distillates from the Kjeldahl process, especially when artificial light is used.

Adding equal quantities of it to 10 cc. each of the following Sörensen standard mixtures,

P_H^+	
7·66	(9 alk. phosphate + 1 acid phosphate) (5·26 borate + 4·74 HCl)
8·30	(9·74 alk. phosphate + 0·26 acid phosphate) (0·30 NaOH + 9·70 glycocoll) (6 borate + 4 HCl)
9·24	(1·68 NaOH + 8·32 glycocoll) (10 borate)
9·63	(2·74 NaOH + 7·26 glycocoll) (2·74 NaOH + 7·26 borate)
10·00	(4 NaOH + 6 borate),

it was seen that the dye behaved in an abnormal fashion, giving colours increasing in depth from yellow to brown, with increasing alkalinity, in the solutions containing boric acid. In the other solutions it gave purple colours

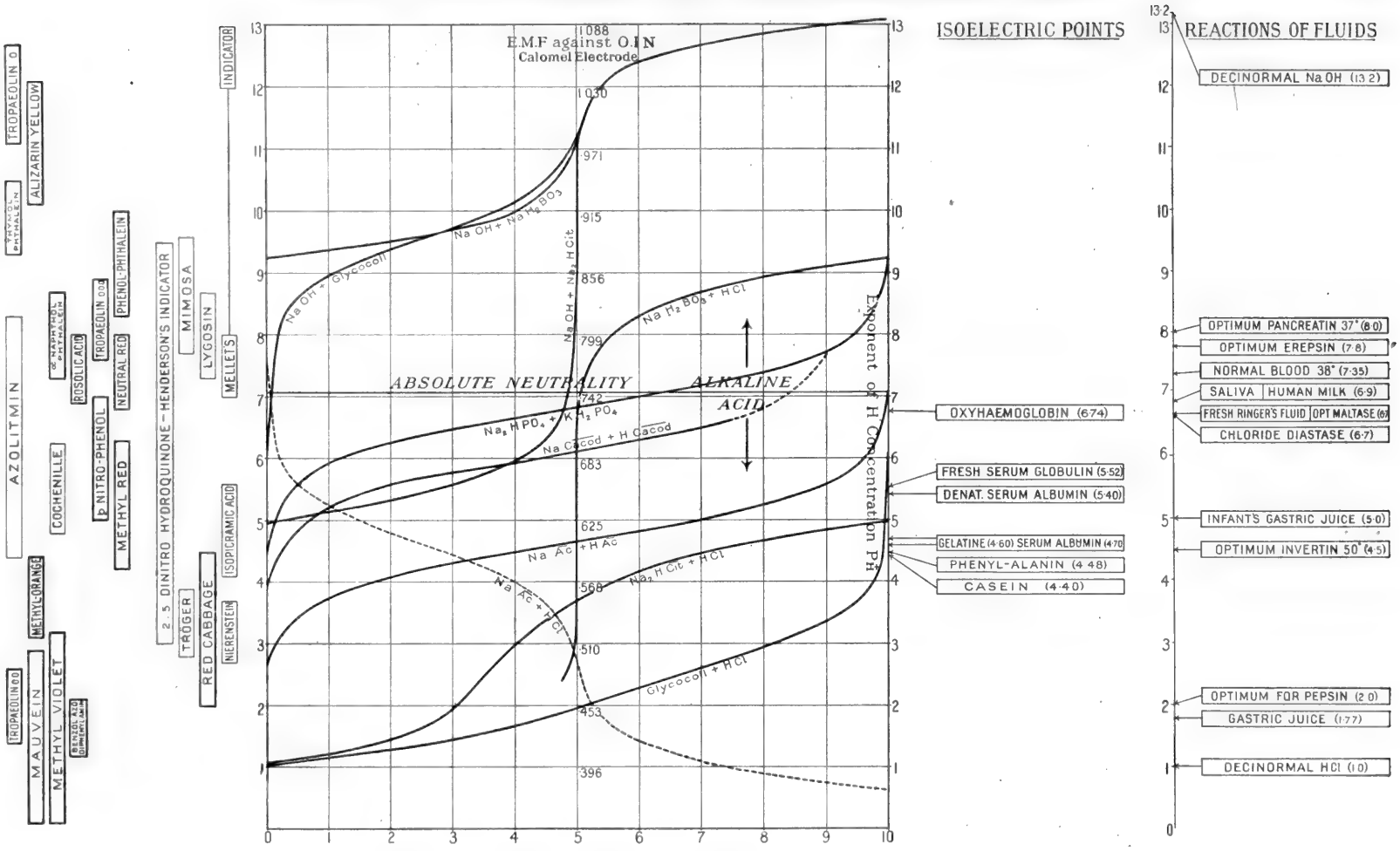
CHART OF HYDROGEN ION CONCENTRATION DATA



PARTS IN TEN OF MIXTURE OF "MORE ALKALINE" OR "LESS ACID" CONSTITUENT
except in the case of the dotted curve ($NaAc + HCl$).

The Sörensen solutions are: 0·10N HCl; 0·10N NaOH; 7·505 g. glycocoll + 5·85 g. NaCl per litre; 11·876 g. $Na_2HPO_4 . 2H_2O$ per litre; 9·078 g. KH_2PO_4 per litre; 21·008 g. citric acid in 1 litre of 0·20N NaOH; 12·404 g. boric acid in 1 litre 0·10N NaOH. The other solutions are: 0·20N sodium acetate, acetic acid, sodium cacodylate, cacodylic acid. For details of plotting of dotted acetate-HCl curve, see notes.

CHART OF HYDROGEN ION CONCENTRATION DATA



except in the case of the dotted curve ($NaAc + HCl$).

The Sörensen solutions are: 0·10N HCl; 0·10N NaOH; 7·505 g. glycocoll + 5·85 g. NaCl per litre; 11·876 g. $Na_2HPO_4 . 2H_2O$ per litre; 9·078 g. KH_2PO_4 per litre; 21·008 g. citric acid in 1 litre of 0·20N NaOH; 12·404 g. boric acid in 1 litre 0·10N NaOH. The other solutions are: 0·20N sodium acetate, acetic acid, sodium cacodylate, cacodylic acid. For details of plotting of dotted acetate-HCl curve, see notes.

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increasing in intensity in a normal and regular fashion with the P_H^+ of the solution.

Such dyes as this, therefore, though they may be used for particular purposes under conditions thoroughly understood, merit no place in any list of approved indicators for H· concentration measurement.

Sörensen made preliminary tests of over 100 indicators but discarded so many (pyrogallol-phthalein, azo-acid-blue, chrysamine, alizarinsulphonic acid, etc.) that only 36 find places in his tabulated results. Of these he was able to recommend 20 for general use. It is significant that neither cochenille, Congo-red, alizarin nor any litmus preparation is included in the list.

In the chart, I have inserted as many as possible of these 20 approved indicators—marked specially—and, in addition, litmus (azolitmin) because it is so much used, and several others which are of interest at present in view of their recent description or for other reasons.

The list of indicators here given, when added to those in the monograph by Sörensen [1912], brings complete to the end of 1914 the whole of the indicators of which notes have been published in the available literature. In cases where a sample of the material could be obtained a test has been made of its range of sensitiveness if this had not been already done. My thanks are due to those authors who have kindly sent me specimens of their preparations. Rectangles representing the sensitive range of some of these indicators are to be found in the chart grouped immediately on the left-hand side of the curves. Where a heavy line has been used in drawing the rectangle the indicator whose name is enclosed has been approved for the colorimetric method.

2 : 5-*Dinitroquinol* [Lawrence G. Henderson and Alexander Forbes, 1910]. This indicator, obtainable from Schuchardt, is extremely useful, as it gives at a glance the approximate reaction of any solution from $P_H^+ = 3$ to $P_H^+ = 9{\cdot}5$. Over this extremely long range it shows a steady change of colour. In dilute acid it is green. If the solution be made progressively more alkaline it commences to assume a yellowish tinge when $P_H^+ = 3$, at absolute neutrality it is a bright reddish brown; when $P_H^+ = 9{\cdot}5$ a purple tint is reached which undergoes no further change with the addition of caustic alkali. The change from brown to purple is very sharp between $P_H^+ = 8{\cdot}3$ and $P_H^+ = 9{\cdot}3$: so is that from green to brown in the region $P_H^+ = 3{\cdot}4$ to $4{\cdot}7$. The convenience of having at hand an indicator working over the ranges of methyl orange, litmus and phenolphthalein will be readily appreciated.

Di-o-hydroxystyryl ketone (*Lygosin*) $CO(CH:CH.C_6H_4.OH)_2$ [Feren Aron, Kolosvar, 1913]. The sodium salt of this indicator is marketed b the Zimmer Factory under the name "natrium lygosinatum." For th sample supplied to me I am indebted to Prof. Aron. I found the range o its change of colour from brownish yellow to green to be exactly that o α-naphthol-phthalein ($P_H^+ = 7{\cdot}3$ to $8{\cdot}7$)—a comparative experiment wit both indicators using the same regulator mixtures confirms this. If mor concentrated solutions of indicator are used the colour in alkaline solutio is the brown-reddish tinge described by the author. Its behaviour wa examined in the series of solutions detailed on page 629. It behaved quit normally except that it was bleached, losing colour entirely after a few hours in every series *except* those containing borate. In solutions containin borate it appeared to retain its colour indefinitely.

1-*Oxy-naphtho-chinomethane* [M. Nierenstein, Bristol. Private communication]. A sample of this indicator was kindly submitted by Dr Nierenstein for examination and its sensitive range was determined. It is colourless in solutions $P_H^+ = 2{\cdot}7$, showing a gradual increase of purple colour to $P_H^+ = 3{\cdot}7$. It is similar therefore to some of the azo indicators described by Sörensen but is of a sharper colour change than any indicator previously described which changes in this region.

6-*Sulpho-α-naphthol*-1-*azo*-m-*hydroxybenzoic acid* [M. R. Mellet, Lausanne, 1910, 1911, 1913]. The sodium salt is a violet black powder very soluble in water. In excess of alkali it is cherry red passing on the addition of acid through blue violet to bright red. Its colouring power is intense, so that quite brilliantly coloured solutions can be used without loss of accuracy in the result. The orange blue change is observed over the range $P_H^+ = 7$ to $P_H^+ = 8$: the second change, violet to red, is seen in solutions comparatively very alkaline—$P_H^+ = 12$ to $P_H^+ = 13$. I am indebted to Prof. Dr Mellet, who informs me that his indicator is still under examination, for a sample with which I have been able to confirm his earlier results.

2:6-*Dinitro*-4-*aminophenol* (*Isopicramic Acid*) [Raphael Meldola and A. J. Hale, 1912]. An alcoholic solution of this indicator is used. From the results of titrations using solutions accurately prepared gravimetrically the authors concluded that "although useless for weak acids it is possibly better than methyl orange for alkalis, of equal sensitiveness to litmus, and better than methyl orange for carbonates."

Using a sample supplied to me by Prof. Meldola I found that in solutions

more acid than $P_H^+ = 4{\cdot}1$ its colour was pink and in solutions more alkaline than $P_H^+ = 5{\cdot}6$ its colour was uniformly yellow. At intermediate reactions the changes of tint are reminiscent of methyl orange.

A sulphonic acid $C_{14}H_{15}N_4SO_3H$ [Julius Tröger and W. Hille, Brunswick, 1903]. This indicator, soluble in water, is not perfectly stable, so that a fresh solution must be prepared from time to time. Like methyl orange it is destroyed rapidly by strong alkali. It is described by the authors as being more sensitive than helianthin (methyl orange) though in its scope of application and colourations it resembles it. The sample supplied to me by the author showed the following colour changes: $P_H^+ = 2{\cdot}78$ orange pink; 2·93 ditto, but much more intense; 3·12 pale yellowish pink; 3·42 very pale brown; 3·76 yellow; 3·92 primrose yellow; 4·00 and upwards greenish primrose yellow. The range of this indicator is entered on the chart as $P_H^+ = 2{\cdot}8$ to $P_H^+ = 3{\cdot}9$. With the same regulator mixtures the methyl orange range—using two samples, one "commercial" and the other purified by repeated recrystallisation—$P_H^+ = 3{\cdot}1$ to 4·4 was confirmed. The conclusions to be drawn from my measurements are that it differs little from methyl orange in sensitiveness, and that its sensitive range is more acid than that over which methyl orange shows its colour changes.

Alizarinmonosulphonic acid [Geo. E. Knowles, 1907; compare Glaser, 1901]. This indicator, to be found amongst those described by Glaser, was discarded by Sörensen [1909, 1, 2] as being useless for the Friedenthal and Salm colorimetric method. Knowles states that it is a good substitute for methyl orange and that it can be used by artificial light. In very dilute solution I found that a sharp colour change took place between $P_H^+ = 3{\cdot}7$ (yellow) and $P_H^+ = 4{\cdot}2$ (pink). A direct comparison showed that it was even more sensitive than methyl orange over this range. Its curious behaviour with borates was noted by Knowles, and has been referred to on page 629.

3-*Amino*-2-*methylquinoline* [O. Stark, Kiel, 1907] is described as an excellent indicator which can replace methyl orange.

Compound obtained when 1 *molecule diazotised* p. *nitroaniline* + 1 *molecule of* 2-*aminonaphthol*-5: 7-*disulphonic acid is boiled with benzaldehyde, hydrochloric acid and water* [J. R. Woods, 1905]. According to the author this indicator—colourless in acid; orange in alkali—is sensitive to CO_2. This suggests a range somewhere on the alkaline side of absolute neutrality.

Dimethyl brown [Emmanuel Pozzi-Escott, 1909]. Yellow in alkaline; brown in neutral and acid solutions.

An indicator of formula $C_{11}H_8O_4$ [H. J. H. Fenton, 1906]. This indicator, and derived substances which may also be used as indicators, would appear, from the author's statements, to be of greater importance as specific reagents for amines than instruments for H˙ concentration measurement.

Indicators from vegetable sources. The discovery of a valuable indicator in red cabbage and the continued use of litmus to-day suggest attention to the accounts of indicators from natural sources. Of those noted below I was able to examine the alcoholic extracts of radish skins and mimosa flowers.

Extract of red cabbage [L. E. Walbum, Copenhagen, 1913, 1, 2]. This indicator is valuable as it works accurately in the presence of proteins, neutral salts, toluene, etc. Its sensitive range is from $P_H^+ = 2$ to $P_H^+ = 4{\cdot}5$.

Radish skins [J. F. Sackur, 1910]. The radish skins were extracted with their own weight of 96 per cent. alcohol. The colour changes observed in this case in passing from normal acid to normal soda were so bewildering in number as to surpass Henderson's indicator in this respect. In addition, the colours changed rapidly although retaining their brilliance and at certain reactions showed a dichroism unequalled by any other solution I have seen not excluding eosin. The use of this extract as an indicator is attended with difficulty on account of the brilliance of these phenomena.

Mimosa flowers [Lucien Robin, 1904]. Yellow dye from mimosa flowers. Its colour change, sensitive range $P_H^+ = 7{\cdot}7$ to $9{\cdot}6$, and behaviour with borates are so strikingly reminiscent of turmeric as to suggest the identity of the colouring matter in the two cases. The two dyes tested with the solutions given on page 629 show that the colour changes of neither depend solely on the H˙ concentration even when borate is not present. In the borate tubes they show brown to black colours of increasing intensity with increasing alkalinity though after some hours this colour fades but at unequal rates, leaving the greatest intensity at $P_H^+ = 9{\cdot}24$.

Iris flowers [A. Ossendowsky, 1903]. Extract of iris flowers is red in the presence of acids; green in the presence of alkalis.

Black pansy flower [E. Pozzi-Escott, 1913]. Alcoholic infusion of crushed black pansy flower: red in the presence of mineral acids passing through bluish violet with successive alkali addition till it becomes green.

Juice of the blueberry [G. N. Watson, 1913]. Alkaline—olive green: acid—rose; "sensitive to CO_2. May be used instead of cochenille or litmus."

Inorganic indicators. There may also be mentioned two "inorganic" indicators descriptions of which have been found.

Cyanogen iodide [J. H. Kastle, 1903]. Cyanogen iodide, potassium iodide and starch are recommended as very sensitive to acids.

Bismuth oxyiodide [C. Reichard, 1912]. This indicator is reported as being not sensitive to CO_2, colourless in alkaline solution and in acid yellow.

SERIES OF MIXTURES OF STANDARD REACTION.

The hydrogen E.M.F.'s of these series have been in some cases very carefully determined. Except when the curves run steep vertically the mixtures they represent are "regulator mixtures," *i.e.* they may be subjected to addition of certain quantities of acids or bases with only minimal changes in $[H^{\cdot}]$.

The seven series originally worked out by Sörensen are plotted on the chart as before. The tables connecting the composition of individual mixtures of these series, their E.M.F.'s against the 0·1 N calomel electrode, and the P_H^+ values calculated therefrom are to be found in the sources mentioned [Sörensen, 1909, 1, 2]. Similar tables referring to the curves added to the original seven in this second edition of the chart are to be found below.

The abscissae of every curve on the chart refer to the volumes in ten of the mixture of the "more alkaline" or "less acid" constituent excepting only the curve which is drawn as a dotted line. This curve refers to mixtures made up in a different fashion as explained on page 637.

Cacodylic acid-sodium cacodylate mixtures. Michaelis and Davidsohn [1912] have employed mixtures of cacodylic acid and caustic soda to obtain solutions of high "reaction inertia" which are not so acid as those obtained by mixing acids of greater dissociation constants with their salts. The solutions are not very stable. The values given in the table below are only approximate but are sufficiently accurate to indicate the range over which these mixtures can be used. They were determined by the electrometric method in these aboratories at room temperature: saturated KCl was used as connecting fluid and no correction for contact potential was made.

Composition of mixture							E.M.F.	*t.*	P_H^+
10 cc. 0·2 N cacodylic acid			+ 0 cc. 0·2 N sodium cacodylate				0·5575	14	3·86
9	,,	,,	1	,,	,,	,,	0·6375	18	5·20
7	,,	,,	3	,,	,,	,,	0·6675	16	5·76
5	,,	,,	5	,,	,,	,,	0·6880	16	6·11
3	,,	,,	7	,,	,,	,,	0·7090	16	6·48
1	,,	,,	9	,,	,,	,,	?	—	?
0	,,	,,	10	,,	,,	,,	—	—	—

The solutions were made by taking successive portions of 20 cc. of a 6·9 per cent. aqueous solution of cacodylic acid (0·5 N), adding 0, 1, 3 cc. of 0·5 N NaOH and then diluting to 50 cc. in each case [compare Michaelis, 1914, p. 186].

Mixtures of acetic acid and sodium acetate,—total acetate 0·20 *normal.* Solutions of this type give hydrogen potentials which change only slightly on dilution. They may be calculated approximately from their composition by a simple formula. Results of such calculations are given by Sörensen [1912] and Michaelis [1910, 1914]. The accurate experimental results for the particular series in which the total acetate is 0·20 normal are given herewith. In the original communication [Walpole, 1914, 1, 2] are given data relating to the extent to which the hydrogen potentials of these solutions change on dilution. The correction for diffusion potential for these solutions, whether diluted or not, is negative and in no case exceeds 1·5 millivolt. It is generally much less.

Acetic acid-sodium acetate mixtures,—total acetate 0·20 *normal.*

Composition of mixture						E.M.F. (fully corrected) against 0·10 N electrode	P_H^+
0·125 cc.	0·20 N	acetic acid +	9·875 cc.	0·20 N	sodium acetate	0·7138	6·518
0·25	,,	,,	9·75	,,	,,	0·6961	6·211
0·375	,,	,,	9·625	,,	,,	0·6853	6·024
0·5	,,	,,	9·5	,,	,,	0·6778	5·894
1	,,	,,	9	,,	,,	0·6593	5·574
1·5	,,	,,	8·5	,,	,,	0·6478	5·374
2	,,	,,	8	,,	,,	0·6393	5·227
2·5	,,	,,	7·5	,,	,,	0·6316	5·093
3	,,	,,	7	,,	,,	0·6256	4·990
4	,,	,,	6	,,	,,	0·6148	4·802
5*	,,	,,	5	,,	,,	0·6046	4·626
6	,,	,,	4	,,	,,	0·5947	4·454
7	,,	,,	3	,,	,,	0·5841	4·272
8	,,	,,	2	,,	,,	0·5712	4·047
9	,,	,,	1	,,	,,	0·5525	3·723
9·25	,,	,,	0·75	,,	,,	0·5450	3·592
9·5	,,	,,	0·5	,,	,,	0·5348	3·416
9·6	,,	,,	0·4	,,	,,	0·5290	3·315
9·7	,,	,,	0·3	,,	,,	0·5225	3·202
9·75	,,	,,	0·25	,,	,,	0·5193	3·147
9·8	,,	,,	0·2	,,	,,	0·5155	3·081
9·85	,,	,,	0·15	,,	,,	0·5105	2·994
9·9	,,	,,	0·1	,,	,,	0·5057	2·912
9·95	,,	,,	0·05	,,	,,	0·4995	2·804
10	,,	,,	—	,,	,,	0·4931	2·696

The mixture marked * is "standard acetate."

Hydrochloric acid-sodium acetate series,—total acetate 0·20 *normal—total sodium* 0·20 *normal.* This series, like the one preceding, has been thoroughly worked out. It has been extended, however, so that it may be conveniently considered in two parts. The first, obtained by adding 0, 1, 2....5 cc. of N hydrochloric acid to 5 cc. N sodium acetate and making up to 25 cc. with water in each case, is the same series as the acetic-acetate series just described except that NaCl is added to each solution in amount equivalent to the acetic acid present. The result of this is to have the total sodium as well as the total acetate 0·20 normal, and each solution is for that reason just a little more acid than the corresponding solution of the series preceding. The second part is obtained by adding 5, 6, 7....10 cc. N hydrochloric acid to 5 cc. N sodium acetate and making up to 25 cc. with water in each case, and these solutions contain increasing quantities from 0 to 0·2 N of

Hydrochloric acid-sodium acetate series,—total acetate 0·20 *normal—total sodium* 0·20 *normal.*

Composition of mixture	E.M.F. (fully corrected) against 0·10 N electrode	P_H^+
0·10 cc. N HCl + 5 cc. N sodium acetate + water to 25 cc.	0·7015	6·31
0·25 „ „ „ „ „	0·6762	5·87
1 „ „ „ „ „	0·6375	5·20
1·5 „ „ „ „ „	0·6235	4·95
2 „ „ „ „ „	0·6126	4·76
2·5 „ „ „ „ „	0·6019	4·58
3 „ „ „ „ „	0·5908	4·39
3·5 „ „ „ „ „	0·5793	4·19
4 „ „ „ „ „	0·5654	3·95
4·25 „ „ „ „ „	0·5564	3·79
4·5 „ „ „ „ „	0·5461	3·61
4·625 „ „ „ „ „	0·5389	3·49
4·725 „ „ „ „ „	0·5297	3·33
4·75 „ „ „ „ „	0·5274	3·29
4·85 „ „ „ „ „	0·5159	3·09
4·875 „ „ „ „ „	0·5129	3·04
4·975 „ „ „ „ „	0·4949	2·72
5 „ „ „ „ „	0·4902	2·64
5·097 „ „ „ „ „	0·4715	2·32
5·222 „ „ „ „ „	0·4550	2·03
5·25 „ „ „ „ „	0·4525	1·99
5·35 „ „ „ „ „	0·4442	1·85
5·475 „ „ „ „ „	0·4372	1·72
5·5 „ „ „ „ „	0·4362	1·71
6 „ „ „ „ „	0·4194	1·42
6·5 „ „ „ „ „	0·4090	1·24
7 „ „ „ „ „	0·4007	1·09
8 „ „ „ „ „	0·3904	0·91
9 „ „ „ „ „	0·3812	0·75
10 „ „ „ „ „	0·3750	0·65

hydrochloric acid in addition to acetic acid and sodium chloride with respect to each of which they are all 0·2 N.

It is necessary in preparing solutions near the middle of this series to standardise the sodium acetate and the hydrochloric acid solutions against each other carefully—and an electrometric method has been devised for this purpose—and in their use to recognise that although the range of possible change of hydrogen potential under ordinary conditions is minimal the "reaction inertia" of such solutions is small. The figures below are taken from the paper referred to.

Care must be taken not to consider more or less concentrated solutions of weak acids as regulator mixtures. The addition of very little strong base leads to the freeing of so many of the anions of the weak acid that its dissociation is reversed with the consequent fall in [H˙], *e.g.* 1 cc. of ·01 N NaOH added to 20 cc. 0·2 N acetic acid will change the P_H^+ value by 0·11.

As the addition of strong base proceeds, however, the reaction inertia rapidly increases until an excellent regulator mixture is obtained. The shape of each of the curves is, in fact, an index of the reaction inertia of the individual members of the series of mixtures the curve represents: the tangent to the curve at any point might well be taken as the measure of the reaction inertia of the mixture represented by that point if a factor were included representing the normality of its constituents so that mixtures of different series should be comparable one with another.

ISOELECTRIC POINTS.

These data are taken from the papers of Michaelis and his collaborators. The actual figure taken in each case is that which had been obtained by the transport method.

Oxyhaemoglobin, $P_H^+ = 6{\cdot}74$ [Michaelis and Takahashi, 1910; Michaelis and Davidsohn, 1912].

Fresh serum globulin, $P_H^+ = 5{\cdot}52$ [Michaelis and Rona, 1910, 1, 2].

Denaturated serum albumin, $P_H^+ = 5{\cdot}40$ and serum albumin, $P_H^+ = 4{\cdot}7$ [Michaelis and Davidsohn, 1911, 2].

Gelatin, $P_H^+ = 4{\cdot}60$ [Michaelis and Grineff, 1912].

Caseinogen, $P_H^+ = 4{\cdot}4$ [Michaelis and Rona, 1910, 1, 2; Michaelis and Pechstein, 1912].

Phenylalanine, $P_H^+ = 4{\cdot}48$ [Michaelis, 1912].

OPTIMUM REACTIONS FOR ENZYMES.

These data, and much accompanying and explanatory matter, are for the most part collected in Michaelis' monograph [1914]. See also Sörensen [1912]. They do not all appear on the chart for lack of space.

The following references are to original papers.

Tryptic enzymes [Kurt Meyer, 1911; Palitsch and Walbum, 1912; Michaelis and Davidsohn, 1911, 1].

Invertin [Sörensen, 1909, 1, 2; Michaelis and Davidsohn, 1911, 3].

Peptic and Ereptic enzymes [Rona and Arnheim, 1913; Sörensen, 1909, 1, 2; Michaelis and Davidsohn, 1911, 4].

Lipase [Davidsohn, 1912, 1, 2; Rona and Bien, 1914, 1, 2].

Catalase [Sörensen, 1909, 1, 2; Michaelis and Pechstein, 1913; Waentig, Percy, and Steche, 1911].

Maltase [Michaelis and Rona, 1913, 1, 2].

Diastase [Michaelis and Pechstein, 1914; Norris, 1913, 1, 2].

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Saliva [Michaelis and Pechstein, 1914].

Gastric Juice [Fränkel, 1905; Michaelis and Davidsohn, 1910].

Infants' Gastric Juice [Allaria, 1908; Davidsohn, 1912, 2].

Intestinal Juice [Auerbach and Pick, 1912].

Blood [Hasselbalch and Lundsgaard, 1912].

Human Milk [Davidsohn, 1913].

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LXIII. THE ESTIMATION OF ALLANTOIN IN URINE IN THE PRESENCE OF GLUCOSE.

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It has been shown by us in a previous communication [1914] that the urea and allantoin in urine can be estimated by Folin's magnesium chloride method; the urea is estimated by means of urease and the difference between the two values is the amount of allantoin present. Glucose in the urine does not affect the determination of urea by means of urease, as shown by Marshall [1913], but it causes a loss of from 9–30 per cent. of the nitrogen of the urea when Folin's magnesium chloride method is used, as shown by Mörner [1903]. The humin which is formed by heating the glucose in urine with hydrochloric acid contains nitrogen [Udransky, 1888]. The same error applies to the estimation of allantoin. Mörner overcame the difficulty in the case of urea by precipitating the glucose with baryta mixture, alcohol and ether, and estimating the urea in the filtrate, but this procedure does not serve for the estimation of allantoin, since it is precipitated with the glucose [Mörner, 1903; Haskins, 1906].

Wiechowski's method for the estimation of allantoin in urine depends upon the precipitation of allantoin as mercury compound after pigments, chlorides and other compounds have been removed, and is not affected by the presence of glucose, but for this method large amounts of urine are required and the estimation takes several days to perform.

The Folin method if it could be adapted to the estimation of allantoin would be of advantage, in that only small quantities of urine are required, which is important when other estimations, *e.g.* of "acetone bodies," have to be performed, and it would be of further advantage in that the results are quickly obtained.

In order to make use of the Folin method it is essential that the glucose be removed from solution. This removal of glucose was found to be a more troublesome process than was anticipated. Several methods were attempted before it was found that the glucose could be completely precipitated by basic lead acetate and sodium hydroxide.

(1) *Removal of Glucose by Fermentation.*

The simplest way of removing glucose seemed to be by fermentation by yeast. Mörner had already tried this method and found it not to be entirely satisfactory. Some preliminary experiments indicated that this method might be possible. The estimation of urea and allantoin was carried out on 5 cc. of urine, and on 5 cc. of urine to which 5 cc. of a 5 per cent. solution of glucose had been added and removed by fermentation. Our data in cc. of 0·1 N ammonia were:

Urine	Urine + Glucose	Urine	Urine + Glucose
15·0 15·3 } 15·15	15·3 15·0 } 15·15	49·4 50·0 } 49·7	49·7 49·6 } 49·55

Further experiments did not give such satisfactory results:

16·2 16·3 } 16·25	15·9 15·3 } 15·7	53·2 53·1 } 53·15	52·2 51·6 } 51·9

It was possible that in these experiments the conditions for the fermentation were not suitable, and it seemed to us that if the conditions for complete fermentation could be found the removal of glucose by the action of yeast would be possible. Numerous experiments were made to test the rate of fermentation of glucose by yeast. 10 cc. of 1, 2, and 3 per cent. solutions of glucose in water were fermented with 1 to 10 cc. of 1 to 5 per cent. suspensions of yeast in water for periods of 6 to 20 hours; the solutions were filtered and the filtrates tested for glucose. The results were extremely variable, as is seen in the following summary:

Series of expts.	Per cent. of glucose solution	Per cent. of yeast suspension added	Number of cc. of yeast suspension required to ferment the glucose	Time of fermentation in hours
1	1	3	4	6
2	1	5	3	6
3	2	1	5	16
4	2	3	4	16
5	2	5	7	6
6	2	5*	5	17
7	2	5	7	17
8	3	3*	6	17
9	3	3	10	20
10	3	4*	6	17
11	3	4	6	20
12	3	5	6	20
13	3	5	10	17

* Small quantities of glucose still left unfermented.

The variability which is particularly noticeable in experiments 3 and 6, and 5 and 7, is mainly accounted for by the use of different samples of yeast, but even when the same sample of yeast was used and the fermentation carried out with the same quantity of glucose dissolved in urine the glucose was not always completely removed in the expected time.

Not only was the rate of fermentation unreliable, probably due to variations in the acidity of the urine, but also it was found necessary to make a correction for the amount of nitrogen introduced into the solution with the yeast.

Though the desired results can often be obtained, there is so much uncertainty in the removal of glucose by fermentation that the method is not a suitable one for practical use.

Some experiments were also made with zymin and with yeast dried according to v. Lebedeff's method and kindly given to us by Prof. Harden. The fermentation of the glucose was much slower, and a still greater correction for the amount of nitrogen introduced in the preparation had to be made.

(2) *Removal of Glucose by Benzoylation.*

Glucose and other carbohydrates give insoluble benzoyl esters when they are acylated by the Schotten-Baumann method. This method was therefore tested after adding glucose to urine and shaking the solution with varying quantities and excess of benzoyl chloride and sodium hydrate. In only one experiment out of 11 was the glucose completely removed. Further in the strongly alkaline liquid there is loss of ammonia from the urine. This method was therefore not serviceable.

(3) *Removal of Glucose by Precipitation with Cupric Hydroxide.*

Salkowski [1872, 1879] observed that glucose was completely precipitated in combination with cupric hydroxide when five molecules of copper sulphate and eleven molecules of sodium hydroxide were added for every molecule of glucose; Yoshimoto [1908] repeated these experiments and extended them to the precipitation of other carbohydrates. We can confirm the results with glucose; the glucose is completely precipitated if the proportions are exactly those given by Salkowski but not otherwise. A slight excess of alkali dissolves the copper hydroxide compound.

Owing to the sensitiveness of the precipitation towards alkali it was not anticipated that its application to the removal of glucose from urine of varying acidity would give the desired result. The precipitate with urine is of large bulk, the filtration is slow and the alkalinity of the solution leads to loss of ammonia during filtration. Glucose was generally found to be present in the filtrate. This method is again not applicable to urine.

(4) *Removal of Glucose with Lead Hydroxide.*

Glucose is not precipitated from solution by lead acetate; it is partially precipitated by basic lead acetate. It is also stated that glucose is removed from solution by basic lead acetate and ammonia, when these reagents are added to diabetic urine in the proportions used in the estimation of aceto-acetic acid and hydroxybutyric acid by Shaffer and Marriott's method [1913]. We have tested urine, to which glucose had been added, for glucose after such a precipitation and have found it still present, so that its precipitation under these conditions is not complete.

It would no doubt be possible to find the exact proportions of these reagents which would completely precipitate the glucose, but since for the purpose of estimating allantoin ammonia cannot be used we have used sodium hydroxide instead. Our experience with copper hydroxide suggested that the precipitation of glucose would be complete if the proportions were:

$$2C_6H_{12}O_6 : 5\,(Pb\,(OH)_2\,.\,Pb\overline{ac}_2) : 10NaOH,$$

and on testing these proportions it was found that the precipitation of glucose was complete. The filtrate from the precipitate shows no reduction or, in a few cases only, a very slight reduction of Fehling's solution.

This precipitation with lead hydroxide is not so sensitive to excess of sodium hydroxide as that with copper hydroxide. A slight excess of sodium hydroxide may be added, and in many experiments as much as 2·5 molecules

of sodium hydroxide were used, and the whole of the glucose removed. A larger excess of the sodium hydroxide leads to solution of the precipitate.

This method can therefore be applied to the estimation of allantoin in urine containing glucose.

The Effect of Basic Lead Acetate and Sodium Hydroxide upon Allantoin.

The estimation of allantoin in urine by the Folin method will be therefore possible if allantoin is not precipitated by the basic lead acetate and sodium hydroxide, or by the glucose lead hydroxide compound. Two series of experiments were therefore made to test if allantoin were so precipitated.

A. 25 cc. of approximately 0·1 N allantoin solution were treated with the amounts of basic lead acetate and sodium hydroxide required assuming that the solution contained 1, 2, 3 and 4 per cent. of glucose.

B. 25 cc. of the same allantoin solution were treated with the same quantities of basic lead acetate and sodium hydroxide in the presence of 1, 2, 3 and 4 per cent. of glucose.

In both cases the volumes were made up to 250 cc., the precipitates filtered off and the nitrogen estimated by Kjeldahl's method in 50 cc. (= 5 cc. of the original solution) of the filtrate. A total nitrogen estimation, C, was also made in 5 cc. of the original solution.

	A. cc. 0·1N NH_3	B. cc. 0·1N NH_3	C. cc. 0·1N NH_3
1 per cent.	2·6	—	2·7
2 ,,	2·6	2·2	
3 ,,	2·5	2·2	
4 ,,	2·5	2·2	
1 per cent.	2·5	2·45	2·75
2 ,,	2·35	2·3	
3 ,,	2·5	2·5	
4 ,,	2·45	2·4	

Though the amount of nitrogen in the filtrate was in all cases less than in the original solution, it cannot be concluded that allantoin is precipitated by basic lead acetate and sodium hydroxide. It is most probable that this difference is due to the precipitation of traces of impurity, such as uric acid, in the allantoin. It is unlikely that the same figure would have been obtained in the series of experiments in which quantities of basic lead acetate and sodium hydroxide varying from 14 to 56 cc. and 4 to 17 cc. respectively were used.

Application of the Method to Urine.

In applying this precipitation of glucose by basic lead acetate and sodium hydroxide to urine the ammonia present in the urine must be removed, as the reagent is alkaline and the ammonia is slowly evolved during filtration. The removal of ammonia presents no difficulty if it be effected by Folin's air current method. The solution which remains can be used for the allantoin estimation, the excess of sodium carbonate being neutralised with acetic acid, using phenolphthalein as indicator, and warming to remove the carbon dioxide before the precipitation of the glucose. Owing to the presence of phosphates, sulphates, pigments and other compounds in urine an excess of basic lead acetate above that required for the amount of glucose present must be added: 25 cc. of basic lead acetate and the corresponding amount of sodium hydroxide have been found to suffice for 25 cc. of dog's urine (when the 24 hours quantity has been diluted to 500 cc.).

In performing the hydrolysis by the magnesium chloride method with large quantities of liquid the water which distils into the special form of condenser is emptied out as required till the concentration is sufficient for the hydrolysis of the urea and allantoin.

The experimental details are as follows:

25 cc. of urine are treated with 1 gram of sodium carbonate and the ammonia estimated by Folin's method; it is not necessary to use paraffin or toluene to prevent frothing if tall cylinders are employed. The solution is washed into a 250 cc. measuring flask, and the sodium carbonate neutralised by carefully adding glacial acetic acid from a burette and warming. To the neutral solution 25 cc. of basic lead acetate + 14 cc. of basic lead acetate for every per cent. of glucose are added. These quantities require 6·2 + 3·4 cc. of 2N sodium hydroxide to precipitate the glucose and to remove any excess of lead acetate. It is advisable to determine the exact amount of 2N sodium hydroxide which is required to precipitate the lead hydroxide from the basic lead acetate by a special experiment, as in making up solutions of the basic lead acetate and 2N sodium hydroxide the solutions have never quite the same concentration.

The caustic soda is added slowly from a burette with constant shaking; the solution is made up to 250 cc. and the precipitate filtered off.

50 cc. of the filtrate should be tested for glucose before proceeding to the allantoin estimation, as it occasionally happens that the glucose is not completely removed. A slight reduction has no effect upon the result. If there

is considerable reduction the removal of the glucose must be repeated in another sample. We are unable to explain why the precipitation sometimes fails, but it seems most probable that it is due to improper neutralisation of the sodium carbonate.

50 cc. of the filtrate (= 5 cc. of urine) are used for the estimation of the urea + allantoin by Folin's magnesium chloride method.

We have tested the method upon urine to which glucose had been added with the following results, a control (= NH_3 + urea + allantoin) having been previously made; the figures are in cc. of 0·1 N ammonia:

Glucose added %	Reduction of filtrate	Urea + allantoin	NH_3 per 5 cc.	NH_3 + urea + allantoin	Difference
1·3	0	42·7	3·0	45·8	−0·1
2·0	+	—	3·0	45·8	—
3·0	0	42·4	3·0	45·8	−0·4
4·0	0	42·9	3·0	45·8	+0·1
1	+	—	1·2	36·2	—
2	slight	35·0	1·2	36·2	0
3	0	35·1	1·2	36·2	+0·1
4	0	34·5	1·2	36·2	−0·5
1·2	very slight	32·3	2·0	34·5	−0·2
2	,,	32·5	2·0	34·5	0
3	0	32·6	2·0	34·5	+0·1
4	0	32·8	2·0	34·5	+0·3
1	very slight	32·4	2·0	34·5	−0·1
2	0	32·0	2·0	34·5	−0·5
3	0	32·3	2·0	34·5	−0·2
4	slight	32·6	2·0	34·5	+0·1
1	very slight	32·5	2·0	34·5	0
2	,,	32·4	2·0	34·5	−0·1
4	,,	32·6	2·0	34·5	+0·1
5	0	32·5	2·0	34·5	0
1	+	—	2·1	39·3	—
2	0	36·8	2·1	39·3	−0·4
3	0	37·0	2·1	39·3	−0·2
4	0	36·8	2·1	39·3	−0·4

The maximum difference is − 0·5 cc., but in many of the experiments there was no difference: a difference of 0·4 cc. is often obtained in two parallel experiments with normal urine; it cannot be regarded as a great discrepancy, as the Folin method is a difficult one to perform.

Analyses of Diabetic Dog's Urine.

We have devised this method for the analysis of diabetic dog's urine and we have been fortunate in being able to use it upon the urine of a dog rendered diabetic by Prof. Starling. The analyses have been performed by

Mr W. W. Reeve, who has carried them out according to our directions. No difficulty was experienced in the removal of the glucose, though the solutions sometimes showed a slight reduction. The output of allantoin of a diabetic dog is greater than that of a normal dog upon a similar diet. The data in cc. 0·1 N ammonia per 5 cc. urine are the following:

Amount of glucose present in %	NH_3	NH_3 + urea	Urea + allantoin	Allantoin
1·0	1·2	29·1	28·8	0·9
5·6	1·4	30·8	31·9	2·5
1·2	0·9	10·8	12·3	2·4
4·5	1·5	28·5	29·0	2·0
4·0	1·5	24·4	25·2	2·3
3·0	2·4	34·1	35·8	4·1
5·8	2·1	35·9	36·4	2·6
3·8	2·9	37·6	38·4	3·7
6·0	2·2	35·7	36·4	2·9
3·0	2·4	32·5	33·7	3·6
3·5	2·9	35·9	36·7	3·7

The fact that these analyses have been made by an independent worker shows that the method is satisfactory.

Summary.

The estimation of allantoin in urine containing glucose can be effected by Folin's magnesium chloride method if the glucose be first removed. The glucose can be removed by precipitation with basic lead acetate and sodium hydroxide if the proportions are $2C_6H_{12}O_6 : 5\,[Pb(OH)_2 . Pb\overline{ac}_2] : 10NaOH$.

The expenses of this research and of the previous one upon the estimation of urea and allantoin have been defrayed by a grant to one of us from the Government Grant Committee of the Royal Society, to whom we desire to express our thanks.

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LXIV. THE GRAVIMETRIC ESTIMATION OF MINUTE QUANTITIES OF PHOSPHORUS.

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In the course of an investigation concerning certain of the phenomena of immunity, a method was required for the estimation of the ether-soluble phosphorus compounds of blood serum. The amount of serum available for the estimation was not more than 10 cc. and it was anticipated that only a few milligrams of the phosphorus compounds in question would be present. The isolation of this small amount and its direct weighing was therefore rejected as a possible method of estimation. An estimation of the phosphorus present appeared to be much more promising, since Neumann's method was known to be accurate for quantities of P_2O_5 as low as 10 to 15 milligrams. It was thought that by suitable modification (the use of decinormal instead of half normal solutions) this process could be used for smaller quantities of phosphorus. For the purpose of working out the method a standard solution of potassium dihydrogen phosphate solution was made such that 5 cc. contained 1 milligram P_2O_5. Addition to this of 10 cc. of the acid mixture (equal parts of nitric and sulphuric acids), and subsequent dilution to about 100 cc. made a solution comparable to one which would be obtained by the Neumann method after oxidation had been completed and the acid residue diluted. A solution prepared in this way was therefore used in all experiments when it was desired to test the applicability of the method to actual working conditions with an unknown substance. In order to obtain rapid and complete precipitation of the phosphorus as ammonium phosphomolybdate it has been found most satisfactory to neutralise a portion of the acid present with ammonia, to add ammonium nitrate and then precipitate with ammonium molybdate at 80°. The titration of this precipitate by the Neumann method using decinormal solutions was found to

be unsatisfactory. The results were from 10 to 20 per cent. too high, when 1 milligram of P_2O_5 was being estimated. On this account the method was rejected as unsuitable, a method with an error of less than 5 per cent. being required.

The next procedure tried was the lead molybdate method of Brearley and Ibbotson [1902], which was devised by them for the estimation of phosphorus in steel. The principle of the method is very ingenious. The ammonium phosphomolybdate precipitate is dissolved in ammonia and the molybdic acid in it is precipitated as lead molybdate, which is capable of being ignited and weighed. This lead molybdate precipitate weighs 142 times as much as the phosphorus originally taken, so that even a tenth of a milligram of phosphorus gives rise to a weighable amount of precipitate. On applying this method to the precipitate of phosphomolybdate obtained in the Neumann method, the results were constantly between 10 and 12 per cent. too high when 1 milligram of P_2O_5 was being estimated. It was found, however, that if the phosphorus was precipitated by ammonium molybdate from a solution containing only ammonium nitrate and nitric acid, no sulphuric acid being present, the lead molybdate method gave very accurate results. The presence of sulphuric acid thus appears to cause precipitation of a small but appreciable quantity of molybdic oxide along with the ammonium phosphomolybdate, and although this causes no marked error when upwards of 20 milligrams of P_2O_5 are being estimated by the Neumann method, the error is magnified when only 1 milligram of P_2O_5 is in question. To overcome this difficulty the ammonium phosphomolybdate precipitate was dissolved in ammonia and reprecipitated in the presence of nitric acid and ammonium nitrate. This proved quite satisfactory and yielded results in which the average error with 1 milligram of P_2O_5 was only about 2 per cent. It was discovered later that the above influence of sulphuric acid in the estimation of phosphorus by the phosphomolybdate method had been observed by Hibbard [1913]. This author also recommends reprecipitation to overcome the difficulty.

Certain other interesting facts have been observed in working out the method finally adopted.

In determining the best amounts of ammonium nitrate and nitric acid to use in reprecipitating the ammonium phosphomolybdate, it was found that although ammonium nitrate in considerable amounts favoured rapid precipitation, the results were always from 6 to 10 per cent. too high unless the amount of nitric acid was largely increased. This in its turn rendered

precipitation less rapid and incomplete. The effect of the ammonium nitrate is due to the ease with which it causes precipitation of molybdic oxide when insufficient nitric acid is present. A determination of the ideal amounts of ammonium nitrate and nitric acid required to produce complete precipitation of 1 milligram of P_2O_5 in 100 cc. of liquid within 20 minutes without concurrent precipitation of molybdic oxide was therefore made; these concentrations were then adhered to in the reprecipitation of the phosphomolybdate.

In addition, it was observed that the use of old ammonium molybdate solution in which a white crystalline deposit had appeared, gave rise to high results. On examination it was shown that such solutions gave rise to precipitation of molybdic oxide in the presence of amounts of nitric acid and ammonium nitrate which did not cause precipitation of molybdic oxide when fresh molybdate solutions were used.

Whilst this work was in progress two methods for the estimation of small quantities of phosphorus were published by Taylor and Miller [1914]. The first method is a modification of the Neumann titration method, and the second is a colorimetric method. In both cases the substance analysed is ashed in the dry condition and the phosphorus subsequently precipitated as ammonium phosphomolybdate. The first method is stated to be accurate to 2 per cent. with amounts of P_2O_5 as low as 1 milligram. The second method is less accurate but can be used for smaller amounts than the first. Both appear to be more cumbersome than the one described in the present paper. Further, it has been found possible by the dry ashing method (which is always preferable to the wet method if it can be adopted), followed by precipitation as ammonium phosphomolybdate and then as lead molybdate, to estimate 0·1 milligram of phosphorus with an error of only 3 per cent. In the following description of the method, the process of precipitating the lead molybdate is given almost exactly as described by Brearley and Ibbotson. An alternative method suggested by them, in which the precipitate contains the phosphorus as lead phosphate in addition to the lead molybdate, has not been found as accurate.

Solutions required.

1. Ammonium Nitrate 50 per cent.
2. Ammonium Acetate 50 per cent.
3. Ammonium Chloride 20 per cent.
4. Lead Acetate 4 per cent.
5. Ammonium Molybdate 10 per cent. (To be made with cold water.)
6. Standard solution of KH_2PO_4; 0·3833 grams per litre; 5 cc. = 1 milligram P_2O_5.

The substance is oxidised in the usual fashion with nitric and sulphuric acids in a Kjeldahl flask. With the small amounts of substance used in the present investigation, rarely more than 10 cc. of the acid mixture were required. The volume of acid left after the oxidation is estimated by pouring water from a measuring cylinder into a similar Kjeldahl flask. The contents of the flask are rinsed into a 500 cc. conical flask which has been marked at the 100 cc. level. 15 cc. of ammonium nitrate are added, and for every cc. of acid left after the oxidation, 0·75 cc. of strong ammonia (S.G. 0·880) in addition. The solution is diluted till the volume is about 100 cc. and is then heated to 80°. 10 cc. of ammonium molybdate are added and the contents of the flask are constantly agitated for about a minute and a half. If precipitation of the phosphomolybdate has not begun, less than one milligram of P_2O_5 is present and 5 cc. of the standard phosphate solution should be added, or precipitation will not be completed quickly enough. The contents of the flask are again shaken, then maintained at 80° for 20 minutes, shaking twice during this period, each time for a minute. The precipitated ammonium phosphomolybdate is filtered off by means of suction on a pulp filter made by shaking a 9 cm. Swedish filter paper with very dilute nitric acid (about 2 per cent.), and pouring the pulp a little at a time on to a 2 cm. perforated porcelain plate in a funnel of suitable size. A 3 inch funnel has been found most convenient. The pulp is well packed down, employing suction by means of a Buchner flask, and is finally pressed down and at the sides with the finger. Prepared in this way the filter always retains the phosphomolybdate precipitate. The flask is rinsed out twice with cold water and the precipitate washed twice on the pulp with cold water using a wash bottle with the jet removed. The precipitate is now dissolved in 6 cc. of dilute ammonia (10 per cent.), which is allowed to filter through into the rinsed-out precipitation flask. The pulp is washed about half a dozen times with water, as much as possible being removed from the pulp each time by suction. For this purpose the stem of the funnel is passed through a rubber stopper bored with two holes. Through the second hole a bent tube passes which can be connected to the pump. The filtrate and washings, which should measure rather less than 100 cc., are now acidified by adding 11 cc. of concentrated nitric acid, and 8 cc. ammonium nitrate added. The liquid is heated to 80° and the phosphorus again precipitated as phosphomolybdate by adding 7 to 8 cc. of ammonium molybdate. The flask is well shaken for from one to two minutes and then allowed to stand at 80° for 20 minutes, with occasional shaking. The precipitate is filtered off on

a pulp filter, prepared as before, washed thoroughly with water and dissolved in 6 cc. of dilute ammonia, the pulp being then washed several times with water. The filtrate and washings are best collected in the washed-out precipitation flask. They are transferred to a beaker, 11 cc. concentrated hydrochloric acid added followed by 10 cc. of lead acetate, and the contents then heated almost to boiling. Meanwhile in the precipitation flask, a mixture of 50 cc. of ammonium chloride and 50 cc. of ammonium acetate is heated to boiling point. When both solutions are hot the solution in the beaker is poured into that in the flask, the beaker is rinsed out with a little hot water, and the precipitation of the lead molybdate facilitated by shaking. The precipitate is filtered off on a Gooch crucible, well washed with hot water, dried, ignited and weighed. The weight of the precipitate in milligrams multiplied by 0·0069 gives the weight of phosphorus in milligrams. The factor 0·0159 gives the weight of P_2O_5. The factors theoretically are 0·00703 and 0·0161. Since the results are on an average 2 per cent. too high, the amended factors have been used when reprecipitation was necessary. When this is not the case the theoretical factors may be used. If 5 cc. of the standard phosphate solution have been added in the first precipitation, then 1 milligram P_2O_5 or 0·437 milligram of phosphorus must be subtracted from the weight found.

The method just outlined has been used for the estimation of the ether-soluble phosphorus compounds of serum. The dry ashing method for the preliminary oxidation was not convenient, because of the large volume of the ether solution of the phosphorus compounds, and the difficulty of evaporating this in a small platinum dish without loss. In cases where the dry ashing method can be used, however, the reprecipitation of the ammonium phosphomolybdate is not necessary. The method of ashing adopted by Taylor and Miller [1914] may be used with advantage. The substance, to which a few drops of saturated sodium carbonate solution have been added, is dried in a platinum dish and ignited. The mass is dissolved in a solution of 8·5 per cent. nitric acid containing 6·5 per cent. ammonium nitrate and after precipitation with ammonium molybdate the phosphorus is determined by the lead molybdate method. It is necessary to know approximately the amount of phosphorus present. For each tenth of a milligram of phosphorus about 25 cc. of the ammonium nitrate-nitric acid mixture must be used for dissolving the mass after ignition and rinsing out the platinum dish. The solutions used in the precipitation of the lead molybdate must be taken in about the same relative proportion, *i.e.* 25 cc. of the ammonium chloride

and ammonium acetate mixture, 2·5 cc. of concentrated hydrochloric acid and 2·5 cc. of 4 per cent. lead acetate solution. When about one to two tenths of a milligram of phosphorus is being estimated it is preferable to use a 100 cc. conical flask, a 1 cm. filter plate and a smaller amount of pulp in the filtration. Usually about a quarter of a 9 cm. Swedish filter paper has been found to be sufficient.

Special emphasis must be laid on the necessity for adhering to the relative concentrations of ammonium nitrate and nitric acid given above, when the phosphoric acid is being precipitated as ammonium phosphomolybdate. It is only in this way that a concurrent precipitation of molybdic oxide is avoided, and much time was lost in the early stages of working out the method through this fact not being appreciated. When Neumann's method of oxidation is used and reprecipitation of the phosphomolybdate is necessary, a trace of molybdic acid in the precipitate appears to be unavoidable, probably due to traces of colloidal matter from the filter pulp facilitating its precipitation. This error has been corrected by a slight reduction in the factor finally used for converting the weight of lead molybdate obtained into the corresponding weight of phosphorus.

The method has so far been used only for the purpose for which it was devised, but there is little doubt that it would serve equally well for the quantitative estimation of phosphorus in most organic substances.

Appended are some of the results obtained by the method both with known and unknown amounts of phosphorus:

Estimation of Known Amounts of Phosphorus.

Single Precipitation Method.			*Reprecipitation Method.*		
Phosphorus taken in milligrams	Phosphorus found	Mean	Phosphorus taken in milligrams	Phosphorus found	Mean
	0·436			0·451	
0·437	0·443	0·439	0·437	0·435	0·445
	0·443			0·453	
	0·434			0·454	
0·175	0·179			0·431	
0·089	0·094			0·448	
	0·091				

Using the factor 0·0069 instead of the theoretical factor 0·007 the mean value becomes 0·437.

Estimation of Unknown Amounts of Phosphorus (Reprecipitation Method).

	Phosphorus found, milligrams		Phosphorus found, milligrams
Solution of crude lecithin in ether 10 cc.	0·66	Ether-soluble phosphorus compounds from 10 cc. ox serum	0·68
	0·66		0·68
	0·66		
5 cc.	0·33		
2 cc.	0·14		
	0·15		

Summary.

A method is described for the gravimetric estimation of fractions of a milligram of phosphorus as low as 0·1 milligram. It is a modification of Brearley and Ibbotson's method for the estimation of phosphorus in steel.

After preliminary oxidation to phosphoric acid, the phosphorus is precipitated under special conditions as ammonium phosphomolybdate. The molybdenum in this precipitate is then estimated as lead molybdate.

The method is rapid and accurate and requires no expensive or special apparatus.

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LXV. THE RATE OF INACTIVATION BY HEAT OF PEROXIDASE IN MILK. I.

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The presence of peroxidase in fresh cow's milk can be detected by the usual reagents for that enzyme. Hydrogen peroxide and *p*-phenylenediamine are considered the most sensitive reagents for the purpose and are mostly employed in testing milk for peroxidase (Storch's reaction). A dark blue-grey colour is imparted almost instantaneously to the milk if the reaction is positive.

That peroxidase is an actual constituent of the milk, and not due to bacterial contamination, is evident from the fact that initially sterile cow's milk, or milk rendered sterile directly after milking by antiseptics like chloroform or toluene, gives a positive reaction. On the other hand, bacterial cultures from the most common organisms in milk fail to react for peroxidase [Lane-Claypon, 1913, p. 11].

Everything seems to point to the fact that the enzyme is associated with the albumin fraction of the milk. Julius Meyer [1910] showed that when caseinogen is brought down by means of trichloracetic acid, acetic acid, natural acidity or calcium chloride, and properly washed, it does not react for peroxidase, while casein brought down by means of rennet gives but a faint reaction. Again, when milk is centrifuged the enzyme remains in the fluid and does not go up with the cream [Lane-Claypon, 1913, p. 12]. Yet when the precipitate brought down from whey by saturating it with ammonium sulphate is redissolved, the solution reacts very intensely for peroxidase [Lane-Claypon, 1913, p. 10].

Like most enzymes, peroxidase can be inactivated by heat, or at least, when the milk is heated beyond certain temperatures for some time no perceptible reaction is manifested when the usual reagents are employed.

This fact Storch found could be utilised as a reliable test for detecting whether the milk had been heated to 80° C.

Van Eck [1911], investigating the process of inactivation of peroxidase in milk by heat, found that it proceeds as a reaction of the first order and, making use of the formula for a unimolecular reaction, was able to obtain a constant. This was confirmed by the author, who further investigated the matter with the view of ascertaining whether the peroxidase reaction in milk could be employed with any certainty as a test for pasteurisation. The temperature coefficient for the inactivation of peroxidase in milk and the influence of acidity and alkalinity and neutral salts on the rate of inactivation were studied.

Experimental Method.

The technique employed was very similar to that used by van Eck. There is no method for the determination of the absolute quantity of peroxidase, and therefore the amount of the enzyme present in the samples was determined relative to that of the raw milk used in the experiment, and expressed as a percentage of the same. It was carried out colorimetrically. For the standard scale 38 test tubes were used, each containing from 0·1 cc. to 5 cc. of raw milk and made up to 10 cc. with boiled milk. The experimental tubes contained 5 cc. of the sample under investigation, and 5 cc. of boiled milk. To each of the tubes, both experimental and scale, 0·3 cc. of 1 % hydrogen peroxide and 0·25 cc. of 2 % *p*-phenylenediamine were added. Half an hour was allowed to elapse between the addition of the reagent and the comparison of the samples with the scale. After that period no perceptible change could be discerned to take place in the shades of the tubes during the time of the estimation. A fresh scale was made up for each experiment and the raw milk employed invariably was of the same sample as the milk used for investigation. New solutions of hydrogen peroxide and *p*-phenylenediamine were also made up every time, for the former is very unstable and the latter, like guaiacum tincture, after remaining for some time in solution, will react with milk without the addition of hydrogen peroxide. The experimental error of this method varies from 5 % when the peroxidase content is high to 2 % when the amount of peroxidase to be estimated is low.

The milk for investigation was heated in a beaker covered with a cork lid, through which passed a thermometer graduated in tenths of a degree and a stirrer, and which was provided with an opening for the removal of

the samples. The beaker was placed in an asbestos-jacketed water-bath, having a layer of liquid paraffin on the surface to diminish the evaporation, and regulated by a toluene regulator. Like the beaker, the bath contained a stirrer driven by a water-motor. In starting the experiment the milk in the beaker was quickly brought up to the desired temperature in a boiling water-bath, and then without loss of time placed in the regulated bath and the stirrers set going. As soon as the temperature in the beaker became constant the first sample was taken. The taking of the samples was then repeated after the desired intervals. In removing the samples warmed pipettes were used in order not to cool down the milk in the beaker. The test tubes containing the freshly withdrawn samples were immediately placed in cooled water so that the temperature of the milk was rapidly brought down. The entire procedure of removing the samples was carried out regularly in ten seconds.

Determination of the Temperature Coefficient.

In order to determine the temperature coefficient of the rate of inactivation by heat of the peroxidase in milk, it was necessary to ascertain the constants for several temperatures from which the coefficients could be calculated. Preliminary experiments showed that heating of milk at 65° C. for four hours made hardly any perceptible impression on the peroxidase content. On the other hand, at 72° C. the inactivation of the enzyme proceeded at a rate much too fast for any reliable results to be recorded. Temperatures of 71°, 70° and 69° were found to be very convenient and were accordingly chosen for the determination of the constants. The following tables give the results obtained for these three temperatures.

TABLE I.

Time of heating in minutes	Temperature of milk	Percent. of peroxidase	Percent. of peroxidase calculated from K = 0·005066
0	69·05° C.	63	77
20	68·95	61	61
40	68·9	49	48·3
60	68·9	37	38·2
80	68·9	31	30·3
101	68·9	25	24
120	68·85	19	19
140	68·85	17	15

$K = \frac{1}{t} \log \frac{C_1}{C_2} = 0{\cdot}005066$ where $t = 100$ mins., $C_1 = 61$, $C_2 = 19$

TABLE II.

Time of heating in minutes	Temperature of milk	Percent. of peroxidase	Percent. of peroxidase calculated from K = 0·01333
0	70·25° C.	61	55·7
10	70·1	41	41
20	70·1	30	30·1
50	70·07	12·5	12
60	70·05	9·5	8·8
70	70·05	6·5	6·5
80	70·05	5·5	4·8

$K = \frac{1}{t} \log \frac{C_1}{C_2} = 0{\cdot}01333$ where $t = 60$ mins., $C_1 = 41$, $C_2 = 6{\cdot}5$.

TABLE III.

Time of heating in minutes	Temperature of milk	Percent. of peroxidase	Percent. of peroxidase calculated from K = 0·02698
0	71·15° C.	37	35·5
5	71·08	26	26
10	71·05	18	19·1
15	71	13	13·9
20	70·98	9·5	10·2
25	70·98	8	7·5
30	70·9	5·5	5·5
35	70·9	4·5	4

$K = \frac{1}{t} \log \frac{C_1}{C_2} = 0{\cdot}02698$ where $t = 25$ mins., $C_1 = 26$, $C_2 = 5{\cdot}5$.

The last column in these tables gives the percentage of peroxidase calculated from the constant (K) obtained from the equation of a unimolecular reaction. The first and last readings were not utilised for calculating the constant, as the possibility of error was more likely in these cases than in any other. In the first sample the temperature is usually not quite constant and the high content of the enzyme makes accurate estimation harder than in the other samples, while in the last sample the experimental error of the colorimetric method is too significant in comparison with the low peroxidase content to be determined. The second reading and the last but one were therefore employed. Considering that the method which was employed for the estimation of the enzyme cannot claim a very high degree of accuracy, the figures calculated from the constants agree well with the figures actually obtained by experiment and this shows clearly that the inactivation of the enzyme proceeds as a reaction of the first order. It is also to be observed that constants obtained by calculating from different data in the tables gave results which approximated to those given above. The error in

the constant is probably between 5–10 %. Fig. 1 gives a graphic representation of the rates of inactivation at the three temperatures.

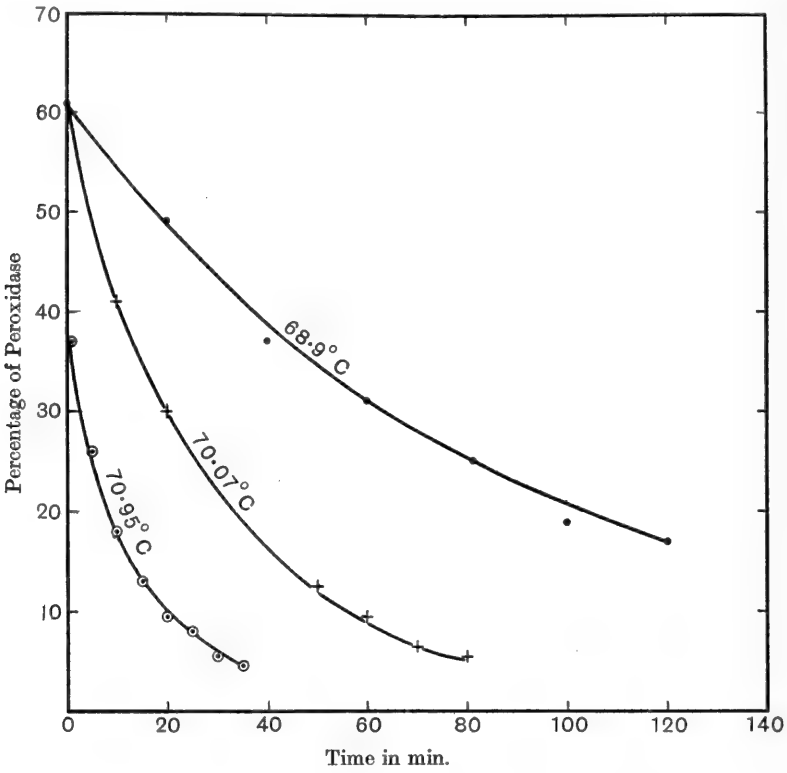

Fig. 1.

Calculating the temperature coefficient from the three constants, we obtain the following figures:

TABLE IV.

Temperature	Constant	Difference in temperature in degrees	Temperature coefficient for one degree
68·9° C.	0·005066	—	—
70·075	0·01333	1·175	2·278
70·95	0·02698	0·875	2·18
70·95	0·02698	2·05	2·236
Mean temperature coefficient			2·231

The mean temperature coefficient is therefore 2·23 per degree. This high value is of the same order as that found by Famulener and Madsen [1908] for the inactivation of tetanolysin and vibriolysin nasik by hot water. For the former the authors obtained the value of 2·4 and for the latter a value

a little under 2 for one degree. Chick and Martin [1910] also found the temperature coefficient for the coagulation of egg albumin by heat to be equal to 1·9 per degree.

The results of Tables I, II and III moreover show the impossibility of applying the peroxidase reaction as a direct test for pasteurisation. The term "pasteurisation" has a very broad application as to the temperature and time of heating the milk. It is not uncommon in some dairies in "pasteurising" to heat the milk up to 80° C. (176° F.), but according to the definition laid down by the Department of Health of the City of New York, pasteurisation consists in heating the milk at not less than

158° F. (70° C.)	for at least	3 mins.
155° F. (68·3° C.)	,,	5 ,,
152° F. (66·7° C.)	,,	10 ,,
148° F. (64·4° C.)	,,	15 ,,
145° F. (62·8° C.)	,,	18 ,,
140° F. (60° C.)	,,	20 ,,

From the constant obtained above for 70° C. it can be calculated that 91·2 % of the original peroxidase would remain after heating the milk for 3 minutes at 158° F. (70° C.). Such an amount of peroxidase could be differentiated with difficulty by the colorimetric method from the full amount of 100 %, even if the original sample of raw milk could be used for the scale. In testing milk, however, for pasteurisation the original raw milk is never available and although different samples of raw milk differ apparently but little in intensity in the peroxidase reaction, the possibility of small variations is by no means excluded. This would make the differentiation still more uncertain. As regards the lower temperature, 20 minutes heating at 60° C. would make no perceptible impression, since, as we have seen, 4 hours at 65° C. makes hardly any difference to the peroxidase content of the milk. It is then evident that the peroxidase reaction cannot be applied at all as a test for pasteurisation. It may, however, serve as an indication whether "pasteurised" milk has been overheated.

TABLE V.

Temperature	Constant	Time in mins. necessary to bring peroxidase down to 10 %	Time in mins. necessary to bring peroxidase down to 50 %
70° C.	0·01333	75	22·5
71	0·02973	33	10·3
72	0·06634	15	4·6
73	0·14802	6·8	2
74	0·33022	3	under a minute
75	0·73673	1·3	,, ,,
80	40·72004	under a minute	,, ,,

Table V gives the periods of time for which the milk must be heated at different temperatures between 70° C. and 80° C. in order to bring down the peroxidase content to 10 % and 50 % respectively. The calculations are made on the assumption that the temperature coefficient remains the same as the temperature rises and the constants in the table are obtained on that assumption. It is clear from the figures that at temperatures beyond 71° C. very short periods of heating produce well marked impressions on the peroxidase content of milk.

Influence of Acidity and Alkalinity on the Rate of Inactivation of Peroxidase in Milk.

Experiments were instituted with the object of studying the effect of acidity and alkalinity on the rate of inactivation of peroxidase in milk. The H ion concentration of the milk was determined by the gas chain method. Unfortunately the determination of H ion concentration in a medium like milk is fraught with many technical difficulties, and the results obtained, though definitely showing the general effect of acidity and alkalinity, are not exact enough for publication in detail. The result arrived at is that a small increase in acidity retards and in alkalinity accelerates the rate of inactivation. Thus the addition of such a quantity of lactic acid as changed the P_H^+ of the milk from about 6·57 to 5·75 brought down the constant at 70° C. from 0·01333 to 0·01086, while the addition of potash which changed the P_H^+ to about 8·26 increased the rate of inactivation at the same temperature to such an extent that the peroxidase content of the milk came down to 2 % in the preliminary heating.

Influence of Salts on the Rate of Inactivation of Peroxidase in Milk.

It was observed by Chick and Martin [1911] that the addition of sodium chloride and ammonium sulphate retarded the rate of coagulation of egg-albumin by heat. It was therefore desirable to ascertain the influence of salts on the rate of inactivation of peroxidase in milk by the same agency. From preliminary experiments it was observed that the addition of sodium chloride greatly retarded the rate of inactivation. In milk made up to the concentration of a N/1 NaCl solution the inactivation of the peroxidase at 70° C. was brought down to a rate too slow for convenient observation, and therefore N/10 and N/20 solutions were used in the actual experiments. The addition of salts alters the tone of the colouration produced by the

peroxidase reaction and salt was therefore added to the scale tubes in the same proportion as to the sample under investigation. Tables VI, VII and VIII illustrate the action of sodium chloride and Fig. 2 shows the same results graphically.

TABLE VI.

(N/10 NaCl.)

Time of heating in minutes	Temperature of milk	Percent. of peroxidase	Percent. of peroxidase calculated from K = 0·002063
0	69·9° C.	72·5	72·5
30	69·9	59	62·9
90	69·9	48	47·4
120	69·9	42	41·1
150	69·9	36	35·6
180	69·9	32	30·9

TABLE VII.

(N/20 NaCl.)

Time of heating in minutes	Temperature of milk	Percent. of peroxidase	Percent. of peroxidase calculated from K = 0·003995
0	70·2	72·5	72·5
31	70·15	50	54·5
60	70·1	41	41·8
90	70	31	31·7
120	70	25	24·1
150	70	20	18·25

TABLE VIII.

(*Normal Milk, no salt added.*)

Time of heating in minutes	Temperature of milk	Percent. of peroxidase	Percent. of peroxidase calculated from K = 0·01721
0	69·9	62·5	62·5
5	70·0	50	51·3
10	70·0	40	42
25	70·1	24	23·2
40	70·1	14	12·8
55	70·2	8	7
70	70·2	4	3·9

Calculating $K = \frac{1}{t} \log \frac{C_1}{C_2}$ from different points on the curves in Fig. 2 we get the following mean coefficients for the corresponding concentrations:

N/10 NaCl, K = 0·002063.
N/20 NaCl, K = 0·003995.
Normal Milk, K = 0·01721.

It is seen from the above figures that the concentration of N/20 salt reduces the rate to about one quarter and N/10 to about one eighth. The constant for the normal milk is rather higher than the one usually obtained for a similar temperature, but the same sample of milk was used for the experiments with the N/10 NaCl and N/20 NaCl and, consequently, these results would also be proportionately higher.

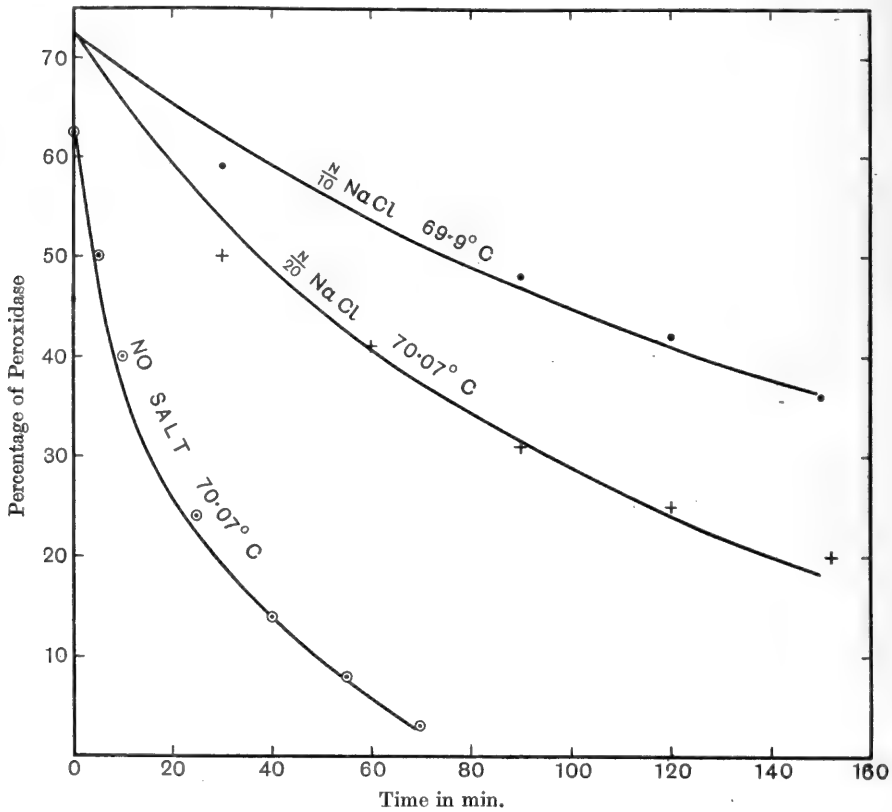

Fig. 2.

The next thing to ascertain was whether other salts had a retarding effect on the rate of inactivation, and if so whether that influence was specific or otherwise. The number of different salts used had to be restricted as some, even in such dilutions as N/20, bring down the caseinogen at 70° C. immediately, or after some time. In all the cases tried the addition of the salts, in concentration of N/20, had a marked retarding effect, although the extent of that influence varied in most cases. The influence of some of the salts is shown by the results in the following tables. Fig. 3 gives curves expressing these results as well as those in Tables VII and VIII.

TABLE IX.

(N/20 KCl.)

Time of heating in minutes	Temperature of milk	Percent. of peroxidase	Percent. of peroxidase calculated from K = 0·00526
0	70·4° C.	65	65
15	70·5	56	54·2
30	70·5	51	45·2
45	70·5	38	37·7
60	70·5	29	31·4
75	70·5	24	26·2
90	70·5	20	21·8

TABLE X.

(N/20 Na_2SO_4.)

Time of heating in minutes	Temperature of milk	Percent. of peroxidase	Percent. of peroxidase calculated from K = 0·00546
0	70·35° C.	77·5	75·5
15	70·32	62·5	62·5
30	70·35	53	51·8
45	70·3	42	42·9
60	70·3	33	35·5
75	70·3	28	29·4
90	70·3	20	24·3

TABLE XI.

(N/20 KNO_3.)

Time of heating in minutes	Temperature of milk	Percent. of peroxidase	Percent. of peroxidase calculated from K = 0·00981
0	70·2° C.	72·5	82·8
15	70·4	59	59
30	70·35	40	42
45	70·4	28	30
60	70·4	20	21·35
75	70·35	16	15·2
90	70·4	10	10·8

Calculating the constants from the curves of Fig. 3 we get the following figures:

N/20 NaCl K = 0·00399.
N/20 KCl K = 0·00526.
N/20 Na_2SO_4 K = 0·00546.
N/20 KNO_3 K = 0·00981.
Normal milk K = 0·0225.

The temperature for the normal milk and the N/20 NaCl was slightly lower than for the remainder. The constant of the normal milk has been calculated for the right temperature with the help of the temperature coefficient. The temperature coefficient for N/20 NaCl has not been deter-

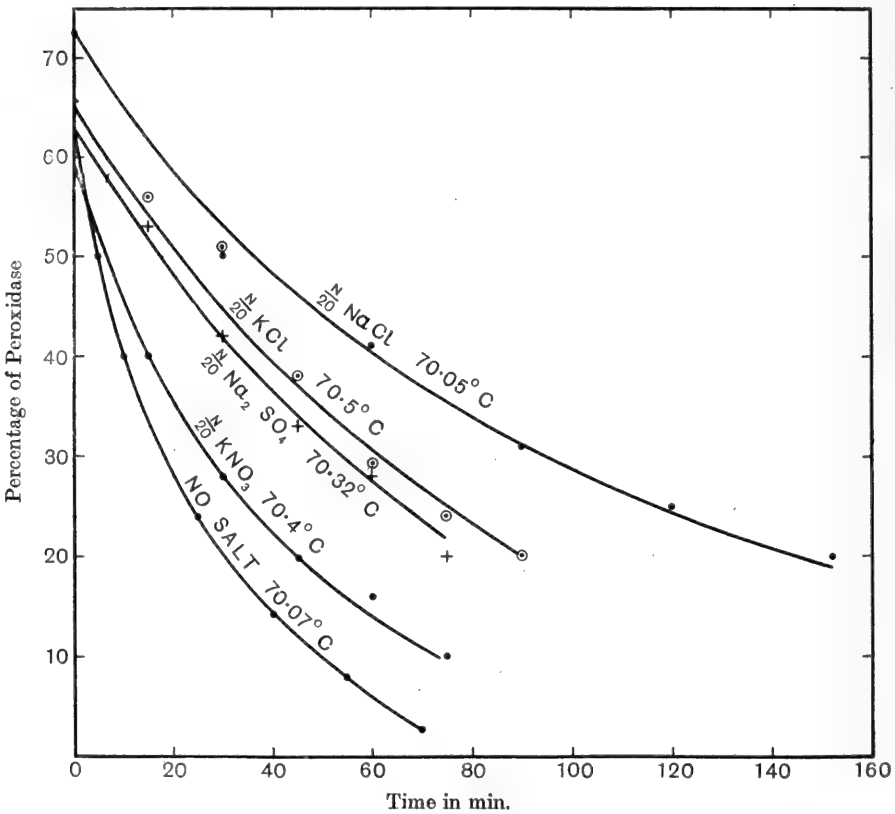

Fig. 3.

mined and consequently no correction could be made for that constant. The results seem to point to the fact that the influence is specific and is not dependent solely on the valency of the ions. KNO_3 and KCl contain monovalent kations and anions, but their constants differ considerably. On the other hand, KCl and Na_2SO_4 contain anions of different valency—the former monovalent and the latter divalent—yet their constants are practically the same.

The Influence of Salts on the rate of Inactivation of Peroxidase in Whey.

The possibility that the salts act on the caseinogen, which then in its altered state influences the rate of inactivation of the peroxidase, was not excluded by the foregoing results and accordingly the effect of the addition of salts to whey was investigated. The whey was prepared by curdling the milk with rennet and filtering it through a cheese-cloth. The experiments were then carried out in the same way as with the milk. The inactivation of the peroxidase in the whey was also observed to proceed as a reaction of the first order but more rapidly than in the milk at the same temperature. The intensity of the peroxidase reaction in the whey was stronger than in the milk, and it was found convenient to dilute it (1 part whey with 3 parts boiled distilled water).

TABLE XII.

(*Diluted Whey, no addition of Salt.*)

Time of heating in minutes	Temperature of milk	Percent. of peroxidase	Percent. of peroxidase calculated from K = 0·01485
0	69·2° C.	72·5	72·5
15	69·35	47	43·4
30	69·3	28	26
45	69·3	14	15·6
60	69·3	9	9·3
75	69·3	5	5·5

TABLE XIII.

(*Diluted Whey and* N/20 NaCl).

Time of heating in minutes	Temperature of milk	Percent. of peroxidase	Percent. of peroxidase calculated from K = 0·00344
0	69·3° C.	72·5	72·5
15	69·3	62·5	64·3
30	69·3	55	57·2
45	69·3	50	50·8
60	69·3	42	45·1
75	69·3	40	40
90	69·3	38	35·5

Tables XII and XIII and Fig. 4 show the influence of N/20 NaCl on the diluted whey. As with milk, the retarding effect is well marked. N/20 KNO_3 was also found to produce a retarding effect.

Whatever the nature of the action of the salts may be, it is thus seen to be independent of the presence of the caseinogen and fat. Further investigation, which is now in progress, is needed in order to ascertain the nature of that action, and whether the enzyme is influenced directly or indirectly by the presence of the salts.

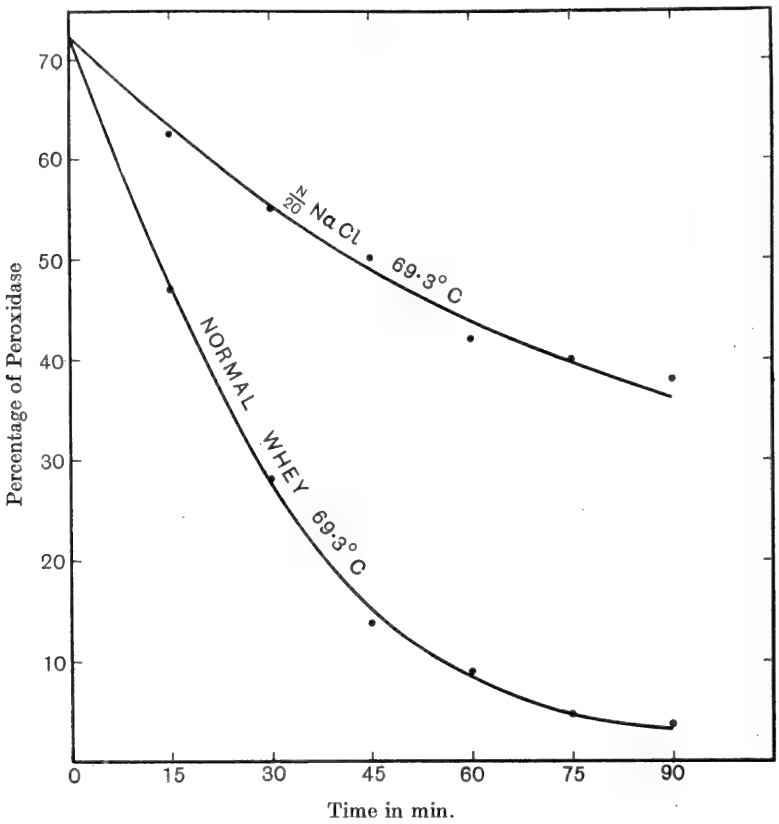

Fig. 4.

Summary.

1. The temperature coefficient for the inactivation of peroxidase in milk by heat is found to be 2·23 per degree centigrade, a value of about the same order as the temperature coefficients for the inactivation by heat of principles like tetanolysin and vibriolysin nasik and for the rate of coagulation of egg-albumin.

2. The rate of inactivation of peroxidase in milk below 70° C. is so small that it does not afford the opportunity of utilising the peroxidase reaction

as a test for pasteurisation. The reaction may, however, indicate whether "pasteurised" milk has been overheated.

3. Small additions of acid retard and of alkali accelerate the rate of inactivation by heat of peroxidase in milk.

4. The presence of salts retards in a pronounced way the inactivation rate. This retardation varies with different salts and is independent of the valencies of their ions.

5. As in milk, the inactivation of peroxidase in whey proceeds as a reaction of the first order.

6. In whey also the presence of salts has a retarding influence on the rate of the enzyme.

In conclusion I wish to express my indebtedness to Professor Harden, F.R.S., for the valuable advice and assistance rendered during this investigation, and to Dr Onodera for helping me with some of the latter part of the experimental work.

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LXVI. LIVER NITROGEN IN ANAPHYLAXIS.

By GEORGE BARGER and HENRY HALLETT DALE.

(*Received Nov. 17th, 1914.*)

It has been clearly demonstrated by the work of Manwaring [1910] and of Voegtlin and Bernheim [1911], that events occurring in the liver have, at least in some species, a predominant importance in producing the train of symptoms known as "anaphylactic shock." The nature of these events, and of the changes in the liver rendering it thus specifically sensitive to a previously injected antigen, has hitherto been obscure. That the events are of such a kind as to produce a hindrance to the portal circulation, has been suggested; and there is a probability that such mechanical factors play at least some part. The recent work of Hashimoto and Pick [1914], however, seemed rich in promise of explanation along other lines. According to these observers, the liver of an animal, rendered anaphylactic by a first small injection of a foreign protein, contains a largely increased store of uncoagulable nitrogenous substances, without change of its total nitrogen from the normal average. The result is an increase of the uncoagulable fraction of the total nitrogen from a normal average of about 8 % to 22 %, or even more. According to their finding, the increase becomes definite two or three days after the sensitising injection, and attains the above-mentioned maximum at about the 14th day, thereafter very gradually declining to a lower, but still supernormal ratio, which they found still maintained as late as the 68th day. They further found that the spleen played an important part, in producing and maintaining this excess of uncoagulable nitrogen in the liver of the anaphylactic animal. Sensitisation performed the day after splenectomy, or followed by splenectomy, was not accompanied by this rise in the uncoagulable nitrogen ratio. Hashimoto and Pick's experiments were made on guinea-pigs, and they point out that this accumulation of non-coagulable nitrogen in the liver has no bearing on the plain muscle sensitisation, which gives to the anaphylactic shock in this species its most characteristic features. Their results, however, if confirmed, have an interesting relation to the

anaphylactic shock seen in such animals as the dog, in which the exclusion of the liver from circulation renders the re-injection of the sensitising antigen without effect.

Hashimoto and Pick's observations were especially suggestive in that they seemed to harmonise with other observations on anaphylaxis and similar conditions. Thus it has been shown, by several workers, that the symptoms of the anaphylactic shock correspond closely with those following the injection of certain products of the partial cleavage of proteins. It has been shown also, that a second parenteral administration of a foreign protein is followed by an increased output of nitrogen in the urine, considerably greater than could be accounted for by the nitrogen-content of the injected substance. If the result of a first injection then, were, as stated, to cause an accumulation of protein cleavage-products in the liver cells, and if these were released into the circulation as the result of the second injection, a possibility would be presented of co-ordinating a number of hitherto imperfectly connected observations. With a view to examining these possibilities further, our ultimate object being a closer analysis of the process of sensitisation and anaphylactic shock in the dog, we have repeated Hashimoto and Pick's experiments; but we find ourselves held up, at the outset of the investigation, by a complete failure to confirm their observations. We are at a loss to account for the discrepancy. Our technique has differed from theirs in no essential particular. We have examined in all 13 guinea-pigs sensitised with injections of horse-serum, varying from 0·1 to 2 cc., and have not found one in which the ratio of non-coagulable to total liver-nitrogen lies outside the range of variation seen in normal controls. The experiments on controls were interspersed among the anaphylactic series, so that seasonal or dietary variation would affect both series alike. The average values in the two series are practically identical.

Being unable to confirm the fundamental observation of the presence of excess of uncoagulable nitrogen in the anaphylactic liver, we were not in a position to study the effect, on the alleged excess, of subjecting the animal to anaphylactic shock. Nor have we, save for casual observations of the rate of autolysis for a few hours at room temperature, which showed no significant difference between normal and anaphylactic liver, made any attempt to follow the further observations of Hashimoto and Pick, since these postulate the accuracy of the main observation, which we fail to confirm. Nor have we, as we had proposed, extended the observations to other species.

The publication of results, which are a mere contradiction of those

obtained by previous investigators, without the possibility or near prospect of the personal communication, which we should have desired, and which might have helped us to an explanation of the discrepancy, is at present an unfortunate necessity.

Experimental.

The observations were made on young, healthy guinea-pigs, varying in weight from 210 to 385 g., taken from a common stock, kept under identical conditions. All the observations were made between August 31st and October 14th and the diet (hay, oats, bran and green stuff) was not varied in composition or quantity throughout the series. Nevertheless, to exclude the influence of seasonal variation, or of any unnoticed change in diet, the observations on normal controls were interspersed among the series on anaphylactic animals.

The anaphylactic series consisted of 13 guinea-pigs, each sensitised by a subcutaneous injection of normal horse-serum; five receiving 0·1 cc., six receiving 0·5 cc., and two receiving 2 cc. each. The livers were examined at an interval varying from 11 to 21 days after the injection. One of the six animals sensitised by 0·5 cc. of horse-serum was killed on the 12th day by a second, intravenous injection of 0·5 cc. of the same serum; death took place within five minutes from typical acute shock, with extreme and permanent distension of the lungs. The others were killed by a blow on the head, and in all cases the animal was bled as completely as possible by severing the blood-vessels in the neck and compressing the abdomen. The 12 normal controls were killed in the same manner.

The pale and nearly bloodless liver was removed rapidly after killing and was immediately cut into portions of suitable size for the different determinations. In a few trial experiments on normal livers we used a method identical in all details with that employed by Hashimoto and Pick; the whole liver, after removing the gall-bladder and large vessels, was pounded in a mortar and then rubbed through fine nickel gauze, portions for estimation being weighed out from the semi-fluid pulp so produced. Such a method provided an ideal safeguard against local variations of composition. At the same time it is troublesome and wasteful—the loss in the meshes of the gauze being such as to make it difficult to obtain four adequately large samples from the liver of a small guinea-pig. Drying is also apt to occur in the thinnish film of material produced. We made a few experiments, therefore, to discover whether local variations of composition occur in the

liver of sufficient magnitude to weaken the accuracy of estimates carried out on different samples cut from a whole liver. We found that the values, whether for total or non-coagulable nitrogen, obtained from different pieces of the same liver, were remarkably concordant—showing at least as good a correspondence as those obtained from different portions of the disintegrated and mixed liver substance. The small error was clearly the error of the Kjeldahl method, and not that of imperfect sampling. We, therefore, abandoned the preliminary disintegration, and simply weighed out in clean, tared beakers as many pieces of whole liver substance as were required, merely avoiding the central portions, where large vessels and ducts are present in undue proportion to the liver cells.

For determining total nitrogen pieces of from 0·5 to 1 g. were usually taken, and these were at once washed from the beakers into Kjeldahl flasks. To each flask were added 20 cc. of strong sulphuric acid, 10 g. of potassium sulphate, and a small fragment of copper gauze. A preliminary heating on a water-bath, before burning, was found useful in diminishing the trouble of bumping.

For determinations of non-coagulable nitrogen portions of 2 to 2·5 g. were usually employed. Two different methods of coagulation were used. One of these gave, with some uniformity, slightly lower values than the other. Estimates on both normal and anaphylactic livers were made by both methods, and comparisons were made of normal and anaphylactic values obtained by the same method.

Method I. This is identical with the method employed by Hashimoto and Pick, except in that disintegration is performed after, instead of before weighing. The sample of liver is transferred with a little distilled water to a glass mortar and rubbed up into as fine a pulp as possible. More distilled water is added and rubbed up with the liver-pulp. After a brief settling the supernatant fluid is poured back into the beaker, the residue of less thoroughly ground fragments subjected to renewed rubbing, more water added and poured off; the process is repeated till nothing remains but a few fine colourless shreds. These are finally washed into the beaker and the mortar and pestle thoroughly washed with several more small quantities of distilled water, which are added to the mixture. The fine emulsion thus obtained was made up, as in Hashimoto and Pick's experiments, to about 70 cc. A pinch of salt was added and the beaker transferred to a boiling water-bath; three drops of 20 % acetic acid were then added from a fine pipette, and heating continued till coagulation was quite complete.

TABLE I.

Number and Date of Experiment	Weight of guinea-pig in g.	Volume of sensitising injection of horse-serum and time since injection	Number of liver-sample	Weight of sample, g.	Determination made (total or non-coagulable nitrogen)	Method for non-coagulable nitrogen	Milligrams of nitrogen	Percentage of nitrogen	Mean	Percentage of non-coagulable in total nitrogen by Method I	Percentage of non-coagulable in total nitrogen by Method II	Percentage of non-coagulable in total nitrogen by Method I, after standing 3 hours
1 Aug. 31st	320	None (control)	1	1·174	Total		35·07	2·99	2·97	10		
			2	1·680	,,		49·35	2·94				
			3	1·047	Non-coagulable	I	3·36	0·321	0·298			
			4	1·630	,,	,,	4·48	0·275				
2 Sept. 1st	2 guinea-pigs, each about 250 grammes. Livers mixed	None (control)	1	2·017	Total		62·37	3·07	3·12	9·2		
			2	1·753	,,		55·65	3·17				
			3	2·709	Non-coagulable	I	7·65	0·282	0·286			
			4	3·025	,,	,,	8·78	0·290				
3 Sept. 3rd	280	None (control)	1	1·593	Total		53·55	3·36	3·36		7·7	
			2	1·738	Non-coagulable	II	4·57	0·263	0·260			
			3	1·880	,,	,,	4·85	0·258				
4 Sept. 8th	390	None (control)	1	3·211	Total		94·92	2·96	2·96		7·5	
			2	2·084	,,		61·74	2·96	0·222			
			3	1·891	Non-coagulable	II	4·2	0·222				
			4	1·790	Lost by accident							
5 Sept. 9th	305	None (control)	1	1·363	Total		48·8	3·58	3·56		8·4	
			2	1·497	,,		53·0	3·54				
			3	1·203	Non-coagulable	II	3·7	0·30	0·30			
			4	1·522	,,	,,	4·6	0·30				
6 Sept. 11th	220	0·1 cc. 11 days	1	1·766	Total		62·37	3·53	3·48		7·9	
			2	0·2638	,, (Micro Kjeldahl)		9·1	3·44				
			3	1·800	Non-coagulable	II	4·95	0·275	0·274			
			4	1·792	,,	,,	4·90	0·273				
7 Sept. 14th	245	0·1 cc. 14 days	1	0·599	Total		19·32	3·22	3·16		7·95	
			2	0·939	,,		29·09	3·10				
			3	1·685	Non-coagulable	II	4·39	0·260	0·251			
			4	1·945	,,	,,	4·71	0·242				
8 Sept. 16th	209	None (control)	1	0·599	Total		19·22	3·21	3·21	8·3		
			2	0·760	,,		24·36	3·21				
			3	2·567	Non-coagulable	I	6·93	0·270	0·267			
			4	2·570	,,	,,	6·82	0·265				

			3	2·032	Non-coagulable	I	5·78	0·219	0·219		
			4	2·933	,,	,,	6·40	0·219			
10 Sept. 17th	280	None (control)	1	0·941	Total		23·5	2·50	2·49	8·7	
			2	0·826	,,		20·5	2·48			
			3	2·012	Non-coagulable	I	4·4	0·219	0·216		
			4	2·207	,,	,,	4·7	0·213			
11 Sept. 17th	251	0·1 cc. 17 days	1	0·928	Total		31·7	3·42	3·48	7·4	
			2	0·657	,,		23·3	3·55			
			3	2·849	Non-coagulable	I	7·1	0·250	0·258		
			4	2·347	,,	,,	6·2	0·265			
12 Sept. 21st	280	None (control)	1	0·873	Total		27·8	3·18	3·15	7·5	
			2	0·983	,,		30·7	3·12			
			3	2·379	Non-coagulable	I	5·65	0·238	0·237		
			4	2·459	,,	,,	5·8	0·236			
13 Sept. 21st	270	0·1 cc. 21 days	1	1·112	Total		40·0	3·60	3·63	7·5	
			2	1·172	,,		43·0	3·67			
			3	2·327	Non-coagulable	I	6·5	0·279	0·273		
			4	2·543	,,	,,	6·8	0·267			
14 Sept. 29th	310	0·5 cc. 11 days	1	1·490	Total		45·0	3·02	3·06	8·7	
			2	1·623	,,		50·4	3·10			
			3	3·054	Non-coagulable	I	8·0	0·264	0·267		
			4	2·151	,,	,,	5·8	0·270			
15 Sept. 30th	290	0·5 cc. 12 days	1	1·548	Total		48·9	3·16	3·22	7·9	
			2	1·265	,,		41·4	3·27			
			3	2·441	Non-coagulable	I	6·15	0·252	0·254		
			4	2·247	,,	,,	5·75	0·256			
16 Oct. 1st	294	0·5 cc. 13 days	1	1·055	Total		41·3	3·90	3·86	7·1	
			2	1·609	,,		61·6	3·82			
			3	2·255	Non-coagulable	I	6·2	0·275	0·275		
			4	2·613	,,	,,	7·2	0·275			
17 Oct. 1st	278	None (control)	1	0·849	Total		30·8	3·62	3·59	6·8	
			2	0·980	,,		34·9	3·56			
			3	2·401	Non-coagulable	I	5·95	0·247	0·244		
			4	2·494	,,	,,	6·00	0·241			
18 Oct. 2nd	385	0·5 cc. 14 days	1	0·978	Total		23·2	2·37	2·38	10	9·1
			2	0·951	,,		22·7	2·38			
			3	2·871	Non-coagulable	I	6·8	0·250	0·238		
			4	2·464	,,	,,	5·6	0·227			
			5	2·077	,,	II	4·5	0·217	0·217		
			6		Lost by accident						
19 Oct. 5th	285	0·5 cc. 17 days	1	0·534	Total		18·3	3·43	3·36		
			2	0·411	,,		13·5	3·29			
			3	2·040	Non-coagulable	II	5·1	0·250			

TABLE I—*continued.*

Number and Date of Experiment	Weight of guinea-pig in g.	Volume of sensitising injection of horse-serum and time since injection	Number of liver sample	Weight of sample, g.	Determination made (total or non-coagulable nitrogen)	Method for non-coagulable nitrogen	Milligrams of nitrogen.	Percentage of nitrogen	Mean	Percentage of non-coagulable in total nitrogen by Method I	Percentage of non-coagulable in total nitrogen by Method II	Percentage of non-coagulable in total nitrogen by Method I, after standing 3 hours
20 Oct. 7th	310	0·5 cc. 19 days	1	0·747	Total		17·92	2·40	2·38	9·4	9·1	12·2
			2	0·704	,,		16·66	2·37				
			3	2·318	Non-coagulable	I	5·25	0·226	0·225			
			4	2·408	,,	,,	5·4	0·224				
			5	2·327	,,	II	5·0	0·215	0·216			
			6	2·205	,,	,,	4·8	0·218				
			7	2·004	,,	I	5·7	0·284	0·291			
			8	2·009	,,	(After 3½ hrs. autolysis)	6·0	0·298				
21 Oct. 8th	275	2 cc. 15 days	1	0·440	Total		16·2	3·70	3·65	8·2	7·5	
			2	0·610	,,		22·0	3·61				
			3	2·244	Non-coagulable	I	6·8	0·303	0·299			
			4	2·242	,,	,,	6·6	0·294				
			5	1·274	,,	II	3·6	0·283	0·275			
			6	1·633	,,	,,	4·35	0·266				
22 Oct. 8th	265	2 cc. 15 days	1	0·593	Total		21·4	3·61	3·59	10·1	8·7	
			2	0·909	,,		32·3	3·57				
			3	1·751	Non-coagulable	I	6·35	0·363	0·363			
			4	2·231	,,	,,	8·1	0·363				
			5	1·659	,,	II	5·3	0·319	0·313			
			6	1·793	,,	,,	5·5	0·307				
23 Oct. 9th	360	None (control)	1	0·594	Total		21·80	3·67	3·65	8·3	7·9	
			2	0·726	,,		26·32	3·63				
			3	1·738	Non-coagulable	I	5·4	0·311	0·302			
			4	1·915	,,	,,	5·6	0·293				
			5	2·158	,,	II	6·2	0·287	0·290			
			6	2·219	,,	,,	6·5	0·293				
24 Oct. 12th	390	None (control)	1	1·135	Total		29·43	2·60	2·64	9·2	8·3	11·2
			2	0·577	,,		15·47	2·68				
			3	2·584	Non-coagulable	II	5·63	0·218	0·218			
			4	2·638	,,	,,	5·75	0·218				
			5	2·505	,,	I	6·2	0·247	0·243			
			6	2·495	,,	,,	5·95	0·239				
			7	2·612	,,	,,	8·4	0·283	0·295			
			8	2·374	,,	(After 3 hrs. autolysis)	8·2	0·307				
25 Oct. 13th	260	None (control)	1	0·785	Total		25·97	3·31	3·27	11·3		
			2	1·155	,,		37·31	3·22				
			3	2·105	Non-coagulable	I	8·2	0·389	0·369			
			4	2·137	,,	,,	7·45	0·349				

The time on the water-bath was in all cases about 30 minutes. The beaker and its contents were then cooled, the coagulum carefully broken up with a rod into fine fragments, and the whole, with repeated washings, transferred to a 100 cc. standard flask, the volume being then accurately adjusted with distilled water. The flask was well shaken and the contents filtered through an ordinary folded filter paper.

Method II. The piece of liver, as soon as it was weighed, was partially coagulated whole, by pouring into the beaker about 10 cc. of boiling distilled water faintly acidulated with acetic acid, heating on the water-bath for one minute and then cooling. By this preliminary coagulation the disintegration in the mortar was rendered much easier. The piece of liver was ground first with a small quantity, and then with the whole of the fluid in which it was coagulated. A fine creamy emulsion was obtained, and, by pouring off after brief settling and regrinding the residue with successive portions of distilled water, a suspension of the whole substance in a very fine state of division was obtained. The fluid was again made up to 70 cc. and definitive coagulation carried out on the same lines as in Method I. The transference of the fluid and coagulum to the standard flask was rendered easier in this case by the finely divided state of the coagulum. The volume was made up to 100 cc. and filtration carried out as in Method I.

Of the filtrate obtained by either method a convenient volume (usually 70 cc.) was taken for Kjeldahl estimation. Hashimoto and Pick stipulate that the filtrate shall be free from any trace of opalescence, though they admit, in a footnote, that opalescent filtrates do not differ materially in nitrogen-content from perfectly clear ones. They suggest that the opalescence is due to glycogen, and their suggestion is certainly correct. It may be said, indeed, that with the majority of livers, worked up with sufficient rapidity, a filtrate free from traces of opalescence is not obtainable. This is especially the case with Method II, in which the preliminary scalding effectively stops post-mortem glycogenolysis. The nitrogen value obtained by Method II is, however, regularly somewhat lower than that obtained from the same liver by Method I. It is clear, then, that this opalescence has no connection with imperfect coagulation, and we have ignored it. Glycogen-rich livers, giving opalescent filtrates, naturally show lower percentages of both total and non-coagulable nitrogen than livers which are practically nitrogen-free; but the ratio of non-coagulable to total nitrogen is not disturbed. In every experiment duplicate samples were worked up.

In several cases the non-coagulable nitrogen was estimated by both Methods I and II—two samples being worked up by each method.

The ammonia resulting from the Kjeldahl combustion was distilled into standard sulphuric acid, 10 cc. N/2 acid being used for a total, 10 cc. N/10 acid for a non-coagulable nitrogen determination. Titration was carried out with N/20 caustic potash, methyl red being used as indicator. The standards were carefully checked on several occasions during the series of experiments. The results are given in Table I.

It will be seen that Method I for determination of the non-coagulable nitrogen regularly gives a somewhat higher percentage than Method II, when both are applied to the same liver. For purposes of comparison, therefore, it is more accurate to consider separately the results obtained by the two methods, as in Table II.

TABLE II.

Percentage of Non-coagulable in Total Nitrogen.

Method I.			Method II.		
Normal	Anaphylactic	Sensitising dose and interval	Normal	Anaphylactic	Sensitising dose and interval
10·0	9·5	0·1 cc. 16 days	7·7	7·9	0·1 cc. 11 days
9·2	7·4	0·1 cc. 17 „	7·5	7·95	0·1 cc. 14 „
8·3	7·5	0·1 cc. 21 „	8·4	—	—
8·7	8·7	0·5 cc. 11 „	8·3	—	—
7·5	7·9	0·5 cc. 12 „	9·2	—	—
6·8	7·1	0·5 cc. 13 „	—	—	—
8·3	10·0	0·5 cc. 14 „	—	9·1	0·5 cc. 14 „
9·2	7·9	0·5 cc. 17 „	—	7·3	0·5 cc. 17 „
11·3	9·4	0·5 cc. 19 „	—	9·1	0·5 cc. 19 „
—	8·2	2 cc. 15 „	—	7·5	2 cc. 15 „
—	10·1	2 cc. 15 „	—	8·7	2 cc. 15 „
Average 8·8	8·5	—	8·2	8·2	—

It is sufficiently clear that, whichever method is used, there is no significant difference between the percentages obtained. We are entirely unable to explain the failure of our animals to show the striking difference exhibited by those of Hashimoto and Pick. That our guinea-pigs were satisfactorily sensitised is clear from the rapid death, in response to a second injection, of the one animal thus tested. Being unable to obtain the accumulation of non-coagulable nitrogenous substances which Hashimoto and Pick described, we are not in a position to investigate it further. Our results seem to us, however, sufficiently clear to indicate that, whatever its origin, the condition described by them had no relation to anaphylaxis.

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