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CONTENTS

No. 1 (May)

No. 2 (August)

Nos. 3 & 4 (December)

I. OCCURRENCE OF RAFFINOSE IN THE SEED OF THE JUTE PLANT (*CORCHORUS CAPSULARIS*).

By HAROLD EDWARD ANNETT.

From the Agricultural Research Laboratory, Dacca, Bengal.

(*Received December 29th, 1916.*)

Raffinose has been shown to occur in beet molasses, cotton seed, barley grains, wheat sprouts and eucalyptus manna. It is now shown to be present in jute seeds.

Preparation. In the course of an examination of jute seed for a glucoside Mr R. S. Finlow, Fibre Expert to the Government of Bengal, obtained a substance, which had a sweet taste, and handed the material to me for further investigation. The jute seed had been finely ground and then exhausted with ether and with petrol. The residue was next exhausted with alcohol. From this alcoholic extract the substance under investigation was obtained as a copious white precipitate on the addition of ether.

The impure substance was dissolved in hot 80 % alcohol. On filtering and cooling, the solution deposited after several days rosettes of white needles. The crystals were collected on a filter and recrystallised from 80 % alcohol. About 3 g. of the pure crystals were finally obtained.

General Physical and Chemical Properties.

The substance had an extremely sweet taste, was very soluble in water, and crystallised from alcohol in characteristic rosettes of white needles. It gave no osazone and did not reduce Fehling's solution. 10 cc. of a 1 % solution gave only 2·5 milligrams of CuO under Brown and Morris's conditions. On hydrolysis with acid or treatment with emulsin or invertase, reducing sugars were formed and then an osazone was readily obtained. It gave Selivanoff's reaction and on boiling with concentrated hydrochloric acid gave a red colour indicating the presence of fructose.

Water of crystallisation. On heating a small portion of the crystals in the steam oven they melted indicating the presence of much water of crystallisation. Some freshly prepared crystals were well washed with absolute alcohol on a small Hirsch funnel under suction and then dried thoroughly between filter paper. About 0·5 g. was then dried *in vacuo* in a toluene bath in presence of phosphorus pentoxide. For the first few hours the temperature was kept below 60° C. but it was finally increased to 105° C. and maintained at that temperature until a constant weight was obtained. This took about 24 hours.

0·5420 g. substance gave water of crystallisation = 15·09 %. For pure raffinose the theory is 15·15 %. A sample of recrystallised raffinose gave 15·54 %.

Specific rotatory power. This had to be determined in a Schmidt-Haensch saccharimeter using ordinary white light. The substance showed no mutarotation.

0·4602 g. of anhydrous sugar was dissolved in water and made up to 50 cc. This gave a reading on the sugar scale of + 6·64. Raffinose should have given + 6·51. The agreement is within the error due to the unfavourable conditions under which the rotation had to be determined.

Action of enzymes. These tests were carried out in conjunction with parallel tests on pure raffinose.

(*a*) *Emulsin.* When emulsin acts on raffinose, cane sugar and galactose are produced according to the equations:

$$C_{18}H_{32}O_{16} \cdot 5H_2O \rightarrow C_6H_{12}O_6 + C_{12}H_{22}O_{11}.$$

Whence 100 parts of raffinose give 30·3 parts of galactose and 57·57 parts of saccharose.

Since $[\alpha]_D^{20}$ for raffinose = + 104°, for galactose = + 81°, and for sucrose = + 66·5°.

30·3 % galactose will give a rotation of 0·303 × 81 = + 24·54°,

57·57 % sucrose will give a rotation of 0·5757 × 66·5 = + 38·28°,

$\therefore$ $[\alpha]_D^{20}$ of the mixture will give a rotation of = + 62·82°,

the $[\alpha]_D^{20}$ falling from + 104° to + 62·82°.

Exp. 1. 1·0103 g. of pure raffinose were dissolved in water and the volume made up to 100 cc. This gave a reading of + 5·94.

To 50 cc. of this solution 0·1 g. emulsin + 0·5 cc. toluene were added. The reading became + 3·50 (corrected for emulsin).

Exp. 2. 1·2280 g. of the jute sugar in the anhydrous condition were dissolved in water and the volume made up to 100 cc. This gave a reading

in the saccharimeter of + 8·51. To 50 cc. 0·2 g. emulsin and 0·5 cc. toluene were added. The reading became + 5·32 (corrected for emulsin).

The reading of the pure raffinose solution after emulsin action should have been 62·8/104 × 5·94 = + 3·59, and that of the jute sugar 62·8/104 × 8·51 = + 5·14.

It must be noted here that with the emulsin used some 10 days were required before a constant saccharimeter reading was obtained.

(*b*) *Invertase + Melibiase.* When invertase acts on raffinose, d-fructose and melibiose are produced.

It can readily be shown that the $[\alpha]_D^{20}$ of raffinose + 104° falls to + 54·4° as a result of the action of invertase. The action of the melibiase in addition to the invertase, produces from one molecule of raffinose, one molecule each of d-glucose, d-galactose and d-fructose. Hence it follows that the $[\alpha]_D^{20}$ of raffinose should fall after the action of invertase + melibiase from + 104° to + 12·5°.

The invertase used in these tests was prepared according to Hudson's method [1914] which gives a highly pure and active product which even in this climate has retained its activity for more than a year. It was made from bottom yeast and contained melibiase.

Exp. 3. To 30 cc. of the pure raffinose solution which had been treated with emulsin (Exp. 1), 0·25 cc. of invertase + melibiase solution was added together with a few drops of toluene. A further fall in rotation was observed and no further fall took place after 24 hours. The original reading of the raffinose solution was + 5·94, the action of emulsin reduced the reading to + 3·50, and the action of invertase further reduced the reading to + 0·87 (corrected for invertase control).

Theoretically the final reading should have been 12·5/104 × 5·94 = + 0·71.

Since the readings were made at about 30°, it is to be expected that the actual reading would be too high owing to the large increase in $[\alpha]_D^{20}$ of fructose caused by rise of temperature.

Exp. 4. 0·9320 g. of roughly anhydrous jute sugar was dissolved and made up to 100 cc. The solution gave a reading in the saccharimeter (200 mm. tube) of + 6·80. To 60 cc. of this solution, 1 cc. of invertase + melibiase solution and 0·5 cc. of toluene were added and the mixture incubated at 37°. The rotation became constant at + 0·94 (corrected for invertase) after 48 hours.

Assuming the sugar to be raffinose this final reading should have been 12·5/104 × 6·80 = + 0·85.

Osazone formation. Exp. 5. 20 cc. of a 1 % solution of the jute sugar were mixed with 0·4 g. of phenylhydrazine hydrochloride and 0·6 g. of sodium acetate and heated in the water bath. No osazone was obtained after 1 hour even on cooling. 0·411 g. of partly dehydrated jute sugar was dissolved in about 4 cc. of water, 0·5 cc. invertase and a few drops of toluene were added, and the mixture incubated at 30° for two days. 5 cc. of a solution containing 1 g. phenylhydrazine hydrochloride and 1·5 g. sodium acetate were then added.

The mixture was heated in a water bath. Under these conditions fructose should form an osazone in 3 minutes, glucose in 4—5 minutes and galactose in 15—19 minutes [1912].

An osazone formed in 3 minutes. After 15 minutes the liquid was filtered and the osazone recrystallised twice from alcohol (Crop 1).

The filtrate was again heated in the water bath for 1 hour when a second crop of crystals was filtered off. These were recrystallised twice from alcohol to form Crop 2. The filtrate was again heated for $\frac{3}{4}$ hour and a third crop of crystals was obtained and recrystallised twice from alcohol (Crop 3).

As a control 0·15 g. each of galactose, glucose and fructose were dissolved in about 4—5 cc. of water and 5 cc. of the phenylhydrazine hydrochloride and sodium acetate mixture added. Three crops of crystals were obtained exactly as with the jute sugar.

Crop 3 in each case was very small in amount.

The melting points of these crops of crystals were taken. Duplicate determinations being done in each case.

They were found to be:

Crop 1. M.P. (*a*) 203, (*b*) 202°.
Crop 2. M.P. (*a*) 202·5, (*b*) 203°·5.
Crop 3. M.P. (*a*) 196·5, (*b*) 195°·5.

The melting points of the crystals from the mixture of the sugars were (corrected):

Crop 1. M.P. (*a*) 204, (*b*) 203°.
Crop 2. M.P. (*a*) 201·5, (*b*) 203°·5.
Crop 3. M.P. (*a*) 193·5, (*b*) 194°·5.

Pure glucosazone under the same conditions had a M.P. (corrected) of 205 to 206°. The M.P. of galactose d-phenylosazone is given as 194—196° [1912].

The crystals were also examined microscopically, after mounting in xylene balsam.

The crystals of Crop 1 from the jute sugar were all of one kind collected

into the characteristic bundles of needles generally seen with phenylglucosazone.

Those of Crop 2 resembled Crop 1, but showed a few square ended yellow leaflets.

Those of Crop 3 consisted mostly of square ended bright yellow leaflets.

The microscopic appearance of the crops of crystals from the artificial mixture of sugars agreed in appearance with that of Crops 1, 2, 3 obtained from jute sugar.

The above experiments prove that the sugar obtained from jute seed is raffinose. By way of confirmation a 1 % solution of the sugar was treated with emulsin in presence of toluene. Emulsin hydrolyses raffinose to galactose and cane sugar. After 10 days the liquid was heated with phenylhydrazine hydrochloride and sodium acetate. An osazone was produced which contained many yellow square ended platelets characteristic of galactose phenylosazone.

The amount of raffinose in jute seed. 200 g. of finely ground seed were well shaken with 500 cc. distilled water, containing 10 cc. toluene, for one hour and filtered.

To 50 cc. of the filtrate 1 cc. of basic lead acetate was added and the liquid filtered. A bright solution was obtained from which lead was removed by means of solid sodium phosphate. This gave a reading in the 100 mm. tube of the saccharimeter of + 8·1.

25 cc. of this solution were slightly acidified by the addition of four drops of acetic acid and then 0·5 cc. invertase + melibiase and 0·5 cc. toluene were added and the mixture incubated at 37° for two days. Four drops concentrated NH_4OH were then added and the liquid filtered. A reading of − 5·2 was obtained in the 100 mm. tube of the saccharimeter.

To 25 cc. of the original water extract (which was faintly acid) 0·5 cc. invertase + melibiase and 0·5 cc. toluene were added and it was then incubated as above. After two days 0·5 cc. of basic lead acetate was added, the liquid being filtered after shaking. Lead was separated from the filtrate with sodium phosphate and the filtrate gave a reading in the 100 mm. tube of + 5·2.

The invertase had thus caused a fall in rotation, measured in the 100 mm. tube of + 8·1 to 5·2 or 2·9 degrees on the sugar scale. For the 200 mm. tube this equals 5·8.

The invertase and melibiase solution will cause a fall in rotation for raffinose of 91·5° from + 104° to + 12·5°.

Assuming that raffinose is the only sugar present in the jute seed capable

of being acted on by invertase, the original rotation due to raffinose for the 200 mm. tube would have been $5{\cdot}8 \times 104/91{\cdot}5 = 6{\cdot}6$; this corresponds to 2·29 angular degrees and gives a content of about 1·1 g. raffinose per 100 cc. of the original water extract from 200 g. jute seed. Since there were 500 cc. of water added; the jute seed contains about 2·25 % raffinose.

Conclusions.

Raffinose has been shown to occur in the seed of the jute plant *Corchorus capsularis*. In the particular seed sample examined, the sugar occurs to the extent of about 2·25 %.

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II. ACCESSORY FACTORS FOR PLANT GROWTH.

By OTTO ROSENHEIM.

From the Physiological Laboratory, King's College, London.

(*Received January 4th, 1917.*)

In his endeavours to find a suitable medium for the growth of the nitrogen-fixing bacteria of the "Azotobacter" group, Prof. Bottomley observed that although these organisms failed to grow in natural peat, they flourished when grown in sphagnum peat which had previously been "rotted" by the agency of ordinary aërobic soil organisms. Sterilised rotted peat, inoculated with a mixed culture of nitrogen-fixing bacteria was subsequently found to produce striking results in the development of pot grown plants, results which could not be ascribed to the manurial contents of the treated peat nor to the beneficial effect of the development of the nitrogen-fixing bacteria. It was found later, indeed, that the presence of these bacteria was not an essential factor. Prof. Bottomley was at a loss to explain these results, which had been confirmed by impartial experienced horticulturists, and in discussion with me suggested an examination of the chemical composition of the treated peat.

Before entering into this necessarily lengthy investigation I desired to convince myself of the activity of the material. In order to eliminate a possible beneficial effect on the soil porosity, etc., due to the increased water-retaining power of the peat-soil mixture, I decided to use an aqueous extract of the material. Although the actual manurial properties of the treated peat are small and its contents of nitrogen, phosphorus and potassium are low as compared with any ordinary fertiliser, I purposely chose an amount of the extract which should exclude its possible manurial effect.

As it was hardly to be expected that the small quantity used in the first experiment could produce any effect, it was my original intention gradually to raise the amount until the minimal effective dose was reached. The surprising result of the experiment to be described, however, made this unnecessary

and led me to the assumption that a substance is present in the treated peat which plays a rôle in plant growth similar to that played by the now generally accepted food accessories in animal growth. Owing to various causes I had no opportunity of continuing this work, and as my experiments, which formed the starting point of Prof. Bottomley's subsequent researches, were necessarily only briefly alluded to by him, a somewhat fuller account seems desirable at this stage.

My experiments were begun in the late autumn of 1913, a season unfavourable for plant growth under ordinary conditions and for that reason more likely to show the effect of any stimulating agent. The only plants I had available at that time for experiments were a number of seedlings of *Primula malacoides*. It was recognised that this primula is by no means an ideal plant for this work, as it is well known to practical gardeners that a considerable variation in its character and rate of growth may occur. An experimental error due to this cause was guarded against by selecting from a large batch of seedlings a considerable number of plants of apparently equal development and by beginning the treatment only at such a relatively advanced stage of their growth that any inequality in their character would have been noticeable. The seedlings were raised during the summer, 1913, and a large number of them pricked out into boxes. Some of the plants were potted up on Oct. 5th in ordinary potting compost (loam, leaf-mould and sand). Shallow porous pots, so-called dwarf alpine pots, of uniform size (12·5 cm. diam. and 500 cc. capacity) were used. A layer of 1·5 cm. washed granite chips served as drainage. On Oct. 24th a series of twelve plants were selected which were as far as possible in a uniform stage of development. Six of these were used as controls, whilst the other six were watered with 60 cc. each of the diluted peat extract (see below). The plants were kept under the ordinary conditions in an unheated greenhouse. During the course of the experiment the outside temperature fell only on a few occasions below freezing point, without affecting the development of the plants.

No difference could be detected between the treated and untreated plants during the first three weeks, and therefore on Nov. 15th three of the treated plants received another dose of 60 cc. of the extract[1]. During the fifth week, however, the difference became very marked indeed. The treated plants not only grew considerably taller, but showed a remarkable luxuriance

[1] It may be mentioned that this second treatment did not produce any additional beneficial effect, an observation which speaks in favour of the view of "accessories."

of growth. They were of a much deeper green and had larger leaves. They produced flower stems in greater abundance and also the size of the individual flowers and the number of whorls was increased. One each of the plants of each series was photographed on Dec. 7th, Pl. 1, Fig. 1, 14th, Fig. 2, and 26th, Fig. 3, and the last photograph shows clearly the increased development of the root system which corresponds to the increase in the foliage. The effect was uniform on all the plants of the series and the control plants also were very uniform in their behaviour.

For the preparation of the extract 15 g. of treated peat, which was kindly put at my disposal by Prof. Bottomley, was left standing for 18 hours with 2 litres of tapwater. 500 cc. of the brown clear solution were decanted and diluted to 1250 cc. The extract contained:

0·045 % Total Solids, 0·011 % Inorganic Substances,
0·034 % Organic Substances, 0·003(2) % Nitrogen.

The plants received therefore in one application the extract of 0·18 g. of treated peat containing 20 mg. of organic matter and 1·9 mg. of nitrogen, i.e. roughly 4 per million nitrogen to soil.

The effect produced on plant growth by this small quantity of soluble substances suggested to me an analogy to that due to "vitamines" or accessory food substances in animal growth and led me to investigate the behaviour of the peat towards reagents which are known to dissolve or precipitate "vitamines." Some of these substances are soluble in alcohol and are precipitated, or carried down in an adsorbed condition, by phosphotungstic acid.

Extracts were made for comparison from garden soil, natural peat and treated peat by allowing 15 g. of each of the materials to stand for one hour with 100 cc. of water. To 20 cc. of each filtrate 2·5 cc. conc. H_2SO_4 in 10 cc. water was added and the volume made up to 50 cc. A brown flocculer precipitate of humic acid formed in the treated peat extract and was filtered off. Equal volumes of a 30 % phosphotungstic acid solution were added to each filtrate. The extract from treated peat gave an immediate copious precipitate, whilst that of natural peat only gave a faint opalescence and the extract of garden soil remained clear.

Extracts were made from the materials in a similar way with boiling alcohol or acetone. The solvent was evaporated and the residue taken up with water. Again a heavy precipitate formed on adding phosphotungstic acid to the extract of the treated peat. It was further noted that a considerable part of the phosphotungstic acid precipitate was insoluble in acetone,

a fact which may be of use for the isolation of the active substance [see Funk, 1916].

It had been suggested by Funk and Macallum [1913] that the blue colour reaction which is given by Folin and Macallum's uric acid and phenol reagents, may serve as an index for the presence of "vitamines" [see also Williams and Seidell, 1916]. I was unable to test the original extract used for these experiments in this direction. A fresh supply of treated peat, whose activity on plants had not, however, been tested and was considered doubtful, did not give the uric acid reaction, but developed a blue colour with the phenol reagent. Prof. Bottomley has since stated that active preparations show the uric acid reaction distinctly.

The chemical examination of the treated peat extract lends therefore strong support to my view which was arrived at from its effect on plant growth and demonstrated the presence of substances similar to the "vitamines" in their general behaviour. At my suggestion the action on plant growth of alcoholic extracts and the fractions obtained from them by phosphotungstic acid were tested in Prof. Bottomley's laboratory. It was found that the active substances were contained in the alcoholic extract and its sub-fractions, and that in minute quantities they exerted a remarkable stimulating effect on plant growth (Bottomley, 1915, 1, 2).

As far as the activity of the treated peat is concerned these observations therefore supply a working hypothesis which is being further tested experimentally. They suggest besides many interesting problems regarding plant nutrition in general, and I am at present engaged in some experiments on the effect on plant growth of the substances contained in yeast and milk, which are known to possess growth promoting properties in animals.

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Fig. 3

Fig. 2

Fig. 1

III. THE PREPARATION OF DESOXYCHOLIC ACID.

By WILLIAM MAIR.

From the Research Laboratory of the Metropolitan Asylums Board, London.

(*Received January 22nd, 1917.*)

In investigating the solvent action of bile salts on "pneumococci" it was found that sodium desoxycholate is ten times as active, in this respect, as sodium cholate, or commercial sodium taurocholate. The remarkable activity of the desoxycholate in this connection makes its introduction into bacteriological technique desirable, and, as it is possible that the salt may also prove to be of value in the prevention and treatment of local pneumococcal infections, it appears to be worth while to place on record a method of preparation of desoxycholic acid which seems to offer certain advantages over those at present in use. The method is in part suggested by the recent work of Wieland and Sorge [1916] who have shown conclusively that the so-called "choleic" acid is a peculiar combination of desoxycholic with stearic (or other fatty) acid, the proportion of the latter being so small that the difference in composition is not appreciable in an elementary analysis.

It is well known that desoxycholic acid can be purified by recrystallisation from glacial acetic acid, and the chief feature of the present method is that this solvent is applied directly to the crude mixture of bile acids.

In detail the method is as follows:—To four litres of ox bile 240 g. of caustic soda are added and dissolved by stirring and heating. The alkaline bile is then boiled gently in an iron digester for about 20 hours. A certain amount of evaporation takes place and the volume should now be between 2 and 3 litres. While still hot it is made neutral to phenolphthalein by adding gradually about 350 cc. strong hydrochloric acid. A flocculent precipitate of silica forms but none of the bile acid is thrown down, provided the solution is sufficiently warm. The amount of silica in ox bile is quite appreciable, amounting to one or two grams per litre. After cooling the bile is filtered. The filtrate is rendered acid to litmus by adding about 50 cc. glacial acetic

acid. The bile acids separate out in a fluid crystalline mass which is at first white but rapidly absorbs pigment. By gentle rotation of the flask the bile acids can be made to adhere in a single mass and can easily be separated by decantation of the mother liquor. The fluid crystalline mass consists of the mixed bile acids holding acetic acid in combination. It is squeezed to get rid of excess of water, and it can then be handled like a mass of putty and so transferred to a flask of about one litre capacity. Rather more than an equal volume of glacial acetic acid (about 600 cc.) is added, and the flask is warmed on the water bath until the whole is dissolved. The solution is poured into a beaker and allowed to stand until crystallisation is complete, usually two or three days. By this procedure a crystallisation of the bile acids from acetic acid of about 60 % strength is obtained. The mixed crystals contain practically all the desoxycholic acid; they also contain an almost equal amount of cholic acid and some fatty acid. The crystals are filtered off, washed free from pigment with 60 % acetic acid, sucked as dry as possible on the filter, and re-dissolved by heating with 300 cc. glacial acetic acid. The solution is now only slightly pigmented, but contains traces of insoluble matter, probably silica. The hot solution is therefore filtered and allowed to stand. The crystals which form now consist chiefly of desoxycholic acid with some admixture of cholic acid. They are separated and pressed dry between porous tiles. They are then dissolved by boiling in about 750 cc. 60 % acetic acid and the hot solution is filtered into a beaker; sometimes a little oily substance remains undissolved and floats on the surface (fatty acid), this can easily be removed after it solidifies on cooling. Next day the crystals are filtered off, washed first with 60 % acetic acid and then thoroughly with water, sucked dry, and dried completely in the hot air oven at 100°. A perfectly white crystalline substance with M.P. between 170° and 175° is thus obtained. It is practically pure desoxycholic acid (M.P. 172°—173°). The yield from four litres of bile was 32 g. with M.P. 172°—173°; from another four litres of bile the yield was 42 g., M.P. 170°.

To prepare the acid from commercial taurocholate, 500 g. of the latter are dissolved in 3 litres of water with 200 g. of caustic soda and the further procedure is as above. The yield from 1000 g. of the taurocholate was 50 g. with M.P. 173°—174°.

As a general procedure for the isolation of the bile acids the method is only recommended where a maximum yield of desoxycholic acid is desired and where it is not necessary to recover a large amount of cholic acid. The cholic acid can, however, be recovered from the purer mother liquors. The

acetic acid is distilled off as completely as possible, finishing the distillation *in vacuo* on the water bath. The residue is treated with a little hot alcohol, and aqueous caustic soda is added till the solution is just alkaline to phenophthalein. The alcohol is boiled off and more water is added. After cooling the cholic acid is precipitated by adding dilute hydrochloric acid. It separates in a fluid crystalline mass containing acetic acid to which it obstinately clings. After pressing out the mother liquor the cholic acid is subjected to prolonged digestion with hot water to remove the acetic acid. On cooling it forms a hard cake which still smells of acetic acid. This is broken up and powdered in a mortar and then dried, first in an exhausted desiccator over caustic soda, and then in the hot air oven at 100°. It is then dissolved in rather more than twice its volume of absolute alcohol by boiling, and is purified by recrystallisation from alcohol.

From the acetic acid mother liquors (second and third recrystallisations) corresponding to 75 g. of desoxycholic acid there was obtained 50 g. of cholic acid and 7 g. of fatty acid.

These figures therefore give the approximate composition of the crystals obtained from the first acetic acid crystallisation from the crude bile acids.

Where a maximum yield of cholic acid is desired it is better to follow Schryver's [1912] procedure as far as the precipitation with magnesium chloride. The insoluble magnesium desoxycholate is then filtered off from the hot solution, sucked dry on the filter and dissolved in hot glacial acetic acid. On cooling and standing desoxycholic acid crystallises out, and the further procedure is as already described.

I wish here to express my indebtedness to Dr Schryver for his kindness in supplying me with samples of the various bile acids prepared by his method.

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IV. ADSORPTIVE STRATIFICATION IN GELS, II.

By SAMUEL CLEMENT BRADFORD.

(From the Science Museum, S. Kensington, S.W.)

(Received February 1st, 1917.)

It has been postulated [Bradford, 1916] that the formation of stratified deposits in gels may be explained by the action of the finely divided particles of the precipitate in adsorbing the solute from the gel in their immediate neighbourhood. The accompanying phenomena are, however, so numerous and of so varied a nature that it is necessary to examine them more in detail and to attempt to demonstrate their complete accordance with the theory.

Von Weimarn [1914] has shown that the form of the precipitate produced in a double decomposition of the type $AB + CD = AC + BD$ is dependent on the number, N, of crystallisation centres produced, determined by the formula:

$$N = \frac{P}{L} K_{AB} \cdot K_{CD} \cdot K_{BD} \cdot K_{AC} \cdot Z \ldots ,$$

where P is the mass of the substance AC to be deposited as precipitate,

L, the solubility of the precipitate,

K_{AB}, K_{CD}, etc., the degrees of aggregation, in solution, of the four components of the reaction, and

Z, the viscosity of the reaction medium.

The value of N is also proportional to the degree of aggregation of the reaction medium and of any other substance, or substances, present therein which do not enter into the double decomposition.

The occurrence of stratification in gels is therefore dependent on N, since it is only in cases where the precipitate is sufficiently finely divided that the adsorbing surface will be large enough to exhaust the solute from the adjacent region of gel. As the value of N decreases, in the presence of sufficient nutrient material, so the size of the individual aggregates, or crystals, will

increase, and the area of adsorbing surface diminish, until there is insufficient surface to produce stratification. On the contrary the greater the value of N, the more gelatinous the precipitate, and the slower the rate of diffusion of the precipitant through the layer of precipitate. So that, with very large values of N, the precipitant cannot permeate the precipitate in time to traverse the exhausted layer of gel immediately beyond, before this is replenished with solute by diffusion from the gel. Precipitation will then be continuous. When in this connection it is remembered that the solution in the gel must be sufficiently dilute to allow of its exhaustion from the region adjacent to the precipitate, giving a small value of P, it will be clear that zone formation can occur only in cases in which L is very small, and within somewhat narrow limits. This is fully confirmed by observation.

Since, however, the already dilute solutions meet in the gel only by diffusion, the value of P will be actually much smaller than that corresponding to the concentration of the hypotonic reagent. Consequently there will be a tendency for the formation of larger crystals than would result from the instantaneous mixing of aqueous solutions.

Under similar conditions Dreaper [1914] obtained large crystals of lead chloride and ferrocyanide by allowing the reacting solutions to diffuse in opposite directions through a capillary tube. Indeed the process of allowing the slow mixing of solutions has long been known as a method for the preparation of crystals of sparingly soluble substances.

The occurrence of such large crystals is a remarkable feature of reactions in gels. This is particularly noticeable in silicic acid gels, frequently observed in agar and more rarely in gelatin. It is thus evident that the gel medium has itself an extraordinary influence on the form of the precipitate resulting from a particular double decomposition, which tends to counteract, in a greater or less degree, the effect of the slow diffusion. Many examples of the influence of the gel medium on the value of N could be given, and it is convenient to consider this in connection with the effect of altering other physical conditions. The original Liesegang reaction with silver bichromate takes place readily in gelatin gels of various concentrations, while in 1 % agar remarkable ribbon-like crystals are formed which are much too large to cause stratification. If, however, the reagents are reversed, so that the silver salt is contained in the gel, beautiful laminae again result. These are, however, almost black and crystalline instead of red-brown and apparently amorphous as in gelatin. This may be attributed to an increased value of N resulting from the effect of a larger mass of that solute which is in the

state of greater aggregation, as in the case of the gelatinous precipitate of lead iodide, produced with basic lead acetate, mentioned below. If potassium chromate be substituted for the bichromate, only detached crystals are formed, without a trace of stratification; though here, of course, the crystals consist of silver chromate instead of bichromate. Returning to the case of the agar gel containing potassium bichromate, if the concentration of the solute be considerably diminished, the size of the crystals rapidly falls and an unmistakable tendency to stratification begins to appear, until, with a concentration of $N/100$ $K_2Cr_2O_7$, two separate bands of tiny rounded brownish black particles are formed at a distance of 2·5 mm. apart. With still smaller concentrations dark reddish brown colorations are noticeable in the weaker part of the gel below the bands, indicating sol formation. On reducing the solubility of the precipitate by mixing the agar gel with half its volume of absolute alcohol, an ordinary concentration of $N/20$ $K_2Cr_2O_7$ gives several bands of tiny feather-like crystals, 2 mm. thick, separated by clear spaces, with indications of sol formation in the lower part of the tube.

The case of lead iodide differs from that of silver bichromate in that beautiful laminations are produced in 1 % agar, and, as stated by Hatschek [1914], only a continuous gelatinous precipitate is formed in gelatin. The statement appears, however, to refer to the concentrations of the reagents used in 1 % agar, viz., 4 % of KI in the gel treated with concentrated $Pb(NO_3)_2$. The value of N in this case is too large. By using $N/20$ KI, and $N/1$ $Pb(NO_3)_2$, or $N/1$ $Pb(C_2H_3O_2)_2$, the precipitate becomes more permeable and stratification occurs in the lower half of the tube, although the precipitate is still much more gelatinous than in agar. A similar gelatinous precipitate is also obtained in agar by treating a gel containing $N/20$ KI with basic lead acetate solution. The number of crystallisation centres is increased by the greater aggregation of the precipitant and the precipitate becomes almost impermeable, so that only a few bands are produced. When the reaction takes place in silicic acid gel containing about 3·5 % of silica, which allows about the same rate of diffusion as in 5 % gelatin and 1 % agar, no stratification is visible, but the salt is deposited in large crystals and growth and dendritic forms. Von Weimarn has shown that growth forms and spherical crystals are conditioned by a value of N which is intermediate between those corresponding to complete crystals and so-called amorphous precipitates.

Lead chromate forms a gelatinous precipitate in agar so that it is only in very dilute concentrations, about $N/100$, that stratification occurs, the

result being then very beautiful. In gelatin the precipitate is too gelatinous to stratify, while in silicic acid crystalline needles are produced. Cupric phosphate gives a gelatinous precipitate in agar, which however becomes distinctly stratified in very dilute concentrations, whereas in silicic acid it yields remarkably beautiful deep blue discs. The basic hydroxide also gives good bands in silicic acid, but has not been obtained, so far, other than as a continuous gelatinous precipitate in test tubes containing agar gel. However, by working with U-tubes, half filled with pure agar gel between the reacting solutions in the limbs above, the value of P is so much reduced where the liquids meet that stratification takes place even with an initial concentration of $N/20$. Zone formation is assisted in this case by the increased facility with which the gel can be exhausted of solute owing to the great dilution in the neighbourhood of the meeting solutions.

From these considerations it will be clear that the formation of a banded precipitate is dependent on the number of crystallisation centres produced, and that this quantity is affected by the properties of the gel in which the reaction takes place. This influence of the reaction medium may be explained by the fact that it is actually a dilute aqueous solution of the gel substance in a high degree of aggregation. The number of crystallisation centres is therefore proportionally increased. Under conditions of sufficient dilution the production of sols would be favoured.

The well-known "protective values" of gelatin, agar and silicic acid are in the proportion which would explain the observed variations in the form of the precipitate. In the masterly paper previously quoted, in which he collects together the great variety of facts which require explanation, Hatschek suggests that this protective action may eventually be found to be connected with the behaviour of different gels.

Sol formation is however unnecessary for the production of a stratified precipitate, and could hardly occur, except momentarily, in normal cases. Dreaper [1914] obtained well-defined bands of copper sulphide in columns of sand, which has no protective action. Although in gelatin, particularly, the instantaneous formation of a sol is possible on the first meeting of the diffusing solutions, it would be coagulated immediately by the rapid increase in the value of P leading merely to a more gelatinous precipitate; and it is noticeable that in cases where sol formation is most probable it is usually most difficult to induce stratification. As Hatschek has clearly demonstrated, supersaturation, in any form, is not adequate to explain the many varied cases of zone formation. Nor does it afford an explanation of the very different

results obtained when the solutions in and above the gel are interchanged, as for example with lead iodide, as previously recorded [Bradford, 1916].

The microscopic observation of reactions in gels has already been carried out by Liesegang [1913] for silver bichromate, and by Hatschek [1914] for lead iodide and chromate. The experiments were made in the plate form. The original descriptions are here reproduced. "On observing the region beyond the last line, for some time one perceives nothing, although, as remarked, silver chromate must be forming. Suddenly a new line appears at a distance of half a millimetre. In distinction from the previous line, which appears dark red by reflected light and black by transmitted light, the new line is yellow and very tenuous. In the course of the next ten seconds it becomes considerably intensified and fiery red in colour, but does not attain the density of the older line for some time. This solidification, which is coupled with a darkening of the colour, is reached in about three minutes." It is convenient to consider here a point raised by Hatschek [1914], in connection with the same reaction. "The drop of silver nitrate diffuses into the bichromated gelatin producing, at first, a band of silver bichromate two or three millimetres wide before ring formation commences, and, even if the concentration of silver nitrate be reduced to 5 %, a similar wide band is still built up." According to the adsorption theory, the silver nitrate diffuses into the gel containing potassium bichromate, forming a ring of precipitate of silver bichromate. This precipitate immediately begins to adsorb solute from the gel, which is precipitated as it collects, with constantly growing surface and increasing adsorbing power. In this way the region of gel next the precipitate tends to become gradually exhausted of potassium bichromate and fresh material diffuses towards this region from the more distant parts of the gel. A concentration gradient is thus set up towards the zone of gel in contact with the adsorbing precipitate, and this region eventually becomes so dilute that no further precipitate is formed. The wide band then ceases to grow. It is evident, therefore, that the formation of this wide band depends on the concentration of the hypotonic reagent in the gel, rather than on that of the precipitant. In the case of U-tube experiments this concentration gradient is already in existence when the diffusing solutions meet, and these are so dilute that band formation generally commences immediately. Considering now Liesegang's description; the silver nitrate slowly diffuses through the previous rings of precipitate into a region from which the potassium bichromate has been adsorbed, and no further silver bichromate is formed (not even as a sol, since this would

be indicated by its colour[1]), until the precipitant meets the bichromate diffusing in from the gel. A faint new ring is then generated. The new precipitate commences to adsorb fresh solute from the gel, which is precipitated as it collects. The density of the ring, and incidentally the adsorbing surface, increases. As the solute is rapidly adsorbed from the thin layer of gel immediately in contact with the precipitate, fresh solute slowly diffuses towards this region, until the layer of gel adjacent to the precipitate again becomes exhausted of solute. The new ring will then cease to grow, and the whole process will be repeated until the available solute in the gel has been precipitated.

Since the adsorbed solute collects on the surfaces of the freshly deposited particles of the precipitate, the value of P/L will be greater there than elsewhere. Consequently precipitation will take place on the surfaces of the grains of the precipitate, which will grow equally in all directions. A further increase in concentration may result in the formation of an additional number of smaller centres, but the individual particles will grow in the same way. Microscopic examination of the rings and bands invariably reveals the spherical or approximately spherical shape of the constituent grains of the precipitate, which frequently reach macroscopic size. In many cases larger particles are seen imbedded in a finer grained precipitate. Hatschek's description of the lead iodide reaction records the observation of the actual growth of such spherical aggregates, which are characteristic of reactions in gels. "Here also the reaction begins with the appearance of a light yellow coloured strip. However, at the same time, numerous points of lead iodide appear on the inner side of the band which grow remarkably while the strip is only increasing slightly in intensity and width. When the isolated grains seem to have reached their full size, the band appears to grow suddenly and become opaque, but it has an essentially coarser structure than the silver chromate ring."

The progress of the lead chromate reaction is exactly the same as that of the lead iodide. The coarser structure of the bands is due to a smaller value of P/L, and allows the precipitant to diffuse through more quickly than in the case of silver bichromate. This has the same effect as if the diffusion gradient of the solute in the gel were less. The precipitant enters a region where the resulting value of P/L is very small, and comparatively few crystallisation

[1] Cases of sol formation which have been observed have always occurred at the end of the reaction, after the solvent has been almost completely extracted from the gel. The appearance is then merely of a diffuse coloration without any indication of stratification.

centres are produced. The excess of precipitant, flowing on, meets with constantly increasing quantities of solute, N grows proportionally, until the precipitated particles appear to form a band, and as their number increases, so their size diminishes. This corresponds exactly to the microscopic appearances, and may be taken as a further direct proof of the existence of a concentration gradient. The isolated particles appear simultaneously with the ring, because it is not until they have reached a certain size that they become separately visible. Actually they may be formed somewhat earlier. They grow by adsorption of nutrient material from the adjacent gel, until this is exhausted, and thickening of the ring cuts off further supplies. By this time the region of the new band has increased in concentration of both the inflowing reagents, resulting, at first, in a denser precipitate and a quicker rate of adsorption and precipitation, until, in consequence of the adsorption, the concentration of the adjacent gel falls once more.

One other point must be mentioned in conclusion. It has been insisted that stratification is a rare phenomenon limited to a few insoluble precipitates. This is not in accordance with the writer's experience. Although experiments have been directed to the examination of the conditions underlying zone formation in a limited number of cases, rather than to the multiplication of specimens, successful attempts have been made to prepare stratified deposits of those substances such as silver chloride, barium sulphate, copper phosphate in agar, and lead iodide in gelatin which have been specifically mentioned as refusing to stratify. No serious attempt to obtain a sufficiently insoluble precipitate in the stratified form has failed, except in the case of aluminium hydroxide in agar in U-tubes, and this was to be expected on account of its extreme insolubility. There appears to be no evidence to show that stratification will not occur provided a precipitate can be obtained in a suitable state of subdivision from solutions of a sufficient dilution: or that the production of such precipitate is other than a question of the influence of the various known physical factors. As opportunity offers, it is hoped, by increasing or diminishing the number of crystallisation centres in various ways, to prepare well stratified precipitates of substances in gels in which they are not ordinarily so obtained.

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V. THE REACTION OF SERA AS A FACTOR IN THE SUCCESSFUL CONCENTRATION OF ANTITOXIC SERA BY THE METHODS AT PRESENT IN USE.

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In a previous communication [1916, 1] I suggested that the practical difficulties so often encountered in the filtration of concentrated antitoxic sera might be due to the fact that hitherto no cognisance had been taken of the reaction of the sera before being concentrated.

With this object in view experiments have been carried out in which the effect of variations in the reactions of the sera have been considered with reference to:

(i) The precipitation of serum proteins by 30 per cent. of saturation with ammonium sulphate.

(ii) The increased precipitation of the serum proteins during the shortened heating process in the Banzhaf (1913) method. [Banzhaf, 1913.]

(iii) The increased precipitability of the serum proteins during the preliminary heating of the plasma as suggested in a previous paper. [Homer, 1916, 2.]

(iv) The practical application of the results obtained from the above investigations.

During the investigation of these points there has been accumulated a considerable amount of data which shows that the reaction of the serum is an important factor in the successful precipitation and heat denaturation of the serum proteins and that, unless the reaction be in some way controlled, apparently inexplicable irregularities are apt to occur in the course of the concentration.

I. The effect of changes in the reaction of serum on the precipitation of the serum proteins by 30 per cent. of saturation with ammonium sulphate.

Several series of experiments were conducted with oxalated plasma from different horses and from batches of pooled plasma or serum made up for concentration.

In each series, separate volumes of the test plasma were treated with known volumes of acid and of alkali. The plasma, thus treated, was brought up to 30 per cent. of saturation with ammonium sulphate by the addition of the necessary volume of a saturated solution of ammonium sulphate. The hydrogen ion concentration of the plasma and of the plasma-ammonium-sulphate mixtures was measured, wherever possible, by means of indicators and Sörensen's solutions in Walpole's colorimeter. The determinations, thus made, were subsequently checked by the electrical method as it was found that many of the indicators were rendered unreliable in the presence of so much protein as there is in serum. The protein contents of the filtrates from the serum mixtures were measured by means of the Zeiss immersion refractometer.

TABLE I.

Showing the influence of changes in the hydrogen ion concentration of the serum-ammonium-sulphate mixtures on the precipitation of serum proteins by 30 % *of saturation with ammonium sulphate.* (*Protein content of the plasma* = 7·92 %. *Hydrogen ion concentration of the saturated solution of ammonium sulphate added to the plasma* = p_{H}^{+} 6·480.)

Volume of normal acid (or equivalent) added to 100 cc. of the diluted plasma	Volume of normal alkali (or equivalent) added to 100 cc. of the diluted plasma	p_{H}^{+} of the diluted plasma to which the acid or alkali has been added, measured by the electrical method	p_{H}^{+} of the filtrate from the serum-ammonium-sulphate mixtures, measured by the electrical method	Residual percentage of protein in solution in the filtrates from the serum-ammonium-sulphate mixtures, measured by means of the refractometer
—	19·6 cc. N . NH_3	—	8·754	5·75
—	13·1 ,,	—	8·551	5·80
—	6·55 ,,	9·945	8·455	5·90
—	3·28 ,,	9·789	8·022	6·00
—	1·96 ,,	9·304	7·745	6·00
—	0·98 ,,	8·500	7·400	6·30
0·00 cc. N . HAc	0·00 ,,	7·430	7·077	6·30
0·38 ,,	—	—	7·000	6·52
1·25 ,,	—	6·450	6·438	6·60
2·80 ,,	—	5·921	6·021	6·60
3·60 ,,	—	5·440	5·579	6·25
3·76 ,,	—	5·225	5·327	6·20
3·99 ,,	—	5·025	5·145	5·87
4·99 ,,	—	4·600	4·972	5·02
6·00 ,,	—	4·560	4·712	4·52

The data with respect to one of the series of experiments have been embodied in Table I; comparable results were obtained with all the samples of serum and plasma investigated.

A study of Table I shows that within certain limits (p_H^+ 5·3 to 7·4) the reaction of the mixtures has little effect on the extent of the precipitation of the serum proteins. Beyond these limits in both directions there is an increased precipitation of protein by the sulphate. It was also noticed that the consistency of the precipitated euglobulin was greatly influenced by the reaction of the mixtures. The agglutination of the precipitate into a granular and readily filterable form proceeded most satisfactorily at a hydrogen ion concentration of about p_H^+ 5·3 to 5·0, i.e. on the slightly acid side of the isoelectric point of euglobulin (p_H^+ 5·5).

The state of aggregation of the Second Fraction precipitate (50 % of saturation with ammonium sulphate) was also dependent on the reaction of the mixture: the conditions most favourable for this precipitation are in the course of investigation.

II. The effect of changes in the reaction of the serum on the increased precipitation of the serum proteins induced during the shortened heating process in Banzhaf's (1913) method.

Several series of experiments were undertaken with samples of oxalated plasma from individual horses and from batches of pooled plasma from different horses.

In each series, to separate volumes of the test plasma contained in stoppered bottles, was added a known amount of acid or of alkali together with the volume of a saturated solution of ammonium sulphate necessary to bring the sulphate content to 30 % of saturation. The hydrogen ion concentration and the protein content of the clear filtrates from each of the experimental liquids were measured in the usual way. Experiments were carried out both with undiluted plasma and with plasma diluted with one-third its volume of water.

The mixtures were heated and then filtered. The protein content of the filtrates was estimated and compared with the corresponding value for the unheated liquids. From the data thus obtained was calculated the percentage increased precipitation of the soluble proteins due to the heating of the mixtures.

Comparable results have been obtained with each of the sera examined

irrespective of their protein content and of their dilution with water. The data with respect to one of the series of experiments have been embodied in Table II and represented graphically in the curve in Fig. 1.

TABLE II.

Showing the influence of changes in the reaction of the sera on the increased precipitation of protein during the heating of the serum-ammonium-sulphate mixtures to a temperature of 61° *and keeping them at that temperature for* 7 *to* 10 *minutes. (Protein content of the plasma* = 8·72 %. *The saturated solution of ammonium sulphate added to the plasma had a hydrogen ion concentration* p_H^+ 6·480.)

Volume of normal acid (or equivalent) added to 100 cc. of plasma diluted with one-third its volume of water	Volume of normal acid (or equivalent) added to 100 cc. of the diluted plasma	p_H^+ of the serum-ammonium-sulphate mixtures determined by the electrical method	Percentage increase in the precipitation of the soluble proteins induced during the heating of the serum-ammonium-sulphate mixtures	Remarks on the filtration of the heated serum-ammonium-sulphate mixtures
—	19·64 cc. N . NH_3	8·754	17·7	Readily, filtrate clear
—	13·04 ,,	8·577	16·4	,, ,, ,,
—	6·52 ,,	8·455	13·4	,, ,, ,,
—	3·28 ,,	8·022	10·0	,, ,, ,,
—	1·96 ,,	7·745	7·0	,, ,, very slightly opalescent
—	0·98 ,,	7·489	9·0	,, ,, ,, ,,
—	0·49 ,,	7·312	7·0	Less readily, filtrate slightly opalescent
0·00 cc. N . HAc	0·00 ,,	7·077	6·3	,, ,, ,, ,,
0·98 ,,	—	7·000	6·8	Slowly, filtrate opalescent
1·96 ,,	—	6·428	3·0	,, ,, ,,
2·94 ,,	—	6·021	2·8	,, ,, ,,
3·60 ,,	—	5·579	0·5	,, ,, ,,
3·76 ,,	—	5·327	18·0	Less slowly, filtrate slightly opalescent
3·92 ,,	—	5·145	20·7	Readily, filtrate clear
4·58 ,,	—	5·001	39·0	,, ,, ,,
4·88 ,,	—	4·972	52·6	,, ,, ,,
5·88 ,,	—	4·712	72·2	,, ,, ,,

It was noticed that where the hydrogen ion concentration of the mixtures was between the values p_H^+ 5·3 and 7·0, the filtration of the heated liquids was slow and the filtrates were markedly opalescent. Throughout these ranges the conversion of soluble into insoluble protein was least. In those cases in which the reaction had been more acid or more alkaline than in the above the filtration of the mixtures was rapid and the filtrates were clear, while the conversion of soluble into insoluble protein was of an appreciable order. On the alkaline side between the ranges p_H^+ 7·0 and 8·7 the conversion gradually rose from 5 to 17 %. On the acid side, as is seen at a glance in the

curve in Fig. 1, the rate of increase was much more marked; it rose from 0·5 to 72 % between the ranges p_H^+ 5·5 and 4·7.

No trouble was experienced with the filtration of the final products obtained in the concentrations of sera in which the reaction of the serum mixtures previous to heating had been adjusted to p_H^+ 5·2 or p_H^+ 5·0. As was however to be anticipated from the character of the filtration of the hot serum mixtures, difficulties were encountered with the end products where the reaction of the serum mixtures had been between the limits p_H^+ 5·3 and 7·0. In these cases the unsatisfactory filtration of the serum mixtures may be ascribed to one of two possible causes; either that between these limits the

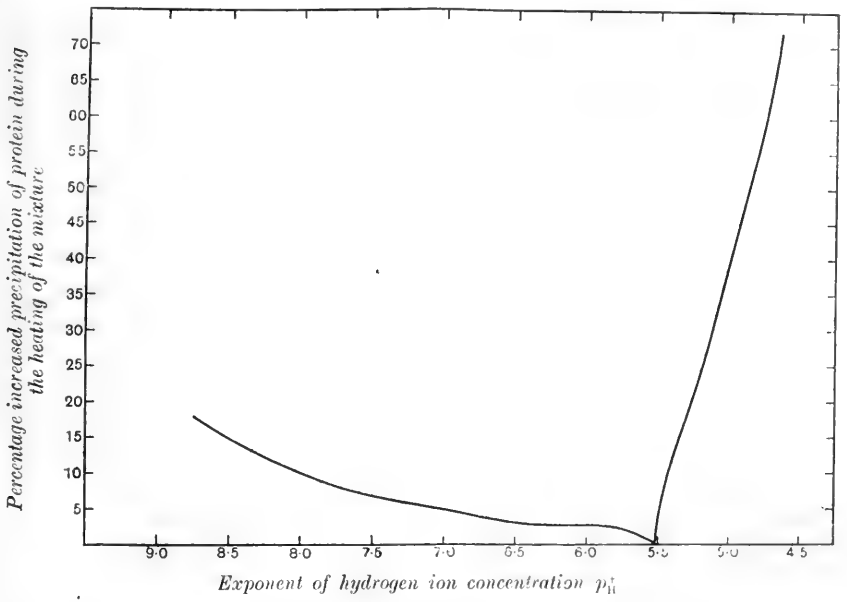

Fig. 1. Curve representing the influence of the reaction of the serum mixtures on the percentage increased precipitation of soluble proteins during the heating of serum-ammonium-sulphate mixtures by Banzhaf's method.

hydrogen ion concentration is unfavourable for the complete agglutination of the precipitated proteins: or that, since near the isoelectric point the heat denaturation of the serum proteins is least, it is possible that in this region the proteins which, under more acid or more alkaline conditions, would be appreciably denaturated are in a transitory, semi-emulsoid, semi-rigid stage and as such impart a slimy consistency not only to the serum mixtures but also to the final products.

It was also found that, with serum mixtures more alkaline than p_H^+ 8·0, there was difficulty with the filtration of the final product even though the filtrates from the heated serum mixtures were clear. In these cases, when these clear alkaline filtrates were acidified to a point p_H^+ 5·5 an opalescence was produced. The presence of a certain amount of precipitated protein which had been kept in the disperse phase by the alkali was thus indicated. The colloidal aggregates thus dispersed escaped precipitation with the First Fraction precipitate but were subsequently precipitated with the Second Fraction precipitate. They impart to the residue from the dialysis of the latter a slimy consistency and thereby considerably impede filtration through filter candles.

III. The effect of changes in the reaction of sera on the increased precipitability of the serum proteins induced during the heating of serum to a temperature of 57° for 6 hours.

During the course of our practical experience with the method suggested by me [Homer, 1916, 2] for the concentration of antitoxic sera it was observed that the heat denaturation of the serum proteins induced during the preliminary heating of the sera varied from 13 to 45 %. Throughout the winter and spring months the values with plasma varied from 28 to 35 % while with carbolised sera values as high as 45 % were reached. During the summer months when the horses were being given a considerable amount of green grass, the denaturation varied from 13 to 20 %.

After a considerable amount of experimental work it was ascertained that these variations were due to differences in the hydrogen ion concentration of the different plasmas and sera. During the summer months, when the low values for the heat denaturation were obtained, the oxalated plasma was slightly less alkaline than that obtained during the winter and spring months.

Samples of plasma from individual horses and from batches of the pooled plasmas from different horses were respectively diluted with one-third their volume of water. The hydrogen ion concentration of the diluted plasma was measured in each case. The reaction of the various samples was adjusted to the value of about p_H^+ 5·6 to 5·9 and also to the value p_H^+ 8·5. The original samples and the adjusted samples were heated in stoppered bottles to a temperature of 57° for 6 hours. The percentage increased precipitability of the serum proteins by 30 % of ammonium sulphate was measured with the aid of the Zeiss refractometer.

The data with respect to these observations have been embodied in Table III. They show that the variations in the extent of the heat denaturation of the serum proteins of individual sera can be obviated by adjusting the reaction of the sera to some fixed value. Thus, when the reaction was adjusted to about p_H^+ 5·6 to 5·9 the denaturation was of the order of 10 %, while at the value p_H^+ 8·5 it was of the order of 40 %[1].

TABLE III.

Showing that the variation in the heat denaturation induced during the heating of different samples of plasma at 57° for 6 hours can be overcome by an adjustment of the reaction of the sera to a fixed degree of acidity or of alkalinity.

Sample No.	Percentage protein content of the plasma	Value for p_H^+ of the diluted plasma	Percentage increased precipitability of the serum proteins during the heating of		
			undiluted plasma	diluted plasma brought, by the addition of ammonia, to the hydrogen ion concentration p_H^+ 8·5*	diluted plasma brought, by the addition of acetic acid, to the hydrogen ion concentration p_H^+ 5·9*
1	8·59	7·648	31·1	—	—
2	8·12	7·503	28·9	44·0	13·0
3	7·80	7·460	26·4	41·0	9·0
4	8·59	7·563	28·4	40·0	12·0
5	6·56	7·430	22·3	41·0	13·0
6	7·57	7·346	23·5	40·5	—
7	7·92	7·346	22·0	—	8·7
8	6·55	7·863	39·0	43·0	13·0
9	6·62	7·346	21·0	41·0	9·0
10	6·60	7·346	23·0	42·0	—
11	8·40	6·813	13·5	40·5	—
12	10·03	6·990	13·5	40·5	—
13	7·15	7·000	17·0	40·0	—

[Similar results were obtained with undiluted plasma.]

* Measured by means of indicators and Walpole's colorimeter.

In order to elaborate the point still further experiments were instituted in which samples of different sera, previous to their being heated, were treated with acid and with alkali to ensure a gradation in the reaction of the sera from p_H^+ 4·2 to 9·9.

Comparable results were obtained with each series of experiments. The typical results from one of these series have been embodied in Table IV and

[1] These adjustments were made with the aid of indicators and Sörensen's solutions in Walpole's colorimeter. A certain amount of difficulty was experienced by me in the matching of colours in serum, especially so, with the indicators available, on the acid side. I subsequently ascertained that, owing to my defective matching, the real values of the hydrogen ion concentration varied from the values aimed at by amounts sufficient to account for the slight variations in the heat denaturation recorded in the Table.

TABLE IV.

Showing the influence of the hydrogen ion concentration of sera on the extent of the heat denaturation of the serum proteins induced during the prolonged heating of serum at a temperature of 57° for 6 hours.

Volume of normal acid (or equivalent) added to 100 cc. of the plasma diluted with one-third its volume of water	Volume of normal alkali (or equivalent) added to 100 cc. of the plasma diluted with one-third its volume of water	p_H^+ of the plasma determined by the electrical method	Appearance of the heated plasma	Percentage conversion of soluble into insoluble protein during the heating of the diluted plasma A	Percentage increase in the precipitability of the soluble proteins by 30 % of saturation with ammonium sulphate B	Percentage destruction of antitoxin during the heating process
—	6·55 cc. N . NH_3	9·945	clear	2·2	55·7	nil
—	4·92 „	9·910	„	2·0	53·5	
—	3·275 „	9·789	„	1·5	46·6	
—	2·62 „	9·477	„	1·5	45·5	
—	1·96 „	9·304	„	2·2	46·5	nil
—	1·31 „	8·870	„	3·7	41·5	
—	0·65 „	8·249	clear liquid and slight deposit	2·2	36·8	
0·00 cc. N . HAc	0·00 „	7·430	„ „ „ „	3·3	22·3	nil
0·63 „	—	6·990	opalescent liquid and filtrate	5·2	10·2	
1·25 „	—	6·450	„ „ „ „	5·2	12·0	nil
1·87 „	—	5·921	„ „ „ „	3·7	13·1	
2·49 „	—	5·440	thin suspension, clear filtrate	6·3	13·1	
3·74 „	—	5·225	„ „ „ „	20·8	25·7	nil
2·50 cc. N . H_2SO_4	—	5·160	„ „ „ „	26·0	35·8	nil
4·17 cc. N . HAc	—	5·025	thick „ „ „	30·0	45·0	
2·52 cc. N . HCl	—	5·000	„ „ „ „	34·0	44·2	
4·99 cc. N . HAc	—	4·600	„ „ „ „	54·0	70·0	nil
3·60 cc. N . HCl	—	4·635	„ „ „ „	54·0	74·0	
3·60 cc. N . H_2SO_4	—	4·643	„ „ „ „	50·0	74·0	
6·74 cc. N . HAc	—	4·560	„ „ „ „	68·0	77·5	nil
4·40 cc. N . H_2SO_4	—	4·262	almost solid	98·0	not fluid enough to be measured	
4·36 cc. N . HCl	—	4·251	„ „	92·0		

With the sera ranging from p_H^+ 9·9 to 5·4 the extent of the changes recorded in *B* was a function of the time of heating, whereas with the more acid sera the change was practically completed within one hour's heating at the specified temperature.

have also been represented graphically in the curves in Fig. 2. Of the two, Curve 1 illustrates the changes in the solubility of the serum proteins during the heating of the serum, while Curve 2 indicates the accompanying changes in the precipitability of the serum proteins by 30 % of saturation with ammonium sulphate.

It was found that, with each of the sera examined, the extent of the changes involved was a function of the hydrogen ion concentration of the serum irrespective of the protein content: the use of undiluted serum or plasma gave rise to results similar to those obtained from experiments with plasma diluted with one-third its volume of water.

The appearance of the heated sera was characteristic throughout the ranges of acidity and alkalinity investigated.

It was found that the heated sera were clear when the reaction of the unheated serum had been more alkaline than p_{H}^{+} 8·3. When the reaction had been between the limits p_{H}^{+} 8·2 and 5·5 the heated sera were characterised by an opalescent appearance or by the presence of a slight deposit of protein. On the acid side of p_{H}^{+} 5·5 the sera were converted, during the heating, into thin suspensions, the semi-solid consistency of which became more marked as the degree of acidity increased, until at p_{H}^{+} 4·2 the serum became practically solid.

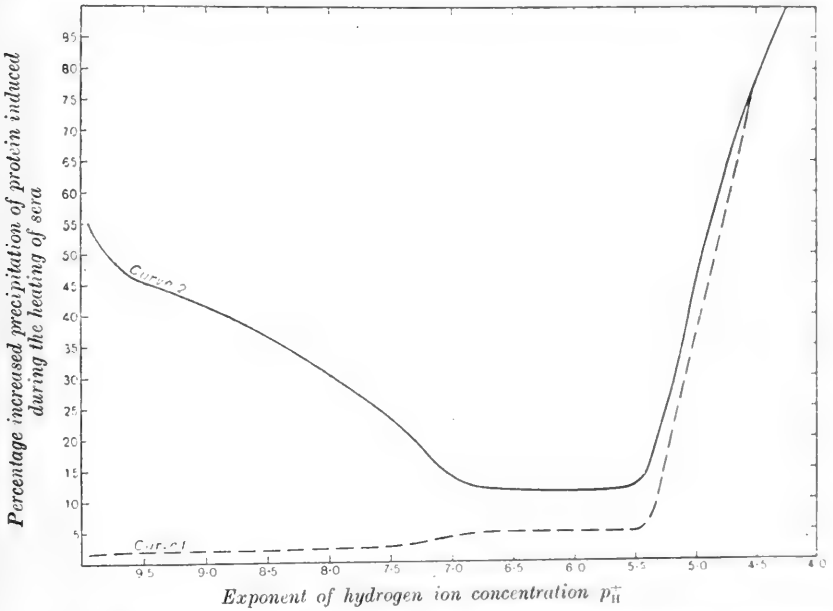

Fig. 2. The curve — — — represents the influence of changes in the reaction of sera on the percentage conversion of soluble into insoluble proteins induced during the heating of sera at 57° for 6 hours.

The curve ———— represents the influence of changes in the reaction of sera on the percentage increased precipitability of the serum proteins by 30 % of saturation with ammonium sulphate induced during the heating of sera at 57° for 6 hours.

From a study of the data given in Table IV, Column A, it will be seen that the conversion of soluble into insoluble protein was of the order of 5 % or less in the sera of which the reaction lay between the ranges p_{H}^{+} 9·9 and 5·5. On the acid side of p_{H}^{+} 5·5 there was marked precipitation of the soluble proteins; thus at a hydrogen ion concentration of p_{H}^{+} 5·4 it was of the order of 6 %, at p_{H}^{+} 5·0 it was 30 %, while at p_{H}^{+} 4·2 it was over 90 %.

From a consideration of Column B in the same table it will be seen that, irrespective of any accompanying conversion of soluble into insoluble protein, there was throughout the whole range investigated a marked increase in the precipitability of the serum proteins by 30 % of saturation with ammonium sulphate: the extent of the increase was a function of the reaction of the serum previous to its being heated.

This increased precipitability was least between the ranges p_{H}^{+} 5·4 and 7·0. On either side of these limits the rate of increase became apparent: the gradient for the increase was more gradual on the alkaline than on the acid side (Curve 2, Fig. 2). As the degree of alkalinity decreased from p_{H}^{+} 9·9 to 8·0 the increased precipitability gradually fell from 45 to 30 %. Between the ranges p_{H}^{+} 7·0 and 5·5 it was of the order of 10 to 15 %; on the acid side it rapidly rose from 25 % at p_{H}^{+} 5·2 to 90 % at p_{H}^{+} 4·2.

It was also interesting to note that, in all cases, there was no appreciable loss of antitoxin during the heating of sera the reaction of which had been adjusted to degrees of acidity or alkalinity within the limits dealt with in the accompanying tables, although under some conditions the extraction of the antitoxin from the heat-denaturated proteins proved a matter of difficulty.

From a study of the curves in Fig. 2 it is apparent that while on the alkaline side the configuration of Curve 1 is quite distinct from that of Curve 2, on the acid side of p_{H}^{+} 5·5 there is practical coincidence. There is, moreover, a marked similarity between the configuration of Curve 2 and that of the curve in Fig. 1. The extent of the increased precipitability on the alkaline side is not so marked in the latter as in the former, but on the acid side the curves, if superimposed, would be almost identical.

The question then arose as to whether the individuality of Curves 1 and 2 (Fig. 2) on the alkaline side indicated some radical difference between the changes respectively involved during the heating of alkaline and acid sera.

In order to elucidate this point samples of the clear heated alkaline sera which showed a 40 % increased precipitability by the sulphate were brought to an acidity of p_{H}^{+} 5·0. Now, had the changes involved during the heating of the alkaline sera been the same as those induced in the acid sera which showed a corresponding percentage increased precipitability, acidification of the clear alkaline sera should have changed the consistency of the latter to that of a thin suspension (*vide ante*, p. 29). A slight opalescence only was formed and the refractometer readings demonstrated that the quantity of protein thus precipitated was inappreciable. The opalescence therefore

probably indicated that a small amount of heat changed protein had been kept in the disperse phase by the excess of alkali (*vide ante*, p. 26).

It was also noticed that the increased precipitability of the proteins of the heated alkaline sera was a function of the duration of the heating process. On the other hand the changes recorded in the acid sera were practically complete after one hour's heating at the specified temperature.

From these observations it is evident that the changes taking place during the heating of alkaline sera are of a different nature from those which occur during the heating on the acid side of the isoelectric point of the globulins. For this reason it is possible that if the heat denaturation be conducted on the acid side, there may be prepared concentrated products which will be less likely to give rise to the troubles of anaphylaxis and of serum sickness than the present products concentrated by methods involving a denaturation of the serum proteins by heating the slightly alkaline sera.

Practical application of the results obtained in Sections I, II and III above.

The results of the investigation detailed above have led us to a better understanding of the causes underlying the irregularities encountered in the concentration of sera by methods involving the heating of the serum previous to the fractional precipitation of the serum proteins by ammonium sulphate.

We are finding that the production of clear and readily filterable end products can only be assured by controlling the reaction of the serum and of the serum mixtures unless certain expedients described below be resorted to.

An account of the investigation undertaken to overcome the causes of irregularities in the results obtained in (A) the Banzhaf (1913) method and later in (B) the method suggested by me [1916, 2] may be of service to those engaged in the concentration of antitoxic sera.

(A) *The regulation of the factors causing irregular results in the concentration of sera by the Banzhaf* (1913) *method.*

It seems probable that the difficulties in filtration so often encountered in this method are due to the presence of incompletely precipitated euglobulin and also of the small amount of protein denaturated during the heating of the serum.

As an outcome of the work recorded above it has been concluded that,

with the published technique, there can be no guarantee for the complete elimination of these disturbing factors, and this for two reasons:

(1) That no cognisance has been taken of the reaction of the serum-ammonium-sulphate mixtures previous to their being heated.

(2) That there is no increased precipitation of the serum proteins during the heating of the serum mixtures.

It is generally recognised that rapid and clear filtration of the hot serum mixtures is one of the essential conditions for the production of readily filterable end products. In this laboratory it has been shown that, for the successful filtration of the hot serum mixtures, there must be assured the agglutination of the particles of precipitated euglobulin into aggregates sufficiently large for their retention on the surface of filter paper. From Tables I and II it is obvious that the success of this operation can only be guaranteed if the reaction of the mixtures be suitably adjusted.

It has been demonstrated that during the heating process advocated in this method the precipitating power of 30 % of saturation with ammonium sulphate is not appreciably enhanced. It is evident, therefore, that the small amount of euglobulin which must always escape precipitation with the First Fraction precipitate will appear in colloidal suspension in the final product. Unless the latter be allowed to stand for a considerable time until this colloidal suspension has settled as a deposit the unprecipitated protein proves a hindrance to filtration and is the cause of the marked clouding of so much of the antitoxic serum concentrated by this method.

The irregularities in the filtration of the serum mixtures can be obviated by an adjustment of the hydrogen ion concentration to the optimum required for the complete agglutination of the particles of "salted out" euglobulin and of the particles of protein denaturated during the heating of the serum. Good results can be obtained by adjusting the reaction to an acidity of about p_{H}^{+} 5·0. As however the determination of the hydrogen ion concentration of the sera by the electrical method is not always practicable in a routine laboratory, an endeavour is being made to work out a simple practical method by which the reaction can be adjusted by the use of suitable indicators.

In the meantime, pending the search for suitable and reliable indicators, it has been shown that the irregularities in the filtration of the serum mixtures can also be obviated by the simple expedient of adding 1·5 to 2 % of sodium chloride to the mixtures previous to their being heated.

It is important to know whether the salt improves the filtration of the hot serum mixtures by virtue of a specific action on the particles of precipi-

tated protein or by an adjustment of the reaction of the medium more nearly to the desired optimum.

In order to elucidate this point duplicate samples of several plasmas were taken. To one of each of the duplicate pairs was added 1·5 to 2 % of sodium chloride. The hydrogen ion concentration of the duplicates was measured. The liquids were then made 30 % of saturation with ammonium sulphate and gradually heated to 61°.

From the results given in Table V it will be seen that the addition of sodium chloride decreases the alkalinity of the serum and of the serum

TABLE V.

Showing the influence of the addition of sodium chloride to the serum on the hydrogen ion concentration of the serum and of the serum-ammonium-sulphate mixtures.

p_H^+ of the plasma diluted with one-third its volume of water = A	p_H^+ of A made 30 % of saturation with ammonium sulphate	Percentage of NaCl added to the diluted plasma	p_H^+ of the salted plasma = B	p_H^+ of B made 30 % of saturation with ammonium sulphate	Remarks on the filtrate from the hot serum-ammonium-sulphate mixtures from	
					A	B
7·485	—	1·5	7·216	—	opalescent	clear
7·408	—	2	7·200	—	,,	,,
7·701	—	1·5	7·485	—	slightly opalescent	,,
7·701	—	4	7·485	—	,, ,,	,,
7·701	—	8	7·485	—	,, ,,	,,
7·347	—	2	7·223	—	opalescent	,,
—	7·250	1·5	—	7·010	,,	,,
—	7·220	2	—	7·000	,,	,,

mixtures. From a comparison with the data given in Table II, it is however obvious that the change in the hydrogen ion concentration induced by the addition of the salt is insufficient to account for the marked improvement in filtration and for the absence of an opalescent suspension in the filtrates.

It seems justifiable, therefore, to conclude that the improvement in filtration is induced by a specific action of the sodium chloride on the particles of precipitated protein whereby their satisfactory agglutination is assured.

Although the salt greatly improves the filtration of the serum mixtures, it cannot completely obviate the difficulties with the final product for it is powerless to ensure the complete precipitation of the euglobulin by 30 % of saturation with ammonium sulphate. Even in the presence of salt the desired increased precipitation of the serum proteins during the heating process does not take place.

In order to guarantee the elimination of euglobulin from the final products there were three courses open, viz.

(i) *The old method of extraction with brine.*

The Second Fraction precipitate containing the pseudo-globulin and antitoxin together with the small amount of attendant euglobulin and heat-denaturated protein was thrown into brine. The pseudo-globulin-antitoxin combination passed into solution while the euglobulin remained undissolved and was separated by filtration. The pseudo-globulin and antitoxin were precipitated by the addition of 0·25 % of glacial acetic acid to the clear filtrate. The precipitate was pressed and dialysed: the end product was crystal clear and its filtration presented no difficulty as it contained no trace of euglobulin or of heat-denaturated protein.

From the practical standpoint this method is not favoured as it involves a considerable amount of extra time and labour.

(ii) *A combination of the prolonged heating process of the Gibson-Banzhaf method with the shortened heating process of the Banzhaf* (1913) *method.*

By the adoption of this course it has been possible to obtain end products which, while showing an increased potency of from 8 to 10 times that of the original serum, do not exhibit the same tendency to form the cloudy deposit so often seen in the sera concentrated by Banzhaf's method. However, recent observations on the factors limiting the extent of the concentration of sera by the fractional precipitation methods at present in use [Homer, 1917], have demonstrated that there is a limit to which the concentration of sera can be carried. It was found that in all cases where the load of antitoxin attached to one gram of protein in the final product threatened to be greater than of the order of 25,000 units, the excess of units beyond this limit were transferred to the First Fraction precipitate.

From these observations it is evident that, for high grade sera (over 600 units per cc.), the above modification, yielding under ordinary circumstances a concentration of 8 to 10 times, cannot be used without apparent loss of antitoxin. The missing antitoxin can be recovered by extraction of the First Fraction precipitate with saturated salt solution.

To provide for such cases a third method of procedure has been employed, viz.

(iii) *The addition of substances such as phenol and its homologues to the serum mixtures previous to their being heated.*

Some years ago MacConkey [1914] observed that, during the heating of carbolised and of etherised sera there was a considerable loss of antitoxin. He subsequently found that the loss was negligible if 1·5 to 2 % of salt had previously been added to the serum.

In this laboratory it has also been demonstrated that, in the presence of salt, there is usually no appreciable loss of antitoxin during the concentration of carbolised sera by Banzhaf's method, if the temperature of the serum mixtures be not raised beyond 61°. At temperatures of 63° and 65° the loss becomes appreciable.

In the routine work, however, it has sometimes happened that, during the concentration of carbolised sera, there were appreciable losses of antitoxin even in the presence of salt. The records showed that in some of these cases the serum had been heated to a temperature of 65°, a procedure which in itself was sufficient to account for the loss. In other cases the records showed that during the concentration there had been removed more than 80 % of the total proteins of the original serum. With such a high removal of the serum proteins a certain proportion of the antitoxic units must be carried down with the First Fraction precipitate instead of with the Second Fraction precipitate. The antitoxin thus brought down with the First Fraction precipitate can only be extracted therefrom with great difficulty; hence the apparent and inexplicable loss of antitoxin under the conditions of the concentration.

It having been previously noticed that the heat-denaturation induced in the prolonged heating of sera to 57° is considerably increased by the addition of phenol, trikresol and cresylic acid to the serum, an experiment was made to ascertain whether the same phenomenon was experienced in the shortened heating process of Banzhaf's method. As this was found to be the case the investigation was carried further to ascertain whether the increased precipitation was due to changes in the hydrogen ion concentration caused by the addition of these substances.

In Table VI have been embodied data to illustrate the influence of phenol and cresylic acid on the precipitation of the serum proteins during the heating of the serum-ammonium-sulphate mixtures by the Banzhaf (1913) method. Similar results have been obtained by other workers in this laboratory from the use of trikresol, ether and chloroform.

From a study of the table it will be seen that while the addition of the above-mentioned substances had no appreciable effect on the hydrogen ion concentration of the sera, there was a marked increase in the precipitation

of protein during the heating and at the same time a marked improvement in the filtration of the hot serum mixtures. The end products were characterised by their clearness and their ease of filtration; they contained no euglobulin.

TABLE VI.

Showing the increased precipitation of serum proteins induced by the addition of phenol and its homologues to the serum-ammonium-sulphate mixtures previous to their being subjected to the shortened heating process of the Banzhaf (1913) *Method.*

Percentage of phenol or its homologues added to the plasma diluted with one-third its volume of water	p_H^+ of the carbolised plasma measured by the electrical method	p_H^+ of the carbolised plasma made 30% of saturation with ammonium sulphate	Percentage conversion of soluble into insoluble protein during the heating process	Remarks on the filtration of the hot serum-ammonium-sulphate mixtures	Loss of antitoxin during the heating process
I. *Plasma containing* 8·74 % *of protein.*					
0·0 phenol	7·748	7·347*	6·3	slow, filtrate opalescent	nil
0·1 „	7·730	7·035†	16·5	more rapid, filtrate slightly opalescent	„
0·2 „	7·730	7·034	23·0	rapid, filtrate clear	„
0·3 „	7·730	7·085	24·0	„ „ „	slight
0·4 „	7·730	7·085	26·0	„ „ „	nil
0·5 „	7·730	7·061	33·0	„ „ „	slight
II. *Plasma containing* 7·92 % *of protein.*					
0·0 phenol	7·460	—	2·0‡	slow, filtrate opalescent	
0·1 „	7·347	—	17·0	rapid, filtrate slightly opalescent	
0·2 „	7·347	—	24·4	rapid, filtrate clear	
0·4 „	7·347	—	27·9	„ „ „	
0·5 „	7·347	—	30·8	„ „ „	
III. *Plasma containing* 10·03 % *of protein.*					
0·0 cresylic acid	7·000	—	1·0	slow, filtrate opalescent	nil
0·35 „ „	7·000	—	27·0	rapid, filtrate clear	„
IV. *Plasma containing* 7·88 % *of protein.*					
0·0 cresylic acid	7·648	—	5·0	slow, filtrate opalescent	
0·30 „ „	7·648	—	26·0	rapid, filtrate clear	

* p_H^+ of the saturated solution of ammonium sulphate added to the plasma = 6·480.
† „ „ „ „ „ „ = 6·010.
‡ „ „ „ „ „ „ = 5·900.

As the addition of phenol, etc., to the serum mixtures previous to their being heated does not cause any alteration in the hydrogen ion concentration it is obvious that the improvement in filtration is due to the specific action of these substances on the proteins themselves, although the mode of action is different from that of sodium chloride. The latter merely serves to stimulate the aggregation of the particles of already precipitated protein, the former induce a considerable increase in the precipitation of the serum

proteins by 30 % of saturation with ammonium sulphate, thereby assuring the complete precipitation of euglobulin at this stage. It is quite possible that the increased heat-denaturation of the serum proteins is brought about by an effect of these substances on the surface tensions of the colloidal particles of dissolved protein whereby their precipitability is considerably increased. Whatever may be the fundamental cause of this change the fact remains that the effect is so marked that, not only is the precipitating power of 30 % of saturation with ammonium sulphate considerably increased, but, in the cases investigated, the agglutination of the aggregates of the precipitated protein is so well defined that the reaction of the serum mixtures is of secondary importance. Moreover, the proteins involved do not seem to enter the intermediate ill-defined stage between the emulsoid and the suspensoid state which proves so great a hindrance to the filtration of the serum mixtures and of the final products.

From the data embodied in Table VI we see that, by the addition of phenol or its homologues, the heat-denaturation of the serum proteins can be regulated to the extent suitable for a particular serum.

For those who prefer to use the Banzhaf method this expedient is recommended until a simple routine method for the adjustment of the reaction of the serum mixtures previous to their being heated has been worked out. The judicious addition of 0·35 to 0·5 % of phenol or of 0·25 to 0·35 % of its homologues to the batches of plasma for concentration will result in the production of clear and readily filterable end products. If the addition of salt is also made a matter of routine, the concentration of the carbolised plasma or sera will not involve undue loss of antitoxin, although a considerable loss may result if the substances in question are added in amounts greater than those suggested.

(B) *The regulation of the factors causing irregular results in the concentration of sera by the more recent methods.* [Homer, 1916, 2.]

It has been found that there was slight trouble with the filtration of the end products from the concentration of sera in which the heat-denaturation had been less than 20 %, that is to say from the concentration of sera the reaction of which lay between the limits p_{H}^{+} 5·4 and 7·0. In these cases the end products contained a small amount of heat-denaturated protein in colloidal suspension.

The occurrence of this difficulty could be prevented by a preliminary adjustment of the serum, previous to its being heated, to a hydrogen ion

concentration of about p_H^+ 7·8 to 8·0. It was inadvisable to increase the alkalinity further for the reasons given (*vide ante*, pp. 30, 31).

Trouble with the final filtration of end products from the concentration of sera of which the reaction fell within the limits p_H^+ 5·4 to 7·0 could also be obviated by the judicious addition of from 0·25 to 0·35 % of phenol or its homologues to the serum previous to the preliminary heating.

It is inadvisable to add the latter substances to more alkaline or to more acid sera than those falling between the aforesaid limits as there is the danger of thereby inducing so great an increased precipitability of the serum proteins that transference of antitoxin to the First Fraction precipitate will occur. Under these conditions there will be an apparent loss of antitoxin during the concentration as the extraction of the antitoxin thus carried down with the First Fraction precipitate is a matter of great difficulty.

It has also been found advisable to adjust the reaction of the serum-ammonium-sulphate mixtures previous to the second stage of the heating process (*ante*, p. 32). It may also be stated that at this stage of the heating the temperature of the serum mixtures is not raised beyond 59°.

From the consideration of the work detailed above it is evident that the reaction of the sera must be taken into account by those engaged in the concentration of antitoxic sera. On the reaction of the serum depends, not only the extent of the heat-denaturation of the serum proteins, but also the successful precipitation of the serum proteins by ammonium sulphate. Unless the reaction be regulated the necessary increased precipitation of the proteins cannot be ensured except by the addition of phenol, etc., to the serum. The latter course, in the hands of inexperienced workers may prove somewhat costly, as these substances favour the destruction of antitoxin.

Although the regulation of the reaction of the serum is proving of material value the possibility must not be lost sight of it being shown at some future date, that, in the concentration of antitoxic sera, there are other, hitherto, unsuspected factors which also influence the operations involved. In any case it is clear that the standardisation and regulation of a concentration process can only be attained by a thorough knowledge of the factors involved.

Summary.

During the course of the investigation it has been demonstrated that:

(1) In the Banzhaf (1913) method the uncertainties in filtration are due to two causes:

(*a*) That no cognisance has been taken of the reaction of the serum.

(*b*) That as the precipitating power of 30 % of ammonium sulphate is not appreciably increased during the heating process, a certain amount of euglobulin escapes precipitation with the First Fraction precipitate and appears in colloidal suspension in the final product.

(2) The uncertainties in the filtration of the hot serum-ammonium-sulphate mixtures in the above method can be obviated by an adjustment of the hydrogen ion concentration. The filtration can also be improved by the addition of sodium chloride to the mixtures, but, in this case, the improvement is due to a specific action of salt on the globulins.

(3) The complete elimination of euglobulin can be assured by:

(*a*) An adjustment of the hydrogen ion concentration of the serum mixtures to the point at which the desired increased precipitation is assured.

(*b*) Brine extraction of the Second Fraction precipitate containing the pseudo-globulin-antitoxin combination together with the small amount of euglobulin which has escaped precipitation with the First Fraction precipitate.

(*c*) Subjecting the serum to a preliminary prolonged heating at a temperature of 57—58° whereby the precipitability of the serum proteins is considerably enhanced.

(*d*) The addition of organic substances such as phenol and its homologues, ether or chloroform which increase the precipitation of the serum proteins, presumably, by virtue of their effect on the surface tension of the protein aggregates in colloidal solution.

(4) The extent of the heat-denaturation of the serum proteins during the heating of serum at 57° for several hours is also influenced by the hydrogen ion concentration of the serum and can be controlled by the adjustment of the latter.

(5) The denaturation induced by heat in alkaline sera apparently does not involve the same type of change as that induced in acid sera.

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VI. FURTHER CONTRIBUTIONS TO THE TECHNIQUE OF PREPARING MEMBRANES FOR DIALYSIS.

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Introduction.

In a previous communication [Brown, 1915] a method was described by which a series of collodion membranes of differential permeability could be prepared. These membranes were for use in aqueous solution. It has been found that the method there described can be extended to the case of other substances (e.g. gelatin, agar, parchment, etc.) and to a large variety of liquids (ethyl alcohol, acetone, chloroform, benzene, etc.). Furthermore by a modification of the method, a series of graded gelatin membranes has been obtained which can be used in aqueous solution.

General Principle.

By way of illustration we may take the case of the collodion technique described in the paper already cited. When films of air-dried collodion (celloidin) are immersed in water, only a negligible imbibition of the latter takes place. When similar films are immersed in absolute (or nearly absolute) ethyl alcohol, a rapid imbibition takes place, the film swelling up, and, if sufficient alcohol be present, disintegrating to form a uniform solution. By immersing the films in an alcohol-water mixture, an intermediate state is reached in regard to imbibition by the collodion, of such a type that the higher the percentage of alcohol used, the greater the resultant imbibition. When the films so treated are placed in water and thoroughly washed, it is found that while a certain amount of shrinkage takes place in the process, the effect of the differential treatment is still strongly marked,

and thus, corresponding to a given strength of alcohol, a collodion membrane possessing a definite permeability and having water as its continuous phase may be prepared.

In the above illustration, the function of the water may be described as being to restrain the imbibition of alcohol by the collodion. For convenience in description we shall speak of "restraining" and "swelling" liquids, the former being exemplified in the above illustration by water and the latter by alcohol.

The general principle may now be enunciated:

Suppose a system consisting of:—Liquid *A*—Membrane substance—Liquid *B*.

If (1) the membrane substance only imbibes a negligible amount of *A*,

(2) the membrane substance strongly imbibes *B*,

(3) *A* and *B* are miscible in all proportions,

then by soaking films of the membrane substance in mixtures of *A* and *B* and subsequently washing in *A*, membranes of graded permeability can be obtained, and of such a type that the higher the percentage of *B* in the mixture the greater is the permeability[1] of the resultant membrane.

The membranes which have been produced by application of the above principle will be described under two headings:

A. Graded membranes for use in the restraining liquid, e.g. collodion in water, gelatin in alcohol. This class comprises the great bulk of the membranes prepared.

B. Graded membranes for use in the swelling liquid. Of this type, only one is at present known, viz., gelatin in water.

[1] In the paper already cited, the effect of the treatment with alcohol-water mixtures was shown by determining the change in the space relationships (e.g. increase in length, increase in thickness, diminution in percentage of dry to total weight), as well as by measurement of the rates of diffusion of particular substances through the membrane. In the case of the membranes dealt with in the present paper, it has not been found possible to carry out the experiments with the same amount of detail, partly on account of the large variety of membranes which have been produced, but mainly because of the impossibility of obtaining at the present time the glassware requisite for carrying out in the case of these membranes experiments on diffusion and dialysis. In the case of the collodion-in-water membranes it was found that high permeability was associated with a low dry weight/wet weight ratio, and *vice versa*, that is, that permeability increased with increased development of the external or continuous phase of the membrane. This correlation has been established in the case of gelatin-in-alcohol and gelatin-in-water membranes, and it is believed will be found to apply to membranes of all descriptions. In order therefore not to overstep the limits of experimentally ascertained fact, the statement in the text should be paraphrased into "the higher the percentage of *B* in the mixture, the lower is the dry weight/wet weight ratio of the resultant membrane, that is, the greater is the amount of its continuous (liquid) phase."

A. Graded Membranes for use in the Restraining Liquid.

Preparation of the Air-dried Films—Practical Notes.

Gelatin. 100 cc. of a 2 % solution of sheet gelatin in water (40—50° C.) is poured over a clean mercury surface in a shallow circular dish of about 20 cm. diameter and allowed to dry completely. The method of preparation by pouring over a flat glass surface was rejected on account of the difficulty of removing the dried film from the glass surface.

Agar. 100 cc. of a 2 % solution is poured over a flat horizontal glass plate (20 cm. diam.) and allowed to dry; when slightly moistened, the film may readily be peeled off. It is then soaked in alcohol (this involving a certain amount of shrinkage) and dried in a current of air. The method adopted in the case of gelatin was found to be impracticable in this case as the drying film invariably broke away from the sides of the vessel and dried with considerable shrinkage in area.

Starch. Only one membrane was prepared as it was found to be too brittle to possess any practical value. It was prepared in the same way as the gelatin membrane.

Collodion. The treatment is the same as in the case of agar films, the film being of course dried off without immersion in alcohol.

Preparation of Graded Membranes.

Films prepared as described above, together with vegetable parchment, were placed in various alcohol-water mixtures for 24 hours, and then transferred to absolute alcohol in which they were washed (with 3 changes) over a period of 2 days. The wet and dry weights of each membrane were then determined in the usual way and with the usual experimental precautions. The figures contained in Table I illustrate the type of result obtained.

TABLE I.

	(Dry weight/wet weight) × 100			
% Alcohol	Parchment	Starch	Gelatin	Agar
0	60·50	62·27	19·83	4·50
40	64·75	77·42	49·07	17·73
60	67·20	87·71	66·07	51·37
80	72·65	—	75·00	77·52
100	97·58	95·14	85·00	90·80

With regard to the above figures it must be remembered that in obtaining the wet weights of the membranes, it is impossible to remove completely the film of alcohol from the surface, and that therefore the figures obtained are all somewhat too low. The percentage error so introduced is obviously greatest in the 100 % alcohol membranes, and least in the case of those at the other end of the series. The error is greatest in the case of the agar and gelatin membranes as these were considerably thinner than the others.

The marked nature of the grading effected is sufficiently emphasised by the above figures not to require further comment. It is most pronounced in the case of the agar and gelatin membranes, and the latter have accordingly been selected as the most favourable substances for purposes of the present technique. Of these agar is preferred on account of the wider range it presents, and on account of the greater ease in its manipulation.

The figures obtained for parchment are anomalous in showing a very slow gradation over the greater part of the range of alcohol-water mixtures and a very sharp gradation towards the higher end of the series. This is considered to be due to the fact that parchment is not a homogeneous substance as it consists of two parchmentised layers with a paper layer between. The observed wet weight of a textile fabric like the latter is higher than the true wet weight as it includes the liquid which lies between the fibres and which is not properly speaking imbibed in the substance of the paper. The high figure obtained for the 100 % parchment membrane is due to the fact that such a membrane is impermeable to alcohol and therefore the internally situated paper cannot be reached by the latter.

The graded effect produced in these membranes can be readily demonstrated by observing the rate at which they stain on immersion in a dye solution. Thus when 40, 60, 80, 100 % membranes of parchment, gelatin and agar are placed in a strong solution of night blue in absolute alcohol for a short time (e.g. 15 minutes), taken out and rapidly wiped with a cloth to remove excess stain, it is seen that in all cases staining of the 100 % membranes is negligible, strong in the case of the 40 % membranes, with intermediate conditions in the case of the 60 and 80 % membranes. *Pari passu* with this graded staining effect runs the capacity of the particular membrane to transmit the dye, though this relationship has, on account of the difficulty of obtaining the requisite glass-ware, only been as yet fully ascertained in the case of the gelatin membranes. Thus in diffusion tests of alcoholic night blue against absolute alcohol with a series of graded gelatin membranes, no trace of the dye had passed through in 24 hours in the case of the 80 and 100 % mem-

branes; a slight passage occurred in the same time in the case of the 60 % membrane; whereas in the 40 % membrane strong coloration of the alcohol took place within a few (e.g. 5) minutes. A similar behaviour can confidently be predicted for the other membranes.

Uniformity and Permanency of the Grading Effect.

With films prepared in the same way the results obtained are markedly uniform. The figures in the following table give as usual the percentage of dry weight in total weight of membrane. The membranes were prepared simultaneously, and all washed in absolute alcohol for 3 days; the wet and dry weights of the first lot *A* were determined immediately, while the corresponding quantities for the second lot *B* were determined after a further 7 days. The ratio of the thickness when wet to original thickness when dry was also determined, and the results are also incorporated in Tables II and III.

TABLE II.

Agar.

	A (3 days in alcohol)		*B* (10 days in alcohol)	
% Alcohol	$\frac{\text{Dry weight}}{\text{Wet weight}} \times 100$	$\frac{\text{Thickness (wet)}}{\text{Thickness (dry)}} \times 100$	$\frac{\text{Dry weight}}{\text{Wet weight}} \times 100$	$\frac{\text{Thickness (wet)}}{\text{Thickness (dry)}} \times 100$
0	4·50	ca. 1900	3·13	ca. 2200
	4·55	ca. 1900	5·18	ca. 1600
40	17·73	ca. 510	19·44	ca. 500
	17·65	ca. 500	18·05	ca. 500
60	51·37	183	53·57	172
	53·40	175	54·14	168
80	77·52	120	78·90	114
	78·22	127	78·94	117
100	90·80	100+	88·33	100
	90·23	100+	87·50	100

TABLE III.

Gelatin.

	A (3 days in alcohol)		*B* (10 days in alcohol)	
% Alcohol	$\frac{\text{Dry weight}}{\text{Wet weight}} \times 100$	$\frac{\text{Thickness (wet)}}{\text{Thickness (dry)}} \times 100$	$\frac{\text{Dry weight}}{\text{Wet weight}} \times 100$	$\frac{\text{Thickness (wet)}}{\text{Thickness (dry)}} \times 100$
0	19·83	ca. 650	17·28	ca. 670
	19·30	ca. 630	18·53	ca. 700
40	49·07	220	52·21	235
	51·75	230	—	—
60	66·07	150	65·22	162
	64·28	150	66·67	160
80	75·00	120	74·47	123
	74·00	130	75·42	125
100	85·00	100	84·00	110
	—	—	—	—

It is thus seen that with similar treatment the membranes obtained show characteristic figures varying by not more than 2—3 per cent. With special precautions in the determination of these figures, it is certain that this range of error could be considerably curtailed.

Examination of the tables also shows that the membranes have not appreciably altered over a period of 1 week. More detailed tests by the staining method have failed to show any alteration over a period of 25 days. Similarly a 40 % gelatin membrane which allowed passage of the dye night blue in a few minutes was found to behave similarly after being kept for a period of 6 weeks. It is thus certain that these membranes preserve their properties with little alteration over a considerable period of time.

The degree of uniformity indicated in the preceding tables is not attainable unless the initial dry films have been prepared in a similar way. In the preparation of the solution from which the films are prepared, increased exposure to heat produces increased imbibition in the 24 hours' exposure to water and the alcohol-water mixtures, with corresponding differences in the dry weight percentage of the finished membranes. In the following table, a comparison is given of two films, one of which (*A*) was prepared by heating the original agar powder suspension to 100° for a few minutes; the other (*B*) by similar treatment at 120° for 20 minutes. It will be observed that the differential effect diminishes with increasing percentage of alcohol.

TABLE IV.

	$\frac{\text{Dry weight}}{\text{Wet weight}} \times 100$		$\frac{\text{Thickness (wet)}}{\text{Thickness (dry)}} \times 100$	
% Alcohol	*A*	*B*	*A*	*B*
0	10·24	4·50	450	1900
40	41·28	17·73	195	510
60	58·93	51·37	152	183
80	75·33	77·52	115	120

The corresponding figures for the ratio of dry to wet weight (in water) after 24 hours' soaking in water are: *A*, 4·4 %; *B*, 1·5 %.

An exactly similar phenomenon occurs in the case of gelatin. This is in agreement with the statement of Wo. Ostwald [1905] that increased heating confers upon gelatin an increased power of imbibing water. It is obvious therefore that in carrying out the present technique the process of preparation of the original films must be standardised.

Generalisation. Graded membranes having been obtained by the above process, the absolute alcohol may be replaced by any liquid which is miscible

with alcohol and which is not imbibed by the dry gel substance. Thus in the case of agar and gelatin, absolute alcohol as continuous phase may be replaced by acetone, benzene, petrol ether, etc., and thus graded membranes prepared for use in these liquids. The process of preparation consists simply of washing out the alcohol by immersion of the membrane in several changes of the particular liquid.

Table V shows such a gradation effected in a series of agar membranes. The figures represent as usual the percentage of dry weight in total weight. All the membranes were prepared by soaking in alcohol-water mixtures, with subsequent transference to absolute alcohol. The experiment was performed in duplicate so that an idea might be obtained of the degree of uniformity realised.

TABLE V.

% of alcohol employed in grading	Eth. alc.	Acetone	Toluene	Benzene	Chloroform	Clove oil	Petrol
0	4·10	4·21	3·89	4·26	2·15	5·00	5·21
	4·23	4·42	3·84	7·93	2·28	5·24	4·49
40	17·88	20·27	18·63	25·00	11·60	25·15	26·55
	17·73	17·00	16·67	23·73	10·97	26·97	23·24
80	74·60	78·22	88·20	78·67	73·21	68·22	87·00
	74·08	76·92	88·42	79·00	74·42	68·60	88·90

It may be remarked that the figures in the horizontal rows of the above table are not comparable with each other. The low figures obtained in the case of the 0 % and 40 % membranes in chloroform are to be explained by the high specific gravity of chloroform. Even if the figures in the horizontal rows be corrected for the different specific gravities of the liquids concerned, it is not certain that they will even then be comparable, as it is possible that the volume of the continuous (liquid) phase of the membrane alters on transference from one liquid to another. An accurate investigation of this type has not yet been attempted.

A detailed determination of the permanence of the graded effect in these membranes has not been carried out. Membranes in clove oil and toluene have been kept for 6 weeks, at the end of which time the grading as far as could be judged from external appearances (thickness) was as pronounced as ever. It is tolerably certain that the permanency of grading demonstrated in the case of the membranes in absolute alcohol is characteristic of these membranes in general.

Membranes prepared by use of other Swelling and Restraining Liquids.

1. *Restraining Liquid.* Throughout the present section alcohol has been used as restraining liquid. Membranes of equal range can be obtained by a similar use of acetone.

In the earlier paper in which the technique of collodion membranes was described, water was employed to restrain the swelling action of alcohol. For this purpose it may be replaced by any other liquid which mixes with alcohol and which is not imbibed directly by the dry collodion. Thus graded collodion membranes have been prepared by use of chloroform, benzene, toluene, and petrol ether as restraining liquids. The range of mixtures available varies in the different cases, the maximum concentration of alcohol which can be used in each case being shown by the following figures:

Alcohol in toluene... ...	30—40 %
Alcohol in benzene ...	40 %
Alcohol in petrol ether...	75—80 %
Alcohol in chloroform ...	90—95 %
Alcohol in water	97—98 %

It is probable that the best series of graded collodion membranes for use in such liquids as benzene and toluene can be prepared by previous manufacture in chloroform and transference to the particular liquid to be employed.

2. *Swelling Liquid.* The scope afforded by the method under consideration is well illustrated by the variety of graded series of membranes which can be prepared from the acetate derivative of cellulose[1]. This substance is soluble in acetone and acetic acid, and swells to a considerable extent in chloroform. It has been found possible to prepare graded membranes by use of any of the following mixtures:

Acetone—water,
Acetone—ethyl alcohol,
Acetic acid—water,
Chloroform—ethyl alcohol,
Chloroform—benzene,
Chloroform—toluene,
Chloroform—petrol ether,

the membranes after being placed in the mixture being finally set up in the

[1] This material was kindly supplied to me by Mr Cross of Messrs Cross and Bevan, New Court, Lincoln's Inn, W.C.

second liquid in each case. Whether, as is probable, these methods are of unequal practical value has not up to the present been determined.

While the method of grading put forward in the present paper is undoubtedly of very wide application, the writer is not prepared to state that where the conditions as laid down on p. 41 are fulfilled, a graded series of any particular membrane substance can always be obtained. Thus it is not certain that graded rubber membranes for work in alcohol can be prepared by use of benzene-alcohol mixtures. As far as preliminary observations went, it appeared that in all cases the differential treatment became obliterated when the membranes were washed in alcohol. The nature of the particular membrane substance therefore plays a part in determining the nature of the result obtained. It is noteworthy that all the substances with which the most marked success has been achieved—agar, gelatin, celloidin—are characteristically plastic substances, whereas rubber is eminently elastic. The difference in effects observed is believed to be correlated with this difference in respect of shear elasticity.

Again, as was stated in the earlier paper dealing with this subject, it was found impossible to produce any effective grading of celloidin by use of acetone-water mixtures. The range of mixtures here available was somewhat limited, 0—60 %, but in addition to that it was observed that the celloidin films towards the upper end of the series tended to disintegrate without the marked continuous swelling which is characteristic of similar films when placed in alcohol. The volume of the continuous phase of such a membrane up to the stage where it was still a manageable membrane was therefore too small to allow of its ever being converted into even a moderately permeable membrane.

At this point it may be opportune to summarise what is considered an ideal relationship in the system:—

Swelling liquid—membrane substance—restraining liquid,

from the point of view of the present technique.

Membrane substance—swelling liquid. The membrane substance should not go into complete solution, or if it does it should reach that state by a process of continuous imbibition. It should preserve a practicable degree of mechanical coherence up to a high degree of swelling. Only in such a case is it possible to prepare highly permeable membranes of practical utility.

Membrane substance—restraining liquid. The membrane substance should not take up anything beyond a trace of the restraining liquid. This condition is nearly realised in the case of celloidin in water, though not sufficiently so

to prevent the passage of water and the simplest salts. In the case of the cellulose-acetate membrane this condition is less closely realised, so that it is doubtful if this substance can be used for the preparation of so-called semi-permeable membranes.

B. Graded Membranes for use in the Swelling Liquid.

Though only one series of such membranes is as yet known (viz. gelatin in water), and though the range of permeability of these is comparatively limited, nevertheless they are considered to possess such practical and theoretical interest as to merit a detailed description. It is known at present for instance that these gelatin membranes can be applied with great effect in the purification of certain enzymes and that they show properties which indicate that they may prove of considerable use in the treatment of phenomena of selective permeability.

Theory of Method.

In all the processes hitherto discussed, the films of membrane substance, once a state of differential swelling had been established by immersion in the series of "swelling-restraining" liquid mixtures, were without further treatment transferred to the pure restraining liquid. It was obvious on theoretical grounds (and was abundantly verified by experiment) that were transference to take place to the swelling liquid, all the membranes would rapidly swell up to much the same degree, and thus the differential effect would be obliterated. If however while the membranes were still immersed in the series of mixtures, the capacity of imbibing the swelling liquid could be suppressed or at any rate reduced to a small amount, it was argued that after such treatment the membranes could be transferred to the swelling liquid without obliteration of the graded effect. The method adopted in the case of gelatin is as follows.

Films of air-dried gelatin are placed in a series of alcohol-water mixtures at 20° C. After 24 hours, a given quantity (1 cc. to 10 cc. of alcohol-water mixture) of 40 % formaldehyde is added to each. After 24 hours' treatment in this solution, the membranes are transferred to water and thoroughly washed; they are then ready for use.

It will be noticed that the addition of the formaldehyde somewhat alters the alcohol-water ratio in the mixture. It is obvious that the process could be carried out without this ratio being altered, but such a refinement of the method has not as yet been deemed to be necessary.

A typical series of results is set forth in the following table.

The gelatin consisted of the ordinary sheet gelatin (as supplied for manufacture of culture media). After treatment as indicated above, the membranes were washed for two days in distilled water.

TABLE VI.

Treatment (% alcohol)	$\frac{\text{Dry weight}}{\text{Wet weight}} \times 100$
0	7·35
20	13·66
40	24·29
60	36·70
80	45·93
100	47·93

The grading may also be illustrated by measuring the increase in length and increase in thickness of the membranes. The following table gives the lengths and thicknesses of strips of commercial sheet gelatin after treatment according to the present technique, the corresponding quantity for the original aid-dried membranes being taken as 100 in each case.

TABLE VII.

Membrane	0 %	20 %	40 %	60 %	80 %
Length	112·5	105·4	103·3	102·2	102
Thickness	390	350	290	225	185

The grading is thus shown markedly by alteration in thickness of the membrane. The effect in lengthening the membrane is comparatively slight. In the case of membranes prepared from films which have dried under tension according to the method described on p. 42, an actual contraction due to the release of this tension has been observed when the membranes are soaked in the intermediate concentrations of alcohol.

By this technique membranes ranging from about 5 % to nearly 50 % of dry substance can be prepared. In no case has a membrane of greater content than 50 % in dry weight been obtained; in other words, it has been found impossible to formalise a membrane so strongly as to prevent it taking up on immersion in water at least an amount of water equal to its own weight. At the other end of the series a limit is placed by the increasing mechanical weakness of the membrane. Membranes of less content in gelatin can readily be prepared by carrying out the imbibition process at a somewhat higher temperature, but their extreme fragility renders them of little practical value.

Test of Uniformity. With similarly prepared dry films, and similar treatment, a very considerable degree of uniformity in the resultant membranes can be attained. This is illustrated in Table VIII which gives the results of an experiment carried out in triplicate.

TABLE VIII.

Alcohol %	0 %	20 %	40 %	60 %	80 %	100 %
$\frac{\text{Dry weight}}{\text{Wet weight}} \times 100$	12·66	17·86	23·86	34·76	44·07	26·43
	13·05	18·08	23·69	34·60	44·09	26·87
	12·17	18·14	23·83	34·72	44·09	26·83

Throughout this series the maximum variation from the mean is less than 0·5 %. This degree of uniformity is however by no means attainable when gelatin films of different origin are employed. A comparison of Tables VI and VIII brings out the essential features of the variations experienced. In both cases crude sheet gelatin as supplied for the manufacture of culture media was employed, that in the latter series being of a different make from the former.

A considerable amount of experimental work has been carried out with a view to determining the range of variation and the means of experimental control. The results may be briefly summarised as follows.

Considerable variations may be shown at the lower end of the series (membranes prepared in water and 20 % alcohol) by different samples of gelatin. This difference must be ascribed to the previous history of the gelatin. Films prepared in the manner described on p. 42 show a higher degree of swelling in water and 20 % alcohol than is shown by the original commercial sheet gelatin itself. This sensitiveness of gelatin to previous heating has already been mentioned.

Within considerable limits, the figures obtained are independent of the initial thickness of the membranes.

In the middle region of the series (40, 60, 80 %) remarkable constancy in results is obtained, no matter what the type of gelatin employed may have been. Thus in several hundred measurements which have been made at one time or another with different samples of gelatin, a maximum variation of about 3 % only has been observed in the dry weight percentage of such membranes.

A remarkable variation is shown by different samples of gelatin in respect of the membranes soaked in 100 % alcohol. This feature is illustrated by

a comparison of Tables VI and VIII. In the former case, the membrane prepared in 100 % alcohol differs only slightly from that prepared in 80 % alcohol; in the latter table it has a much lower gelatin ratio. This is due to the fact that in the latter case only the outer layers of the membrane are formalised, the inner mass being highly swollen and unformalised. By lengthening the formalising period, this result can be avoided. In any case it may be noted that very little practical advantage is obtained by extending the series beyond the 80 % mixture.

Length of Imbibition Period. Though a slow imbibition in water and the lower concentrations of alcohol can be demonstrated over several days, nevertheless the effect so produced is so slight as to render an imbibition period of over 24 hours unnecessary.

Length of Formalising Period. It has been seen that in the case of membranes in 100 % alcohol, a period of 24 hours may be insufficient for formalising. In the case of membranes in 0—80 % alcohol, only a slight difference could be detected between membranes which had been formalised for 4 days and those which had had 1 day's formalisation. Thus 24 hours may be considered ample for the process. Table IX, giving a comparison of 6, 10, and 24 hours' formalising, shows that the period allotted (24 hours) cannot safely be reduced.

TABLE IX.

Formalising period	Membrane					
	0 %	20 %	40 %	60 %	80 %	100 %
6 hrs.	6·93	12·43	20·80	30·82	37·31	32·94
10 „	7·24	12·93	21·64	33·33	40·56	42·03
24 „	7·33	13·24	23·20	36·48	44·32	45·83

Permanence of Graded Effect. Unlike the membranes treated of in Section A, the membranes of the present section are known to undergo a definite and appreciable change when kept in water. This consists in a slow uptake of water, so that the figures representing the gelatin ratio undergo a steady decline. The amount of this effect will appear from Table X.

The membranes were prepared in the usual way, i.e. with 24 hours' imbibition in the alcohol-water mixtures, and 24 hours' formalising. The first wet weight readings were taken after 3 days; and then further readings taken at intervals of 7 days. From preliminary experiments it was known that the absolute dry weight of formalised gelatin films when kept in water does not appreciably alter. Therefore the relative gelatin content of the membranes at any time can be determined by measuring the wet weight at the particular time and the dry weight at the end of the experiment. The figures in the table are as usual the percentage ratio of dry weight to wet weight.

TABLE X.

Membrane	After 3 days	After 10 days	After 17 days	After 24 days	After 31 days	After 38 days	After 45 days	After 66 days
0 %	7·45	7·53	6·61	5·80	5·42	5·55	6·33	7·10
20 %	14·66	14·13	13·20	12·33	12·28	12·20	12·20	13·00
40 %	23·62	22·62	21·00	19·70	18·84	17·83	17·00	16·51
60 %	35·67	34·01	32·00	29·55	28·13	26·83	25·50	23·26
80 %	44·90	42·00	39·42	36·00	35·00	33·00	31·60	30·00
100 %	45·95	42·13	40·00	37·31	34·82	32·81	30·63	26·71

It will be seen that in the case of the 80 % alcohol membrane, the ratio of dry to wet weight decreases by about 2·5 % per week; that the weekly decrease in the case of the 40 % alcohol membrane is about 1 %. The membranes at the lower end of the series show a very gradual decrease. The slight increase shown towards the end of the experiment may have been due to a certain amount of bacterial contamination which appeared in the later stages of the experiment.

Attempts were made to reduce the rate of change indicated above, but so far without success. Thus membranes which were exposed for 24 hours to the action of formalin of full strength (40 % formaldehyde) in addition to the ordinary formalising treatment were found to behave in very much the same way as those which had only received the ordinary treatment. This property must therefore for the present be regarded as inevitable, and its presence must be remembered in all applications of these membranes.

The rate at which these membranes are degraded is considerably increased by the presence of acid and alkali. Tables XI and XII illustrate this effect.

TABLE XI.

Effect of Acid.

	Membranes in water		Membranes in $n/100$ H_2SO_4	
Membrane	After 1 day	After 15 days	After 1 day	After 15 days
0 %	7·91	8·22	7·60	5·56
20 %	13·00	12·50	14·87	10·62
40 %	24·67	22·30	23·00	15·39
60 %	34·67	30·35	34·74	21·77
80 %	42·53	35·70	42·57	28·40

TABLE XII.

Effect of Alkali.

	Membranes in water		Membranes in $n/100$ NaOH	
Membrane	After 1 day	After 15 days	After 1 day	After 15 days
0 %	7·28	7·73	7·00	5·00
40 %	22·74	21·00	24·00	15·63
60 %	37·44	33·04	37·46	24·67
80 %	45·80	40·00	46·00	32·18

With increasing strength of acid or alkali, it is highly probable that the rate of degradation is further increased. The instability of the membranes under such conditions renders them therefore unsuitable for work in acid or alkaline media. It may also be mentioned incidentally that membranes of gelatin cannot be used in the presence of acid pepsin, as they are rapidly attacked and totally destroyed.

General Remarks on the Application and Uses of these Membranes.

In applying these membranes to work on diffusion and dialysis it is to be kept in mind that in any series of membranes prepared from films of the same initial thickness, the more permeable members of the series are much thicker than those at the other end of the series. It will be observed that in this respect the technique of the present writer differs essentially from that described by Bechhold [1907]. The latter impregnated filter papers with solutions of various strengths of gelatin in water, or of celloidin in acetic acid, allowed to drain for a given time, and fixed, the former by immersion in 2—4 % formalin solution, the latter by immersion in water. It is obvious that by reason of viscosity the film which remains on the filter paper after drainage will be thicker in the case of the higher gel concentrations than in the case of the lower ones, and that therefore the apparent diminution of permeability of the membranes with higher gel content may be due in part simply to their greater thickness. If therefore the membranes described in the present paper be used in an investigation of the relationship between the "pore size" of a membrane and its capacity for allowing passage to substances in solution, it will be necessary to proceed in such a way as to ensure equal thickness in the resulting membranes. The initial dry films must thus be made correspondingly thinner when it is desired to use them for the preparation of the more permeable membranes. It is obvious that by a process of trial and error this experimental difficulty can readily be overcome.

The gelatin-in-water membranes described in the present paper compare favourably with the similar membranes prepared according to Bechhold's technique in respect of the much wider range (up to 50 % gelatin) of permeability and of the readiness by which they can be characterised. Bechhold's method was to standardise each particular lot in relation to its behaviour to a standard haemoglobin solution. The membranes of the present technique are standardised simply by determining the ratio of dry to wet weight of a

sample portion. The determination of this ratio is difficult if not actually impossible in the case of Bechhold's membranes on account of the presence of the supporting matrix of filter paper. It should also be noted that in Bechhold's technique, the concentration of gelatin in the final membrane may not be that of the gelatin solution originally employed. As the process of formalisation is a comparatively slow one, the gelatin membrane may swell considerably before that process is complete. It has for instance been found that membranes with as low a gelatin percentage as 5 % may be obtained by directly immersing air-dried films in 4 % formaldehyde at 20° C. Thus when in Bechhold's technique a filter paper impregnated with 10 % gelatin is placed in 4 % formaldehyde (even at 0° C.) it is not in the least certain that it formalises as such; on the contrary there is every probability that it swells during the process, producing a membrane of lower gelatin content and greater thickness. This consideration adds weight to the criticism as regards thickness emphasised above.

The method adopted by the present writer to characterise the collodion-in-water membranes was to state the concentration of alcohol in the mixture used in their preparation. In the case of the membranes dealt with in this paper (chiefly gelatin and agar), it has been shown that certain other factors (especially the thermal history of the gel substance) play a somewhat pronounced rôle. Unless therefore it be found possible and convenient to adhere to standard material, it would appear advisable to state, in addition to the alcohol concentration employed in the manufacture of the membrane, the ratio of dry weight to wet weight of the latter as determined on a sample portion.

Methods of attaching these membranes to glass-ware for purposes of diffusion and dialysis experiments will be described in a subsequent paper.

As regards the scientific or other interest of the large variety of membranes described in Section A of the present paper, it is obviously impossible to speak with certitude at the present stage, but it is plain that through them the scope of investigation by means of the processes of diffusion (in particular, dialysis) and adsorption is considerably extended. The present writer has begun an investigation by means of these membranes of the relation between the power of a membrane to adsorb a particular substance and its power to transmit it by diffusion, that is, of the relation existing between adsorption and permeability.

The practical aspects of this technique may also be mentioned. In the case of substances which are not readily separable from each other by dialysis

in water, it may be possible to find a solvent in which dialytic separation is practicable. An illustration of such is afforded in the case of Congo red and night blue dyes. Whereas no practicable separation can be obtained in aqueous solution, both substances being highly colloidal in water, the blue dye can be dialysed away with considerable ease in alcoholic solution, and thus a separation effected.

Lastly, these membranes can be used, not only in a large variety of liquids, but also in mixtures of the same. It is believed that in this feature there lies the possibility of an interesting investigation. Thus if a particular substance is crystalloidal in a particular solvent and colloidal in another, then it is probable that it shows intermediate characters in mixtures of the two solvents. It would thus be possible to vary the colloidal nature of the particular substance at will, and to determine its properties (diffusibility, degree of adsorption) in each case with a variety of membranes in equilibrium with the solvent mixture.

As regards the series of gelatin-in-water membranes treated of in Section B, it is already possible to speak with more definiteness. These membranes cover a portion only of the range covered by the collodion-in-water membranes, being, so far as is known at present, permeable to all electrolytes and to such simple colloids as safranin, neutral red, etc. As regards more colloidal substances, investigation has already shown that the order in which substances arrange themselves in reference to their diffusive capacity through gelatin is not the same as in the case of collodion membranes. One well authenticated case is afforded by the behaviour of starch and night blue solutions. It has been found possible to prepare a collodion membrane which completely prevents passage of night blue while definitely allowing passage to starch, whereas gelatin membranes have been prepared which allow slow passage of night blue but completely hold back a starch solution. It is hoped when this subject is further pursued that such a behaviour will be shown by a variety of substances (especially dye substances), so that a method may be evolved of attacking the problem of selective permeability in its purely physical relationships.

These gelatin-in-water membranes have been found to show properties which indicate that they may prove of service in the technique of enzyme purification. Membranes at the lower end of this series allow of the diffusion of the enzymes diastase and cytase, while those at the upper end hold them back. It is hoped to publish shortly an account of an investigation relating to the subject of enzyme purification.

Lastly, in relation to the phenomena of selective permeability, it would appear that certain dialytic processes can be carried out with greater effectiveness by means of these gelatin membranes than with membranes of collodion. Thus preliminary observations have shown that the dialytic separation of a starch-dextrin mixture can be carried out with greater rapidity with certain gelatin membranes than with any of the collodion series.

Summary.

The general principle by which membranes of graded permeability may be prepared is as follows:—

Suppose a system consisting of:—Liquid *A*—Membrane substance—Liquid *B*.

If (1) the membrane substance only imbibes a negligible amount of *A*,

(2) the membrane substance strongly imbibes *B*,

(3) *A* and *B* are miscible in all proportions,

membranes of graded permeability for use in the liquid *A* can be prepared by immersing films of the membrane substance in mixtures of *A* and *B* and transferring to liquid *A*. In this way are prepared graded membranes of collodion in water, chloroform, benzene, etc.; of agar and gelatin in alcohol, acetone, benzene, etc.; of cellulose acetate in water, alcohol, benzene, etc. Graded membranes for use in *B* can be prepared only when a method exists of suppressing the capacity of the membrane substance to imbibe *B*, this suppression to be brought about at the stage where the membranes are still immersed in the mixture of *A* and *B*. In this way graded membranes of formalised gelatin for use in water have been prepared.

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VII. THE SYNTHESIS OF RACEMIC Pr-2-METHYLTRYPTOPHAN.

By GEORGE BARGER and ARTHUR JAMES EWINS.

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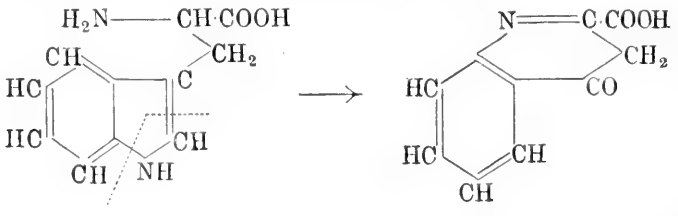
(*Received March 5th, 1917.*)

The mechanism of the changes involved in the complete oxidation of tryptophan in the animal body is quite unknown, partly no doubt on account of the difficulty of synthesising indole derivatives, which might be presumed to be intermediate stages in the degradation of this essential amino-acid. The only known product of its transformation is kynurenic acid which has so far been observed in the dog, the rabbit and the rat. (Note by F. G. Hopkins in a paper by Asayama [1916].) According to Homer [1915] and to Asayama kynurenic acid is not a product on the main line of the destructive breakdown of tryptophan, but in spite of this it is desirable that the mechanism of transformation should become known. "It is possible to picture the conversion of tryptophan to kynurenic acid in many different ways" [Dakin, 1912, p. 72]; the first question is whether the single nitrogen atom of kynurenic acid is represented by the indole- or by the amino-nitrogen of its precursor. Ellinger [1904], when he first identified the precursor, accepted the latter alternative, considering tryptophan to be α-amino-α-scatoleacetic acid; kynurenic acid was at that time regarded as being 4-hydroxyquinoline-3-carboxylic acid. The recognition of the true constitution of tryptophan rendered Ellinger's view less likely, until it was shown by Homer [1913] that kynurenic acid is 4-hydroxyquinoline-2-carboxylic acid. This correction of Camps' formula seems to us to re-establish a strong probability in favour of the elimination of the indole-nitrogen, as follows:



On the other hand it has been considered that the pyrrole ring is enlarged to a pyridine ring by the introduction of a carbon atom between the 2 and 3 positions, for which there is a good deal of analogy *in vitro*. Possible mechanisms have been discussed, e.g. by Dakin [1912], but the subsequent recognition of the fact that in kynurenic acid the carboxyl group adjoins the nitrogen atom, has made it necessary to assume, on this hypothesis, that the carboxyl group (oxidised side chain of tryptophan) wanders from the 3-to the 2-carbon atom, which is unlikely.

If by feeding a substituted tryptophan, a substituted kynurenic acid were obtainable, it would be possible to decide between the two hypotheses. A substituent in the benzene ring would be most suitable for this purpose, but is difficult to introduce, and we have therefore contented ourselves for the present with the preparation of a tryptophan methylated at the α-carbon atom of the pyrrole ring (*Pr*-2) which substance is relatively easily synthesised. We were led to this choice by the fact that 2-methylindole (methylketole) is the most readily obtainable indole derivative, and by the observation, that from this substance 2-methylindole-3-aldehyde is obtainable by Gattermann's hydrocyanic acid method in an unexpectedly good yield. From this aldehyde the synthesis followed closely that of tryptophan by Ellinger and Flamand [1907]. The yields were better throughout, probably owing to the protective action of the methyl group on the pyrrole ring, but this very circumstance seems to prevent the rupture of this ring in the animal organism, for, in the only feeding experiment undertaken so far, the urine contained an indole derivative, but neither kynurenic acid nor a similar substance. As we are unable to continue the work at present, we give the details of the synthesis below.

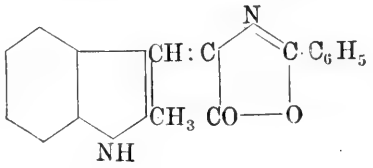
Preparation of 2-methylindole-3-aldehyde.

This substance has been previously obtained by Plancher and Ponti [1907] who acted on 2-methylindole with chloroform and sodium ethoxide, and by Angeli and Marchetti [1907], who obtained it from 2-methylindole and amyl formate, in the presence of sodium ethoxide. We find that Gattermann's aldehyde synthesis is applicable to the present case, and, as in the case of phenols, it is far preferable to the Tiemann-Reimer reaction. 21·5 g. of 2-methylindole was added to 150 cc. of dry ether, 19·2 cc. of anhydrous hydrocyanic acid and 21·5 g. of zinc chloride, freshly fused and powdered; hydrogen chloride was passed in, first for $2\frac{1}{2}$ hours at 0° and finally for $\frac{1}{2}$ hour at 45°. The almost colourless crystalline solid which had separated was filtered off,

washed with ether, boiled for a few minutes with water containing a little ammonia to neutralise traces of adherent acid, and filtered off after cooling. The crude product amounted to 23·9 g., and was somewhat pigmented. After recrystallisation from boiling xylene, in which the pigment is little soluble, we obtained a slightly coloured product melting at 202°; yield 20 g. or 75 % of the theoretical.

The aldehyde sublimes wholly at 180° in the vacuum of a Gaede pump without melting and when the resulting needles were crystallised from benzene flat acicular prisms were formed, M.P. 202—203°. Like Plancher and Ponti we also observed cube-like crystals.

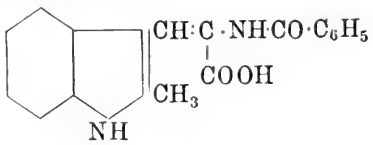
Azlactone of Pr-3-*Methylindolyl-α-benzoylamino-acrylic acid.*



17·3 g. of the aldehyde, 22·3 g. hippuric acid, 8·84 g. anhydrous sodium acetate and 39·3 cc. acetic anhydride were heated for 15 minutes on the water bath. The lactone began to separate when the solution was still hot. In a preliminary experiment the reaction product was extracted with boiling water, as indicated by Ellinger, but as we obtained hardly any unchanged aldehyde, we found it simpler to crystallise at once from glacial acetic acid, obtaining thus 30·5 g. of brownish red prismatic plates, melting at 204—207°. Ellinger and Flamand crystallised their lactone from chloroform, of which one molecule was retained as chloroform of crystallisation. Our lactone was not very soluble in chloroform and the air dry crystals retained an indefinite quantity, 10·6 %, rather less than half a molecule. The orange yellow glistening prisms so obtained sintered at 202°, and melted at 211°.

Analysis: 0·1318 dried at 100°; 0·3666 CO_2; 0·0580 H_2O. C = 75·8; H = 4·89.
Calculated for $C_{19}H_{14}O_2N_2$, C = 75·5, H = 4·63.

Pr-2-*Methylindolyl-α-benzoylamino-acrylic acid.*

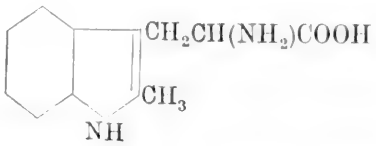


By repeated boiling with 100 times its weight of 1 % sodium hydroxide the lactone was hydrolysed to the above acid. After crystallisation of the precipitated acid from 70 % alcohol we obtained pale yellow prismatic needles, M.P. 221—222°, in a yield of 75 % of the theoretical.

Analysis: 0·1149 dried at 100°; 0·2994 CO_2; 0·0525 H_2O. C = 71·1; H = 5·1.

Calculated for $C_{19}H_{16}O_3N_2$, C = 71·2, H = 5·0.

Pr-2-*Methyltryptophan*



4 g. of the above acid were dissolved in 120 cc. of absolute alcohol and heated on the water bath with 12·5 g. of sodium in the course of half an hour. Then 20 cc. of absolute alcohol were added and the solution was boiled for 20 minutes, the flask being immersed in a salt bath. After adding 15 cc. of water, the removal of the benzoyl group was completed by a further boiling for 45 minutes. On pouring into 5 volumes of water the solution was washed with ether, acidified to Congo-red, left in the cold room over night, filtered, washed with ether and ethyl acetate, made up to 5 % with sulphuric acid and precipitated with Hopkins' reagent. After decomposing the mercury precipitate with hydrogen sulphide and treatment with lead carbonate in the usual way, about 60 % of the theoretical yield of an amorphous product was obtained, which yielded sphaerocrystals from 50 % alcohol, but could not be purified by crystallisation.

We therefore treated the concentrated aqueous solution of the substance with a slight excess of warm saturated picric acid solution in water. The gummy picrate which separated, afterwards became partially crystalline. It could be crystallised from picric acid solution (in water it dissociates) but better from methyl alcohol and petrol, which yielded orange red plates, melting at 173°.

Analysis: 0·1000 dried at 100°; 0·1775 CO_2; 0·0364 H_2O. C = 48·4; H = 4·0.

Calculated for $C_{12}H_{14}O_2N_2$, $C_6H_3O_7N_3$, C = 48·3, H = 3·8.

Regeneration from the picrate by means of sulphuric acid, washing with ether, and quantitative removal of the sulphuric acid by baryta was not successful. We therefore used the calculated quantity of nitron in dilute acetic acid solution. After filtration from the nitron picrate the solution was evaporated to dryness; the residue was dissolved in a little hot water, boiled with charcoal

and filtered. The amino-acid now crystallised satisfactorily. On recrystallisation from methyl alcohol and ether we obtained colourless prisms, melting variously at 263—273°, according to the rate of heating. The methyltryptophan crystallises with one molecule of methyl alcohol, which is retained *in vacuo* over sulphuric acid, but is given off at 110°.

Analysis:

0·1118 dried at 110°; 0·2861 CO_2; 0·0669 H_2O. C = 65·7; H = 6·25.

Calculated for $C_{12}H_{14}O_2N_2$, C = 66·05; H = 6·4.

0·1489 dried *in vacuo*, lost at 110° 0·0180 = 12·1 %.

0·0719 dried *in vacuo*, gave 0·1627 CO_2 and 0·0459 H_2O. C = 61·7; H = 7·1. $C_{12}H_{14}O_2N_2$, CH_3OH requires C = 62·4; H = 7·2; CH_3OH = 12·8 %.

Subsequently we found that the methyltryptophan could be crystallised at once from methyl alcohol and ether, if the crude substance was precipitated a second time by Hopkins' reagent. In this way we obtained a yield corresponding to slightly over 40 % of the theoretical.

Pr-2-Methyltryptophan is readily soluble in water, hardly soluble in absolute ethyl alcohol. It gives a strong reaction with triketohydrindene hydrate. With excess of bromine water a precipitate is produced, but no coloration. Perhaps we may conclude from this, that the bromine reaction of natural tryptophan consists in an attack on the (unsubstituted) α-carbon atom of the indole ring. Methyltryptophan gives with Hopkins and Cole's reagent a greenish blue coloration in dilutions up to 1 : 500. The reaction is therefore very much less sensitive than with the non-methylated amino-acid. In very concentrated solutions the ring test with sulphuric and glyoxylic acids gives at first a reddish purple coloration, which on mixing the two layers becomes deep blue.

The methyltryptophan we obtained has a sweet taste, probably due to its being racemic, for natural *l*-tryptophan is tasteless, *d*-tryptophan is sweet.

Behaviour of methyltryptophan in the dog.

0·5 g. of the substance, dissolved in 10 cc. of water, was given by a stomach tube to a puppy weighing 2 kilos[1]. The urine collected during 48 hours after the experiment, was purified by addition of half its volume of 10 % barium chloride containing 5 % ammonia, and kynurenic acid found to be absent,

[1] This experiment was kindly carried out for us by Dr H. H. Dale.

on estimation by Capaldi's method. The urine gave no reaction in the cold with glyoxylic and sulphuric acids, but on heating with 60 % sulphuric acid a dirty green colour was developed, not given by normal urine. We therefore removed the excess of barium, concentrated, acidified and extracted with ether, which solvent did not remove the substance giving the green coloration with hot sulphuric acid. We then evaporated to dryness and extracted the residue with ethyl acetate. The extract contained principally urea, but on destructive distillation of the residue, the vapours gave the pyrrole reaction with pine wood and the residue gave a reddish coloration with picric acid in aqueous solution. It appears possible therefore that methyltryptophan undergoes in the dog a change, which leaves the indole ring intact.

In a subsequent control experiment[1] the puppy was given 0·5 g. tryptophan and yielded 20—30 mg. of kynurenic acid, M.P. 280°.

Conclusion.

Further feeding experiments are required, but as long as they are negative it is impossible to draw definite conclusions as to the mechanism of the production of kynurenic acid. We think, however, that on the whole our result is in favour of the view that in natural tryptophan the pyrrole ring is eliminated, and that in methyltryptophan this elimination is prevented by the protective action of the methyl group. If the pyridine ring of kynurenic acid were formed by enlargement of the pyrrole ring, we might expect that methyltryptophan should yield a methylkynurenic acid, just as 2-methylindole yields 3-chloroquinaldine, when acted on by chloroform and alcoholic sodium hydroxide.

We desire to express our indebtedness to Mr W. W. Starling for valuable assistance in the preparation of the compounds described.

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Plancher and Ponti (1907). *Atti R. Accad. dei Lincei* [5], **16**, i, 130.

[1] This experiment was kindly carried out for us by Dr H. H. Dale.

VIII. THE CONDITIONS OF ACTIVATION OF WASHED ZYMIN AND THE SPECIFIC FUNCTION OF CERTAIN CATIONS IN ALCOHOLIC FERMENTATION.

By ARTHUR HARDEN.

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(*Received March 12th, 1917.*)

It was observed by Oppenheimer [1915] that the addition of a small amount of a pyruvate (1 per mille) or of pyruvic acid (1 %) to a mixture of Lebedev's maceration juice (prepared from dried yeast) and glucose caused an increase in the rate of fermentation of the glucose, and that a similar, but much smaller, acceleration was produced by the addition of acetaldehyde (1 : 200,000). Neuberg [1915, p. 75] somewhat later made a similar observation and extended it to the fermentation of fructose, mannose, cane sugar and maltose and also found [1915, p. 83] that other α-ketonic acids produced an analogous effect. He further attempted [Neuberg and Schwenk, 1915] to activate yeast-juice, which had been freed from coenzyme by dialysis, and zymin (acetone-yeast), rendered inactive by washing, by adding to them salts of individual α-ketonic acids and a phosphate. In this, however, he was unsuccessful, but by adding a mixture of salts of a large number of α-ketonic acids (pyruvic, α-ketobutyric, α-ketoisovaleric, α-ketocapronic, phenylglyoxylic, phenylpyruvic, *p*-hydroxyphenylpyruvic, hydroxypyruvic, oxalacetic and α-ketoglutaric) along with dipotassium hydrogen phosphate, he succeeded in obtaining a small amount of fermentation (of the order of 5 cc. of CO_2 from excess of sugar by the action of 20 cc. of inactivated maceration juice or 2 g. of inactivated zymin), and concluded that this mixture could in part replace the coenzyme.

On repeating these experiments it was found that zymin, which had been prepared from top-yeast and rendered inactive by thorough washing, was readily activated by potassium pyruvate in presence of a suitable concentration of a phosphate. The experiments were then extended to acetaldehyde, since this substance is the immediate product of the decomposition

of salts of pyruvic acid by the carboxylase of the zymin, which, as has been previously shown [Harden, 1913; Neuberg and Rosenthal, 1913] is not inactivated by washing. The first experiments were carried out in the presence of sodium phosphate and were unsuccessful, but it was subsequently found that, when the sodium phosphate was replaced by the potassium or ammonium salt, acetaldehyde activated the zymin in the same manner as the pyruvate.

It thus appears that potassium and ammonium ions play some special part in the process of fermentation in which they cannot be replaced by sodium ions. The presence of sodium ions is not in itself inhibitory, since activation occurs in their presence, provided only that potassium or ammonium ions are also present. This observation complements in an interesting manner the discovery by Adolf Mayer [1874] that potassium phosphate cannot be replaced by the sodium salt in a synthetic culture medium for yeast.

The fact that washed zymin can be activated by acetaldehyde in presence of a phosphate is of considerable interest from several points of view. In the first place it is consistent with, and may even be regarded as strong evidence in favour of, the theory now held by many investigators [for literature see Harden, 1914] that acetaldehyde is an intermediate product in alcoholic fermentation and is reduced in that process to alcohol by hydrogen liberated at a previous stage of the decomposition. According to this view the acetaldehyde would fulfil the function of an activator by serving as an acceptor for the hydrogen and would thus enable the reaction to start. It is possible however that other reducible substances would act in the same way and this would to some extent lessen the weight of the evidence in support of the acetaldehyde theory although it would be in agreement with the view that a hydrogen acceptor is a factor necessary for fermentation. This question will form the subject of further experiments as soon as circumstances permit.

These considerations lead directly to the question whether acetaldehyde can be regarded as constituting the coenzyme of yeast-juice. There is much in favour of this idea, but in view of the possibility that some other reducible substance may play the same part as acetaldehyde it is impossible to come to a definite conclusion without further investigation respecting the actual presence of acetaldehyde in yeast-juice and the activation of yeast-juice freed from coenzyme by dialysis, ultrafiltration and continued fermentation. As regards washed zymin, acetaldehyde certainly acts in all respects as a coenzyme, provided that phosphate and potassium or ammonium ions are present in suitable concentrations.

A second consideration is that the possibility of reactivating washed zymin by means of acetaldehyde and a phosphate affords a means of examining the effect of various cations and anions on the initiation and course of the fermentation and this opens a field of enquiry which may be expected to yield interesting results.

The stimulating effect of pyruvate and acetaldehyde on fermentation by yeast-juice observed by Oppenheimer and by Neuberg, also becomes of great interest in the light of these results and will form the subject of a further communication.

Experimental.

The zymin used in these experiments was prepared from a top-fermentation yeast employed in an English brewery by the usual method of treatment with acetone. It was washed five times with 6 parts of water in the manner previously described [Harden and Young, 1911] each washing lasting $\frac{3}{4}$ hour, and was then made up to a given volume with water. The same sample of zymin was used throughout the experiments quoted so that the results of experiments done with different batches are roughly comparable. All the fermentations were carried out in presence of toluene at 25°, the solutions being previously saturated with CO_2 at this temperature and incubated for 10—15 minutes before readings were made. The carbon dioxide evolved was collected and measured in the apparatus previously described [Harden, Thompson and Young, 1910] and the figures given are cc. of gas at atmospheric pressure and temperature.

Experiments with zymin.

I. The activation of washed zymin by potassium pyruvate and potassium phosphate and the effect of the replacement of these salts by the corresponding sodium salts.

14 g. of zymin were washed and made up to 80 cc.

In each experiment 10 cc. of this suspension (1·75 g. zymin) were used along with 1 g. of glucose and water to a total volume of 20·6 cc. To five quantities were added:

Exp. 1. 2 cc. 0·5 M K_2HPO_4.
„ 2. 2 cc. 0·5 M K_2HPO_4 + 3 cc. 1 % pyruvic acid (as K salt).
„ 3. 3·3 cc. 0·3 M Na_2HPO_4 + 3 cc. 1 % pyruvic acid (as Na salt).
„ 4. 1·6 cc. 0·3 M Na_2HPO_4 + 3 cc. 1 % pyruvic acid (as Na salt).
„ 5. 10 cc. boiled washings.

Time		Evolution of CO_2 in cc.				
h.	m.	1	2	3	4	5
1	5	0·5	12·6	1·9	2·1	11·1
2	5	0·5	20·4	2·1	2·8	17·6
20	20	2·9	55·8	2·5	6·6	69

In presence of the sodium salts (Nos. 3 and 4) little more CO_2 was evolved than in the control (No. 1) in the absence of coenzyme, whereas in the presence of the potassium salts (No. 2) the fermentation is almost as vigorous as in the presence of boiled washings (No. 5).

II. The activation of zymin by acetaldehyde and potassium phosphate or ammonium phosphate and the effect of the replacement of these salts by sodium phosphate.

A. *Exp.* 6. This was carried out along with the foregoing series. To the mixture of zymin glucose and toluene were added 1 cc. acetaldehyde solution (0·7 %) + 2 cc. 0·5 M K_2HPO_4.

Time		Evolution of CO_2 in cc.	
h.	m.	Control No. 1 (above)	6
1	5	0·5	13·3
2	5	0·5	19·8
20	20	2·9	52·4

B. 10 cc. of washed zymin suspension and water to 20·6 cc. To six quantities were added:

Exp. 7. 10 cc. boiled washings + 1 g. glucose.
„ 8. 3 cc. 0·5 M K_2HPO_4 + 1 g. glucose.
„ 9. 3 cc. 0·5 M K_2HPO_4.
„ 10. 3 cc. 0·5 M K_2HPO_4 + 1 g. glucose + 1 cc. acetaldehyde.
„ 11. 2 cc. 0·5 M K_2HPO_4 + 1 g. glucose + 1 cc. acetaldehyde.
„ 12. 1·6 cc. 0·3 M Na_2HPO_4 + 1 g. glucose + 1 cc. acetaldehyde.

Time		Evolution of CO_2 in cc.					
h.	m.	7	8	9	10	11	12
	55	14·1	0·2	0·5	14·2	11·7	0·9
1	55	25·1	0·6	1·1	29·6	19·5	1·6
3	0	33·2	0·7	1·3	37·7	23·6	2·3
18	10	89	0·8	1·3	80·1	51·6	2·3

The amount of sodium phosphate added was only half the equivalent of the K salt because a previous experiment had shown that even in the presence of a small concentration of K ions, no fermentation occurred in presence of the full equivalent.

C. 10 g. zymin to 60 cc. 10 cc. of zymin suspension + 1 g. glucose were taken. Total vol. = 20·6 cc. To three lots were added:

Exp. 13. 10 cc. boiled washings.
„ 14. 2 cc. 0·5 M K_2HPO_4.
„ 15. 2 cc. 0·5 M $(NH_4)_2HPO_4$ + 1 cc. acetaldehyde.

Time		Evolution of CO_2 in cc.		
h.	m.	13	14	15
1	10	8·1	1·4	6·5
3	25	16·1	1·5	15·6
20	35	39·8	1·5	34·6

Here again acetaldehyde and potassium phosphate (Nos. 10, 11) or ammonium phosphate (No. 15) produce a good fermentation whereas acetaldehyde and sodium phosphate (No. 12) are without action.

III. Negative effect of potassium ions and acetaldehyde in absence of mineral phosphate.

Six quantities of 10 cc. of zymin suspension + 1 g. glucose + water to 20·6 cc. were taken and to these were added:

Exp. 16. 10 cc. boiled washings.
„ 17. 3 cc. 0·5 M K_2HPO_4 + 1 cc. acetaldehyde.
„ 18. 3 cc. M $KHCO_3$ + 1 cc. acetaldehyde.
„ 19. 3 cc. M $NaHCO_3$ + 1 cc. acetaldehyde.
„ 20. 1·6 cc. 0·3 M Na_2HPO_4 + 1 cc. acetaldehyde.
„ 21. 0·8 cc. 0·3 M Na_2HPO_4 + 1 cc. acetaldehyde.

Time		Evolution of CO_2 in cc.					
h.	m.	16	17	18	19	20	21
1	10	11·7	14·4	2·1	1·3	0·8	1·5
2	10	18	28·3	2·3	1·7	1·4	1·9
18	10	69	74·9	3·3	3·8	1·5	2

This shows that K ions and acetaldehyde in the absence of phosphate (No. 18) are incapable of producing fermentation and further that the same is true of Na ions (No. 19) and that the reduction of the concentration of sodium phosphate does not render it efficacious as an activator (No. 21).

Experiments with dried yeast.

Since dried yeast can be inactivated by washing [Euler and Bäckström, 1912] it can readily be used for experiments on activation. It is however not so satisfactory as zymin since, probably owing to the presence of a certain proportion of living cells, some samples yield irregular results. The yeast was dried in the air at 37° and then ground in a coffee mill. It was washed in a similar manner to the zymin.

IV. Effect of the pyruvates and phosphates of potassium and sodium.

20 g. yeast, washed four times and made to 100. 15 cc. yeast suspension + 1 g. glucose + 1 cc. toluene; water to 30·6 cc.; five quantities were taken.

Exp. 22. 5 cc. 1 % pyruvic (as K salt).
„ 23. 5 cc. 1 % „ „ + 1·5 cc. 0·5 M K_2HPO_4.
„ 24. 5 cc. 1 % „ „ + 2·5 cc. 0·3 M Na_2HPO_4.
„ 25. 5 cc. 1 % „ (as Na salt) + 1·5 cc. 0·5 M K_2HPO_4.
„ 26. 5 cc. 1 % „ „ + 2·5 cc. 0·3 M Na_2HPO_4.

Time		Evolution of CO_2 in cc.				
h.	m.	22	23	24	25	26
	20	2·6	9·7	15·6	13·1	1·4
1	10	5·6	16·2	22·8	20·2	2·7
2	5	6·7	24·3	—	—	3·9
3	5	7·1	34·1	—	—	5·2
19	20	9·9	71·1	—	—	8·6

Here the substitution of sodium for potassium salts (No. 26), in equivalent amounts has resulted in the complete absence of any evolution of CO_2 beyond

that in the control in the absence of phosphate (No. 22). That the presence of sodium ions is not seriously harmful is shown by a comparison of Nos. 21 and 25 with No. 23.

V. Effect of substituting sodium phosphate for potassium phosphate in presence of acetaldehyde.

The dried yeast was treated as in IV. 15 cc. yeast suspension + 1 g. glucose + 1 cc. toluene; water to 30·6 cc.

Exp. 27. 1·5 cc. 0·5 M K_2HPO_4.
„ 28. 1·5 cc. 0·5 M K_2HPO_4 + 1·4 cc. 1 % acetaldehyde.
„ 29. 2·5 cc. 0·3 M Na_2HPO_4 + 1·4 cc. 1 % acetaldehyde.

Time		Evolution of CO_2 in cc.		
h.	m.	27	28	29
1		1·5	25·5	2·9
3		1·5	41·8	3·2
5	30	1·7	58·3	3·8
21	45	4·4	86·3	6·1

The contrast here between the action of equivalent quantities of sodium and potassium phosphate is very marked.

VI. Effect of the addition of various chlorides in presence of sodium phosphate and acetaldehyde.

Dried yeast, treated as above. 15 cc. of yeast suspension + 1 g. glucose + 1 cc. toluene + 0·5 cc. 0·3 M Na_2HPO_4 + 5 cc. 0·1 % acetaldehyde; water to 30·6 cc. Four quantities.

Exp. 30. 1·5 cc. M KCl.
„ 31. 1·5 cc. M NH_4Cl.
„ 32. 1·5 cc. M NaCl.
„ 33. Control.

Time		Evolution of CO_2 in cc.			
h.	m.	30	31	32	33
1	15	7·7	7·8	0·3	1·9
2	15	10·6	11·1	2·5	2·8
18		11·6	13·4	3·3	3·3

This shows that whereas the addition of KCl (No. 30) or NH_4Cl (No. 31) to a mixture containing inactivated dried yeast, acetaldehyde and sodium phosphate causes a definite, though small, degree of fermentation, that of NaCl (No. 32) has no effect, the amount of CO_2 evolved being the same as in the control (No. 33).

Summary.

1. In the presence of potassium phosphate, zymin and dried yeast, which have been inactivated by washing, can be activated by the addition of a pyruvate or acetaldehyde.

2. A specific difference in relation to alcoholic fermentation exists between the ions of sodium on the one hand and of potassium and ammonium on the other.

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IX. ON A NEW TYPE OF CHEMICAL CHANGE PRODUCED BY BACTERIA. THE CONVERSION OF HISTIDINE INTO UROCANIC ACID BY BACTERIA OF THE COLI-TYPHOSUS GROUP.

By HAROLD RAISTRICK.

From the Biochemical Laboratory, Cambridge.
Report to the Medical Research Committee.

(*Received March 29th, 1917.*)

The investigation of the products of the putrefactive decomposition of proteins, commenced by Nencki, Baumann, Brieger and E. and H. Salkowski, has more recently been continued by Ellinger, Ackermann and others, who have investigated the bacterial decomposition of the amino-acids. These decomposition products, varied in nature as they are, are rendered all the more interesting because of the very marked pharmacological and toxicological action exhibited by some of them, e.g. tyramine (*p*-hydroxyphenylethylamine and histamine (*β*-iminazolylethylamine).

By far the most common of all the chemical changes induced in amino-acids by bacterial action is simple decarboxylation, i.e. the production of an amine from an amino-acid by the loss of CO_2.

$$R . CH_2 . CH(NH_2) . COOH \rightarrow R . CH_2 . CH_2 . NH_2 + CO_2.$$

Ellinger [1900] proved that the bases putrescine and cadaverine described by Brieger owe their origin to the decarboxylation by putrefactive bacteria of ornithine and lysine respectively. By the use of the same bacteria Ackermann [1910, 1] obtained a 42 % yield of histamine (*β*-iminazolylethylamine) from histidine. His medium contained peptone, glucose, salts and histidine. Barger and Walpole [1909] isolated tyramine (*p*-hydroxyphenylethylamine) from putrid horse flesh and showed that it was derived from tyrosine by the loss of CO_2. More recently Sasaki [1914] obtained a 78 % yield of the same base by the action of *B. coli communis* on a medium containing tyrosine, glycerol, and inorganic salts. These are only a few of a number of similar

results. In fact almost all the amines which might be expected to result from the known amino-acids have been obtained by bacterial action.

Of somewhat less interest, since the products are usually non-toxic, is the change brought about by bacteria which is known as "deaminisation," i.e. the production of a saturated acid and ammonia from the amino-acid.

$$R . CH_2 . CH(NH_2) . COOH + H_2 \rightarrow R . CH_2 . CH_2 . COOH + NH_3.$$

Thus "deaminisation" in the usually accepted sense of the word *is accompanied by reduction.* Baumann [1879] found that when tyrosine is digested with putrefying pancreas it splits up into ammonia and *p*-hydroxyphenylpropionic acid. In their investigation of the constitution of tryptophan Hopkins and Cole [1903] subjected this amino-acid to the action of the Rauschbrand bacillus and of *B. coli communis.* In each case the bacteria, when grown *anaerobically* in a medium containing Rochelle salt and gelatin in addition to the amino-acid, produced an acid identical with Nencki's scatole-acetic acid, which was subsequently shown by Ellinger to be indolepropionic acid. In the research referred to above Ackermann [1910, 1] found in addition to histamine, a small amount of β-iminazolylpropionic acid. δ-Aminovalerianic acid, first isolated by E. and H. Salkowski [1883] from putrid fibrin, was shown by Ackermann [1910, 2] to be produced by the partial deaminisation of ornithine.

In all the cases quoted it will be noticed that, in addition to the amino-acid under observation the medium also contains large amounts of other organic substances, the presence and nature of which have probably an influence on the end-products. Thus Ackermann has shown that the bacterial decarboxylation of pure amino-acids is favoured by the presence of peptone in the medium. Mellanby and Twort [1912] also observe that the production of β-iminazolylethylamine from histidine by a bacillus, which they isolated from the alimentary canal, is inhibited if the medium is slightly acid or if it contains glucose.

The gradual breakdown of the side chain in amino-acids containing a cyclic nucleus, as seen in the bacterial decomposition of tyrosine and of tryptophan, also illustrates the marked effect of external conditions on the end-products. Baumann [1879, 1880, 1, 2] found that in the putrefactive decomposition of tyrosine *p*-hydroxyphenylpropionic acid is first formed, which in presence of air breaks down further into *p*-hydroxyphenylacetic acid, *p*-cresol and, to a less extent, into phenol. Hopkins and Cole [1903] found a similar breakdown with tryptophan. Under anaerobic conditions

both the Rauschbrand bacillus and *B. coli* gave an abundant production of indolepropionic acid and only slight traces of indole. Aerobically, however, the putrefactive bacteria, or *B. coli*, gave a good yield of indole and indoleacetic acid, traces of scatole, but no indolepropionic acid.

In commencing a study of the action of the bacteria of the Coli-Typhosus group on different amino-acids it seemed desirable therefore, in order to avoid any possible secondary reactions, not to introduce any organic compounds into the medium in addition to the amino-acid under observation. To this end the amino-acid—in this case, histidine—was dissolved in Ringer's solution (mammalian) and this medium used for inoculation. In such a simple medium the bacteria did not grow well but this difficulty was to some extent overcome by sowing thickly and by long continued incubation.

In the preliminary stages a medium of the following composition was made up:

Pure histidine hydrochloride equivalent to 0·2 % of the free base.

2 cc. per cent. of 0·04 % solution of phenolsulphonephthalein to act as indicator.

Ringer's solution.

Normal soda solution until P_H after boiling is 7·35.

This was tubed, sterilised, and inoculated with 24 hour old cultures (from agar slopes) of (i) *B. coli communis*, (ii) *B. typhosus*, (iii) *B. paratyphosus A*, (iv) *B. paratyphosus B*, (v) *B. enteritidis* (Gaertner), (vi) *B. dysenteriae* (Flexner). Even after ten days' incubation at 37·5° there was no change in the reaction of the tubes containing *B. coli*, *B. typhosus*, and *B. paratyphosus A*, though the other three organisms had produced a marked alkalinity, which became intense after 20 days' incubation. After this time *B. typhosus* showed a slight alkalinity but there was still no change in the reaction of the tubes containing *B. coli* and *B. paratyphosus A*.

In order to prove whether the alkalinity were due to amine production the experiment was repeated on a larger scale using *B. paratyphosus B* as the organism for inoculation. Experimental details are given later in the paper. A substance was isolated from the products of bacterial action, which gave a crystalline picrate. It was found impossible to isolate any β-iminazolylethylamine picrate from this, but it was shown to be the picrate of urocanic acid (β-iminazolylacrylic acid). This would arise from histidine according to the following equation:

$$C_3H_3N_2 . CH_2 . CH(NH_2) . COOH - NH_3 = C_3H_3N_2 . CH - CH . COOH.$$

i.e. *by deaminisation without subsequent reduction* to β-iminazolylpropionic acid. The production of an unsaturated acid from an amino-acid is a type of bacteriological change which so far as is known has not been previously observed.

The history of the occurrence of urocanic acid is very interesting. It was first isolated by Jaffé [1874] in 1874 from the urine of a dog—hence its name: greatly to its owner's chagrin the dog disappeared after some days and was never recaptured. Although Jaffé and others investigated the urine of a number of dogs and men it was not re-discovered until 1898 when Siegfried isolated it once more from a dog's urine [1898]. These are the only instances on record of its isolation from urine. Since it was continually present in both cases in fairly large amounts (2—3 g. per day) it appears that these two dogs presented a rare anomaly of metabolism. In 1912 Hunter [1912] was able to identify a substance which he obtained by prolonged tryptic digestion of caseinogen as urocanic acid, and by direct comparison with a synthetic specimen of β-iminazolylacrylic acid obtained by Barger and Ewins [1911] proved that the two were identical. Inasmuch as Hunter only obtained the acid from one tryptic digest—subsequent repetition of the experiment failing to give him any urocanic acid—he concluded that it had probably arisen from bacterial contamination although he was unable to observe its formation by any of the bacteria which he tried. The results described in this paper seem to bear out this presumption. It is also possible that the two dogs, whose urine contained urocanic acid, may have been suffering from some bacterial infection of this type, though Jaffé states that his dog was apparently perfectly healthy.

It now became of interest to prove whether this type of change is characteristic of *B. paratyphosus B* to the exclusion of the other members of the group and whether it is peculiar to the particular strain which was employed. Two other different strains of *B. paratyphosus B* have been tried and each of them brought about the same change. It was also shown that the property is possessed to a greater or less extent by the other members of the group, i.e. *B. coli*, *B. typhosus*, *B. paratyphosus A*, *B. enteritidis* (Gaertner) and *B. dysenteriae* (Flexner). The reaction is thus not restricted to those members which produce an alkaline reaction. In fact the largest yield of urocanic acid was obtained with *B. paratyphosus A*.

Experimental.

The composition of the medium for inoculation, which was the same throughout, was as follows:

2·7 g. pure histidine monochloride (= 2 g. of free base).
12·9 cc. normal soda, to neutralise the HCl of the histidine monochloride.
1000 cc. Ringer's solution (mammalian).

900 cc. of this medium were introduced into each of six litre flasks, and the whole sterilised by steaming for 45 minutes on three consecutive days. Agar slopes were sown over the whole surface with 24 hour pure cultures in tryptic broth, the bacteria used being (i) *B. coli communis*, (ii) *B. typhosus*, (iii) *B. paratyphosus A*, (iv) *B. paratyphosus B*, (v) *B. enteritidis* (Gaertner), (vi) *B. dysenteriae* (Flexner). After 24 hours' incubation the growth was washed off the agar slopes with sterile Ringer solution, emulsified by agitating with the platinum needle, and introduced into the flasks taking all precautions to prevent contamination. The growth from five agar slopes was added to each flask. The flasks were placed in an incubator at 37·5°, those containing the first three organisms being allowed to remain for 40 days, while the remainder were incubated for 28 days.

The investigation of the products of bacterial action was the same in each case. After sterilisation, the solution was filtered through kieselguhr, neutralised with HCl, and evaporated to dryness on the water bath. The dried residue was ground up, thoroughly extracted with boiling methyl alcohol, the alcoholic extract evaporated to dryness, and the residue taken up with a little water. This solution was then precipitated completely with saturated aqueous picric acid, the precipitated picrate filtered off and recrystallised once from a minimal amount of boiling water. A small amount was reserved for M.P. determination, and the greater part ground up with a little water (5—10 cc.), acidified strongly with conc. HNO_3 (about 2 cc.) and the picric acid completely extracted by shaking with ether. In this way the very slightly soluble nitrate of urocanic acid was obtained which was washed with alcohol and ether, dried, weighed, and converted into the free acid by the addition of one equivalent of pure sodium bicarbonate to a concentrated aqueous solution of the nitrate. The free urocanic acid readily separated out, was filtered off and recrystallised from hot water with the addition of a very small amount of animal charcoal.

In the case of all the six bacteria used, I succeeded in isolating the picrate and nitrate of urocanic acid and also free urocanic acid itself.

Urocanic acid picrate is only slightly soluble in cold water and crystallises well from hot water. When not quite pure it forms rosettes of needles, which on further recrystallisation change to well-defined golden yellow prisms. The M.P. varies considerably with the rate of heating and so for identification the M.P.s of the six different specimens were determined in pairs, M.P. tubes containing two different specimens being attached to the same thermometer. In each case the different specimens melted at 212—215° according to the rate of heating. Barger and Ewins give for the picrate of β-iminazolylacrylic acid, M.P. 213—214°.

In a later experiment from 13·9 g. of pure histidine monochloride submitted to the action of a different strain of *B. paratyphosus B* for 47 days there were isolated 3·4 g. of urocanic acid picrate (M.P. 212—213°).

Analysis: 0·1100 g. gave 18·5 cc. N at 19° and 752 mm. = 19·31 % N.

Calculated for $C_6H_6O_2N_2 . C_6H_3O_7N_3$, N = 19·07 %.

Urocanic acid nitrate. In each case this salt was obtained, which Jaffé and later Hunter consider characteristic of urocanic acid. It forms a white crystalline precipitate which under the microscope appears as "bent sickle-shaped plates whose ends appear to be frayed or eaten away. Frequently many such plates are combined to form cross- or rosette-shaped aggregates" (Jaffé).

The yield of the nitrate varied largely with the particular organism used, which may have been due to a number of factors—unequal inoculation, growth or duration of incubation. From 2·7 g. histidine monochloride (= 2 g. free histidine) in each case the following amounts of urocanic acid nitrate were obtained: *B. coli* 0·72 g.; *B. typhosus* 0·10 g.; *B. paratyphosus A* 1·30 g. (= 58 %); *B. paratyphosus B* 0·37 g.; *B. enteritidis* (Gaertner) 0·13 g.; *B. dysenteriae* (Flexner) 0·64 g.

The M.P., which also varies with the rate of heating, was determined in the same manner as that of the picrate. In each case the two samples melted simultaneously at 195—197° with explosive decomposition.

Barger and Ewins give for a synthetic specimen M.P. 198°.

Urocanic acid. The free acid crystallises readily from hot water. On allowing a hot solution to cool slowly long iridescent needles were obtained, while on cooling more quickly the acid crystallised as a mass of very fine needles. The M.P. of urocanic acid as given by different observers varies within wide limits. Jaffé gave 212—213°, Siegfried 229°, Barger and Ewins 235—236°, Hunter 231—232° (corr.). As Hunter observes, the M.P. varies

greatly with the rate of heating, which may explain the discrepancies. The six different specimens obtained were compared, in pairs, and were found to melt simultaneously at 226—230° according to the rate of heating. All the specimens gave the red coloration with sodium *p*-diazobenzenesulphonate and all reduced alkaline permanganate in the cold—proving the presence of an unsaturated linking.

Analysis:

0·1672 g.; 0·2543 g. CO_2; 0·0862 g. H_2O.

0·4763 g. dried at 110° lost 0·0988 g. H_2O.

0·1139 g. of substance dried at 110° gave 20·3 cc. moist N at 18·5° and 758·4 mm.

	Found	Calculated for $C_6H_6O_2N_2.2H_2O$
C	41·49	41·38
H	5·73	5·75
N	20·57	20·30
H_2O of crystallisation ...	20·74	20·69

Summary.

Histidine (β-iminazolyl-α-aminopropionic acid) is converted into urocanic acid (β-iminazolylacrylic acid), in a medium consisting of Ringer's solution + histidine, by the bacteria of the Coli-Typhosus group, i.e. *B. coli communis*, *B. typhosus*, *B. paratyphosus A*, *B. paratyphosus B*, *B. enteritidis* (Gaertner), *B. dysenteriae* (Flexner). This is the first recorded instance of the bacteriological conversion of an amino-acid into an unsaturated acid.

It is a great pleasure to express my indebtedness to Professor F. Gowland Hopkins and to offer to him my sincerest thanks for his advice and encouragement during the progress of this research.

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X. ON THE OXIDATION OF AMINO-ACIDS AND OF RELATED SUBSTANCES WITH CHLORAMINE-T.

By HENRY DRYSDALE DAKIN.

(*Report to the Medical Research Committee.*)

(*Received April 24th, 1917.*)

Reference has already been made [1916, 1, 2] to the oxidation of certain amino-acids with hypochlorite and with chloramine-T. The latter term is the abbreviated name for sodio-*p*-toluenesulphochloroamine, a substance which appears to constitute a useful addition to the small class of neutral oxidising agents. These experiments seemed worth extending, partly because interesting products of oxidation were likely to be met with and also as preliminary to a study of the mode of antiseptic action of substances of the chloroamine group. In the case of glycine, alanine, leucine, and α-aminophenylacetic acid it was found that the first product of oxidation, using one molecule of oxidising agent, was an aldehyde, as is usually the case with hypochlorite oxidation, as shown by Langheld. On using two molecules of chloramine-T for the oxidations, nitriles were formed, sometimes in good yield. With histidine, no aldehyde[1] could be isolated, but an excellent yield of cyanomethylglyoxaline was obtained.

The present communication deals especially with the oxidation of the neutral salts of glutamic, aspartic, and methylaspartic acids and asparagine, valine and isoleucine.

Glutamic acid may be considered first since the reaction is less complex than with the other substances.

On acting on a dilute aqueous solution of the mono-sodium salt of glutamic acid (1 mol.) with chloramine-T (1 mol.) an excellent yield of the semi-aldehyde of succinic acid (β-aldehydopropionic acid) is obtained. It is most readily separated and identified by means of its nitrophenylhydrazone, but the substance itself may be isolated and distilled although with considerable

[1] It would appear that Langheld's observations on the formation of iminazoleacetaldehyde by the action of hypochlorite on histidine require confirmation.

loss, as described by Harries and Alefeld [1909]. The reaction may be expressed as follows:

$$COOH \cdot (CH_2)_2 \cdot CH(NH_2) \cdot COOH \rightarrow COOH \cdot (CH_2)_2 \cdot CH(NHCl) \cdot COOH \rightarrow COOH \cdot (CH_2)_2 \cdot CHO + NH_4Cl + CO_2.$$

Using two molecules of chloramine-T to one of the glutamic salt, the reaction takes a different course with formation of large quantities of β-cyanopropionic acid, but only negligible traces of succinic acid. The formation of β-cyanopropionic acid is noteworthy since the preparation of this acid has long been unsuccessfully attempted; the methods which would ordinarily be chosen for preparing the substance not having given the desired result.

β-Cyanopropionic acid is a strong monobasic acid, which is readily obtained in crystalline form and with a satisfactory yield. It is likely that it is formed as the result of the decomposition of a dichloroamino-acid, although the latter unstable substance was not isolated.

$$COOH \cdot (CH_2)_2 \cdot CH(NH_2) \cdot COOH \rightarrow COOH \cdot (CH_2)_2 \cdot CH(NCl_2) \cdot COOH \rightarrow COOH \cdot (CH_2)_2 \cdot CN + CO_2 + 2HCl.$$

The constitution of the cyano-acid was definitely fixed by its quantitative conversion into succinic acid on treatment with concentrated hydrochloric acid and by its reduction to γ-aminobutyric acid by sodium and alcohol. The latter substance was obtained in very small yield but its formation is of interest since it has been obtained by the bacterial decomposition of glutamic acid.

$$COOH \cdot CH_2 \cdot CH_2 \cdot CN \begin{cases} \nearrow COOH \cdot CH_2 \cdot CH_2 \cdot COOH \\ \searrow COOH \cdot CH_2 \cdot CH_2 \cdot CH_2 \cdot NH_2. \end{cases}$$

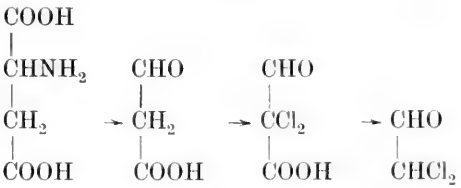
Aspartic acid. The oxidation of aspartic acid, or rather its neutral sodium salt, proved to be a more complicated process. On adding chloramine-T in the proportion of one, two or three molecules to sodium aspartate (1 mol.) in 1–5 % aqueous solution, an immediate reaction took place with precipitation of toluenesulphonamide and liberation of ammonia and carbon dioxide. On heating the filtered solution with phenylhydrazine or semicarbazide or with aromatic orthodiamines, derivatives of glyoxal were obtained in large amounts. The best yields were obtained when between two and three molecular proportions of the chloramine-T were used and with the higher limit the yield of carbon dioxide closely approached two molecules. This carbon dioxide undoubtedly came from the two terminal carboxyl groups, leaving the two adjacent carbon groups to furnish the glyoxal derivatives. The apparent direct oxidation of the (CH_2) group to CHO seemed surprising,

and eventually another explanation was found. On oxidising asparagine with chloramine-T, among other products a substance was isolated which proved to be dichloroacetamide. This at once indicated the probability that the hydrogen of the (CH_2) group in aspartic acid, on oxidation with chloramine-T was replaced by chlorine. This proved to be the case and the substance yielding the glyoxal derivatives was identified as dichloroacetaldehyde. Similarly it was found that methylaspartic acid,

$$COOH \cdot C(NH_2)(CH_3) \cdot CH_2 \cdot COOH,$$

on oxidation gave derivatives of methylglyoxal which originated from dichloroacetone.

It appears probable that the half aldehyde of malonic acid is the first product of the oxidation of sodium aspartate with chloramine-T and that the former substance in neutral or alkaline solution undergoes chlorination at the methylene group, giving the half aldehyde of dichloromalonic acid which then loses carbon dioxide to give dichloroacetaldehyde.

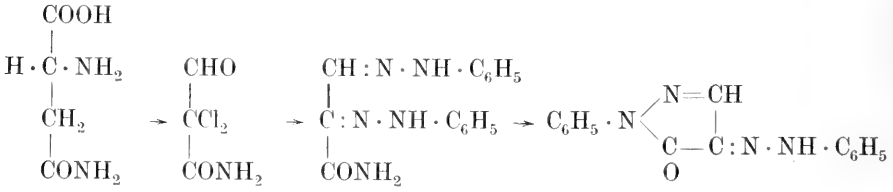


The ready chlorination of the half aldehyde of malonic acid is not surprising. Apparently chloramine-T chlorinates free acetaldehyde much less readily than is observed in the present reaction, so that it appears improbable that acetaldehyde is a primary product of oxidation which subsequently undergoes chlorination, although the formation of acetaldehyde from aspartic acid by oxidation with sodium hypochlorite or hydrogen peroxide is well established. Unlike glutamic and many other amino-acids, which yield nitriles on oxidation with two molecules of chloramine-T, no indications were obtained of the presence of cyanoacetic acid among the oxidation products of aspartic acid. On the other hand, the presence of a small quantity of a volatile compound, soluble in ether, giving a strong diazo-reaction, was noted. This substance has not yet been identified but appears to be a glyoxaline derivative. The same compound appears to be formed by the interaction of dichloroacetaldehyde and ammonia, and as both of these latter substances are formed in the oxidation of sodium aspartate by chloramine-T, its formation is in a measure accounted for. The action of ammonia on dichloroacetaldehyde invites further study.

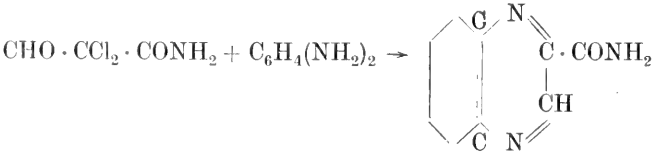
Asparagine. On oxidising aqueous solutions of asparagine with chloramine-T, a reducing substance is formed which, on treatment with phenylhydrazine and other bases, gives derivatives of mesoxalic semi-aldehyde. The substance was not isolated in the pure state but was characterised by a number of derivatives, all of which indicated that its constitution might be represented by some such formula as $CHO \cdot CCl_2 \cdot CO \cdot NH_2$, *i.e.* the semi-aldehyde of dichloromalonamide. In addition, as already mentioned, a considerable quantity of dichloroacetamide was isolated from the oxidation products. Neither formaldehyde nor acetaldehyde was found in appreciable amounts.

On treating solutions containing the semi-aldehyde of dichloromalonamide with phenylhydrazine acetate in the cold, an oily precipitate is first formed due to the formation of the mono-hydrazone, but on warming, chlorine is removed and the whole is converted into a finely crystalline compound, namely the amide of mesoxalic semi-aldehyde *bis*-phenylhydrazone. On heating the latter compound with dilute hydrochloric acid, it is converted into 4-benzeneazo-1-phenyl-5-pyrazolone already obtained by Knorr and by Will.

The changes may be indicated as follows:



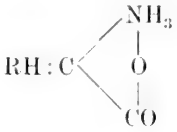
Another reaction of the semi-aldehyde of dichloromalonamide, which may be of practical value, is the fact that it reacts with various orthodiamines to give the amides of quinoxaline-carboxylic acids. These amides as well as many of the corresponding mono-carboxylic acids appear to have been hitherto inaccessible.



Reference must be made here to Langheld's observations on the action of sodium hypochlorite upon asparagine. He states that the product of the reaction is the hitherto unknown half-aldehyde of malonic acid which he did not isolate but characterised as a phenylhydrazone melting at 239–240°. Satisfactory analyses for this compound were recorded by Langheld (see

experimental section) but as a matter of fact the compound is the *bis*-phenylhydrazone of mesoxamide semi-aldehyde described above. Direct comparison of the compounds, as well as their mixed melting point and conversion into 4-benzeneazo-1-phenyl-5-pyrazolone prove this conclusively. Langheld's description of the half aldehyde of malonic acid and its hydrazone must therefore be abandoned.

It is a somewhat striking fact that so far as at present observed, chloramine-T does not react at all readily with amino-acid esters. This is the more surprising in view of the pronounced basic properties of the esters. It is possible that chloramine-T reacts with the cyclic betaine form of amino-acids,



rather than with the carboxylic form.

In the experimental portion are included experiments demonstrating the formation of isobutyric aldehyde from valine and of methylethylacetaldehyde from isoleucine on oxidation with chloramine-T. These reactions are typical of those previously encountered and present nothing essentially new.

An addendum to the experimental portion gives an account of an interesting substance obtained during the oxidation of carbohydrates by chloramine-T together with a number of its derivatives. The substance proved to be the hitherto unknown benzaldehyde-*p*-sulphonamide. The production of this compound is rather curious. It appears to be produced by the action of chloramine-T upon *p*-toluenesulphonamide, the latter substance being formed by the oxidising action of chloramine-T upon the sugar. The apparent anomaly is thus presented of chloramine-T oxidising its own reaction product. The formation of benzaldehyde-*p*-sulphonamide appears worthy of record since this substance could not be readily synthesised by the usual methods. The other products derived from the glucose are complicated and need further study, although small quantities of saccharic acid have been definitely identified, while little gluconic acid is found, although pentoses are present in noteworthy amount.

Experimental Part.

Oxidation of glutamic acid.

Formation of β-aldehydopropionic acid. This substance is readily formed when neutral sodium glutamate is treated with one molecular proportion of chloramine-T. Glutamic acid (1·47 g.) was dissolved in 100 cc. of hot N/10 sodium hydroxide and the solution cooled to air temperature. Solid chloramine-T (2·9 g.) was next added, which readily dissolved, *p*-toluenesulphonamide being then rapidly precipitated. The reaction did not readily proceed to completion in the cold but on gently warming to 50° a steady evolution of ammonia and carbon dioxide took place and active chlorine, tested for with potassium iodide and acetic acid, rapidly disappeared. The solution was then chilled and the sulphonamide removed by filtration. The filtrate on treatment with nitrophenylhydrazine (1·7 g.) dissolved in hot dilute hydrochloric acid gave 2·1 g. of the nitrophenylhydrazone of β-aldehydopropionic acid compared with a theoretical yield of 2·37 g. The substance was purified by recrystallisation from boiling water and crystallised in platelets which when rapidly heated melted at 185–187°. On analysis the substance was found to contain 17·9 % nitrogen (Dumas) compared with a calculated value of 17·7 for $C_{10}H_{11}O_4N_3$. In the original paper by Harries and Alefeld [1909] this compound was described as melting at 158° and this value later was corrected by Alefeld [1909] who gave 175°.

A second oxidation was carried out with larger quantities of glutamic acid (5·41 g.), but otherwise under similar conditions. After filtering off the sulphonamide, the solution was concentrated under reduced pressure and the filtered concentrated solution acidified with dilute sulphuric acid and extracted thoroughly with ether in a continuous extraction apparatus. On distilling off the ether and distilling the residue under a pressure of 2 mm. a small amount of the oily β-aldehydopropionic acid was obtained as described by Perkin [1899] and by Harries and Alefeld, but by far the greater part of the aldehyde had undergone oxidation during the rather protracted manipulations, so that much succinic acid, m.p. 185°, was obtained in the second fraction. The aldehyde was characterised as nitrophenylhydrazone, m.p. 185–187°, and semicarbazone, m.p. 177–178°.

Formation of β-cyanopropionic acid. Glutamic acid (7·35 g.) was dissolved in 250 cc. 0·2 N sodium hydroxide and chloramine-T (28·4 g. = 2 mols.) added to the cooled solution. The mixture was kept at 35° for a couple of hours, then cooled and filtered from the precipitated sulphonamide. The

filtrate was thoroughly extracted with ether in a continuous extraction apparatus to remove remaining traces of sulphonamide, then acidified with sulphuric acid and again extracted to separate the cyano-acid. The second ether extract on evaporation gave 4·75 g. of crude cyanopropionic acid which readily solidified on standing a short time. The substance was almost pure save for a trace of succinic acid which was removed by dissolving the dry residue in a little absolute alcohol, then adding chloroform and a little petroleum ether. The succinic acid was readily precipitated and filtered off and the filtrate on concentration gave a crystalline mass of pure β-cyanopropionic acid which was purified by washing with a little chloroform. The acid is colourless, crystallises in stout prisms and melts at 48–50° without decomposition.

Analysis: 0·1587 g.; 0·2817 g. CO_2; 0·0733 g. H_2O.
0·1058 g.; 13·2 cc. moist N at 20° and 762 mm.

	C	H	N
Found %	48·3	5·1	14·2
Calculated for $C_4H_5O_2N$ %	48·5	5·1	14·1

β-Cyanopropionic acid is a strong acid, freely soluble in most organic solvents, except petroleum ether, and extremely soluble in water, although not deliquescent. 0·111 g. of the acid required 11·1 cc. of N/10 sodium hydroxide to neutralise to phenolphthalein, compared with a calculated value of 11·2 cc. The sodium salt is very soluble in water and aqueous solutions of it are not precipitated by lead acetate, silver nitrate or mercuric chloride.

On evaporation of β-cyanopropionic acid with concentrated hydrochloric acid on the water-bath, it is quantitatively converted into succinic acid, m.p. 185°. The succinic acid was obtained pure on washing the residue with a little cold water to remove ammonium salts and then crystallising from dilute hydrochloric acid.

The reduction of β-cyanopropionic acid was carried out by dissolving 5 g. of the acid in 75 cc. of alcohol and rapidly adding an excess of metallic sodium (7 g.). The mixture was diluted with water, neutralised with dilute sulphuric acid and the alcohol evaporated off on the water-bath. The aqueous solution was then precipitated with phosphotungstic acid in 5 % sulphuric acid. A fine, heavy precipitate was obtained, which after standing was decomposed in the usual way with barium hydroxide. The free γ-aminobutyric acid obtained on evaporation after removing excess of barium hydroxide, was dissolved in hot alcohol and then precipitated with chloroplatinic acid, when the characteristic orange yellow platinum salt, melting

at 218–220°, was obtained. (Pt 31·4 %, calculated 31·7 %.) The yield of γ-aminobutyric acid was very poor, amounting to about 5 % of the theoretical amount. It is not unlikely that the conditions of the reduction might be advantageously modified.

Oxidation of aspartic acid.

Aspartic acid was neutralised with 0·1 or 0·2 normal sodium hydroxide and oxidised with chloramine-T, as already directed for glutamic acid, but no essential difference in the nature of the products was noted when either one, two or three molecular proportions of the oxidising agent were used. Quantitative estimations of the carbon dioxide formed in the process showed that 1 gram-molecular proportion of sodium aspartate gave 0·65, 1·43 and 1·8 molecular proportions of carbon dioxide when treated respectively with 1, 2 and 3 gram-molecules of chloramine-T. In each case a volatile aldehyde was present, which proved to be chiefly, if not exclusively, dichloroacetaldehyde.

On treating the filtrates from the toluenesulphonamide with phenylhydrazines either before or after distillation, glyoxal hydrazones derived from dichloroacetaldehyde were readily obtained in fair yield. For example, 1·33 g. aspartic acid was dissolved in 100 cc. 0·1 N sodium hydroxide and treated with 2·84 g. (1 mol.) chloramine-T. The reaction was prompt but active chlorine persisted for some time at room temperature so the mixture was incubated at 35° for half-an-hour. The mixture, which had an odour suggestive of a little isonitrile, was then chilled and the toluenesulphonamide filtered off. On warming the filtrate with excess of phenylhydrazine acetate 0·7 g. of glyoxal *bis*-phenylhydrazone was obtained. Similar experiments with two and three molecular proportions of chloramine-T gave yields of 1·4 and 1·2 g. respectively.

The glyoxal *bis*-phenylhydrazone was crystallised from alcohol and melted at 170–171°. It was free from chlorine and gave the following results on analysis:

0·1025 g.; 0·2650 g. CO_2; 0·0540 g. H_2O.

	C	H	N
Found	70·5	5·85	23·5
Calculated for $C_{14}H_{14}N_4$ %	70·6	5·88	23·5

On similar treatment with *p*-bromophenylhydrazine hydrochloride, glyoxal *bis*-bromophenylhydrazone melting at 240° was obtained, while *p*-nitrophenyl-

hydrazine hydrochloride gave the very characteristic glyoxal *bis*-nitrophenylhydrazone crystallising from pyridine in scarlet needles melting at about 315°.

The dichloroacetaldehyde was also converted into glyoxal *bis*-semicarbazone on digesting the aldehyde solution with excess of semicarbazide hydrochloride and sodium acetate. This reaction has already been observed by Kling [1909]. The product did not melt at 270° and was identical with the substance prepared directly from glyoxal.

Finally, the dichloroacetaldehyde was converted into β-dichlorolactic acid as follows: aspartic acid (13·3 g.) was neutralised with soda and oxidised with chloramine-T (56·8 g. = 2 mols.) as before described. After filtration, the ammonia formed in the reaction was neutralised by addition of acetic acid and the mixture distilled. The distillate was treated with hydrocyanic acid and hydrochloric acid according to the directions of Grimaux and Adam [1880], and after standing over night the nitrile was hydrolysed by boiling. On extraction with ether β-dichlorolactic acid crystallising in platelets and melting at 76–77° was readily obtained.

It is noteworthy that the distillate from the aspartic acid oxidations, especially when free ammonia was not neutralised, gave intense red colour reactions with diazobenzenesulphonic acid in sodium carbonate solution. The same phenomenon was observed when pure dichloroacetaldehyde was distilled with aqueous ammonia, and it seems probable that some glyoxaline synthesis had occurred. The constitution of the compound remains to be determined.

Oxidation of asparagine.

Asparagine was oxidised with chloramine-T in aqueous solution, no addition of alkali being necessary. The reaction commences rapidly at room temperature, with precipitation of toluenesulphonamide. When one or two molecular proportions of chloramine-T are used, all trace of active chlorine disappears on warming the mixture to 30° for about half-an-hour, but with three molecules of chloramine-T the reaction for active chlorine usually persists for a long time even though unchanged asparagine may be present in solution. The products of the reaction are ammonia, carbon dioxide, dichloroacetamide and a non-volatile aldehyde which was not obtained pure, but which is probably the semi-aldehyde of dichloromalonamide,

$$CHO \cdot CCl_2 \cdot CONH_2,$$

from which a variety of derivatives were obtained. The latter will be first described. About 25 % of the theoretical amount of the aldehyde, as judged

by its derivatives, was formed when one molecule of chloramine-T was used, about 50 % with two molecules, less with three and almost none with four. The yield of carbon dioxide was about 0·3 molecule for each molecule of oxidising agent.

Mesoxamide semi-aldehyde bis-*phenylhydrazone,*

$$CH : (N \cdot NHC_6H_5)C : (N \cdot NH \cdot C_6H_5) \cdot CONH_2.$$

Asparagine (6·6 g.) was dissolved in hot water (150 cc.), the solution cooled to room temperature and chloramine-T (28·4 g.) added. The reaction went smoothly, and after standing for half-an-hour at 30°, all active chlorine had disappeared. The mixture was cooled and the precipitated sulphonamide removed by filtration and the filtrate mixed with phenylhydrazine (10 g.) dissolved in excess of acetic acid. For a few moments there was no precipitation, then an unattractive-looking oil began to separate in the cold, but on warming on the water-bath, a finely granular reddish precipitate was formed. The hydrazone was filtered off and crystallised from boiling ethyl acetate. It forms light yellow prisms and needles melting sharply at 250–252°. The yield of hydrazone was 4·5 g.

Analysis: 0·1210 g.; 0·2841 g. CO_2; 0·0587 g. H_2O,
0·1500 g.; 33·2 cc. moist N at 19° and 750 mm.

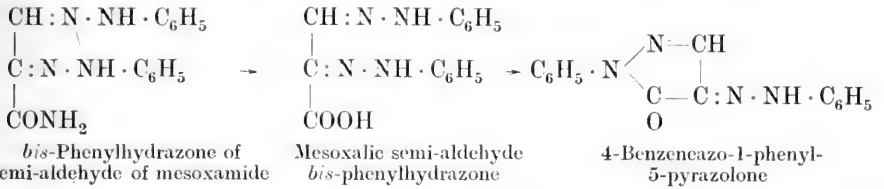
	C	H	N
Found %	64·0	5·38	25·0
Calculated for $C_{15}H_{15}ON_5$ %	64·1	5·32	24·8

The hydrazone is very soluble in warm glacial acetic acid, and moderately soluble in alcohol, ethyl acetate and acetone.

This substance is identical with the compound obtained by Langheld [1909] by the action of sodium hypochlorite upon asparagine to which he erroneously assigned the constitution of a mono-phenylhydrazone of the unknown amide of malonic semi-aldehyde. Langheld gave the melting point of the compound as 239–240° and gave analyses supporting his hypothesis. The calculated values for $C_9H_{11}N_3O_2$ are C 61·04 %, H 6·21 %, N 23·75 %, while Langheld found C 60·81 %, H 5·95 %, N 24·05 %. However, direct comparison of the product obtained according to Langheld's description and the substance above described proved their identity and the agreement of Langheld's values for the carbon and hydrogen with the mono-hydrazone, remains unexplained. The melting point of the product obtained according to Langheld's description was 250–252° and was unchanged on mixing with

the *bis*-phenylhydrazone above described and on elementary analysis his compound gave results practically identical with those for the latter substance. On treatment with acids both products gave 4-benzeneazo-1-phenyl-5-pyrazolone.

4-Benzeneazo-1-phenyl-5-pyrazolone. This substance was obtained on dissolving the above described *bis*-phenylhydrazone of the semi-aldehyde of mesoxamide in hot alcohol, adding about 5 % concentrated hydrochloric acid and boiling for two hours under a reflux condenser. On cooling, fine orange reddish needles melting at 150° were obtained, identical with the product obtained by Knorr [1888] and Will [1891] by the action of acetic anhydride or alcoholic hydrochloric acid on mesoxalic semi-aldehyde *bis*-phenylhydrazone. The reaction may be represented as follows:



bis-Phenylhydrazone of semi-aldehyde of mesoxamide — Mesoxalic semi-aldehyde *bis*-phenylhydrazone — 4-Benzeneazo-1-phenyl-5-pyrazolone

The constitution of the osazone from the oxidation products of asparagine is satisfactorily established by this reaction.

Mesoxamide semi-aldehyde bis-p-*bromophenylhydrazone.* This substance was obtained in the same way as the *bis*-phenylhydrazone by using an excess of *p*-bromophenylhydrazine hydrochloride at water-bath temperature. The solid product was filtered off, washed with water and a little warm alcohol. It was recrystallised from a mixture of glacial acetic acid and alcohol and formed golden yellow rosettes of needles melting sharply with decomposition at 274–275°. The substance is sparingly soluble in alcohol or ethyl acetate but readily soluble in acetic acid.

Analysis:	0·1782 g.; 24·2 cc. moist N at 15° and 752 mm.
Found	15·7 % N.
Calculated for $C_{15}H_{13}ON_3Br_2$	15·9 % N.

Mesoxamide semi-aldehyde bis-p-*nitrophenylhydrazone.* This substance was prepared like the corresponding bromine derivative. 2 g. of asparagine when oxidised with chloramine-T (8·4 g.) gave, when treated with 3 g. nitrophenylhydrazine hydrochloride, 1·4 g. of the nitrophenylhydrazone. The compound was obtained as a deep scarlet red precipitate which was washed with alcohol and ether. It is very sparingly soluble in alcohol, ether, acetone, amyl alcohol or pyridine, but can be crystallised from boiling nitrobenzene.

It forms deep red prismatic needles melting at about 340°. A trace of the substance treated with sodium hydroxide and alcohol gives an intense blue solution, a reaction which appears to be characteristic of two adjacent nitrophenylhydrazine groups.

Analysis: 0·1500 g.; 30·5 cc. moist N at 21° and 750 mm.

Found	22·7 % N.
Calculated for $C_{15}H_{13}O_5N_7$	22·7 % N.

Mesoxamide semi-aldehyde bis-*semicarbazone.*

$$CH : (N \cdot NH \cdot CONH_2) \cdot C : (N \cdot NH \cdot CONH_2) \cdot CONH_2.$$

A part of the solution obtained from the oxidation of asparagine with two molecules of chloramine-T, as previously described, was concentrated under reduced pressure, then cooled and filtered from a little toluenesulphonamide. The filtrate was gently warmed with excess of semicarbazide hydrochloride, sodium acetate and a few drops of acetic acid. The semicarbazone was slowly precipitated as a heavy white crystalline substance. It is very sparingly soluble in alcohol or water and when recrystallised from either solvent is obtained in the form of fine felted needles melting at 240°.

Analysis: 0·1072 g.; 42·5 cc. moist N at 17° and 750 mm.

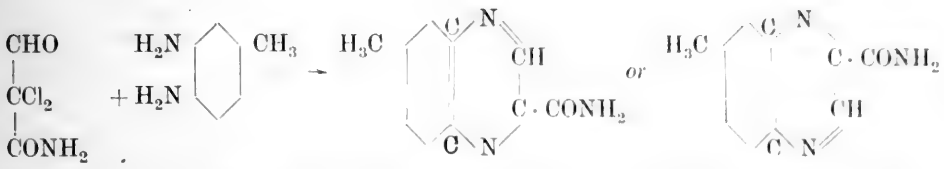
Found	45·1 % N.
Calculated for $C_5H_9O_3N_7$	45·5 % N.

Amide of 7-methylquinoxaline-2 or 3-carboxylic acid. The solution containing the oxidation products from 5 g. of asparagine prepared in the usual way, was treated with excess (3 g.) of 3 : 4-tolylene diamine. The mixture was acidified decidedly with acetic acid and then warmed on the water-bath for ten minutes. Sodium carbonate was then added to neutralise the acid and the precipitated quinoxaline was allowed to settle out. After a short time the product was filtered off, washed with a little alcohol to remove a small amount of resinous impurity and then crystallised from hot 90 % alcohol. The substance forms glistening plates which appear colourless under the microscope but which are slightly greyish brown in bulk. An alcoholic solution is light yellow in colour. The melting point of the recrystallised substance is 286–287°. The yield was poor, being only about 15 % of the calculated amount.

Analysis: 0·1117 g.; 0·2625 g. CO_2; 0·0483 g. H_2O.

	C	H
Found %	64·1	4·8
Calculated for $C_{10}H_9ON_3$ %	64·2	4·8

The formation of the quinoxaline compound may probably be represented as follows:



Other aromatic orthodiamines react similarly, but the products have not yet been characterised.

Separation of dichloroacetamide from oxidation products of asparagine.

Asparagine (5 g.) was dissolved in water (100 cc.) and oxidised at 20–25° with chloramine-T (25 g.). After two or three hours almost all active chlorine had disappeared and the solution was filtered from toluenesulphonamide and concentrated under reduced pressure to about 30 cc. A small additional separation of sulphonamide was filtered off. The filtrate was next extracted for several hours with ethyl acetate and the solvent was then removed under diminished pressure. Some of the aldehyde compound already discussed is found in the extract but much remains in the aqueous portion. The clear ethyl acetate extract, which often crystallised, was dissolved in a little hot water, treated with a little charcoal, filtered and concentrated slowly. Fine prismatic needles were thus obtained which were purified by dissolving in a little dry ether, filtering from a trace of insoluble matter and allowing the solution to evaporate spontaneously. In this way about a gram of large stout prisms of dichloroacetamide, melting at 96–98°, was obtained. The substance showed all the reactions characteristic of this compound and gave the following results on analysis: C 19·5, H 2·4, N 11·0, calculated for $C_2H_3ONCl_2$, C 18·9, H 2·34, N 10·9.

Oxidation of methylaspartic acid.

Methylaspartic ester was prepared by the action of potassium cyanide and ammonium chloride upon ethyl acetoacetate according to the directions of Zelinsky, Annenkoff and Kulikoff [1911]. The ethyl ester, as already mentioned, did not react at all readily with chloramine-T. The free acid was prepared by hydrolysing 25 g. of the ester with 40 g. barium hydroxide and 250 cc. of water. The mixture was heated two hours on the water-bath, barium salts were then removed exactly by sulphuric acid and the free acid crystallised from the concentrated aqueous solution.

The acid (7·35 g.) was neutralised with 50 cc. normal sodium hydroxide in 450 cc. of water, and chloramine-T (28·4 g.) was then added. In a few seconds separation of sulphonamide began and the solution became moderately warm. Active chlorine rapidly disappeared from the solution and the odour of dichloroacetone became marked. The solution was neutralised with acetic acid and distilled, when oily drops of dichloroacetone collected in the distillate. It was identified by its reactions with phenylhydrazine, Fehling's and alkaline silver solutions. Another portion was warmed with excess of *p*-nitrophenylhydrazine in dilute sulphuric acid, when a 30 % yield of characteristic methylglyoxal-*bis*-*p*-nitrophenylhydrazone was obtained. The substance was dissolved in boiling pyridine and crystallised by adding an equal volume of absolute alcohol and was again crystallised from nitrobenzene and dried *in vacuo* at 150°. It melted at 300–302° and gave the following result on analysis:

0·1345 g.; 29·5 cc. moist N at 20° and 758 mm.

Found ... ... 24·9 % N.

Calculated for $C_{15}H_{14}O_4N_6$ 24·6 % N.

Oxidation of valine.

Valine (1·17 g.) was oxidised in neutral 1 % aqueous solution with chloramine-T (1 mol.). The reaction was rather slow and the mixture required warming at 37° for an hour and a half before active chlorine disappeared. The solution was then neutralised with acetic acid and the volatile isobutyric aldehyde distilled off into nitrophenylhydrazine sulphate solution. The characteristic orange yellow nitrophenylhydrazone (2·3 g.) was crystallised from alcohol and melted at 132–133°. This derivative has already been described [Dakin, 1908]. The substance was analysed with concordant results.

On carrying out the oxidation with two equivalents of chloramine-T, qualitative evidence of the formation of isopropyl cyanide was easily obtained but the yield was too small for satisfactory identification. The formation of this cyanide is analogous to the conversion of leucine into isobutyl cyanide previously referred to in the introduction.

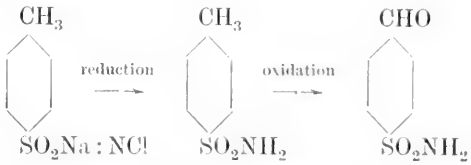
Oxidation of isoleucine.

Synthetic isoleucine (2·62 g.) was oxidised with chloramine-T (5·7 g.) as described in the case of valine. The reaction seemed to go smoothly and ammonia and carbon dioxide were promptly noticeable. The volatile methylethylacetaldehyde was rather difficult to characterise since most of

the hydrazine and other derivatives were oily or crystallised with difficulty. Nitrophenylhydrazine was found to give a crystalline derivative, but it was very soluble in most solvents and difficult to crystallise satisfactorily. The aldehyde was therefore oxidised to methylethylacetic acid with moist silver oxide. The silver salt on analysis was found to contain 51·8 % of silver compared with a calculated value of 51·6.

*Benzaldehyde-*p*-sulphonamide and its derivatives.*

On heating 5 % glucose solutions at water-bath temperature with one, two or three equivalents of chloramine-T, oxidation occurs rather slowly and toluenesulphonamide is precipitated. On treating the filtrate at room temperature with phenylhydrazine acetate, a voluminous yellow precipitate is obtained which at first was thought to be a sugar derivative. The substance on examination proved to be the hydrazone of benzaldehyde-*p*-sulphonamide. The yield of product was considerable, *e.g.* 3 g. glucose when oxidised with 9 g. chloramine-T gave 1·3 g. of pure hydrazone. Apparently the aldehyde is produced from *p*-toluenesulphonamide by oxidation with unchanged chloramine-T; at any rate, its production is noted when the two substances are heated together in aqueous solution.



Subsequently it was found that the aldehyde itself could be separated with ether by prolonged extraction of the aqueous filtrate referred to above. The ether solution on evaporation gave a mixture of the aldehyde with *p*-toluenesulphonamide. The pure aldehyde was most readily obtained by decomposing its "anil" derivative obtained by adding aniline, dissolved in alcohol, to an aqueous extract of the ether residue, or direct to the crude filtrate from the toluenesulphonamide.

p-*Sulphonamidobenzylideneaniline* $(SO_2NH_2) \cdot C_6H_4 \cdot CH : N \cdot C_6H_5$.

This substance, obtained as just described, by adding an alcoholic solution of aniline to an aqueous solution containing benzaldehyde-*p*-sulphonamide, is precipitated as a greyish white precipitate, which on crystallisation from boiling water is obtained as a felted mass of silky crystals melting sharply at 208°. It has the usual properties of anils and is readily decomposed by acids.

Analysis: 0·1168 g.; 0·2563 g. CO_2; 0·0470 g. H_2O;
0·1381 g.; 12·6 cc. moist N at 16° and 766 mm.

	C	H	N
Found %	59·9	4·5	10·7
Calculated for $C_{13}H_{12}O_2N_2S$ %	60·0	4·6	10·8

Benzaldehyde-p-*sulphonamide.* On warming *p*-sulphonamidobenzylideneaniline with ten parts of 10 % sulphuric acid for a few minutes on the water-bath, the aldehyde is readily set free. On repeated extraction with ether, the aldehyde is slowly extracted and is obtained as a crystalline residue on evaporation of the ether. The crude substance is recrystallised from boiling water, filtered from a trace of oily impurity, and is obtained in the form of shining colourless plates and needles melting at 122–124°.

Benzaldehyde-*p*-sulphonamide is readily soluble in warm water, moderately soluble in cold water. It is readily soluble in methyl or ethyl alcohol and acetone but much less soluble in ether. It is sparingly soluble in chloroform, insoluble in petroleum. The aldehyde appears to be quite stable when exposed to the air and undergoes oxidation with some difficulty.

Analysis: 0·1153 g.; 0·1915 g. CO_2; 0·0388 g. H_2O,
0·1000 g.; 6·8 cc. moist N at 17° and 764 mm.

	C	H	N
Found %	45·3	3·7	7·8
Calculated for $C_7H_7O_3NS$ %	45·4	3·8	7·6

Benzaldehyde-p-*sulphonamide hydrazone.* On adding hydrazine hydrochloride to an aqueous solution of benzaldehyde-*p*-sulphonamide a gelatinous precipitate of the hydrazone is obtained which becomes granular on gentle warming and standing. It is very sparingly soluble in boiling water but may be crystallised from 80 % alcohol in which it is moderately soluble. It forms flat shining plates and needles melting at 288–290°.

Analysis: 0·1029 g.; 0·1608 g. CO_2; 0·0405 g. H_2O.

	C	H
Found %	42·6	4·4
Calculated for $C_7H_9O_2N_3S$ %	42·2	4·5

Benzaldehyde-p-*sulphonamide phenylhydrazone.* The formation of this substance has already been described. It is obtained as greenish gold shining plates melting with decomposition at 244–245° when crystallised from hot alcohol in which it is sparingly soluble.

Analysis: 0·1733 g.; 0·3568 g. CO_2; 0·0755 g. H_2O.

	C	H
Found %	56·2	4·8
Calculated for $C_{13}H_{13}O_2N_3S$ %	56·7	4·7

Benzaldehyde-p-sulphonamide semicarbazone. This substance was obtained by adding excess of semicarbazide hydrochloride and sodium acetate to an aqueous solution of the aldehyde. The semicarbazone crystallises from water, in which it is sparingly soluble, in the form of characteristic twin prisms and melts at 250° 251° with a little sintering a few degrees below. It is not readily soluble in either acid or alkali.

Analysis: 0·1801 g.; 0·2590 g. CO_2; 0·0665 g. H_2O.

	C	H
Found %	39·2	4·1
Calculated for $C_8H_{10}O_3N_4S$ %	39·6	4·1

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XI. A RAPID PROCESS FOR THE ESTIMATION OF THE HIGHER FATTY ACIDS AND SOAPS IN FAECES.

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(*Received April 25th, 1917.*)

In connection with an investigation on the aetiology of rickets at present being carried out under the Medical Research Committee, the necessity was experienced of a rapid and accurate method for the determination of fat in the faeces. That such a method would prove of value in clinical work is obvious, as it would facilitate the regulation of the diet of patients under treatment. It was therefore with this end in view that the present work was commenced. Various processes for the estimation of the fat content were tried. The results obtained with the staining method were far from accurate and this was rejected.

The acid amyl alcohol separation used for milk did not give a satisfactory result owing to the difficulty of obtaining an average of the faeces when made liquid with water, as sedimentation of the insoluble matter rapidly took place which carried down with it some of the fat.

On further consideration it was thought that the first step in the determination of the fat content should be the use of a suitable solvent. A 2 % alcoholic solution of caustic potash was employed since by this treatment all the fat is converted into the corresponding soap from which the cloud of fatty acid is displaced and determined by the nephelometer [Kober and Graves, 1915].

The only work which I can find directly bearing on the present investigation is by Laws and Bloor [1916] who extract the faeces with acidified alcohol and ether and seem to obtain fairly consistent results, which they claim to be within 5 % of those obtained by older methods.

By employing an acid medium for extraction it is however possible that there may be, on dilution, a recombination of the calcium and magnesium with some of the fatty acids, thus giving a low result.

Method.

(1) *Total Fatty Acids, i.e.* free and combined as neutral fats and soaps.

An average sample of the faeces is taken, thoroughly mixed and 1 g. weighed on to a fat-free filter paper, the material being spread out so as to form a thin layer. It is then introduced into a conical flask, 50 cc. of 2 % alcoholic potash solution are added and the flask is connected with a reflux condenser. Boiling is continued for about 15 minutes, by which time all the fat becomes saponified. The solution is cooled and decanted into a 200 cc. flask, made up to the mark, thoroughly mixed and a quantity filtered into a dry beaker. Of the filtrate 2 or 5 cc., depending on the quantity of fat present, are pipetted off and placed in a 100 cc. measure, 5 cc. of hydrochloric acid are added and the whole made up to 50 cc. with distilled water and mixed.

The standard is prepared as nearly as possible at the same time by taking 2 or 4 cc. of a standard fatty acid solution (1 cc. = 0·0005 g. fatty acids) made by saponifying oleic acid[1] 50 %, palmitic acid 25 % and stearic acid 25 % with alcoholic potassium hydroxide, and treating with hydrochloric acid in the same way as the sample. Both volumes are then made the same and the measures are set aside for about five minutes to allow the clouds of suspended fat to develop and the clouds are then compared in the nephelometer. The quantity of fat in the faeces can then be calculated. There is no colour in the sample tube when the cloud is precipitated, which makes the comparison of the fields very easy.

Results.

(1) *Test of the nephelometric process.* To ensure a good average for the test the faeces were dried, hence the high percentage of fatty acids. This was done by slow evaporation with alcohol on the steam-bath, care being taken not to overheat.

A. Sample from a child with rickets.

(1) Nephelometric method { reading of standard ... ... 60 ; ,, ,, sample (average) ... 68 }

$$\therefore \frac{60 \times 0{\cdot}002}{68} = \text{quantity of fatty acids in amount of sample used}$$

$$= 17{\cdot}60\ \%\ \text{fatty acids.}$$

(2) By Soxhlet's extraction = 17·68 % ,, ,,

[1] It was found that oleic acid was necessary to produce the fat cloud. Dilute soap solutions of palmitic and stearic acids when acted on by HCl flocculated and settled as a curd, but on addition of the slightest trace of oleic acid the cloud so formed remained in complete suspension indefinitely.

A. Two days later.

(1) Nephelometric method { reading of standard ... ... 60 ; „ „ sample (average) ... 81 } = 14·8 % fatty acids.

(2) By Soxhlet's extraction ... ... = 14·7 % „ „

B. Child with rickets.

(1) By nephelometer { reading of standard ... 60 ; „ „ sample (average) 59 } = 20·3 % fatty acids.

(2) „ Soxhlet's extraction ... ... = 19·9 % „ „

(3) „ ethereal extraction after liberation of the fatty acids by HCl ... ... = 20·0 % „ „

B. Two days later.

(1) By nephelometer { reading of standard ... 60 ; „ „ sample (average) 65 } = 18·4 % fatty acids.

(2) „ Soxhlet's extraction ... ... = 17·9 % „ „

(3) „ ethereal extraction after liberation of the fatty acids by HCl ... ... = 18·0 % „ „

It will be seen that the results are well within the limits of experimental error.

For accurate work in cloud reading the nephelometer is indispensable. Nesslerising tubes may however be substituted, and when used with care give a fair degree of accuracy. When using the comparison tubes all that is necessary is to produce the cloud in a similar quantity of the sample, as described above, and, using a second tube, run in from a burette the standard fatty acid solution, diluted if necessary, until the densities of the clouds are alike. From the number of cc. used the quantity of fatty acids in the faeces can readily be found.

Example:

(*a*) cc. of standard soap sol. used = 4·0; 20·0 % fatty acids.

(*b*) „ „ „ „ = 3·7: 18·7 % „ „

By Soxhlet's extraction ... ... 19·9 % „ „

(2) *Soaps.* (i) Sodium and potassium soaps (soluble soaps).

For the estimation of the soluble soaps it is only necessary to extract with distilled water and proceed exactly as above. Of course a larger quantity of the sample must be taken as there is a very small percentage of fat combined

in this form. The average amount in the samples I have examined is about 0·2 % calculated as fatty acid.

(ii) Calcium and magnesium soaps (insoluble soaps).

There is no direct nephelometric method for the estimation of these insoluble soaps in the faeces. It is necessary to know the ether-soluble fatty acids, as well as the total fatty acids and the water-soluble soaps.

Example:	Sample A	Sample B
Total fatty acids by nephelometer[1] ...	18·4 %	20·3 %
Fatty acids by Soxhlet	17·9 %	19·9 %
= Total soaps (by difference)	0·5 %	0·4 %
Water-sol. soaps by nephelometer ...	0·2 %	0·2 %
= Ca and Mg soaps (by difference) ...	0·3 %	0·2 %

With regard to the time necessary to complete a series of these estimations it was found that from eight to ten samples could be undertaken in a day. Thus the process offers a very great advantage over the tedious method of extraction with ether.

Another point in favour of the method is that all the fatty material is extracted whether combined as neutral fats, fatty acids or soaps and estimated as total fatty acids. This overcomes the possibility of incomplete ether extraction which is a well-known difficulty.

Conclusions.

By the saponification process and the use of the nephelometer very rapid and accurate determinations of the fatty acid content in faeces can be made. To ensure good results the following points require attention.

(*a*) The clouds in the sample and standard should be precipitated at the same time.

(*b*) The same amount of HCl should be added to each.

(*c*) The same time (about five minutes) should be allowed for the clouds to form.

(*d*) A standard should be used which gives readings fairly close to those of the sample analysed.

The application of the method to substances other than faeces is being investigated.

[1] I have evidence in work on fat metabolism in rickets, as yet unpublished, to show that there is very little neutral fat present in the faeces. The fatty acids are for the most part free, with a very small quantity combined as soaps (see above).

I have to thank Professor Noël Paton for the advice he gave me during this work. I am also indebted to Mr David Burns who provided the nephelometer from a grant by the Carnegie Trustees.

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XII. THE DISTRIBUTION OF NITROGEN IN BEER.

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(*Received May 25th, 1917.*)

The value of beer as a food has been for long a subject of controversy. There can be no doubt that the alcohol and sugars have a certain value as sources of energy, but whether the nitrogenous compounds are of any value has never been fully investigated.

In the analysis of beer it is customary to determine the nitrogen content, to multiply this by 6·25 and to express the result as protein—a totally fallacious procedure.

In the Report of the Committee of the Royal Society on the Food Supply of the United Kingdom it is stated "that the nitrogen compounds of beer have been little studied," and so incomplete is our knowledge as regards their nature that the committee merely made a guess in assuming that something between 50 and 100 % of the nitrogen is in the form of protein or amino-acids which are utilisable in the body.

The object of the present paper is to throw further light upon the nature of these nitrogenous compounds.

Fate of the Proteins of Barley during the Brewing Process.

As a result of the malting, part of the protein in the grain is acted on by proteolytic enzymes which break it down into simpler substances. These along with a little soluble protein pass into solution during the mashing but the greater part is left in the brewers' grain. During the processes of boiling, hopping and subsequent cooling of the wort some of this protein becomes precipitated.

The amount of nitrogen thus finally left in solution is termed by the brewer "permanently soluble nitrogen." By the action of the yeast during fermentation a further quantity of soluble nitrogen is removed by these organisms using a certain proportion as food.

A further amount of protein is removed after the beer is made and this process is carried out to ensure that the beer may remain "bright."

The following table is taken from analyses by Bungener and Fries [quoted by Chapman, 1912, p. 14]. It shows the increase in the soluble nitrogen in the production of malt from barley.

TABLE I.

	Barley	Malt
Total nitrogen	1·690 %	1·580 %
Nitrogen as albumin	0·161	0·230
,, peptone	0·040	0·060
,, amides	0·206	0·534
Total soluble nitrogen	0·355	0·642

Thus it will be seen that in barley there is about 20 % of the total nitrogen in a soluble form, while in malt almost 40 % exists in this form. Of this 40 %, which during the period of mashing goes into solution, a certain proportion as already stated remains in the wort as "permanently soluble nitrogen." A large amount of research work on these bodies has been undertaken by Horace Brown [1907].

He accounts for almost 66 % of this "permanently soluble nitrogen" as the following table details:

TABLE II.

Ammonia nitrogen	3·5 %
Malt-albumose nitrogen	20·0
Malt-peptone nitrogen	31·0
Amide and amino-nitrogen	8·5
Nitrogen due to organic bases ...	4·0
Balance (unclassified)	33·0
	100·0

The 4 % of nitrogen due to organic bases comes from betaine and choline, and allantoin (glyoxyldiureide) was also present on the wort (Horace Brown).

The proportion of nitrogen capable of being assimilated by the yeast was ascertained by Horace Brown. By successive croppings of yeast and the estimation of the nitrogen content of the wort before and after fermentation he found that almost 60 % could be accounted for by the yeast action. This was a laboratory experiment and the action of the yeast was continued much longer than it is in practice. However the same test was carried out under brewing conditions with the result that the assimilated nitrogen varied between 20 and 36 % of the permanently soluble nitrogen.

From the foregoing it will readily be seen that the protein content of the beer or ale will not be appreciable when it reaches the consumer.

Application of Protein Tests.

1. The biuret test was tried both directly and on the isolated protein bodies. In every case negative results were obtained. The same result has also been obtained by Horace Brown.

2. Millon's reagent gave a negative result.

3. The ninhydrin test was also negative.

4. The xanthoproteic test alone gave a positive result.

Table III gives the percentage of so-called protein in different ales, calculated from the total nitrogen as given in the second column [Chapman 1912].

TABLE III.

	Proteins	Total nitrogen
London Mild Ale	0·33 %	0·052 %
Burton Pale Ale	0·50	0·080
English Strong Ale	0·91	0·145
Dublin Stout	0·75	0·120
American Ale	0·57	0·091

The only figures for the distribution of nitrogen in beer which I have been able to find are by Voltz, Förster and Baudrexel [1910] who give an analysis of North German dark beer used by them in their investigation of the absorption and general action of beer.

TABLE IV.

Alcohol	4 %
Extract	5·7

Analysis of Extract.

Ash	3·44
Organic	96·56
Nitrogen × 6·25	6·36 = 1·01 % N.
Ethereal extract	0·13
Nitrogen-containing substances:	
Protein	42·7
Amide-N.	57·3

METHODS.

(1) **Total Nitrogen.** This was determined by the Kjeldahl method using 10 cc. of the sample. The Kjeldahl-Jodlbauer [see Sutton, 1904, p. 87] modification was tried but no advantage was gained since no nitrogen seems to exist as nitrates or nitrites.

(2) **Protein Nitrogen.** 25 cc. of the sample were evaporated to half bulk and then cooled to about 10°, 10 % tannic acid solution containing 2 % phosphoric acid (about 4 vols. to 1 of sample) was added, shaking the while.

until no further precipitate formed. The protein mass was allowed to flocculate, then filtered off on nitrogen-free filter paper and washed with the precipitating solution. The nitrogen in the precipitate was then determined by the Kjeldahl method.

Nitrogen found $\times$ 6·25 = Total Protein.

Tannic acid solution used by itself as a precipitant, may bring down bases along with the protein but this is prevented by the solvent action of the phosphoric acid. By too lavish use of tannic acid an error may be incurred, (it is a case of the mutual precipitation of two colloids) with consequent loss of protein [Barger 1914, p. 117].

Other methods of taking out the proteins were tried but it was found that the above gave the most satisfactory results. The lead acetate precipitation was investigated. This salt has a well recognised power of throwing proteins out of solution, especially if a little of the tribasic acetate is added. The results with this were far from satisfactory as the following figures will show:

Protein nitrogen by tannic acid method	0·021 %
,, ,, acetate method	0·011 %

The failure of lead acetate to precipitate all the protein is difficult to explain, unless it be that the metallic salts of these plant proteins are more soluble than those in the corresponding animal series. Further light on this point would be interesting.

The use of (*a*) copper acetate or (*b*) phosphotungstic acid as a precipitating agent is open to serious objection as both precipitate purine and basic bodies. These were therefore rejected.

(3) Amino-acid Nitrogen. The estimation of the nitrogen combined in this form was effected by the nitrous acid method of van Slyke [1912]. 5 cc. of the ale or beer were run into the deaminising flask and this was acted on by 20 cc. of the nitrous acid mixture. Numerous duplicate estimations were performed and an average of the whole was taken. Amide-nitrogen is included in the figures given.

A full description of the process employed by previous workers for the separation of the amino-acid and amide-nitrogen is given by Horace Brown [1903].

(4) Purine Nitrogen. (*a*) *Qualitative Tests.* The presence of purine bodies was proved by the following tests:

1. The murexide test.
2. Precipitation by ammoniacal silver nitrate and magnesia mixture.
3. Precipitation after adding copper sulphate and sodium bisulphite.

4. The intense blue colour produced on adding phosphotungstic acid reagent (Folin) to the material made alkaline with sodium carbonate. This Folin considers characteristic of uric acid (trioxypurine).

(*b*) *Quantitative Tests.* The estimation of the uric acid content was carried out by the new method of Folin and Denis [1913] for its estimation in urine. It involves the precipitation as a silver magnesium compound with subsequent treatment by the phosphotungstic acid reagent and comparison of the colour produced with a standard. This method was used in the case of dark coloured beer and stout where the original colour of the material would have interfered greatly with the delicacy of the reaction. It was however found that in light ales or beers quite accurate results were obtained by direct treatment with Folin's reagent, but that care had to be exercised not to allow too much time to elapse after the addition of the reagent owing to the liability of the colour to fade on standing longer than five minutes. Of the beer under examination 2 cc. were therefore drawn off and placed in a test tube; 5 cc. of saturated sodium carbonate solution were next added and then from ten to twenty drops of the phosphotungstic acid. The mixture was shaken together and slightly heated to facilitate the reaction. This was washed into a Nesslerising-tube to about 25 cc.

The standard was prepared in a similar tube by mixing together 20 cc. of water, 5 cc. of saturated sodium carbonate solution and about 20 drops of the phosphotungstic acid, a lithium carbonate solution of uric acid (1 cc. = 1 mg. uric acid) was then delivered from a burette into the tube containing the standard until the depth of colour in each was alike. Both volumes were then made the same. These were further compared in a Dubosecq colorimeter and any necessary adjustment made. The number of cc. of the standard solution used multiplied by 0·333 gives the equivalent in trioxypurine nitrogen[1].

(*c*) *Xanthine-like purines.* These were determined by a modification of Salkowski's [1895] method for urine. The proteins were removed by lead acetate, the lead removed by H_2S gas. The phosphates were removed from the filtrate by magnesia mixture. Ammoniacal silver nitrate was then added and the resulting precipitate was filtered off and suspended in water, decomposed with H_2S and filtered. The filtrate was evaporated to a syrup and the residue treated with a little water containing 2 % of sulphuric acid which dissolves the xanthine bases leaving the uric acid practically insoluble.

The purine bases were reprecipitated by ammoniacal silver nitrate and

[1] A table giving the purine content of some beers and ales is given by C. von Noorden [1907].

either estimated by the quantity of silver in the precipitate or by the quantity of nitrogen given by the Kjeldahl method. For this work the nitrogen in the precipitate was estimated.

Xanthine seems to form the greater bulk of these more soluble purines. Confirmatory tests were given by the base liberated from the silver compound. The following may be quoted:

(*a*) Strecker's test. On evaporating the residue on a watch glass with a drop of nitric acid a yellow residue remains which turns reddish yellow on addition of potassium or sodium hydroxide.

If ammonia be substituted for the fixed alkali, no violet coloration is obtained. This is a distinction from uric acid.

(*b*) The residue was soluble in nitric acid and on crystallising, characteristic nitrate crystals were formed.

(*c*) Solutions of the residue gave a yellow crystalline precipitate with sodium picrate; at first it seemed to be amorphous, then assumed the form of round bunches of crystals.

Hypoxanthine, adenine and guanine were tested for, but none of these was found. Methylpurines were in every case absent.

The proportion of nitrogen existing in the different forms of combination found by the above processes, *b* and *c*, varies greatly. Thus the nitrogen as trioxypurine may be as much as 90 % of the total purine nitrogen or as low as 15 or 20 %, the sum being completed by the nitrogen combined in the xanthine-like bodies.

The "purine nitrogen" reported in Tables V to VIII is the sum of the nitrogen found by the above two processes *b* and *c* (p 105). This gives the total purine nitrogen.

(5) Total solid matter. This was estimated by drawing off 10 cc. from a good average sample of the beer or ale in question. It was then placed in a tared evaporating basin and evaporation continued on a water bath until dry. It was then transferred to a vacuum desiccator and dried until constant in weight.

(6) Carbohydrate. The amount of carbohydrate was determined after precipitation of the protein using Wiley's mercuric nitrate method [see Allen 1914, p. 157]. 25 cc. of the material were placed in a 100 cc. flask, 10 cc. of mercuric nitrate solution were added and the whole thoroughly mixed. When the froth settled, water was run in up to the mark on the neck and the contents again shaken. No allowance need be made for the precipitate in this case as it is so very small. When the supernatant liquid

was clear, a little (about 30 cc.) was filtered off through a fine paper (Green's 602). The concentration of sugars was then found by the polarimeter the whole being calculated to dextrin.

The results obtained by calculating the rotation all to dextrin are slightly low, about 0·3 % where a 3 % dextrin content is given. This is due to the maltose present having a slightly less specific rotation than dextrin.

The formation of an osazone derivative was tested for. Maltose was proved by the formation of maltosazone crystals, but no glucosazone was found.

Levulose was tested for, but negative results were obtained (Selivanov's resorcinol test). This sugar is very easily fermented by yeast whereas dextrin is not acted on, hence the latter is, on an average, a little above 66 % of the total carbohydrate present, the remainder being maltose [Chapman 1912].

Results.

The following tables show the results of analyses of some typical beers, ales and stouts. The volume of each sample is noted.

TABLE V.

Beer (light).

	Tennent's Lager Beer		Draught Beer	
Total nitrogen	—	0·040 %	—	0·57 %
Protein nitrogen	0·006 %	—	0·021 %	—
=Total protein	—	0·038	—	0·133
Amino-acid nitrogen	0·017	—	0·014	—
Purine nitrogen	0·016	—	0·019	—
Undetermined nitrogen	0·001	—	0·003	—
Total solid matter	4·89 %		3·47 %	
Carbohydrate (dextrin)	2·46		1·30	
Specific gravity	1006·8		1006·1	
Bottle contained	250 cc.		550 cc.	

TABLE VI.

Beer (strong).

	Bass & Co.'s Pale Ale (A)		Bass & Co.'s Pale Ale (B)	
Total nitrogen	—	0·084 %	—	0·082 %
Protein nitrogen	0·011	—	0·016	—
=Total protein	—	0·074	—	0·102
Amino-acid nitrogen	0·036	—	0·040	—
Purine nitrogen	0·039	—	not done	
Undetermined nitrogen	0·004	—		
Total solid matter	6·37 %		6·20 %	
Carbohydrate (dextrin)	3·06		3·00	
Specific gravity	1009·4		1009·0	
Bottle contained	330 cc.		265 cc.	

TABLE VII.

Ales.

	Younger's Edinburgh Pale Ale		Worthington's Indian Pale Ale	
Total nitrogen	—	0·039 %	—	0·063 %
Protein nitrgoen	0·010 %	—	0·010 %	—
=Total protein	—	0·063	—	0·063
Amino-acid nitrogen	0·018	—	0·016	—
Purine Nitrogen	0·010	—	0·033	
Undetermined nitrogen	0·001	—	0·004	—
Total solid matter	4·89 %		4·92 %	
Carbohydrate (dextrin)	2·46		2·24	
Specific gravity	1008·3		1007·0	
Bottle contained	535 cc.		330 cc.	

TABLE VIII.

Porter.

	Allsopp's Special Stout		Guinness's Extra Stout	
Total nitrogen	—	0·056 %	—	0·113 %
Protein nitrogen	0·012 %	—	0·029 %	—
=Total Protein	—	0·080	—	0·185
Amino-acid nitrogen	0·019	—	not done	
Purine nitrogen	0·020	—		
Undetermined nitrogen	0·005	—		
Total solid matter	5·90 %		—	
Carbohydrate (dextrin)	2·05		—	
Specific gravity	1009·9		1010·0	
Bottle contained	305 cc.		310 cc.	

(All the above analyses were made in duplicate.)

Table IX shows the percentage on the total nitrogen of the nitrogen combined in various forms.

TABLE IX.

	Tennent's Draught	Bass's	Younger's	Worthington's	Allsopp's	Guinness's
Protein N in total N	15·1	37·0	13·2	25·8	15·9	21·4	25·0
Amino N ,,	42·5	24·5	35·7	46·0	25·4	33·8	—
Purine N ,,	40·0	33·2	46·4	25·8	52·4	35·7	—
Undetermined N in total N	2·4	5·3	4·7	2·4	6·3	9·1	—
	100	100	100	100	100	100	—

Consideration of results.

(1) From a study of Tables V to VIII it will be seen how close the figures for the total nitrogen are, to those given in Table III from which previous workers have calculated the total protein. According to the present investigation only about 22 % of the total nitrogen exists as protein.

(2) Horace Brown [1907] in his researches on malt extract obtained a mixture of crystalline substances. These proved to be asparagine (amino-succinamide), tyrosine (*p*-hydroxyphenyl-*a*-aminopropionic acid) and leucine (isobutyl-*a*-aminoacetic acid).

These amino-acids will ultimately find their way into the beer and it is probable that they may be the source of the amino-nitrogen.

(3) As regards the purines there is some evidence to show that these bases may be derived directly from the malt, hops, and yeast. A. C. Chapman has isolated adenine, hypoxanthine and xanthine from these but he does not give data as to the percentage of the bases.

With regard to the malt and yeast the presence of purine bases in these seems to be fairly well established. As far as the present investigation is concerned xanthine (dioxypurine) and uric acid (trioxypurine) were present in comparatively large quantities. Further, since these have been found in the present work and allantoin has been found in wort by previous workers [Brown 1907] it is possible that an addition to the purine nitrogen may result from the hydrolysis and oxidation of nucleic acid [Mathew, 1915, p. 729], partly from the grain and partly from the yeast.

Examination of the Undetermined Nitrogen.

(1) *Alkaloids.*

On extracting a quantity of the beer made alkaline by sodium hydroxide with chloroform and evaporating off the solvent, a clear oil was obtained which turned brown on exposure to air, and assumed an odour very like that of tobacco juice from a pipe. It seemed also to be very volatile as it had to be dried in a desiccator for fear of loss which rapidly took place on drying at 100°.

A. Colour tests on the isolated alkaloid.

(i) A pale red colour was immediately produced by a drop of concentrated sulphuric acid in the cold. This colour was discharged on addition of a trace of nitric acid.

(ii) Sulphomolybdic acid gave a blood red colour in the cold which became darker on heating.

(iii) On evaporation with nitric acid a yellow colour was produced which turned rose pink on addition of alcoholic potash.

(iv) A deep pink colour was produced on heating with concentrated hydrochloric acid. This colour is discharged on addition of alkali.

B. The alkaloid dissolved in hydrochloric acid and in nitric acid quite freely and was liberated on addition of excess of caustic soda giving off the same odour as it had when extracted. It was proved by experiment that this alkaloid was not precipitated from its solutions by the tannic acid mixture used for the protein extraction, it is therefore another source of nitrogen.

The alkaloid seems to belong to the pyridine group. Many of the reactions are typical of "coniine" which has been found in hops by Chapman [1914]. The following table shows the percentage of alkaloid in a few samples of beer etc. examined.

TABLE X.

	Alkaloid
Draught Beer, Paterson & Co. ...	0·007 %
Younger's Edinburgh Ale	0·004
Allsopp's Stout	0·010
Guinness's Stout	0·012

(2) *Bases.*

These were taken out with a solution of phosphotungstic acid. The precipitate was filtered off and suspended in water, decomposed with barium hydroxide and filtered. The barium was removed by a current of CO_2 gas and the precipitate filtered off. Purines were then precipitated by ammoniacal silver nitrate solution, allowed to settle and filtered. The excess of silver was removed from the filtrate by hydrochloric acid and the solution concentrated to a syrup. Alcoholic mercuric chloride solution was added which precipitated the bases. This precipitate was decomposed with H_2S gas and the filtrate again concentrated. Picric acid solution was added and the whole set aside to crystallise. Bunches of yellow needles were formed which were fairly insoluble in water and had a melting point a little over 181°. This corresponds to the commonly occurring plant base, betaine, which has been found by previous workers in wort, see page 102. There was however an extremely small percentage of this in beer, only 0·005 % of the picrate, on an average, being isolated.

Conclusions.

1. The protein nitrogen of the beers analysed, varies from 13·2 to 37·0 % of the total nitrogen, *i.e.* the protein content of beer varies from 0·038 to 0·185 %.

2. The nitrogen as amino-acid varies from 25 to 46 % of the total nitrogen, *i.e.* the amino-acid nitrogen of beer varies from 0·014 to 0·040 %.

3. There is a varying proportion of nitrogen combined as purine compounds which in some cases exceeds that combined as amino-acid nitrogen. It varies from 25·8 to 52·4 % of the total nitrogen or from 0·010 to 0·039 % of the beer itself.

4. The undetermined nitrogen of these beers varies from 2·4 to 6·3 % of the total nitrogen, *i.e.* the percentage on the beer itself varies from 0·001 to 0·005.

5. The percentage of undetermined nitrogen is not quite made up by the addition of the nitrogen combined as alkaloid and base.

In the case of Allsopp's stout there is only 0·0011 % of nitrogen combined as the former and the stouts seem to contain the largest amount of alkaloid.

Of basic nitrogen there is only 0·0002 %, which is negligible.

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XIII. THE VISCOSITY OF SOLUTIONS.

By SVANTE ARRHENIUS.

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1. *Theory of Einstein.* In recent years a great number of investigations, of theoretical or of experimental scope, has been carried out regarding the viscosity of solutions by different scientists, so that it seems possible to arrive at more definite ideas on this important question.

The chief theoretical investigations in this line are due to A. Einstein [1906, 1911] and von Smoluchowski [1916]. Einstein deduced in 1906 and 1911 the following formula:

$$\eta = \eta_0 (1 + 2{\cdot}5\, \phi) \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots (1)$$

in which η is the viscosity of a solution containing the volume fraction ϕ of a dissolved substance in unit volume of a solvent with the viscosity η_0 at the same temperature. This formula is only valid for very dilute solutions for which $\sqrt[3]{\phi}$ may be neglected in comparison with 1. Further it is supposed that the dissolved particles are of spherical form and of great magnitude compared with the molecules of the solvent. The formula is deduced for the case of suspensions—*e.g.* so-called colloidal solutions of sulphur or of gamboge—but as there is a continuous transition from suspensions to genuine solutions, Einstein did not hesitate to apply his formula to solutions of cane sugar in water although the sugar molecules are probably not of spherical form.

2. *Experiments of Bancelin.* The said formula has been examined experimentally on suspensions of gamboge by Bancelin [1911, 1, 2] in the laboratory of Perrin. He found a good agreement with the theory, as the coefficient of ϕ was found equal to 2·7 (Bancelin gives 2·9, which is probably a misprint) whereas the theory demands 2·5. Still better is the agreement if we use the formula (2) below, as is seen from the following table:

% (volume)		Viscosity . 10^5		
measured	corrected	obs.	calc. 1	calc. 2
0	0	1016	1016	1016
0·09	0·09	1018	1018	1018
0·24	0·241	1023	1022	1022
0·33	0·331	1025	1025	1024
0·53	0·533	1029	1030	1029
0·66	0·665	1033	1033	1033
1·05	1·061	1044	1044	1043

ERRATA

page 115 line 16 from bottom, *for* Equér *read* Egnér.
118 last line, *for* solvent *read* solute.
120 line 7 from top, *for* disaggregation *read* aggregation.
128 line 9 from top, *for* 5·00 *read* 500.
130 line 9 from top, *for* great *read* greater.
133 line 11 from top, *for* Equér *read* Egnér.

for the same percentage, which is also demanded by the theory of Einstein. Further this theory demands that the coefficient 2·5 shall be independent of the viscosity, η_0, of the solvent. Bancelin determined the viscosity of suspensions of gamboge in an aqueous solution of glycerol with a viscosity fifteen times that of water. His results were wholly concordant with the theoretical predictions. Experiments with methylene blue gave consistent results.

3. *Experiments of Mifka.* Some experiments of Mifka [1911] on suspensions of silver, gold and copper, prepared in water according to the method of Bredig, indicate that these suspensions possess the same viscosity as the solvent at the same temperature. The concentrations of these solutions were only 4·25, 3·48, 2·79 resp. of the thousandth part of 1 % (volume). so

XIII. THE VISCOSITY OF SOLUTIONS.

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0·53	0·533	1029	1030	1029
0·66	0·665	1033	1033	1033
1·05	1·061	1044	1044	1043
2·11	2·155	1074	1074	1072

The measured percentage is given by Bancelin, the corrected one gives the volume of the gamboge granules, when the volume of the water is put equal to 100. This corrected value is, as easily seen, proportional to the molecular percentage of the gamboge. In the logarithmic formula (2) the concentration is determined in this manner (cf. § 5). The calculated values below calc. 1 are found if we suppose the constant θ in formula (2) to be 0·0111, corresponding to a value 2·56 of the constant in formula (1), whereas the figures below calc. 2 correspond to $\theta = 0{\cdot}01086$ or 2·5 in Einstein's formula. 2·56 corresponds to an empirical determination of the said constant. The agreement may be regarded as perfect in both cases.

Bancelin has made experiments with granules of different diameters, namely 0·3, 1·2 or 4 μ, and he always found the same value of the viscosity for the same percentage, which is also demanded by the theory of Einstein. Further this theory demands that the coefficient 2·5 shall be independent of the viscosity, η_0, of the solvent. Bancelin determined the viscosity of suspensions of gamboge in an aqueous solution of glycerol with a viscosity fifteen times that of water. His results were wholly concordant with the theoretical predictions. Experiments with methylene blue gave consistent results.

3. *Experiments of Mifka.* Some experiments of Mifka [1911] on suspensions of silver, gold and copper, prepared in water according to the method of Bredig, indicate that these suspensions possess the same viscosity as the solvent at the same temperature. The concentrations of these solutions were only 4·25, 3·48, 2·79 resp. of the thousandth part of 1 % (volume), so

that their viscosities ought not to differ more than about 0·01 % from that of the solvent according to Einstein's theory. This difference falls wholly within the errors of observation (here about 0·05 %), so that it may be maintained that these experiments are in accordance with Einstein's theory. The conclusion, which is sometimes given, that metallic suspensions in general possess the same viscosity as the solvent, would of course not be warranted, if it were possible to prepare much stronger solutions than those investigated by Mifka.

4. *Woudstra's determinations.* Some experiments of Woudstra [1908] on suspensions of silver in water, containing *in maximo* 0·49 g. per litre, which corresponds to 0·00464 % by volume, are said to have given viscosities that *in maximo* exceeded the viscosity of water by 4·57 %, whereas the theory of Einstein demands only 0·012 %. Woudstra's experiments were carried out by means of colloidal silver prepared according to Muthmann's method and dialysed during three weeks. His values are the following. c is the concentration in mg. silver per litre, ϕ the corresponding value in volume %—the specific gravity of silver taken as 10·53—η the viscosity, $\eta_{\text{calc.}}$ found from formula (2).

c	$\phi \cdot 10^5$	η	$\eta_{\text{calc.}}$
490	464	1·0457	1·0072
385	365	1·0098	1·0056
337	319	1·0057	1·0049
289	273	1·0045	1·0041
190	180	1·0021	1·0028
93	88	1·0013	1·0014

The values are calculated on the supposition that the relative viscosity of a solution containing 0·01 vol. % is 1·0154, whereas Einstein's formula gives 1·00025, *i.e.* a sixty times smaller value of the difference from η_0 (put = 1). We shall find later, that such great deviations occur—they are probably due to a highly flocculated state of the suspended particles, similar to that of aluminium hydroxide, precipitated from a dissolved aluminium salt by means of an alkali. The most concentrated solutions give much higher viscosities than those calculated. This discrepancy vanishes with time. Thus the viscosity of the most concentrated solution was diminished after standing thirty-seven days to 1·0077, or nearly the calculated value. An addition of salts also diminishes the viscosity of the solution.

5. *The logarithmic formula.* We have seen above that Bancelin found that the viscosity of a gamboge suspension in glycerol may be written $\eta_1\eta_2$ if η_1 is the viscosity of the glycerol solution without gamboge and if η_2 is the

viscosity of a suspension of the same volume percentage of gamboge in pure water, when the viscosity of water at the same temperature is put equal to unity. This result, which is in accord with Einstein's theory, naturally suggests the idea that if two aqueous solutions of two substances possess the same relative viscosity η, then the viscosity of an aqueous solution containing both substances in the same volume ought to be η^2. Now the two substances may be identical. If we then carry our deductions further we find that the viscosity η of an aqueous solution of the concentration c ought to be subject to the condition

$$\log \eta = \theta c \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots(2)$$

where θ is a constant and the viscosity of water at the same temperature is put equal to unity. This is the logarithmic formula found by myself empirically in 1887 [Arrhenius, 1887]. For dilute solutions, which I investigated at that time, it is indifferent whether the concentration is determined in per cent. of mass, per cent. of volume or molecular per cent.—in reality I used volume per cent. But when greater concentrations are examined, it is, as Kendall [1913] showed, better to express c in molecular percentage. It is remarkable that a similar assertion is true regarding other properties of solutions, *e.g.* osmotic pressure. In fact Kendall proved that the logarithmic formula is very nearly followed by mixtures in all proportions of liquids, which do not chemically react upon each other, if the concentration is expressed in molecular percentage. Egner [1917] has recently made a series of determinations on mixtures of two fluids and corroborated the conclusion of Kendall.

This statement is of great importance for controlling Einstein's formula (1). It is only valid for extremely dilute solutions and, as Smoluchowski states "it would demand an enormous work of calculation to carry the approximation further according to the method of Einstein." I have therefore used the logarithmic formula for calculating the experiments of Bancelin and in this manner I found the value 2·56 for the coefficient in formula (1) instead of the value 2·7 calculated by Bancelin, whereas Einstein's theory demands 2·5. In this case the size of the "molecules" of the gamboge is so enormous that their number in a suspension of 2·11 vol. % may be neglected in comparison with the number of the molecules of the solvent water. The molecular percentage may therefore without any sensible error be assumed to be proportional to the quantity of gamboge divided by the quantity of water in the solution examined. The same is true for all suspensions or emulsions

or solutions of albuminous substances or, in general, for what are called colloidal solutions.

6. *Suspensions of sulphur, examined by Odén* [1913]. In general it has not been possible to prepare suspensions of higher concentration, except for sulphur, and it is of great value for us that Odén has determined the viscosities of such suspensions, which contained up to 50 g. sulphur in 100 cc. I have shown [1916] that these suspensions are subject to the logarithmic formula (2) within the limits of the errors of observation.

The formula of Einstein is not applicable to the calculation of these concentrated solutions. Therefore Smoluchowski tried to construct an empirical formula with two new arbitrary constants. This formula has no greater interest; it may suffice to give, under $\eta_{\text{Smo.}}$, the tabulated values calculated by means of this formula in order to represent the observed values, $\eta_{\text{obs.}}$, for submicroscopical suspensions of sulphur (diameter of particles about $0{\cdot}1\,\mu$). These observed values are the mean values for three temperatures, 5°, 20° and 40°. Below $\eta_{\text{Ein.}}$ are written figures calculated by means of formula (1) where the specific gravity of the sulphur is taken equal to 1·95; below $\eta_{\text{Arrh.}}$ figures founded on formula (2). c gives the weight (grams) of sulphur in 100 cc. of the suspension, c_1 the corresponding weight in 100 cc. of water. c_1 is proportional to the molecular percentage of the sulphur. k is the calculated constant in formula (1), which ought to be 2·5 in extreme attenuation. The viscosity of water is put equal to 1.

c	c_1	$100\,\phi$	k	$\eta_{\text{obs.}}$	$\eta_{\text{Ein.}}$	$\eta_{\text{Smo.}}$	$\eta_{\text{Arrh.}}$
5	5·13	2·63	3·84	1·101	1·066	1·079	1·103
12·51	13·38	6·58	4·35	1·286	1·164	1·238	1·290
25·02	28·76	13·16	5·38	1·708	1·329	1·656	1·730
50·03	67·72	26·33	10·25	3·700	1·657	4·000	3·635

The deviation of k from Einstein's value 2·5 as well as a comparison of $\eta_{\text{Ein.}}$ with $\eta_{\text{obs.}}$ indicate how far the figures of Odén differ from the formula (1). Smoluchowski's formula gives in reality the limiting value 2·5, but its agreement with the observed values is not very satisfactory, the mean deviation reaching 4·2 %. On the other hand the figures in the last column, calculated by means of formula (2) with only one arbitrary constant give an agreement within the errors of observation, the mean difference being only 0·9 %. We may conclude from this that the limiting value of k for $c = 0$ is 3·72, *i.e.* 1·5 times greater than Einstein's theory demands.

7. *Influence of hydratation.* In the calculation of Odén's figures by means of formula (2) it has been supposed that no water is chemically bound to the

spherules of sulphur; in reality we have no indication at all that sulphur enters into compounds with water, all chemical evidence is against such a supposition. Therefore the good concordance between formula (2) and the experimental results is of a very high value in this case. This concordance is still better for the observations regarding amicroscopical sulphur (diameter of the particles about $0{\cdot}01\,\mu$) in which case the mean difference is only 0·5 % for seven observations in the same concentration interval as above, when η changes in the proportion 1 to 5.

In other cases there is high probability that a certain quantity of water becomes bound to the dissolved substance, *e.g.* if we investigate albuminous substances in aqueous solution. In such cases the molecular percentage has a higher value than if no water were bound, and the increase of this quantity is proportionally greater the higher the concentrations treated, if we suppose that each gram of the protein binds the same quantity of water at all concentrations. This hypothesis is true for very dilute solutions, *i.e.* solutions which contain very few gram-molecules per litre. As the proteins examined have not been more concentrated than about 30 % and their molecular weight is of the order of magnitude of about 10,000, the highest concentrations have not been more than about 0·03 normal. We may therefore suppose that their tendency to bind water is nearly constant and make our calculations on that ground.

In such cases we have to determine two arbitrary constants in the formula (2) namely not only the constant θ but also the quantity n of water bound to one gram of protein substance for the calculation of the molecular concentration. I have carried out some such calculations for some proteins, examined by Miss H. Chick [Chick 1914; Chick and Lubrzynska 1914], and furthermore determined the specific gravity of these substances from that of their aqueous solutions and calculated the value of k in formula (1). The results are given in the following table:

Substance	Sp. gravity	θ	n	k
Egg albumin	1·36	0·0184	0·7	2·95
Serum albumin	1·375	0·0207	1·5	2·14
Euglobulin	1·372	0·0625	2·2	4·91
Pseudoglobulin	1·402	0·0402	1·2	4·83
Sodium caseinogenate	1·414	0·1127	1·0	15·1

Hatschek, who [1916, 1] has tried to discredit the value of formula (2) in this journal, has still conceded that it, "when applied to Miss Chick's viscosity figures, fits with remarkable accuracy, and the values of θ and n are constants over the whole range with slight exceptions."

8. *Influence of temperature on the value of* $\theta = d \log \eta / dc$ *for dilute solutions.* In order to show how associated substances, which form aggregates together with the solvent, behave when the temperature changes, I have calculated some figures of Noack [1886] regarding the relative viscosity of two aqueous solutions of ethyl alcohol containing 3·38 mol. % (= 8·21 weight %) of alcohol or 27·17 mol. % (= 12·55 weight %) of water and of two solutions of acetic acid containing 4·96 mol. % (= 14·82 weight %) of CH_3COOH and 4·77 mol. % (= 1·78 weight %) of water. As units of η for the strong solutions are taken the values for the most concentrated solutions, measured by Noack, viz. solutions containing 99·28 mol. % (= 99·72 weight %) of alcohol and 99·34 mol. % (= 99·8 weight %) of acetic acid. For the weak solutions the unit viscosity is that of water at the temperature indicated in the table.

	Ethyl alcohol		Acetic acid	
Temp. °C.	3·38 mol. %	82·83 mol. %	4·96 mol. %	95·23 mol. %
0	1·582	1·632	1·374	1·380
10	1·489	1·506	1·350	1·213
20	1·410	1·411	1·327	1·138
30	1·343	1·344	1·304	1·108
40	1·284	1·288	1·279	1·106
50	1·234	1·248	1·262	1·120
60	1·191	1·216	1·234	1·146

For the alcoholic solutions the relative viscosities decrease with increasing temperature. This decrease is more pronounced at lower temperatures than at higher ones. In the interval 0°–60° the total decrease of θ is about in the proportion 2·5 : 1. The decrease is much less for the acetic acid solution of 4·96 mol. % so that θ sinks only in the proportion of about 1·5 : 1 and for the solution of 4·77 mol. % water in acetic acid we find a minimum of η and thereafter, between 40° and 60° an increase with temperature, which seems to indicate that here no sensible thermal dissociation takes place. The viscosity measurements show a very pronounced maximum at a concentration of 77 weight % at all temperatures between 0° and 60°. It is probable that this maximum belongs to a definite compound, namely the monohydrate of acetic acid, $CH_3COOH + H_2O$, which corresponds to 76·93 weight %. The acetic acid of 95·23 mol. % (= 98·52 weight %) may therefore be regarded as a solution of this monohydrate in acetic acid. At lower temperatures some loose aggregates of the solvent (acetic acid) exist, which cause the diminution of θ with increasing temperature as in the other three cases treated above, but at higher temperatures these loose aggregates have nearly wholly disappeared, so that the solvent has a constant composition.

The alcoholic solutions behave in a quite different manner. The maximum viscosity belongs at 0° to a solution of 36 weight % (= 18·05 mol. %) and at 60° to a solution of 51 weight % (= 28·94 mol. %). At intermediate temperatures there is a continuous change between these two values—perhaps at higher temperatures the composition of the solution with a maximum viscosity approaches to 33·3 mol. %, corresponding to the hydrate $C_2H_5OH + 2H_2O$.

Most dilute aqueous solutions are characterised by a positive $d \log \eta/dc$ decreasing with increasing temperature. The same behaviour may be expected for dilute solutions in alcohols.

For mixtures of non-associated liquids, for instance ethyl ether (viscosity 0·00230 at 25°) and carbon disulphide (viscosity 0·00358 at 25°), $\log \eta$ is, in general, a linear function of the molecular concentration, and therefore a constant value is found for $d \log \eta/dc$ in the whole range from $c = 0$ to $c = 100$. If c represents the concentration of the more viscous component θ is positive; but if c represents that of the less viscous component θ acquires a negative value. Normally the absolute value of θ for such solutions decreases with increasing temperature.

9. *Associating substances.* Very different and much more complicated results are obtained if we investigate substances, which become dissociated through dilution, which often present maxima of viscosity at certain concentrations. A typical substance of this kind is ethyl alcohol, which consists of strongly associated molecules in a concentrated state, but, as the freezing point determinations indicate, of simple molecules, probably associated to a certain quantity of water, in very dilute aqueous solutions. I therefore give in the following table some determinations of Horiba [1910, 1912] on the viscosity of aqueous solutions of ethyl alcohol at 25°.

c	c_1	η	$\log \eta$	$\log \eta/c_1$	$\log \eta_2/c_2$
0	0	1	0	(0·0440)	−0·000847
3·65	1·46	1·151	0·0611	0·0418	−0·000240
7·84	3·224	1·339	0·1268	0·0394	0·000435
14·88	6·403	1·708	0·2325	0·0363	0·00158
27·49	12·92	2·344	0·3696	0·0286	0·00327
40·35	20·93	2·617	0·4178	0·0200	0·00423
54·56	31·96	2·552	0·4068	0·01273	0·00473
63·95	40·92	2·375	0·3757	0·00918	0·00493
69·25	46·84	2·233	0·3489	0·00745	0·00497
79·50	60·24	1·924	0·2842	0·00472	0·00502
98·33	95·8	1·276	0·1058	0·001104	0·00502
100	100	1·2153	0·0847	0·000847	(0·00502)

c is the concentration in per cent. of weight, c_1 the molecular percentage

of alcohol, $c_2 = 100 - c_1$ that of water. For the calculation of c_1 the molecular weights 46 for alcohol and 18 for water have been used.

The viscosity decreases when the dissolved molecules form greater aggregates. Therefore the quotient $\log \eta/c_1$ also decreases with increasing *concentration* c_1. The binding of water to the alcohol molecules would have an opposite effect, *i.e.* increase the viscosity on *dilution*, but the influence of the disaggregation predominates. If we regard the series from the other side, the water behaves in the same manner. The water molecules associate with each other when the concentration of water, c_2, increases. For this case we have to choose the viscosity of pure alcohol as unit, *i.e.* to put $\eta_2 = \eta/1{\cdot}2153$. The figure 1·2153 for pure alcohol is not given by Horiba but is calculated from the data of Thorpe and Rodger [1894]. We also see that $\log \eta_2/c_2$ decreases with increasing c_2. But this decrease is very small till we reach concentrations $c_2 = 100 - c_1$ of about 60 % (mol.). The two named effects here nearly compensate each other.

In similar cases, when chemical changes occur on dilution we are not entitled to expect $\log \eta/c$ to remain constant, when the concentration changes. Only for solutions so dilute that further dilution does not change their composition may such constancy be expected. For this case we have then to look for the value,

$$(d \log \eta/dc)_0 = \theta \qquad (2\,a)$$

where the index 0 indicates infinite dilution or concentration zero, as the characteristic constant θ. For alcohol dissolved in water θ is about 0·044 and for water dissolved in alcohol θ is about equal to 0·00502 if we calculate with molecular concentrations.

10. *Solutions of nitrocellulose.* This is precisely the manner in which Baker [1913] treated his data regarding the viscosity of solutions of nitrocellulose in different solvents. He expresses the concentration (c) in grams per 100 cc. of solution. As instance I give below Baker's Table II of solutions in acetone at 25°. I have supposed that the molecular weight of nitrocellulose is so great, that the number of its molecules may be neglected in comparison with that of the acetone molecules, and by means of the specific gravity I have calculated the number of grams of nitrocellulose in 100 g. of acetone. This number is written under c_1 and is very nearly proportional to the molecular percentage of the nitrocellulose. The viscosity of acetone at 25° is given as $\eta = 3{\cdot}15 \,.\, 10^{-3}$ C.G.S. units. This number is taken as unit for η in the table.

c	c_1	η	$\log \eta$	$\log \eta/c_1$
0	0	1	0	(2·2)
0·2212	0·2809	3·88	0·589	2·096
0·5261	0·6778	18·7	1·273	1·878
0·7922	1·008	56	1·748	1·734
1·058	1·349	143	2·160	1·602
1·640	2·094	711	2·852	1·358
2·348	3·008	5050	3·703	1·230
3·865	4·782	53300	4·727	0·949

Just as for alcohol the quotient $\log \eta/c_1$ sinks with increasing concentration and viscosity. But the quotient diminishes only in the proportion 2·2 : 1, when η increases in the proportion 1 : 50,000, whereas for the alcohol solution in water the same decrease of the quotient is followed by an increase in η only in the proportion 1 : 2·6.

Just as with the alcohol, it is generally supposed that nitrocellulose in a concentrated state consists of molecular aggregates, which are in this case very large. On solution in acetone or some other solvent these aggregates are partially broken down and in higher degree the greater the dilution. This explains why $\log \eta/c_1$ decreases with increasing concentration. It is evidently not possible to explain this behaviour by supposing that the nitrocellulose binds a part of the solvent, for in this case, and if the dissolved molecules do not alter their composition with concentration, the quotient $\log \eta/c_1$ would diminish with increasing concentration. Now Hatschek has tried [1916, 1] to calculate the quantity n of the solvent bound to 1 g. of nitrocellulose and found a negative value of about − 30 cc. We quite agree with him that this result "is devoid of any physical meaning." But therefrom to conclude that formula (2) has no physical meaning, as Hatschek does, seems the less cautious as Baker himself in calculating his experiments has on purely empirical grounds been led to the formula (2 *a*), derived from formula (2), which Baker seems not to have known. Baker's investigation may therefore be regarded as giving a very strong confirmation of formula (2), in direct opposition to Hatschek's opinion. Baker has found that the constant θ in formula (2 *a*) decreases with increasing temperature, which is characteristic of solutions of substances which form molecular complexes with the solvent. It is therefore probable that this very general feature also prevails for solutions of nitrocellulose, a conclusion to which Baker was also led from other considerations. This is also indicated through the contraction on dissolving "blasting soluble nitrocellulose" in acetone, when the total volume diminishes by about one-third of the volume of the dissolved substance.

The specific gravity of nitrocellulose is 1·634 according to Escales. If we recalculate the figures given by Baker for $d \log \eta/dc$, where c is expressed in grams per cc. and natural logarithms are used, so that we obtain the corresponding values for volume per cent. and in common logarithms, we get the value of θ in formula (2). In the case tabulated above Baker has the figure 6·22, which reduced in the said manner gives $\theta = 4{\cdot}42$, *i.e.* 406 times as great as the value demanded by Einstein's theory. The values given by Baker for different solutions fall between 3·22 and 10·45 corresponding to values of θ between 2·29 and 7·43 which are 210 and 684 times as great as the theoretical value calculated by Einstein.

11. *Solutions of rubber.* Hatschek has also used some determinations by Schidrowitz and Goldsbrough [1909, 1, 2] on rubber solutions for calculations by means of which he tries to attack formula (2). Hatschek uses the following figures regarding hard Para rubber in benzene. η is the relative viscosity, that of the solvent being taken as unit. The concentration c is given in grams per 100 cc. and these cannot be recalculated to cc. in 100 cc. benzene, because "for the rubber solutions the densities are not available." The value of $\log \eta/c$ is therefore "approximate only but in view of the low concentrations the possible error is certainly slight."

c	η	$\log \eta$	$\log \eta/c$	$\log \eta_{\text{calc.}}$	$\eta_{\text{calc.}}$
0·25	2·5	0·40	1·60	0·39	2·5
0·50	6·8	0·83	1·66	0·78	6·0
1·00	32·3	1·51	1·51	1·56	36·3

As $\log \eta/c$ is greater in the second case than in the first one, a calculation of n, *i.e.* the quantity of benzene bound to 1 g. of rubber, gives a positive value $n = 14{\cdot}4$. The inverse is true if we compare the second and the third measurement, they give $n = -21{\cdot}5$, which is evidently physically impossible. Against this it must be said that the whole calculation is illusory, as the magnitude of the experimental errors is not considered. From the measurements of Kirchhof [1914] quoted below it is very probable that these errors may reach 10 or 12 % and, as the figures given above show, it is possible to reconcile these data of Schidrowitz and Goldsbrough with the demands of formula (2) within the said limit of errors. Hatschek in his calculations uses logarithms with five decimals, which correspond to an accuracy of 0·003 %. It is therefore not to be wondered at that his results become highly "erratic."

Kirchhof has given a great number of measurements on the viscosity at 20° of solutions of indiarubber in different solvents. The concentration ranges between 0·5 and 3 % (g. in 100 cc.) and the viscosities are expressed

in that of the corresponding solvent as unit. His results are given in the following table, corresponding to Kirchhof's Table IV. The data in the table are relative viscosities, observed and calculated by means of formula (2). Kirchhof also gives no specific gravities of his solutions, the calculation is therefore only approximate as in the preceding case.

Solvent	0·5 %	1 %	2 %	3 %
Gasolene, obs.	1·9	4·3	—	94·0
calc.	2·1	4·47	—	89·1
Benzene, obs.	2·1	4·7	23·5	97·3
calc.	2·16	4·7	21·9	102·2
Carbon tetrachloride, obs.	2·6	7·5	—	211·3
calc.	2·45	6·03	—	219
Tetrachloroethane, obs. ...	2·5	6·9	—	168
calc....	2·40	5·76	—	191
Pentachloroethane, obs. ...	3·0	8·7	46·0	284·5
calc....	2·6	6·76	45·7	309

The last figure for pentachloroethane is given by Kirchhof as 213·5 but a recalculation of the original observations in Table I and Table IV gives 284·5.

Kirchhof has made experiments with different pressures from 10 to 60 cm. of water. For distilled water he has found the times of flow to be 50·6 seconds if the pressure was 10 cm. and 10·0 seconds if it was 60 cm. As the times of flow are supposed to be strictly proportional to the viscosity they ought to be in inverse proportion to the pressures and the first figure should be 60 sec. if the second figure is right. Still worse is the agreement for gasolene where the time of flow is 32·2 sec., instead of 45·6 sec., and for benzene 28·6 instead of 40·8. The last figures, 45·6 and 40·8 respectively, are six times the time of flow at a pressure of 60 cm. water. This great disagreement, reaching in the three cases 18·6, 38·5 and 44·2 %, depends upon the very short time of flow at the higher pressure (10·0, 7·6 and 6·8 seconds). It is very much to be regretted that Kirchhof has preferred just these high pressures (60 cm. water), for the determinations given in his Table IV, reproduced above. It must therefore be said that the calculated figures agree with the observed ones within the errors of observation, although the difference in one case is nearly 25 % (this may of course be due to an accidental error). The figures of Schidrowitz and Goldsbrough do not contain any data from which the errors of observation might be estimated, but they very much resemble those of Kirchhof. Therefore I find it possible or even probable that, as said above, their figures agree satisfactorily with the demands of the logarithmic formula, especially as they characterise their experiments as "preliminary."

If we now calculate the values of θ in formula (2) for these substances we find:

	θ	A
Hard Para rubber in benzene ...	1·435	132
Indiarubber in gasolene	0·618	57
,, ,, benzene	0·637	59
,, ,, carbon tetrachloride	0·74	68
,, ,, tetrachloroethane ...	0·722	67
,, ,, pentachloroethane	0·788	73
,, ,, trichloroethylene ...	0·584	54

The specific gravity of the rubbers is not given, it is supposed to have been 0·95. Below A is written how many times the θ values exceed the theoretical value deduced from formula (1). This ratio is not as great as for the solutions of nitrocellulose, but still they are amongst the largest observed.

12. *Hatschek's formula for solvatation* (*hydratation*). Hatschek [1911, 1912, 1, 2] has given a formula for calculating the quantity of solvent, which is bound to 1 g. of the dissolved substance. The unbound quantity of the solvent is supposed not to exceed 70 % of the total volume. The formula is very simple, it has the form:

$$\eta_0/\eta = 1 - \sqrt[3]{\phi} \quad \text{.....(3)}$$

where η, as usual, is the viscosity of the solution, η_0 that of the solvent and $100\,\phi$ is the volume per cent. of the dissolved substance with its associated solvent. Regarding this formula Smoluchowski says: "It may be an interpolation formula of a certain use in many cases, but it is probably of no general signification, as it is impossible to give it a rational foundation." Till now its practical application has been very scant. Hatschek gives himself [1916, 1] an example when he says: "If the association factor is calculated, say for the 0·5 % concentration of the first example (0·5 % Para rubber in benzene $\eta = 6{\cdot}8$; cf. § 11) we find that about 62 % of the total volume of solvent is already associated with the solute. A 1 % solution would therefore be impossible if the association factor were constant. Therefore the association factor must be lower for the 1 % solution. There is certainly nothing inherently improbable about decreasing association with increasing concentration." Now we must confess that the formula has a very reduced interest if it does not give a constant association factor. Hatschek has himself emphasised the high value of Miss Chick's calculations, because his formula gives nearly constant results for the association factor (cf. below).

Kirchhof has used formula (3) for calculating his figures and has not observed the difficulties which occur in nearly all the results. A glance at the figures in his Table V gives no good prognosis for the use of formula (3).

The results of Miss Chick, which are regarded by Hatschek as the best proof of his formula, are considered by herself to be very encouraging. I give below a typical example, the calculations regarding the viscosity of pseudo-globulin. c is the concentration in per cent. of weight, η the relative viscosity in regard to the solvent water, B is the volume occupied by 1 g. dissolved substance as hydrate calculated according to formula (3) and said to be nearly constant, when the volume, p, of the hydrated molecules exceeds 50 % of the total volume—p is given in per cent. of the total volume.

c	η	p	B	
3·61	1·40	2·3	0·64	
5·95	1·85	9·7	1·603	
8·32	2·59	23·2	2·716	
10·04	3·43	35·6	3·441	
11·82	4·70	48·8	3·990	
14·06	8·05	68·8	4·585	
16·43	13·74	79·7	4·627	Mean value 4·50
18·45	23·31	87·6	4·499	(4·496)
20·37	38·79	92·4	4·272	

The formula should be valid if p exceeded about 50 %. Instead of that we find that B, which ought to be constant, increases strongly until p reaches about 80 %. This increase is also very pronounced from $p = 50$ % on, which corresponds to the interpolated value $B = 4{\cdot}06$, although Miss Chick says that "the value v/c ($= B$) remained constant when the disperse phase occupied more than one-half the total volume." At $p = 74{\cdot}06$ is a peculiar point, for if the hydrated molecules are spherical, they are tightly packed there, so that the spheres touch each other on all sides. Above this point formula (3) cannot be valid. We find that at higher values of p this deficiency of the formula is manifested by a decrease of B with increasing concentration. At higher concentrations than $c = 20$ % B would decrease still more rapidly. Through this circumstance the function B passes through a maximum, in the neighbourhood of which its value is approximately constant, and this is then said to be a proof that the formula is correct. I think it would be more justifiable to say that the formula gives unreliable results in the neighbourhood of the said point. According to this it seems clear that Smoluchowski has overestimated the practical value of formula (3). The hyperbolical form of equation (3) makes it unfit to represent the regularly continuous course of η with concentration.

13. *Viscosity of rice-starch suspensions.* From Odén's investigations on colloidal sulphur it is evident that the viscosity of its solutions

increases more rapidly with concentration than according to a linear formula:

$$\eta = \eta_0 (1 + k\phi) \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots (1\,a)$$

where k is a constant, which according to Einstein ought to be 2·5. Hatschek [1910, 1911, 1913, 1916, 2] has also deduced a similar formula, in which k has the value 4·5. Regarding this deduction, Smoluchowski makes the remark, that "it is erroneous (unrichtig) and consequently the formula, with $k = 4{\cdot}5$, lacks any general signification." Hatschek believed that the discrepancy between his formula and Odén's experiments depended upon disturbances due to the use of capillary viscosimeters in the determinations. He therefore made some experiments in collaboration with Miss Edith Humphrey [1916] with a modification of the viscosimeter of Couette. The substance examined was rice-starch in granules of 0·003 mm. diameter, suspended in a mixture of toluene and carbon tetrachloride. The calculated viscosities showed a very great dependence on the velocity of rotation of the instrument. The calculated viscosity of the solvent increased by about 10 %, when this velocity increased in the proportion 1 to 5. With the suspensions the inverse was true, a 6 % suspension diminished its apparent viscosity by about a third for the said increase of velocity. Further a kind of hysteresis took place, so that the viscosity of the solvent seemed to be about 5 % greater if the velocity decreased, than if it increased. Evidently this cannot be right for mixtures of such liquids as toluene and carbon tetrachloride—for coagulating solutions a kind of hysteresis in the opposite direction is often observed; cf. § 14. It must be said that the apparatus used was far inferior to the capillary viscosimeter. Hatschek is of the opinion that the measurements with the highest velocity used, 125° per second, are the most reliable. He has given the results in curves and I have taken the following figures from measurements on the curve signed 125 of his fig. 3.

Volume %		Relative viscosity			
given	corrected	H.	measured	calculated	Difference %
0	0	1	1	1	—
2	2·041	1·3	1·288	1·275	−1·0
4	4·167	1·6	1·647	1·642	−0·3
6	6·384	2·1	2·109	2·137	+1·3

The corrected value of the volume per cent. is expressed in the volume of the solvent taken as 100, in order that it should be proportional to the molecular concentration. Below H. are written the figures estimated by Hatschek. From these figures he concludes that the linear formula (1 a) is valid up to

4 % of volume, but not to 6. It is clear that, if more than one decimal had been used, this assertion would have proved to be false unless errors of observation as great as 3 % are admitted. But under such circumstances the accuracy is quite insufficient for examining the validity of formula (1 *a*). On the other hand we learn from the last two columns that the logarithmic formula (2) represents the observations with a much higher accuracy, and well within the limits of the errors of observation. It must therefore be said that these experiments confirm the observations of Odén, which also obey the logarithmic formula, instead of showing them to be incorrect. The same cannot of course be said regarding the linear formula (1 *a*).

θ in formula (2) is in this case 0·0517, *i.e.* 4·76 times greater than the value demanded by Einstein and 2·7 times greater than that expected by Hatschek.

14. *Irregularities.* In working with emulsions with a tendency to coagulate we meet with many difficulties. Such a case has been investigated by Freundlich and Ishizaka [1913]. They prepared a solution of $Al(OH)_3$ according to the method of Crum and coagulated it by means of $K_4C_6N_6Fe$. m designates the quantity of $Al(OH)_3$, expressed in grams of Al_2O_3 per litre, η the relative viscosity at 25°.

m	η	log η	log η/m	$\eta_{calc.}$	A
0·187	1·154	0·0622	0·333	1·135	543
0·28	1·231	0·0902	0·322	1·210	526
0·48	1·408	0·1485	0·310	1·386	507
1·12	2·069	0·3158	0·282	2·140	461

As seen from these figures log η is nearly proportional to the concentration m and the calculated values of η agree tolerably well with the observed values. But there is a pronounced tendency of log η/m to decrease with increasing concentration. The coagulating solution looks, to begin with, just like the uncoagulated, but its viscosity increases slowly, and at first after several hours, if the solution is not too concentrated, the flocculation becomes visible. It is supposed that the maximal viscosity is reached before this point, but that is not at all proved and in all cases it is probably rather difficult to determine this maximal viscosity, which is tabulated above under η. Further this viscosity is, for more concentrated solutions, dependent on the mechanical treatment. Thus the maximal viscosity 2·069 of the last solution changes to 1·62 after shaking. Under such circumstances it is clear that deviations between the observed and the calculated values may occur. The maximal viscosity depends also in a high degree on the coagulating power of the salt added as is seen from the following table for different salts of potassium or ammonium. The solution used was always the same with $m = 0{\cdot}88$.

Coagulating salt	η	Potassium salicylate	1·929
Potassium nitrate	1·423	,, sulphate	1·998
,, thiocyanate	1·423	,, ferrocyanide	2·000
Ammonium chloride	1·481		

From the specific gravity, 3·85, of Al_2O_3 I have calculated the specific gravity of $Al(OH)_3$ under the assumption that no change of volume takes place on hydration. With the figure 2·21, found in this way, I have calculated how many times, A, the θ-value for 1 vol. % $Al(OH)_3$, exceeds the theoretical figure 0·01086. As seen, A reaches the value of about 5·00. Probably the floccules correspond to a much higher degree of hydration than that expressed by the formula $Al(OH)_3$, so that A should be considerably diminished.

Similar remarks may be made regarding some determinations of Bingham and Durham [1911], in which small additions of potassium chloride increased the viscosity of suspensions of infusorial earth, in one case by 60 %, or diminished that of a suspension of clay by about 37 %, whereas one drop of acetic acid increased the viscosity of a graphite suspension by 81 %. The influence increases with the concentration of the suspension. Evidently we have here to do with a change in the state of flocculation. These suspensions do not at all follow the formula (2) for higher concentrations. Probably the flocculation makes the suspended particles cling to the walls of the viscosimeter tube, thereby narrowing it. We must therefore use formula (2 a). In this case it is sufficient to calculate A from the values of not too concentrated suspensions. For infusorial earth (3·2 vol. %) A increases with temperature, as is seen from the following table:

Aqueous suspensions		Alcoholic suspensions	
Temp. ° C.	A	Temp. ° C.	A
25	3·03	30	2·59
45	3·37	45	2·83
65	3·74	65	3·97
75	3·82		

For china clay (in 2·48 % suspension) the corresponding figures are:

Temp. ° C.	A
35	6·33
45	6·28
65	6·70

For graphite (Acheson's "aquadag," 0·4 vol. %) the figures are rather irregular and give a decrease of A, from $A = 7·12$ at 30° to $A = 6·55$ at 65°.

For solutions of indiarubber the irregularities are perhaps still greater than in the last named cases as Fol [1913] has shown. The viscosity of a solution diminishes, when it is allowed to flow repeatedly through the tube of the viscosimeter. This corresponds to the behaviour of the suspensions of $Al(OH)_3$ on shaking. But if we make a solution of a given quantity of rubber in a given quantity of benzene and shake this solution, its filtrate, taken after 24 hours, has a greater viscosity than if it had not been shaken. This depends upon a disintegration of small solid particles on shaking, so that they pass through the filter. But the increase of the viscosity is greater than corresponds to the increased concentration. This peculiarity is probably connected with the circumstance that the parts of the rubber which enter at first into solution give a lower viscosity than the parts dissolved later on. The viscosity of a rubber solution diminishes on standing.

From what has been said it seems evident that it is necessary to use the greatest precaution if we wish to draw theoretical conclusions from measurements on the viscosity of substances which exhibit such irregularities, specially when the measurements were made at a time when these irregularities were much more imperfectly known than they are to-day.

15. *The values of k in formula* (1 *a*). We have seen above that Bancelin's experiments gave a brilliant confirmation of Einstein's theory, demanding $k = 2{\cdot}5$. Further the experiments of Mifka are well in concordance with this theory. But all other experiments on colloidal solutions, quoted above, give too high values of k.

To begin with, the experiments of Odén for colloidal sulphur give the following values:

For submicroscopical solutions (diam. of granules $100\mu\mu$)	at 5°,	$k = 3{\cdot}60$.
,, ,, ,, ,, ,, ,,	20°,	3·67.
,, ,, ,, ,, ,, ,,	40°,	3·88.
For amicroscopical solutions (diameter $10\mu\mu$)	at 25°,	4·73.

For all the other suspensions or emulsions examined (with exception of serum albumin, an exception perhaps due to errors of observation) the values of k have been found to exceed 2·5, in many cases a hundredfold and more. The question arises: what is the cause of this discrepancy? Smoluchowski has pointed to the probable cause. He says: "For the sphere this value (k) has evidently a minimum value, as it by virtue of its rolling movement has influence upon the smallest possible volume of the liquid in its state of shearing." Therefore the coefficient 2·5 in Einstein's formula applied to

colloidal solutions must be regarded as a minimum value. But there is evidently no sufficient ground to replace it by a higher value as Hatschek proposes.

There is another peculiarity, which Smoluchowski has not observed, namely that k in formula (1 a) decreases with increasing diameter of the spherical particles, as is seen from Odén's measurements. Now we know that the Brownian movement of the suspended particles increases, when their dimensions are diminished. Therefore the same quantity of smaller particles will come in contact with a great number of molecules of the solvent through their speedier movement, than the same quantity of bigger particles. This circumstance will also increase the value of A when the dimensions of the particles decrease.

On the same ground, as the Brownian movement increases with temperature, the value of A or of k will increase with temperature. This effect is very pronounced in Odén's figures. It is due not only to the movement of the suspended particles, but also to that of the molecules of the solvent. This must be true for particles of any form and it is seen from the experiments on suspensions of clay and ground infusoria that this statement is true. When molecular aggregates of the solvent are formed their disaggregation with increasing temperature has the inverse effect.

For substances which are hydratated θ decreases with rising temperature. This depends upon the partial dissociation of the hydratated molecules with increasing temperature, whereby the volume concentration of the dissolved or suspended "molecules" diminishes. This seems to be the case with most aqueous solutions and Miss Chick has observed it for the solutions of protein substances. The low value of $k = 2{\cdot}14$ for serum albumin is probably due to errors of observation, which have caused a too high value of the hydratation factor, n. The decrease of A with increasing temperature for graphite suspensions is perhaps due to a diminished agglutination of the particles, for it is not probable that graphite forms hydrates.

The hydratation has a great effect on the viscosity at higher concentrations and causes positive deviations from formula (2), if we calculate the values as if no hydratation took place. We have not found any instance of such positive deviations for solvents which are non-associated in contradistinction to the highly associated water. Therefore it is not probable that these non-associated solvents in the cases examined hitherto, especially with solutions of india-rubber or nitrocellulose, enter into molecular aggregates with the dissolved molecules, as is maintained by Hatschek on the ground of calculations by

means of his formula (3). But probably many alcoholic solutions behave as aqueous ones. On the other hand suspensions of infusorial earth in alcohol, as well as similar suspensions of infusorial earth, clay or graphite in water show a θ-value, that increases with temperature. In this case we have no hydratation nor alcoholatation of the suspended particles. The very high viscosity of these suspensions in greater concentrations, which is followed by a very pronounced deviation from formula (2) depends on a formation of large aggregates between the suspended particles themselves, which form a kind of felt which obstructs the capillary tube. Similar cases will probably be very common in agglutinating or coagulating suspensions in higher concentrations.

The very large values of θ or A found in most cases have a similar explanation. Every chemist knows fairly well the voluminous flocculi of hydrated alumina which on desiccation crumble to nearly insensible granules. It is quite natural to suppose that the high value of A, about 500, in this case depends on this voluminous nature. The hydrated particles may further, as Smoluchowski says, possess a spongy structure or be formed as spherolites consisting of very fine needles radiating from a centre. The high values of A found for solutions of indiarubber or nitrocellulose suggest a similar form for the corresponding aggregates, which may also cling to the walls of the viscosimeter tube and cause similar deviations from formula (2) as for suspensions of clay or infusorial earth or graphite. Many examples of such a behaviour in more concentrated solutions are found in the memoirs of Schidrowitz and Goldsbrough. This behaviour seems very probable for indiarubber in the dissolved state, the solutions being extremely sticky. Regarding the similar property of moist clay, on which its high plasticity depends, Le Chatelier [1914, p. 481] has drawn attention to the circumstance that it is a consequence of the fine laminated structure of kaolin. The same may be true for graphite which crystallises in thin hexagonal scales. Probably its high lubricating power is connected with this property. Evidently we have here to do with capillary forces which attain a predominant influence for laminated substances in the form of very fine dust.

Hatschek has also made the objection against formula (2) that it has no theoretical foundation. Lees [1900, 1901] has given such a one (cf. also § 5). But it is also to be remembered that the most "rational" laws have originally been built up from purely empirical foundations.

16. *Summary.* The formula of Einstein, as applied to colloidal solutions, represents a limiting law. It has been confirmed by the experiments of

Bancelin. But in most cases the viscosity is much higher than this law demands. The anomalies depend on the Brownian movement, on the non-spherical form of the suspended particles, on formation of aggregates with the solvent especially on hydratation or on sticking together of suspended particles, circumstances which all increase the viscosity. The logarithmic formula (2) is found to be valid for true solutions as well as for pseudosolutions (emulsions and suspensions). Deviations from this formula are caused through chemical changes occurring on dilution.

The chemical changes occurring on mixing solvent and solute are the following:

(1) The solvent may be associated and its complex molecules may be dissociated by the addition of the solute. This process is followed by a diminution of the viscosity. A rise of temperature exerts also such a dissociating influence, whereby a diminution of θ in formula (2) results.

(2) A dissociation of the molecules of the solute has an opposite influence, so that the viscosity increases thereby. This corresponds to a higher viscosity of suspensions with small spherical granules, compared with that of a similar suspension with larger granules. If the suspended particles possess a laminated structure, they form felty aggregates, which in higher concentrations raise the viscosity.

(3) Association between molecules of the solute and of the solvent increases the viscosity. In this case the dissociating influence of rising temperature diminishes θ in formula (2).

Objections made against the validity of the logarithmic formula, raised in this journal by Hatschek, do not bear a close analysis. Experimental attempts by Hatschek and Miss Humphrey to disprove this formula have wholly failed. Hatschek's formula for calculating the degree of binding between solvent and solute not only lacks theoretical foundation but is also incompatible with experimental facts.

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XIV. THE ESTIMATION OF SUCCINIC ACID.

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The original method employed by Pasteur for the separation of succinic acid from other acids produced during fermentation consisted in extracting the calcium salts with a mixture of alcohol and ether. The calcium succinate is insoluble and the salts of certain other acids dissolve. The method as now usually employed consists in adding to a concentrated solution of the calcium salts in water, sufficient alcohol to bring the final concentration to 85 %. The amount of succinate is estimated from the determination of the calcium in the precipitate.

The ordinary method is liable to serious errors, and probably the experience of inconstant results has led certain authors to discard it in favour of a method based on the use of some other salts such as the salts of barium. As a matter of fact however the errors to which the Pasteur method, as generally used, is liable, do not arise from any unsuitability of the calcium salt, but from other causes which are as follows:

(1) Loss of succinic acid during concentration. Succinic acid volatilises even from a solution which is alkaline to phenolphthalein. The loss may easily be 7 %.

(2) The solvent action of 85 % alcohol containing traces of peptone on calcium succinate. 0·15 g. of peptone in 100 cc. of the solution may introduce an error of 10 to 15 %.

(3) The presence of any substance which prevents the crystallisation of the calcium succinate.

(4) Acidity of the alcohol employed. The alcohol must be neutral.

(5) Loss through washing the precipitate.

The freshly formed precipitate of calcium salts is flocculent and in the presence of certain impurities such as peptone this condition persists. In the absence of impurities the precipitate gradually becomes crystalline. This change may be taken as an indication that the separation is quantitative. The errors referred to above may be avoided by attending to the following details.

In the case of the fermentation products studied by the writer, lactic acid is the only acid which remains to be separated from succinic acid after the volatile acids have been removed, but the method described here applies equally well to cases where other acids are present, provided the calcium salts of such acids are soluble in 85 % alcohol.

The mixture of succinic and lactic acids obtained by extracting the acidified solution of the non-volatile products of fermentation by ether, in a suitable continuous extraction apparatus, is dissolved in about 100 cc. of hot water and chalk added in excess. The solution should not be boiled as loss of succinic acid will occur by volatilisation. The neutralisation takes place slowly owing to the presence of the anhydride of lactic acid.

The final neutralisation is effected by adding a solution of calcium hydroxide after the addition of phenolphthalein. The solution is not neutralised from the start by lime water to avoid excessive dilution. The solution is kept hot during at least an hour to ensure the thorough conversion of the anhydride into calcium lactate, and the solution, distinctly pink, is filtered into a graduated flask of 200 cc. capacity. Of the filtrate 50 cc. is used to estimate the calcium corresponding to the total acids, *i.e.* succinic plus lactic and possibly other acids, the calcium being precipitated as oxalate and the oxalic acid equivalent of the calcium determined by titration with a standard solution of potassium permanganate. A suitable portion of the remaining solution is concentrated slowly to a volume of 13 cc.

This is done in a 110 cc. graduated flask in an oven kept at a little over 100°. Five or six hours are required for the concentration of 50 cc. of the solution. The author employs a series of tared 110 cc. graduated flasks and continues the evaporation until the flasks contain the required 13 g. of water. Alcohol is then added to make up the volume to 110 cc. which in the case of 96 % alcohol brings the final concentration to 85 %. The flask is well shaken and allowed to stand until the flocculent precipitate has become crystalline. This may require till next day but takes place in a few hours if the solution is free from impurity. The solution is filtered into a 100 cc. graduated flask, and after removal of the alcohol, the calcium is estimated in

this 100 cc. This calcium figure multiplied by 11/10 gives the amount of calcium corresponding to the acids of the soluble calcium salts, and the amount of succinic acid is obtained by subtracting the figure thus obtained from that previously found for the total acidity. The error of the experiment need not exceed 1 % of the weight of succinic acid present.

Should the amount of acids present be small, the estimation of the total calcium need not be made on a separate sample but after filtering the 100 cc. of the alcoholic solution the remaining 10 cc. of alcohol together with the precipitate of calcium succinate may be dissolved in water and the calcium determined. From this figure the amount of calcium corresponding to 10 cc. of the alcoholic solution is deducted, the difference representing the calcium in the precipitate[1].

The following example of the separation of a mixture of lactic and succinic acids may be given.

A mixture was made of 40 cc. of a solution of lactic acid (corresponding to 59·4 cc. N/10 lactic acid) and 30 cc. of a solution of succinic acid (corresponding to 26·25 cc. N/10 succinic acid) and the 70 cc. was extracted in a continuous apparatus by ether during nine hours. The extracted acids were treated as described above and the volume of the solution of the calcium salts made up to 200 cc. 50 cc. of this solution was brought to the boil and precipitated by oxalic acid. The calcium oxalate precipitate required for oxidation 21·1 cc. N/10 permanganate solution. The total acidity found therefore corresponds to 84·4 cc. instead of 85·6 cc. There is here an error of 1·5 %.

Another 50 cc. was evaporated to 15 cc. in a tared graduated flask and the volume made up to 110 cc. with 98 % alcohol. 100 cc. of the filtrate after removal of alcohol was precipitated by oxalic acid and yielded calcium corresponding to 13·15 cc. N/10 permanganate solution. This corresponds to 14·45 cc. for 110 cc. of the alcoholic solution. The total lactic acid is therefore 57·8 cc. N/10, which figure deducted from 84·4 gives 26·6. The amount of succinic employed was 26·25.

[1] *Note on the estimation of calcium by the permanganate method.* It is necessary for me to point out that the procedure which certain authors (Cahen and Hurtley, *Biochem. Journ.* 1916, **10**, 308) have adopted in connection with the well-known method of estimating calcium oxalate by permanganate is to be strictly avoided. The correct method of procedure is to filter the calcium oxalate upon asbestos in a suitable vessel, such as the ordinary Gooch crucible, and, after washing it, to decompose it by sulphuric acid in the ordinary way. The washing takes a few seconds and there is certainly no need for the seven washings of which these authors speak. But whatever procedure is adopted for collecting and washing precipitates, there can be no justification for boiling paper with sulphuric acid solution and titrating this with permanganate. Nothing must be oxidised except the oxalic acid.

This shows that all the manipulations can be carried out with an error less than 2 %.

The actual separation of the two acids need not involve an error of more than 1 %, as will be seen from Appendix, Ex. 2 (4, 5, 6).

Appendix.

Experiments demonstrating the magnitude of the errors referred to in the communication.

Ex. 1. Loss of lactic and succinic acids during the evaporation of the slightly alkaline solutions of the calcium salts.

Pasteur in describing the original method indicates the necessity of slow evaporation to avoid loss of succinic acid. It seemed to the writer of interest to know whether this loss occurred even when the solution was kept alkaline throughout the evaporation.

200 cc. of a solution of calcium lactate corresponding to 55·2 cc. N/10 acid, was evaporated on the water bath in an open vessel, the solution being kept alkaline throughout by the addition from time to time of a solution of calcium hydroxide; the dried residue was dissolved in water and the solution filtered from calcium carbonate. The filtrate contained calcium corresponding to 52·75 cc. of lactic acid. This represents a loss of 2·45 cc. N/10 lactic acid, or approximately 5 %.

Under the same conditions 36·6 cc. N/10 succinic acid lost 2·85 cc. or 8 % of the succinic acid employed.

To avoid such errors the evaporation should be made as directed in this communication in a long necked graduated flask in an oven regulated so that the temperature of the air is about 110°; under these circumstances there is no loss.

Ex. 2. The effect of peptone.

Peptone may introduce a grave error. It prevents the precipitation of calcium succinate by 85 % alcohol. Unneutralised peptone will dissolve calcium succinate readily, but the experiments here described refer to peptone which has been neutralised by boiling with chalk.

Into each of six tared graduated 110 cc. flasks were introduced 10 cc. of a solution of calcium lactate corresponding to 12·95 cc. N/10 lactic acid, and 10 cc. of a solution of calcium succinate corresponding to 6·85 cc. of N/10 succinic acid.

In addition

to No. 1	was added	peptone	0·170 g.
,, 2	,,	,,	,,	0·023
,, 3	,,	,,	glycerol	0·70
,, 4	,,	,,	glucose	0·42

Absolute alcohol was added to each to make the volume up to 110 cc.

The calcium in solution in 100 cc. of the filtrate, was determined in each case.

No.	Substance added	$KMnO_4$ N/10 for 100 cc. of filtrate	Total calcium in solution	Calcium in precipitate corresponds to
1	Peptone, 0·17	13·8 cc.	15·1 cc.	4·7 cc.
2	,, 0·023	12·8	14·1	5·7
3	Glycerol, 0·70	12·2	13·4	6·4
4	Glucose, 0·42	11·75	12·9	6·9
5	Control	11·75	12·9	6·9
6	,,	11·90	13·1	6·7
		Succinic acid employed		6·85

This experiment shows that glucose does not affect the precipitation. There is a small effect due to glycerol, but the amount here added is about ten times that which would be present after a fermentation even if the whole of the glycerol were allowed to remain as an impurity. The error due to this cause is therefore negligible. On the other hand even 0·023 g. of peptone in 100 cc. of the fluid, may introduce an error of 15 % upon the amount of succinic acid estimated.

Summary.

By avoiding the errors described in this communication and following accurately certain details, succinic acid may be estimated in the presence of lactic acid, by the Pasteur method, with an error not greater than that of ordinary volumetric analysis.

In conclusion I beg to express my very best thanks to Professor Fernbach in whose laboratory this work was done.

XV. THE DISTRIBUTION OF THE FATTY ACIDS IN THE MILK FAT OF THE COW AND SHEEP.

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The variation in the character of the fat of cow's milk with advance of lactation, change of diet and other factors has received probably more attention at the hands of the investigator than any other aspect of the composition of milk. A careful examination of the formidable mass of literature in which this work is recorded reveals, however, that very little guidance has yet been obtained as to the fundamental causes underlying the changes observed. The prime reason for this comparative sterility of the work has undoubtedly been the difficulty of carrying out more than a superficial examination of the milk fat. No problem presents greater difficulties to the analyst than the quantitative separation and determination of a mixture of fatty acids, and the acme of difficulty is reached in the case of the most complicated mixture of fatty acids which is found in butter fat. For this reason investigators have mostly been content to limit their attention to the determination of various more or less well-defined groups of acids, such as steam-volatile, or water-soluble or unsaturated acids, etc., whereby naturally only a very imperfect record of the variations in composition has been obtained.

Efforts have not been lacking, however, to obtain a complete expression of the composition of the fat by the determination of each individual acid present, but in most cases the methods employed have been admittedly very imperfect, and in some cases assumptions have been made which subsequent research has proved to be incorrect. There is even a doubt whether the qualitative composition of butter fat has yet been fully explored, but there is clear evidence that the only acids present in more than minimal proportions are the unsaturated acid, oleic acid, and the eight saturated acids of the acetic series, representing the members with an even number of carbon atoms from C_4 to C_{18}, viz. butyric, caproic, caprylic, capric, lauric, myristic, palmitic, and stearic acids.

In the work reported in the present communication an attempt has been made to devise a method more precise than any previously used for the analysis of this complex mixture of acids.

Up to the present, most of the methods advanced for the quantitative analysis of this mixture have been based upon the difference in solubility of the several salts in certain solvents at given temperatures. Since these methods have proved more or less unreliable and have given discordant results in the hands of different workers we decided to examine the possibilities of a separation of the mixed methyl esters obtainable from butter fat. A detailed account of the method eventually devised will be found in the Experimental Part of this communication, and may be briefly summarised here.

A weighed amount of dry butter fat is converted to methyl esters by heating in ethereal solution with excess of methyl alcohol containing a small percentage of hydrogen chloride. The mixed esters are carefully separated from the reaction mixture and then fractionally distilled three or four times, in each case the distillation being made under atmospheric pressure until a temperature of 150°–160° is reached, and the fractionation subsequently completed under a pressure of about 15 mm.

The mixture of esters is thereby resolved into a series of fractions each assumed to contain only two saturated esters and one unsaturated ester, the latter being further assumed to be methyl oleate. Evidence in support of these assumptions will be found on a later page. From the iodine absorption value and the saponification value of each fraction it is then possible to calculate the weight of each ester contained in it (see later). Finally in summing up these results for the individual fractions the weight of each acid in the original weight of butter fat is arrived at.

In carrying out the fractional distillation under the conditions described in the Experimental Part it will be found that distinct fractions tend to collect round about the following temperatures:

Fraction	Boiling point	Fraction composed of
(*a*)	120°/760 mm.	Butyrate + Caproate
(*b*)	150°/760 mm.	,, ,, + a small proportion of oleate
(*c*)	70°/15 mm.	,, ,, + a small proportion of oleate
(*d*)	105°/15 mm.	Caproate + Caprylate + a small proportion of oleate
(*e*)	135°/15 mm.	Caprylate + Caprate + a small proportion of oleate
(*f*)	150°/15 mm.	Caprate + Laurate + a small proportion of oleate
(*g*)	175°/15 mm.	Laurate + Myristate + considerable oleate
(*h*)	202°/15 mm.	Myristate + Palmitate + a large proportion of oleate
(*i*)	210°/15 mm.	Palmitate + Stearate + a large proportion of oleate
(*j*)	over 212°/15 mm.	Stearate + Dihydroxystearate + a large proportion of oleate

The temperature registered by the thermometer during the distillation of the final fraction (*j*) varies considerably in different experiments, owing to the fact that this fraction is usually a small one, and that slight decomposition occurs during its distillation. If the esterification has been carried out successfully, however, no difficulty will be experienced in the distillation, and the decomposition involved will be so slight that no appreciable loss of ester will result. On an average, the *total* loss of esters incurred during an experiment amounts to less than 1 % of the weight of fat taken for analysis.

Haller [1906, 1907, 1908] states that the fractional separation by this method proceeds satisfactorily up to methyl laurate, but it was found impossible to obtain even the earlier fractions free from unsaturated material. Similar results have been recorded by Miss Smedley [1912] who concluded from a study of the iodine values of the various fractions, that unsaturated acids other than oleic acid exist in butter fat. In several instances she found that the iodine value of the fraction containing methyl caprate was appreciably greater than those of the fractions immediately preceding or following it, and accordingly the presence of a decylenic acid seemed probable, though she failed to isolate the compound. As we also repeatedly observed a local maximum iodine value for the fraction boiling at 120°/15 mm. (cf. p. 142) experiments were carried out with the object of identifying the unsaturated body present in this fraction. These, unfortunately, were inconclusive, the only positive result obtained being the isolation, from the product of an oxidation experiment with the fraction, of a small quantity of material, which from its crystalline appearance and melting point (130°) was regarded as dihydroxystearic acid.

Some indirect evidence of the possibility of methyl oleate being present in the lower boiling fractions, however, was obtained from the following experiment. Pure specimens of butyric, caproic, caprylic, capric, lauric, myristic, palmitic and stearic acids were obtained, and, before use, proved to be free from unsaturated material by the fact that no iodine absorption resulted when they were treated with Wijs' reagent. Weighed amounts of the above acids were then mixed with a known weight of pure oleic acid, so as to give a mixture of acids approximately the same as that occurring in butter fat. The acid mixture was then esterified in the usual way, the esters fractionated in the same manner as those prepared from butter fat, and after three fractionations the iodine value of each fraction was determined. In the following table are given details of

the final fractionation and the weights and iodine values of the individual fractions:

Fraction	Temperature ° C.	Pressure mm.	Weight g.	Iodine value g.
1	65–130	760	0·29	1·867
2	–150	760	0·33	2·523
3	75– ?	15	1·27	1·730
4	–120	15	0·90	4·324
5	–140	15	1·18	5·823
6	–147	15	1·70	2·320
7	–155	15	2·90	2·840
8	165–170	15	8·61	3·630
9	–185	15	14·12	8·250
10	–200	12	14·77	39·12
11	–202	12	16·72	57·51
12	197–199	9	18·14	68·00
13	–205	9	6·80	72·61
14	Residue	—	1·80	71·50

As methyl oleate is the only unsaturated compound which can possibly be present, it is evident from the above results that it distils over with the lower esters. Further, a distinct local maximum iodine value is indicated corresponding to the fraction boiling between 120°–140° at 15 mm., so that the results, though at a lower level, are perfectly analogous to those obtained from an examination of butter fat. Accordingly it is reasonable to conclude that oleic acid is the only unsaturated acid present in the butter fat, and support is given to this view by the fact that so far all attempts to isolate or identify any other unsaturated body have proved unsuccessful.

If this be granted, it is possible, as indicated later (p. 151), to determine the mean molecular weight of the saturated esters present in each fraction: and these results, taken in conjunction with the observed boiling points for the respective fractions, point to each fraction containing, in addition to the proportion of methyl oleate, two saturated esters, one of these being present in large excess. Thus, for example, the saturated portion of the first fraction is largely methyl butyrate, a little methyl caproate being also present: the second fraction is largely caproate with a little butyrate or caprylate: and so on. Working on this principle, that there are present in each fraction, besides a proportion of methyl oleate, two saturated esters, one of which largely predominates, the amount of each ester present can be estimated from the mean molecular weight of the saturated esters by use of the following formulae:

$$x_1 = \frac{m_1}{m} \cdot \frac{m_2 - m}{m_2 - m_1} \cdot w \text{ and } x_2 = w - x_1,$$

where m is the mean molecular weight of the saturated esters in the fraction,

,, w is the weight of the saturated esters in the fraction,

,, x_1 ,, ,, ,, ester, whose mol. wt. is m_1,

,, x_2 ,, ,, ,, ,, ,, ,, m_2.

By summing up the results obtained in this way for all the fractions, the total amount of each ester present is arrived at, and hence the percentage can be calculated.

Although the above method for arriving at the composition of the fat is liable to many sources of error, it has been found to yield consistent results when duplicate analyses of the same sample were carried out (see p. 145). This, of course, is no guarantee that the percentage of any particular acid found in a fat by this method is the correct one, but such was found to be the case when a more rigorous test was applied. When the artificial mixture of acids mentioned in the preceding pages was subjected to this method of analysis, the results obtained showed a remarkably close agreement with the theoretical values.

These are tabulated below for comparison:

Acid	Wt. of acid taken g.	Wt. of esters cal. from wt. of acid taken g.	Wt. of esters found by analysis g.
Butyric	4·34	5·03	5·026
Caproic	1·69	1·89	1·854
Caprylic	1·10	1·21	1·240
Capric	1·30	1·41	1·467
Lauric	3·80	4·06	3·994
Myristic	20·50	21·76	22·108
Palmitic	14·50	15·30	14·950
Stearic	1·26	1·32	1·416
Dihydroxystearic	—	—	0·210*
Oleic	40·00	41·98	41·675
	Total weight	93·96 g.	93·940 g.

* 0·21 g. dihydroxystearic ester would be produced from 0·188 g. oleic ester, therefore total weight of methyl oleate found = 41·863 g.

It would appear, from the above results, that the method is sufficiently reliable for all practical purposes for the detection of significant differences in composition between different fats. We have indeed already applied it for the comparison of the fat obtained from "first runnings" of milk with that from "last runnings" or "strippings" from the same cows. Reference to the summary on p. 145 will show that these two fats showed differences that were much larger than those obtained with duplicates of the same fat. The greatest differences noted are in the amounts of myristic, palmitic and

stearic esters, there being nearly 9 % less myristate in fat of "first runnings," but 6 % more palmitate, and nearly 3 % more stearate. The "first runnings" fat also yielded a larger proportion of methyl oleate, as was indicated by the iodine values.

Duplicate complete analyses of the fat obtained from ewe's milk gave quite a good agreement throughout (cf. p. 145), but the mean composition of ewe's milk fat thus arrived at showed marked differences from the average analysis of cow's milk fat, especially with regard to the percentages of the lower fatty acids. The amount of methyl butyrate was considerably higher than in cow's milk fat, the caproate somewhat lower, while the caprylate and caprate were between four and four and a half times as great. Without further evidence it is impossible to say whether these differences are characteristic or purely accidental.

With regard to the actual percentages of the several acids present in cow's milk fat, the results obtained by different observers vary within wide limits. Although this is only to be expected with a mixture such as butter fat, where so many factors contribute to the variation, still the great differences in the amounts, for example, of lauric acid, or stearic acid, reported as being present in butter fat, must be due mainly to the method of analysis employed. The following table (p. 145) gives a summary of results obtained by several workers, and there is added for comparison the results of the analyses, which are detailed in the present communication (pp. 147–162).

As far as the lower fatty acids are concerned the percentages found by us are about a mean of those hitherto published. The values for the butyrate tend to be a little high, and this is probably accounted for by the special precautions taken to secure efficient condensation when working with the lowest boiling fractions. The amounts of lauric acid found are considerably higher than was found by Browne, whose results are commonly quoted as authoritative. That butter fat does contain a considerable proportion of lauric acid was demonstrated by the following experiment. A fraction of methyl esters, obtained from butter fat, was found by the above method to consist of methyl caprate, methyl laurate, and methyl oleate approximately in the ratio 13 : 100 : 8. Three grams of this were hydrolysed, and the resulting acids isolated. The semi-solid mass obtained on cooling was spread on a porous plate, which soon absorbed the liquid oleic and capric acids, leaving behind fully two grams of a pure white solid acid. This weight agrees closely with the estimated amount of lauric acid present, and the solid after recrystallisation from spirit was identified by its melting point (43°) as lauric

Composition of Butter Fat.

Observer			Butyric acid	Caproic acid	Caprylic acid	Capric acid	Lauric acid	Myristic acid	Palmitic acid	Stearic acid	Dihydroxy-stearic acid	Oleic acid
			%	%	%	%	%	%	%	%	%	%
Molt [1907, p. 281]				7·40			—	—	20·00	40·93	—	26·53
Koefoed [1891]			1·37	1·83	0·46	1·83	7·32	20·13	25·62	1·83	—	31·11
Bell [1890, p. 48]			6·13		2·09		—		49·46		—	36·10
Duclaux [1886]			3·38–3·65	2·0–2·26	—	—	—	—	—	—	—	–
Blyth [1903, p. 340]			3·49	2·40	0·80		—	—	47·50		—	40·40
Browne [1899]			5·45	2·09	0·49	0·32	2·57	9·89	38·61	1·83	1·00	32·50
Fleischmann and Warmbold [1907]	Sample 1		5·00	2·00	0·15	—	—	—	52·12	—	—	35·36
	„ 2		5·00	2·00	0·15	—	—	—	51·10	3·35	—	33·30
	„ 3		4·32	2·16	0·67	—	—	4·46	48·94	5·54	—	28·81
	„ 4		4·32	2·16	0·67	—	—	10·00	42·75	2·00	—	33·00
Siegfeld [1907]	Sample 1		3·53	2·50	1·03	—	—	25·35	17·18	—	—	42·75
	„ 2		3·58	1·26	2·87	—	—	22·94	20·84	—	—	40·53
	„ 3		3·27	1·73	1·89	—	—	30·70	16·89	—	—	37·98
	„ 4		3·35	1·68	2·21	—	—	26·00	20·96	—	—	38·09
Jensen [1905, 1, 2]			3·92	1·88	—	—	—	—	—	—	—	—
Richmond [1914, p. 42]			3·43	3·25	0·51	1·77	6·94	19·14	24·48	1·72	—	33·60
Crowther and Hynd	Cow's milk fat	Dairy butter	4·45	1·45	0·99	1·10	3·55	20·13	15·24	1·08	0·68	45·47
		First runnings	4·30	1·98	1·11	1·51	5·08	10·38	17·47	5·93	0·30	46·49
		Last runnings	4·06	1·48	1·37	0·96	6·40	18·78	11·78	3·19	0·16	41·31
		Average	4·27	1·64	1·16	1·19	5·01	16·43	14·83	3·40	0·38	44·42
	Sheep's milk fat	1st analysis	6·60	1·06	4·18	4·72	4·73	13·20	13·87	4·56	0·36	40·61
		2nd analysis	6·48	1·29	4·27	4·86	4·67	14·28	12·81	4·22	0·18	40·72
		Mean	6·54	1·18	4·23	4·79	4·70	13·74	13·34	4·39	0·27	40·67

acid. It seems from the above result that the usually accepted idea that only traces of lauric acid are present in butter fat must be modified. At any rate one cannot accept Caldwell and Hurtley's statement [1909], that the amount of lauric acid in butter fat is so small that none can be isolated from a pound. At the same time their observation, that palmitic acid is present in much smaller proportions than usually assumed, while myristic acid is more abundant, is borne out by our results, which in this particular also agree well with those of Siegfeld [1907]. It is with regard to stearic acid, however, that the greatest discrepancies have been reported. Many observers state that this acid either does not occur in butter fat, or is present only in very small amount. Thus Lewkowitsch [1913, p. 558] found only 0·49 % stearic acid in the insoluble fatty acids of butter fat, and Hehner and Mitchell [1896] also found very small proportions of stearic acid, varying from 1·3 to 3·6 %, and in some cases none at all. Koefoed [1891] and Browne [1899] both quote 1·83 % stearic acid, and the latter author writes:

"A noteworthy fact is the low percentage of stearic acid in butter fat.

This is somewhat contradictory to the statements which we find given by many authorities on the subject, who regard stearic acid as one of the principal acids in butter fat, it being placed by some writers as high even as 40 %. We believe that these statements should be modified somewhat; when we consider that oleic acid of molecular weight 282 constitutes over one-third of the insoluble acids from butter fat, it is self-evident that the percentage of stearic acid (mol. wt. 284) must be small in comparison with that of its lower homologues to give a mean molecular weight of 261 to the whole mixture....The low percentage of stearic acid should rank with the high percentage of soluble fatty acids, as one of the characteristic differences between butter and other animal fats."

In view of the above, it is surprising to find later evidence, which shows that the percentage of stearic acid in butter fat is considerable. Thus, Miss Smedley [1912] concluded that, of the total fatty acids from 10 to 15 % was stearic, and quite recently Holland, Reed and Buckley [1916] estimated that the proportion of stearic acid in the insoluble fatty acids from butter varied from 6·93 to 22·33 %, according to the type of fodder, and probably also to the individuality and period of lactation. Though the factors mentioned would, no doubt, influence the amount of stearic acid present, the variation seems extraordinarily well marked, and one feels inclined to doubt the applicability of Hehner and Mitchell's method to this particular analysis, especially as Lewkowitsch has pointed out that this method yields entirely unreliable or at best capricious results in a large number of cases where mixtures of stearic acid with acids other than palmitic and oleic are involved. Even if only palmitic and oleic acids be present, the results are still very uncertain; for example in the case of the fatty acids of cotton seed oil it was impossible to detect stearic acid by this method, even if 5 or 8 % of pure stearic acid had been added to the mixed fatty acids.

The average proportion of stearic acid obtained by us is 3·4 % for cow's butter fat, and 4·39 % for sheep's butter fat. The small amounts of dihydroxystearic acid found are regarded as oxidation products of a portion of the oleic acid, and not present originally. If these amounts be converted to oleic acid, the total oleic acid thus found agrees with that calculated from the iodine value of the original fat.

In the literature several references are found relating to the presence in butter fat of traces of acids other than those tabulated here. If such exist, they will probably be found amongst the higher fractions, the investigation of which we hope will form the subject of a future communication. In the

meantime, however, we intend to apply the method now described for a comparative study of fats obtained from different sources, and of milk fats produced under different conditions.

Experimental.

In the following pages full details are given of the method employed for the analysis of a sample of butter fat prepared from butter purchased at a local dairy. The general procedure followed was the same for the other materials analysed for which, therefore, only a condensed account is given.

I. *Analysis of Cow's Butter Fat.*

Preparation of the fat for analysis.

The butter was placed in a beaker, and melted by warming at a temperature not above 50°. As soon as the water and curd had separated, leaving the upper layer of fat perfectly clear, it was decanted through a folded filter paper, which had previously been thoroughly dried in the steam oven. The filtered fat was then ready for analysis. If not required at once it should be preserved in the cold away from light and air. In melting the butter a high temperature must be avoided, but at the same time the duration of heating should be reduced to a minimum.

Preparation of the methyl esters from butter fat.

From preliminary experiments it was found that Haller's [1906, 1907, 1908] acid method of esterification was preferable to that employed by Bull [1900, 1906], but to render the process quantitative, several precautions must be taken. These will be mentioned in the account which follows.

136·11 g. clarified fat were weighed off accurately into a round-bottomed litre flask and mixed with 214 cc. absolute methyl alcohol containing 2·5 % hydrogen chloride, and 323 cc. pure dry ether. The flask used was fitted to a fairly long double-surface condenser by means of a ground-glass joint, and the condenser carried a delivery tube, which was bent twice at right angles, and led into a series of U-tubes, surrounded by a freezing mixture of ice and salt. The esterification mixture was then heated for twelve hours on a water-bath at a temperature just sufficient to keep the liquid refluxing gently. After allowing to cool thoroughly, the U-tubes were detached, and the small quantity of liquid (10 to 15 cc.), which had collected, transferred to a litre graduated flask (*A*). The tubes were washed out with a little pure dry ether, and the washings also added to flask (*A*). The liquid, which had collected

in these tubes, was mainly ether, but at the same time an appreciable quantity of methyl ester (methyl butyrate) was always found to be present. Consequently, if this precaution was not taken, loss of this ester resulted.

To the ether-alcoholic solution of esters in the reaction flask, slightly more than the calculated amount of barium carbonate was added to remove the free hydrogen chloride. Though the mixture was frequently shaken, neutralisation proceeded slowly, but finally all the mineral acid was removed as indicated by Congo paper. The flask was then fitted with a five-pear still head and the ether and methyl alcohol distilled off, using a long double-surface condenser, and collecting the distillate in a flask cooled in a freezing mixture. The distillate, which contained in addition to ether and alcohol the greater part of the butyric ester, was then transferred to flask (*A*), the collection flask being washed out with ether, and the washings also added to (*A*). The residue, which consisted of methyl esters, glycerol, barium chloride, the excess of barium carbonate used, and the traces of solvents, was transferred by means of ether to a separating funnel and shaken with brine. This caused the ethereal solution of esters to separate out on top free from glycerol which was held in the salt solution. The process was repeated three times, and the separated ethereal solution then dried over anhydrous freshly ignited magnesium sulphate. After standing over night the magnesium sulphate was filtered off through a large Gooch crucible prepared in the usual way, and washed well with dry ether.

Fractional distillation of esters.

A 250 cc. Claisen distilling flask was fitted with a dropping funnel and thermometer, a portion of the dried ethereal solution of esters introduced through the funnel, and the ether distilled off carefully on the water-bath, the distillate being again collected in a cooled receiver, so as to avoid any loss of esters. As the ether evaporated, more solution was added by means of the tap-funnel until the whole had been introduced, and finally the flask and funnel were washed with pure dry ether. When all the ether had been distilled off, the receiver was removed, its contents transferred to flask (*A*), and in its place was fitted a "Perkin fractionation triangle." The tap-funnel was removed from the straight neck of the Claisen flask, which was now fitted with a cork, carrying a tube drawn out to a *very* fine capillary, and reaching to the bottom of the flask. The distillation of the esters was then carried out, at first at ordinary pressure, and then *in vacuo*, the distilling flask being heated by means of an asbestos air-bath. After the fraction boiling

about 140°/15 mm. had been collected the condenser was dispensed with, and the residual esters transferred with ether to a smaller Claisen flask, the side limb of which was connected directly with the receiver.

The first fraction that distilled over consisted mainly of ether, and consequently was added to the litre flask (*A*), which contained all the solvent alcohol and ether recovered from the process. The volume of this was now made up to the mark with pure alcohol (or ether) and the amount of methyl ester in solution determined as described below.

It was found that, as a rule, the fractionation must be repeated three or four times before a sufficient separation was effected. Appended are the details of the final (third) fractionation in the experiment described:

Fraction	Temperature ° C.	Pressure mm.	Weight g.
1	65–130	atmospheric	1·6690
2	–150 53– 75	atmospheric 15	1·9662
3	–110	15	1·3396
4	–135	15	1·0886
5	–155	15	2·4600
6	–185	15	14·1852
7	–200 184–190	15 13	18·3872
8	–197	13	18·1542
9	–202	13	18·1230
10	–202	12	16·7630
11	–206	12	18·6558
12	–211	12	10·0762
13 (residue)	—	—	6·7827
	Total weight of fractions		129·6507

The temperatures quoted for the various fractions indicate the points at which the first and last drops contained in the respective fractions came over.

On comparison it will be seen that the boiling points given on p. 140 fall between these temperatures, and that it was about these points that the bulk of the fraction distilled. For example, in the above fractionation fraction 3 corresponds to (*d*) on p. 140, fraction 5 to (*f*), fractions 7 to 11 (inclusive) to (*h*). The collection of the last named in five fractions was necessitated simply by the relatively large volume of distillate obtained at this stage, collection-tubes of uniform size being used for general convenience.

Determination of methyl butyrate volatilised with solvents.

A 250 cc. conical flask was fitted with a cork carrying a dropping funnel and a delivery tube, the latter being connected with a condenser. 20 cc. of alcoholic KOH solution (roughly N/2) were transferred to the flask and

100 cc. of the ether-alcoholic solution from flask (*A*) placed in the funnel, the stem of which, drawn out to deliver small drops, reached almost to the surface of the potash solution. The flask was then heated on the water-bath, and the ether-alcoholic solution allowed to drop in slowly, so that the volume in the flask remained about the same. When all the solution had been added the distillate was transferred to the funnel, and the distillation repeated. All the ester in the original solution was thus hydrolysed, and after cooling the contents of the flask a few drops of phenolphthalein were added, and the excess of potash titrated with standard (roughly N/2) hydrochloric acid.

A blank experiment was carried out at the same time, using a mixture of 50 cc. pure ether and 50 cc. neutral alcohol in place of the ether-alcoholic solution of ester, and proceeding exactly as described above. From the difference between the two titres the amount of ester can be calculated if the reasonable assumption be made, that it consists solely of methyl butyrate. In all cases the mean of two closely agreeing determinations was used in calculating the results.

Appended are the details for the analysis here described:

	cc. HCl (N/1 × 0·497) required	
	a	*b*
Blank experiment	31·89	31·86
100 cc. ester solution	21·05	21·00
Difference	10·84	10·86
Mean titre	10·85 cc.	

Hence weight of methyl butyrate in solution (1000 cc.)

$$= 10{\cdot}85 \times 10 \times 0{\cdot}497 \times 0{\cdot}1021 \text{ g.}$$

$$= 5{\cdot}507 \text{ g.}$$

If this weight be now added to the 129·651 g. of esters obtained in the fractionation, a total of 135·158 g. of esters is arrived at as having been obtained from 136·11 g. of butter fat.

Examination of the individual fractions.

(1) *Iodine value.* The iodine value of each fraction was determined, using the method of Wijs. [Cf. Leathes 1910, pp. 67–69.]

The Wijs solution was always made up two or three days before being required for analysis, and before use its strength was determined by checking it against the thiosulphate solution, the latter being standardised by titration with weighed quantities of pure iodine.

From the iodine absorption of each fraction, the weight of methyl oleate present in it was then calculated, on the assumption that no other unsaturated compound was present.

The results obtained are summarised in the following table:

10 cc. Wijs reagent (= 21·92 cc. thiosulphate) were used in each case.

1 cc. thio. = 0·01257 g. iodine.

Fraction	Weight taken	Thio. used	Δ	Iodine value	Weight of methyl oleate in fraction
	g.	cc.	cc.	g.	g.
1	0·1775	21·76	0·16	01·13	0·022
2	0·2224	21·56	0·36	02·03	0·047
3	0·2642	20·59	0·33	06·33	0·106
4	0·2805	19·10	2·82	12·63	0·160
5	0·3017	19·35	2·57	10·70	0·307
6	0·2797	18·63	3·29	14·78	2·443
7	0·2564	15·81	6·11	30·07	6·294
8	0·2982	13·65	8·27	34·85	7·375
9	0·3257	9·56	12·36	47·70	10·080
10	0·2929	8·26	13·66	58·61	11·450
11	0·2559	8·83	13·09	64·26	13·970
12	0·2721	7·32	14·60	67·43	7·912
13	0·2904	9·25	12·67	54·84	4·335
		Total weight of methyl oleate			64·501

(2) *The saponification value.* The usual method for determining the saponification value of a fat was followed, that is, a weighed portion of each fraction was heated with excess of approximately semi-normal alcoholic potash, and the excess then estimated by titration with standard (0·497 N/1) hydrochloric acid, using phenolphthalein as indicator.

As all the fractions, except the last, were perfectly colourless liquids, the solutions for titration were only faintly coloured, and hence it was possible to obtain a very sharp end-point, thus giving results of considerable accuracy. A blank experiment was always carried out at the same time, and under identical conditions, and the difference between the two titres thus obtained corresponded to the fatty acids contained in and liberated during the process from the weight of esters taken for analysis.

The actual saponification numbers of the several fractions were not calculated, as the real object of the experiment was the determination of the mean molecular weights of the *saturated esters* present in each fraction. This was arrived at in the following way.

Having found the iodine value of each fraction, the amount of methyl oleate (b)[1] contained in the weight of esters (a) taken for the analysis of each fraction can readily be calculated, and the number of cc. of the standard hydrochloric acid (b') equivalent to these amounts of methyl oleate can then be obtained. If these titres for methyl oleate (b') be subtracted from the titres found for the total esters (a'), titres corresponding to the amounts of

[1] The index letters refer to the first table on p. 152

saturated esters (c') are arrived at, and hence the mean molecular weights of the saturated esters in each fraction can be determined, as the weights of the saturated esters (c) are also known. The complete results are tabulated below.

Fraction	(a) Weight taken	(b) Weight of oleate	(c) Weight of saturated esters	(a') Titre	(b') Titre for oleate	(c') Titre for saturated esters	Mean molecular weight of saturated esters
	g.	g.	g.	cc.	cc.	cc.	
1	0·5237	0·0069	0·5168	6·21	0·04	6·17	108·55
2	0·6023	0·0143	0·5880	9·22	0·09	9·13	129·6
3	0·6520	0·0517	0·6003	8·11	0·35	7·76	155·7
4	0·6701	0·0987	0·5714	7·23	0·67	6·56	175·3
5	0·7225	0·0901	0·6324	6·97	0·61	6·36	200·1
6	1·6495	0·2841	1·3654	13·80	1·93	11·87	231·45
7	1·7611	0·6028	1·1583	13·35	4·09	9·26	251·65
8	1·6410	0·6669	0·9741	12·32	4·53	7·79	251·7
9	1·6894	0·9392	0·7502	12·32	6·39	5·93	254·4
10	1·7109	1·1685	0·5424	12·11	7·94	4·17	262·05
11	1·6915	1·2520	0·4395	11·88	8·51	3·37	262·55
12	1·6942	1·3110	0·3832	11·88	9·05	2·83	270·4
13	1·6895	1·0800	0·6095	11·30	7·34	3·96	309·7

Fraction	Total weight	Weight of methyl oleate	Weight of saturated esters
	g.	g.	g.
1	1·6690	0·022	1·647
2	1·9662	0·047	1·920
3	1·3396	0·106	1·233
4	1·0886	0·160	0·928
5	2·4600	0·307	2·153
6	14·1852	2·443	11·742
7	18·3872	6·294	12·093
8	18·1542	7·375	10·779
9	18·1230	10·080	8·043
10	16·7630	11·450	5·313
11	18·6558	13·970	4·686
12	10·0762	7·912	2·164
13	6·7827	4·335	2·448
Totals	129·6507	64·501	65·149

Weights of Individual Esters in each Fraction.

Fraction	Butyric	Caproic	Caprylic	Capric	Lauric	Myristic	Palmitic	Stearic	Dihydroxy-stearic	Oleic
	g.	g.	g.	g.	g.	g.	g.	g.	g.	g.
Volatile with solvents	5·507	—	—	—	—	—	—	—	—	—
1	1·194	0·453	—	—	—	—	—	—	—	0·022
2	0·270	1·650	—	—	—	—	—	—	—	0·047
3	—	0·090	1·143	—	—	—	—	—	—	0·106
4	—	—	0·320	0·608	—	—	—	—	—	0·160
5	—	—	—	0·993	1·160	—	—	—	—	0·307
6	—	—	—	—	4·072	7·670	—	—	—	2·443
7	—	—	—	—	—	7·640	4·453	—	—	6·294
8	—	—	—	—	—	6·774	4·005	—	—	7·375
9	—	—	—	—	—	4·382	3·661	—	—	10·080
10	—	—	—	—	—	1·389	3·924	—	—	11·450
11	—	—	—	—	—	1·149	3·537	—	—	13·970
12	—	—	—	—	—	—	2·130	0·034	—	7·912
13	—	—	—	—	—	—	—	1·493	0·955	4·335
Totals	6·971	2·193	1·463	1·601	5·232	29·004	21·710	1·527	0·955	64·501

Final Summary.

Acid	Weight of ester	Ester	Acid*
	g.	%	%
Butyric	6·971	5·16	4·45
Caproic	2·193	1·62	1·45
Caprylic	1·463	1·08	0·99
Capric	1·601	1·19	1·10
Lauric	5·232	3·80	3·55
Myristic	29·004	21·46	20·13
Palmitic	21·710	16·08	15·24
Stearic	1·527	1·13	1·08
Dihydroxystearic	0·955	0·71	0·68
Oleic	64·501	47·74	45·47

* Strictly speaking the numbers given represent the weights of the respective acids obtainable from 100 g. of the mixed methyl esters, but must approximate very closely to the percentages in the actual fat.

II. *Analysis of Artificial Mixture of Fatty Acids.*

Details of the final distillation, and the iodine value of each fraction have already been given (p. 142).

Estimation of methyl butyrate:

Total ester required for saponification KOH = (86·9 × 0·497) cc. N/1 HCl.

Therefore total weight of methyl butyrate = 86·9 × 0·497 × 0·1021 = 4·41 g.

Total weight of esters obtained = 89·53 + 4·41 = 93·94 g.

„ „ „ calculated = 93·97 g.

Analysis of Fractions.

Fraction	Iodine value	Mean molecular weight of saturated esters	Total weight of fraction	Weight of methyl oleate in fraction	Weight of saturated esters in fraction
	g.		g.	g.	g.
1	1·87	102·0	0·29	0·004	0·286
2	2·52	103·55	0·33	0·007	0·323
3	1·73	129·3	1·27	0·025	1·245
4	4·32	136·9	0·90	0·045	0·855
5	5·82	160·2	1·18	0·080	1·100
6	2·32	190·4	1·70	0·046	1·654
7	2·84	214·9	2·90	0·096	2·804
8	3·63	238·2	8·61	0·365	8·245
9	8·25	243·3	14·12	1·361	12·759
10	39·12	260·0	14·77	6·747	8·023
11	57·51	270·9	16·72	11·230	5·490
12	68·00	270·9	18·14	14·400	3·740
13	72·61	297·1	6·80	5·766	1·034
14	71·50	319·9	1·80	1·503	0·297
		Totals	89·53	41·675	47·855

Weights of Individual Esters in each Fraction.

Fraction	Butyric	Caproic	Caprylic	Capric	Lauric	Myristic	Palmitic	Stearic	Dihydroxy-stearic	Oleic
	g.	g.	g.	g.	g.	g.	g.	g.	g.	g.
Volatile with solvents	4·410	—	—	—	—	—	—	—	—	—
1	0·286	—	—	—	—	—	—	—	—	0·004
2	0·302	0·021	—	—	—	—	—	—	—	0·007
3	0·028	1·217	—	—	—	—	—	—	—	0·025
4	—	0·616	0·239	—	—	—	—	—	—	0·045
5	—	—	1·001	0·099	—	—	—	—	—	0·080
6	—	—	—	1·368	0·286	—	—	—	—	0·046
7	—	—	—	—	2·703	0·101	—	—	—	0·096
8	—	—	—	—	1·005	7·240	—	—	—	0·365
9	—	—	—	—	—	12·100	0·659	—	—	1·361
10	—	—	—	—	—	2·667	5·356	—	—	6·747
11	—	—	—	—	—	—	8·905	0·325	—	11·230
12	—	—	—	—	—	—			—	14·400
13	—	—	—	—	—	—	0·030	1·004	—	5·766
14	—	—	—	—	—	—	—	0·087	0·210	1·503
Totals	5·026	1·854	1·240	1·467	3·994	22·108	14·950	1·416	0·210	41·675
									equal to	0·188
										41·863
Theory	5·030	1·893	1·207	1·406	4·066	21·760	15·299	1·322	—	41·986

III. *Analysis of Butter Fat from "First Runnings" of Milk. July* 27–31, 1914.

The sample was prepared from the mixed "first runnings" (*i.e.* first drawn milk) of several cows, the milk drawn representing about one-quarter of the total yield of the cows. The whole of the milk thus obtained was passed through a cream separator, the cream churned without delay, and the butter thus obtained used for the analysis.

The fat gave a Reichert-Wollny number of 28·37 and an iodine value of 45·65. The latter corresponds to 50·68 % of oleic acid in the fat.

Complete analysis:

Weight of fat taken 157·54 g.
Volume of methyl alcohol containing 2·5 % HCl 248 cc.
Volume of ether 375 cc.

Final (3rd) Fractionation.

Fractions from 2nd fractionation introduced	Fraction	Temperature °C.	Pressure mm.	Weight g.
1, 2 →	1	65–130	760	2·3970
3 →	2	–150	760	1·4453
4 →		53– 78	15	
	3	–110	16	1·3808
5, 6, 7 →	4	–133	14	1·6900
	5	–160	14	2·6844
	6	–180	13	10·7606
8, 9 →	7	–188	13	13·3677
	8	–196	14	14·3442
	9	–205	14	18·3769
10, 11 →	10	200–203·5	13	16·5646
	11	–205·5	13	15·1847
12, 13 →	12	–208	13	16·1208
	13	206·5–210	13	18·9202
	14	–280	13	14·7388
			Total weight	147·976

Estimation of methyl butyrate:

Total ester required for saponification KOH = 123·5 × 0·523 cc. N/1 HCl.
Therefore total weight of methyl butyrate = 123·5 × 0·523 × 0·1021 = 6·59 g.
Total weight of esters obtained = 147·976 + 6·59 = 154·566 g.

Analysis of Fractions.

Fraction	Iodine value g.	Mean molecular weight of saturated esters	Weight of methyl oleate in fraction g.	Weight of saturated esters in fraction g.
1	0·24	117·8	0·007	2·390
2	1·08	125·15	0·018	1·427
3	3·28	141·6	0·053	1·328
4	8·06	166·7	0·159	1·530
5	12·73	182·0	0·408	2·276
6	13·89	221·0	1·746	9·015
7	19·20	236·45	2·997	10·370
8	28·06	257·2	4·701	9·643
9	37·59	264·35	8·067	10·310
10	48·01	271·6	9·289	7·276
11	55·95	272·2	9·920	5·265
12	60·34	286·5	11·360	4·761
13	68·03	289·1	15·040	3·880
14	65·26	302·2	11·235	3·504
		Totals	75·000	72·975

Weights of Individual Esters in each Fraction.

Fraction	Butyric	Caproic	Caprylic	Capric	Lauric	Myristic	Palmitic	Stearic	Dihydroxy-stearic	Oleic
	g.	g.	g.	g.	g.	g.	g.	g.	g.	g.
Volatile with solvents	6·590	—	—	—	—	—	—	—	—	—
1	0·909	1·481	—	—	—	—	—	—	—	0·007
2	0·206	1·221	—	—	—	—	—	—	—	0·018
3	—	0·721	0·607	—	—	—	—	—	—	0·053
4	—	—	1·001	0·530	—	—	—	—	—	0·159
5	—	—	0·283	1·993	—	—	—	—	—	0·408
6	—	—	—	—	6·546	2·469	—	—	—	1·746
7	—	—	—	—	1·860	8·510	—	—	—	2·997
8	—	—	—	—	—	4·148	5·495	—	—	4·701
9	—	—	—	—	—	1·904	8·406	—	—	8·067
10	—	—	—	—	—	—	6·823	0·453	—	9·289
11	—	—	—	—	—	—	4·812	0·453	—	9·920
12	—	—	—	—	—	—	1·843	2·918	—	11·360
13	—	—	—	—	—	—	1·100	2·780	—	15·040
14	—	—	—	—	—	—	—	3·011	0·492	11·235
Totals	7·705	3·423	1·891	2·523	8·406	17·031	28·479	9·615	0·492	75·000

Summary.

Ester	Wt. isolated	Ester	Acid
	g.	%	%
Butyric ...	7·705	4·99	4·30
Caproic	3·423	2·22	1·98
Caprylic ...	1·891	1·22	1·11
Capric	2·523	1·63	1·51
Lauric	8·406	5·44	5·08
Myristic ...	17·031	11·02	10·38
Palmitic ...	28·479	18·43	17·47
Stearic	9·615	6·22	5·93
Dihydroxystearic	0·492	0·32	0·30
Oleic	75·000	48·55	46·49
Total weight	154·565		

IV. *Analysis of Butter Fat from "Strippings" (July 27–31, 1914).*

The sample was prepared from the mixed "strippings" (last-drawn milk) of the same cows, and same milkings as furnished the sample of "first runnings," and was dealt with in precisely the same way.

The fat gave a Reichert-Wollny number of 29·38, and an iodine value of 44·02, the latter corresponding to 48·88 % of oleic acid in the fat. The fat was thus slightly richer in total volatile acids and slightly poorer in oleic acid than the fat of the "first runnings."

Complete analysis:

Weight of fat taken 158·10 g.

Volume of methyl alcohol containing 2·5 % HCl 248 cc.

Volume of ether 375 cc.

Final (3rd) Fractionation.

Fractions from 2nd fractionation introduced	Fraction	Temperature ° C.	Pressure mm.	Weight g.
1*	1	65–130	760	0·7170
2*	2	(–150 / (53– 77	760) / 17)	1·5583
3*	3	–110	17	1·0808
4*	4	–130	17	1·7800
5*	5	–140	17	1·7644
6, 7, 8 →	6	–160	18	1·4912
	7	–185	18	15·1776
9, 10 →	8	189–193	18	12·0946
	9	–201	19	13·8648
11 →	10	199–203	17	13·3750
	11	–206	17	14·4274
12, 13, 14 →	12	–208	17	16·7288
	13	–213	17	14·0042
	14	211–213	17	17·1292
15 →	15	–220	17	18·0496
	16 (residue)	—	—	5·3868
			Total weight	148·6297

* Fractions 1–5 of second fractionation were not redistilled.

Estimation of methyl butyrate:

Total ester required for saponification KOH = (122·8 × 0·523) cc. N/1 HCl.

Therefore total weight of methyl butyrate = 122·8 × 0·523 × 0·1021 = 6·557 g.

Total weight of esters obtained = 148·630 + 6·557 = 155·187 g.

Analysis of Fractions.

Fraction	Iodine value g.	Mean molecular weight of saturated esters	Weight of methyl oleate in fraction g.	Weight of saturated esters in fraction g.
1	0·15	112·9	0·001	0·716
2	0·55	122·6	0·010	1·548
3	1·63	135·0	0·021	1·060
4	4·90	153·9	0·102	1·678
5	12·16	172·9	0·250	1·514
6	11·65	196·8	0·203	1·288
7	11·41	223·8	2·022	13·156
8	19·16	235·7	2·704	9·390
9	24·64	246·1	3·990	9·875
10	30·33	251·9	4·848	8·527
11	40·94	252·35	6·898	7·529
12	56·74	268·7	11·090	5·639
13	63·73	269·0	10·420	3·584
14	68·21	285·4	13·650	3·479
15	68·91	288·1	14·620	3·430
16	66·86	304·8	4·208	1·179
		Totals	75·037	73·592

Weights of Individual Esters in each Fraction.

Fraction	Butyric	Caproic	Caprylic	Capric	Laurie	Myristic	Palmitic	Stearic	Dihydroxy-stearic	Oleic
	g.	g.	g.	g.	g.	g.	g.	g.	g.	g.
Volatile with solvents	6·557	—	—	—	—	—	—	—	—	—
1	0·398	0·318	—	—	—	—	—	—	—	0·001
2	0·343	1·205	—	—	—	—	—	—	—	0·010
3	—	0·844	0·216	—	—	—	—	—	—	0·021
4	—	0·215	1·463	—	—	—	—	—	—	0·102
5	—	—	0·648	0·866	—	—	—	—	—	0·250
6	—	—	—	0·748	0·540	—	—	—	—	0·203
7	—	—	—	—	8·175	4·981	—	—	—	2·022
8	—	—	—	—	1·918	7·472	—	—	—	2·704
9	—	—	—	—	—	8·287	1·588	—	—	3·990
10	—	—	—	—	—	5·295	3·232	—	—	4·848
11	—	—	—	—	—	4·550	2·979	—	—	6·898
12	—	—	—	—	—	0·236	5·403	—	—	11·090
13	—	—	—	—	—	0·115	3·469	—	—	10·420
14	—	—	—	—	—	—	1·481	1·998	—	13·650
15	—	—	—	—	—	—	1·136	2·294	—	14·620
16	—	—	—	—	—	—	—	0·908	0·271	4·208
Totals	7·298	2·582	2·327	1·614	10·633	30·936	19·288	5·200	0·271	75·037

Summary.

Ester	Wt. isolated	Ester	Acid
	g.	%	%
Butyric ...	7·298	4·70	4·06
Caproic	2·582	1·66	1·48
Caprylic ...	2·327	1·50	1·37
Capric	1·614	1·04	0·96
Laurie	10·633	6·85	6·40
Myristic ...	30·936	19·93	18·78
Palmitic ...	19·288	12·43	11·78
Stearic	5·200	3·35	3·19
Dihydroxystearic	0·271	0·17	0·16
Oleic	75·037	48·36	41·31
Total weight	155·186		

V. *Analysis of Butter Fat from Ewe's milk.*

The sample was prepared from the mixed milk of several ewes of the flock at the Manor Farm, Garforth, in the spring of 1914.

The fat was separated by churning, and dealt with in precisely the same way as the samples of cow's butter fat.

The fat gave a Reichert-Wollny number of 29·41, and an iodine value of 41·41.

Complete analysis:

Weight of fat taken 162·16 g.

Volume of methyl alcohol containing 2·5 % HCl 280 cc.

Volume of ether 250 cc.

Final (4th) Fractionation.

Fraction	Temperature °C.	Pressure mm.	Weight g.
1	–150	760	0·65
2	–160	760	2·30
3	55 – 77	16	1·25
4	–110	15	3·46
5	–130	15	8·15
6	–143	15	1·31
7	–155	15	4·97
8	–185	14	10·88
9	–190	13	16·83
10	–196	12	15·46
11	–201	12	17·14
12	–204	12	19·08
13	–207	12	15·72
14	–210	12	18·16
15	–220	12	9·10
16	–300	12	4·81
		Total weight	149·27

Estimation of methyl butyrate:

Total ester required for saponification KOH = (248·35 × 0·523) cc. N/1 HCl.
Therefore total weight of methyl butyrate = 248·35 × 0·523 × 0·1021 = 12·26 g.
Total weight of esters obtained = 149·27 + 12·26 = 161·53 g.

Analysis of Fractions.

Fraction	Iodine value g.	Mean molecular weight of saturated esters	Weight of methyl oleate in fraction g.	Weight of saturated esters in fraction g.
1	0·075	125·7	0·001	0·649
2	0·365	145·7	·010	2·290
3	0·55	146·8	·008	1·242
4	1·525	159·8	·062	3·398
5	3·34	177·6	·318	7·832
6	3·72	187·8	·057	1·253
7	3·70	206·9	·214	4·756
8	8·64	228·5	1·098	9·782
9	17·19	245·7	3·378	13·452
10	28·30	254·3	5·110	10·350
11	44·59	269·2	8·926	8·214
12	53·55	273·9	11·935	7·145
13	64·73	285·7	11·882	3·838
14	68·54	288·1	14·456	3·704
15	70·61	298·8	7·503	1·597
16	69·01	316·5	3·876	0·934
		Totals	68·834	80·436

Weights of Individual Esters in each Fraction.

Fraction	Butyric	Caproic	Caprylic	Capric	Lauric	Myristic	Palmitic	Stearic	Dihydroxy-stearic	Oleic
	g.	g.	g.	g.	g.	g.	g	g.	g.	g.
Volatile with solvents	12·260	—	—	—	—	—	—	—	—	—
1	0·083	0·566	—	—	—	—	—	—	—	0·001
2	—	0·909	1·381	—	—	—	—	—	—	0·010
3	—	0·446	0·796	—	—	—	—	—	—	0·008
4	—	—	3·146	0·252	—	—	—	—	—	0·062
5	—	—	2·093	5·739	—	—	—	—	—	0·318
6	—	—	—	1·161	0·092	—	—	—	—	0·057
7	—	—	—	1·084	3·672	—	—	—	—	0·214
8	—	—	—	—	4·416	5·366	—	—	—	1·098
9	—	—	—			11·522	1·930	—	—	3·378
10	—	—	—	—	—	5·522	4·828	—	—	5·110
11	—	—	—	—	—	0·211	8·003	—	—	8·926
12	—	—	—	—	—	—	6·062	1·083	—	11·935
13	—	—	—	—	—	—	1·593	2·245	—	11·882
14	—	—	—	—	—	—	1·227	2·477	—	14·456
15	—	—	—	—	—	—	—	1·554	0·043	7·503
16	—	—	—	—	—	—	—	0·371	0·563	3·876
Totals	12·343	1·921	7·416	8·236	8·180	22·621	23·643	7·730	0·606	68·834

Summary.

Ester	Wt. isolated g.	Ester %	Acid %
Butyric ...	12·343	7·64	6·60
Caproic	1·921	1·19	1·06
Caprylic ...	7·416	4·59	4·18
Capric	8·236	5·10	4·72
Lauric	8·180	5·06	4·73
Myristic ...	22·621	14·01	13·20
Palmitic ...	23·643	14·64	13·87
Stearic	7·730	4·79	4·56
Dihydroxystearic	0·606	0·38	0·36
Oleic	68·834	42·63	40·61
Total weight	161·530		

VI. *Analysis of Butter Fat from Ewe's milk.*

Repetition of V with a further portion of same sample.

Complete analysis:

Weight of fat taken 164·35 g.

Volume of methyl alcohol containing 2·5 % HCl 273 cc.

Volume of ether 250 cc.

Final Fractionation.

Fraction	Temperature ° C.	Pressure mm.	Weight g.
1	−140	760	0·89
2	−155 55 − 77	760 12	2·82
3	− 80	12	3·72
4	−110	11	5·65
5	−140	11	7·12
6	−155	11	4·93
7	−185	11	17·56
8	184 −190	11	17·63
9	−192	11	17·54
10	−195	11	18·33
11	−199	11	17·71
12	−201	11	18·32
13	−215	11	12·45
14	Residue	—	6·94
	Total weight		151·61

Estimation of methyl butyrate:

Total ester required for saponification KOH = (227·9 × 0·523) cc. N/1 HCl.
Therefore total weight of methyl butyrate = 227·9 × 0·523 × 0·1021 = 12·17 g.
Total weight of esters obtained = 151·61 + 12·17 = 163·78 g.

Analysis of Fractions.

Fraction	Iodine value g.	Mean molecular weight of saturated esters	Weight of methyl oleate in fraction g.	Weight of saturated esters in fraction g.
1	0·01	124·9	0·001	0·89
2	1·62	145·0	0·053	2·77
3	3·00	154·0	0·131	3·59
4	3·74	169·7	0·247	5·40
5	4·42	190·2	0·368	6·75
6	6·50	210·4	0·374	4·56
7	11·40	235·9	2·337	15·22
8	25·09	247·1	5·166	12·46
9	38·97	261·3	7·981	9·56
10	48·01	271·0	10·280	8·05
11	61·70	277·0	12·760	4·95
12	67·63	286·6	14·470	3·85
13	70·18	299·1	10·200	2·25
14	69·53	303·3	5·636	1·30
		Totals	70·004	81·60

Weights of Individual Esters in each Fraction.

Fraction	Butyric	Caproic	Caprylic	Capric	Lauric	Myristic	Palmitic	Stearic	Dihydroxy-stearic	Oleic
	g.	g.	g.	g.	g.	g.	g.	g.	g.	g.
Volatile with solvents	12·170	—	—	—	—	—	—	—	—	—
1	0·135	0·755	—	—	—	—	—	—	—	0·001
2	—	1·167	1·603	—	—	—	—	—	—	0·053
3	—	0·449	3·141	—	—	—	—	—	—	0·131
4	—	—	2·929	2·471	—	—	—	—	—	0·247
5	—	—	—	5·610	1·140	—	—	—	—	0·368
6	—	—	—	0·518	4·042	—	—	—	—	0·374
7	—	—	—	—	3·007	12·213	—	—	—	2·337
8	—	—	—	—	—	9·977	2·483	—	—	5·166
9	—	—	—	—	—	2·751	6·809	—	—	7·981
10	—	—	—	—	—	—	7·734	0·316	—	10·280
11	—	—	—	—	—	—	3·618	1·332	—	12·760
12	—	—	—	—	—	—	1·477	2·373	—	14·470
13	—	—	—	—	—	—	—	2·168	0·082	10·200
14	—	—	—	—	—	—	—	1·066	0·234	5·636
Totals	12·305	2·371	7·673	8·599	8·189	24·941	22·121	7·255	0·316	70·004

Summary.

Ester	Wt. isolated	Ester	Acid
	g.	%	%
Butyric ...	12·305	7·51	6·48
Caproic	2·371	1·45	1·29
Caprylic ...	7·673	4·69	4·27
Capric	8·599	5·25	4·86
Lauric	8·189	5·00	4·67
Myristic ...	24·941	15·23	14·28
Palmitic ...	22·121	13·51	12·81
Stearic	7·255	4·43	4·22
Dihydroxystearic	0·316	0·19	0·18
Oleic	70·004	42·75	40·72
Total weight	163·774		

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XVI. THE OXIDATION OF THE ALKALI BUTYRATES BY HYDROGEN PEROXIDE WITH THE PRODUCTION OF SUCCINIC ACID.

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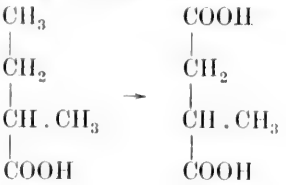
The oxidation of butyric acid in the form of its ammonium salt has been studied by Dakin [1908]. He slowly distilled a solution of ammonium butyrate containing a slight excess of ammonia with two molecular proportions of 3 % hydrogen peroxide. To identify an unstable intermediate product—acetoacetic acid—he allowed ammonium butyrate and hydrogen peroxide to act upon each other at 37°. Dakin supposes that the attack on the fatty acid occurs both at the α- and β-carbon atoms, the main attack being at the latter. The attack at the α-carbon atom will lead to the production of propionic aldehyde, propionic acid, acetaldehyde, acetic and formic acids: the attack at the β-carbon atom will yield acetoacetic acid, which will readily break up into acetone and carbon dioxide. Dakin was able to prove the formation of all the substances mentioned, and he claims that the yield of acetone is as high as 50 % of that theoretically possible.

In a paper recently published by Hurtley [1916] the theory of the oxidation of the fatty acids by attack at the β-carbon atom has been subjected to some criticism on the ground of its inadequacy. It was there suggested that the oxidative attack occurred simultaneously at many points in the long chains of carbon atoms which are present in all the common higher fatty acids and in particular that the attack might occur at the terminal methyl group. This is the more likely because Dakin and Herter [1907] have shown that when hydrogen peroxide attacks the benzene ring it does not show the discrimination which is shown by the usual substituting agents: thus, when it attacks benzoic acid it yields a mixture of all three hydroxybenzoic acids. Direct evidence that oxidation of fatty acids occurs at the methyl group in the animal body is not available. Only one piece of evidence, as far as the authors know, exists showing that it occurs at the methyl group when a fatty acid is oxidised by hydrogen peroxide, and this is furnished by Raper

[1914], who found that, when α-methylbutyric acid is oxidised by hydrogen peroxide, methylsuccinic acid is one of the products of the oxidation.



This surely indicates attack at the terminal methyl group. We have therefore taken up the question of the oxidation of the fatty acids by hydrogen peroxide and starting with butyric acid we have obtained results which lead us to suppose that the methyl group is a principal point of attack in this kind of oxidation and also encourage us to think that it will be worth while to study other fatty acids from this point of view. At first, following Dakin, we used the ammonium salt of butyric acid, but although the oxidation of this salt appears to take place more quickly than that of the sodium or potassium salts we have discarded it and now work with the sodium salt only, because:

(1) The ammonium salt gave rather strongly coloured products.

(2) It always left the product acid at the end of the reaction.

(3) In the body it is more probable that the sodium or potassium salts and not the ammonium salts are those that are oxidised.

(4) On submitting a liquid containing ammonium butyrate to steam distillation, both ammonia and butyric acid pass over with the steam.

To show this we distilled 200 cc. of a solution of ammonium butyrate containing 8·8 g. of butyric acid in a current of steam at such a rate that 50 cc. collected in about four minutes, collecting six fractions of 500 cc. each. In the table of results the first column gives the butyric acid in terms of normal sodium hydroxide: 50 cc. of the distillate were treated with a measured excess of normal sodium hydroxide, boiled till all ammonia was expelled and titrated with normal acid and phenolphthalein. These results are a little high. The second column gives the ammonia also in terms of normal sodium hydroxide: 50 cc. of the distillate were treated as in a Kjeldahl determination. This column gives the ammonia exactly.

Fraction	Butyric acid as cc. N NaOH	Ammonia as cc. N NaOH
1	32	36
2	23	19
3	14	12
4	10	8
5	8	6
6	8	5
Residue	19	14

It will be seen that the first distillate is alkaline and all the rest and the residue are acid in reaction. We wished to be able to distil our products without fear of obtaining ammonia in the distillate.

Our method of working is different from that of Dakin. After many preliminary experiments we adopted the following procedure: 1 g.-molecular proportion of sodium butyrate, equivalent to 4·4 g. of butyric acid, was dissolved in water and 3, 6, or 12 g.-molecular proportions of hydrogen peroxide (20 volume H_2O_2 for the first two and 40 volume for the third) were added and the mixture made up to 190 cc. in all cases. In most of our experiments the mixture was heated at 65° in a flask with an efficient reflux condenser, until all, or all but a trace, of the hydrogen peroxide had disappeared. Temperature, as would be expected, has an exceedingly marked effect on the duration of the reaction: thus with 3 g.-molecules of peroxide at 37° the products are practically the same as at 65°, but the times required are 168 and 66 hours respectively. At 100° the reaction appears to be complete in four hours, but we observed that at this temperature much oxygen was given off and the product was much discoloured. At the end of an experiment with three atoms of oxygen the product is neutral: with six atoms of oxygen the reaction is acid: with twelve atoms of oxygen the reaction is again neutral, or slightly alkaline.

At the end of an oxidation the solution is neutralised, if it is not neutral. In one part aldehydes plus acetone are determined (*a*) directly by addition of standard iodine and caustic soda and titration of excess of iodine by standard thiosulphate in the usual way; (*b*) by distilling a part acidified with acetic acid exactly as in the Messinger-Huppert process. The other part is evaporated to dryness on the water-bath: acidified with a slight excess of normal sulphuric acid, and distilled in steam until all volatile acids are expelled: this usually requires 3000 cc. of distillate. This distillate is titrated and called volatile acids. The residue from this steam distillation is then extracted for 72 hours with ether in a continuous apparatus such as that described by one of us [Hurtley, 1916; see also Plimmer, 1915, p. 598]. After distilling off the ether the liquid is evaporated to dryness in a weighed platinum dish, the solid residue is dried at 100° and weighed. We call this weight the yield of crude acid. It is practically all succinic acid; the colour is usually slightly brown and it has a smell recalling that of pyruvic acid. A small portion of it is very sparingly soluble in water. To purify and identify the succinic acid it was redissolved in water, boiled with animal charcoal, filtered and crystallised. The crystals obtained were still slightly yellow

and were not improved by repeating this process of purification nor by recrystallising from alcohol. They melted at 185°, the melting point of succinic acid, and the melting point was not changed on mixing with succinic acid (Nos. 1, 2 and 3).

The acid was finally obtained pure by recrystallisation from pure concentrated nitric acid and then from water (No. 4).

Analysis:

				C	H
1.	0·2513 g.	0·3875 g. CO_2	0·1170 g. H_2O	42·06	5·17
2.	0·2408 g.	0·3713 g. ,,	0·1092 g. ,,	42·06	5·05
3.	0·2264 g.	0·3484 g. ,,	0·1059 g. ,,	41·97	5·19
4.	0·2345 g.	0·3502 g. ,,	0·1070 g. ,,	40·72	5·07
Calculated for succinic acid ...		... ...		40·67	5·09

The acid crystallises like succinic acid: in neutral solution it gives a buff coloured precipitate with ferric chloride. Of this acid 0·1672 g. required 14·4 cc. N/5 NaOH which corresponds to a molecular weight of 116: theory 118.

One g. of the acid was digested at 60° with 3–4 g. of acetyl chloride for three hours; on cooling it deposited beautiful crystals of succinic anhydride which melted at 119°–120°.

It is inconceivable that succinic acid is produced from butyric acid without the formation of intermediate products, and we have some evidence of the presence of such substances. The succinic acid before crystallisation from nitric acid gave high figures for the percentage of carbon and hydrogen; the acid also gave Schiff's test for aldehydes, and it reduced ammoniacal silver. All these observations would be explained by assuming that before crystallisation from nitric acid the acid contained a small amount of the half aldehyde of succinic acid. The following table will give some idea of the yield of succinic acid, of aldehydes and acetone taken together, and of carbon dioxide. In order to be able to calculate a yield for the succinic acid it is necessary to form an idea of the butyric acid left over and also of that part of it which has been converted into other products than succinic acid. At the end of one of our experiments the solution will contain (*a*) unchanged sodium butyrate, (*b*) sodium salts of lower fatty acids, (*c*) aldehydes (acetic and propionic as Dakin [1908] has shown) and acetone, (*d*) sodium succinate. If after neutralisation the liquid is evaporated to expel aldehydes and acetone, then acidified and distilled in steam, the distillate should give on titration, expressing the result as butyric acid, the sum of the unchanged butyric acid

and the other volatile fatty acids. Of the fatty acids in the distillate other than butyric, every molecule of propionic must correspond to one molecule of butyric acid oxidised. It is unlikely that acetic acid would arise from acetoacetic acid in our experiments, but it might arise from the oxidation of acetone. Some acetic acid must also be formed from the oxidation of propionic acid, but in each of these cases one molecule of acetic acid would correspond to one molecule of butyric acid oxidised. In the case of formic acid it is true that this acid might be formed in different ways so that each molecule of formic acid in our distillate would not represent one molecule of butyric acid oxidised, but perhaps more nearly one-third of a molecule. By far the greatest part of our volatile fatty acid is unchanged butyric acid and certainly it contains only a little formic acid, for in working up 250 g. of sodium salts from the steam distillate we obtained only a little carbon monoxide on treatment with pure concentrated sulphuric acid. We may therefore take the difference between the initial butyric acid and the titration of the volatile acid, expressed as butyric acid, as a measure of the butyric acid which has been converted into aldehydes, acetone and succinic acid, so that if we calculate the yield of succinic acid only on this difference we shall almost certainly be underestimating it. In the table the yield of succinic acid is so estimated.

From the table it is seen that the yield of succinic acid is variable even when the conditions appear to be the same; the only explanation we can offer of this phenomenon is that in some cases there may have been a greater loss of oxygen than in others. Nevertheless in spite of these differences certain results appear perfectly clearly:

1. A yield of 50 % and over of succinic acid can be obtained by oxidising sodium butyrate in neutral solution by hydrogen peroxide at 65°. It is therefore established that under these conditions a fatty acid such as butyric can be and is attacked to a very great extent at the methyl end of the chain.

2. The yield is much diminished in less concentrated solutions. In experiment number 2 when the volume is 1000 cc. instead of 720 cc. the yield of succinic acid is only 20 %.

3. When the concentration of the oxidising agent is doubled the yield of succinic acid is increased with respect to the butyric acid taken, but it is practically the same with respect to butyric acid oxidised. More acid is oxidised and the time taken is nearly doubled.

4. When the peroxide is quadrupled a more marked change occurs in the yield of the several products and in the time required to use up the

peroxide. The reaction is now complete in 23 hours; the amount of butyric acid oxidised is double the amount oxidised with three atoms of oxygen; the yield of succinic acid is greatly reduced while that of the carbon dioxide is enormously increased. Perhaps it is a fair inference to draw that under these conditions the succinic acid is formed, but a large part of it is oxidised as it is produced.

Number of experiment	Time in hours	Weight of butyric acid taken in g.	Total volume of solution in cc.	Hydrogen peroxide in atoms of available oxygen	Difference between column 3 and volatile acids calculated as butyric acid in g.	Weight of crude succinic acid in g.	Yield per cent. of theory of crude succinic acid	CO_2 evolved in g.	Aldehyde plus acetone calculated as butyric acid in g.	Remarks
1	48	17·6	720	3	3·08	2·13	51·5	—	—	At 65°
2	48	17·6	1000	3	5·63	1·54	20·3	—	—	,,
3	66	4·4	190	3	1·14	0·48	31·6	0·36*	—	,,
4	66	4·4	190	3	1·07	0·43	30·1	0·40*	—	,,
5	66	4·4	190	3	1·41	0·47	25·1	—	0·65	,,
6	66	4·4	190	3	0·75	0·37	36·3	—	0·50	,,
7	72	4·4	190	3	1·10	0·50	33·6	0·37*	—	,,
8	72	4·4	190	3	1·02	0·46	33·6	—	0·64	,,
9	72	4·4	190	3	0·75	0·51	50·7	0·54*	—	,,
10	72	4·4	190	3	0·79	0·51	48·4	—	0·60	,,
11	72	4·4	190	3	0·79	0·52	49·2	0·44	—	,,
12	66	4·4	190	3	1·23	0·48	29·3	—	0·71	,,
13	72	4·4	190	3	1·41	0·52	27·2	0·37	—	,,
14	168	4·4	190	3	1·23	0·32	19·5	—	0·47	At 37°
15	72	17·6	720	6	6·69	3·18	35·5	—	—	At 65°
16	114	4·4	190	6	1·63	0·72	32·8	0·48*	—	,,
17	114	4·4	190	6	1·54	0·69	33·2	—	1·10	,,
18	114	4·4	190	6	1·50	0·77	38·0	—	—	,,
19	114	4·4	190	6	1·36	0·71	38·8	—	—	,,
20	114	4·4	190	6	1·32	0·67	37·8	0·88	—	,,
21	114	4·4	190	6	1·89	0·72	28·5	—	1·16	,,
22	114	4·4	190	6	1·72	0·75	32·5	1·12	—	,,
23	672	4·4	190	6	0·79	0·71	67·3	—	0·73	At 37°
24	22	4·4	190	12	2·46	0·56	16·8	2·59	—	At 65°
25	23	4·4	190	12	2·46	0·51	15·4	—	0·80	,,
26	23	4·4	190	12	2·46	0·50	15·2	2·72	—	,,

* Determined by passing the gas through baryta water and titrating.

A number of carbon dioxide determinations have been made, some by the use of baryta water and others by direct weighing; with care both methods give practically the same result. We do not find that our hydrogen peroxide contains more than a trace of carbon dioxide. Dakin found that his specimens contained a great deal. We have found however that the hydrogen peroxide acts upon rubber stoppers producing sulphur dioxide, which is absorbed by the potash used for absorbing the carbon dioxide—but this action is quite slow and only affects experiments which are continued for many hours.

In the last column but one are given some determinations of aldehyde plus acetone which are calculated to butyric acid. All the figures in this column were obtained by distilling an aliquot part of the oxidised product exactly as in a Messinger-Huppert acetone determination. We have also made some determinations by titrating after Messinger-Huppert without previous distillation. There was always a marked difference between the two results, a difference we attribute to the presence of less volatile aldehydes or aldehydo-acids.

We have also attempted to make separate determinations of aldehydes and acetone using Tollens' reagent for oxidation of the aldehydes. The following table gives some of our results. The amount of butyric acid used for the experiments and the volume of the solution are the same in all the experiments, namely 4·4 g. of the acid neutralised by sodium hydroxide, and 190 cc.

Hydrogen peroxide in atoms of available oxygen	Aldehydes + acetone expressed as g. of butyric acid		Acetone expressed as g. of butyric acid	Yield* % of theory
	By distillation	By direct titration		
3	0·76	1·02	0·206	20
3	0·73	1·06	0·407	39
6	0·94	1·46	0·457	32
6	0·73	1·43	0·290	20†
12	0·79	—	0·250	11
12	0·84	0·96	0·323	14
12	0·86	0·93	0·217	10

* The yields are calculated on the *average* value of the butyric acid oxidised in each class of experiment because the volatile acids were not determined in all these experiments.

† Experiment at 37°.

From this table it is seen that by our method of oxidation we cannot approach Dakin's yield of 50 % of acetone. We believe our highest figure of 39 % is probably too high. Yet we believe that our method of working is a nearer approach to the conditions which prevail in the animal body than Dakin's method of actual distillation.

Butyric acid in the form of its sodium salt may be broken down by oxidation in three ways by hydrogen peroxide, namely by attack at each of its carbon atoms except that one present as carboxyl. Of these three modes of attack that at the terminal carbon atom results in the production of succinic acid; that at the β-carbon atom results in the production primarily no doubt of acetoacetic acid which is broken down to acetone and carbon dioxide; that at the α-carbon atom probably yields propionic aldehyde, then the acid, and so on. Of these three we believe the

attack at the methyl group to be by far the most important. Butyric acid is easily oxidised in the body; of the intermediate products of its oxidation by hydrogen peroxide succinic acid is known to be easily oxidised in the body. Thus v. Longo [1878] took 13 g. of sodium succinate in two portions in 24 hours; no trace of the acid could be found in the urine. Marfori [1896] shows the same for animals. Batelli and Stern [1911] have shown that the acid is oxidised by nearly every tissue of the body. Acetoacetic acid on the contrary is difficult of oxidation in the body [see Hartley, 1916, p. 380]. Propionic acid is known to be easily oxidised by the body. We believe therefore that in the body butyric acid is mainly attacked at the terminal carbon atom. If this is the case the same should be true of the higher fatty acids, and we propose to seek for evidence of this mode of attack in the case of some fatty acids higher in the series than butyric acid.

SUMMARY.

It is shown that a yield of succinic acid of over 50 % of the theoretical can be obtained by the oxidation of sodium butyrate by hydrogen peroxide.

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XVII. THE ALLEGED ANTINEURITIC PROPERTIES OF α-HYDROXYPYRIDINE AND ADENINE.

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(Received June 5th, 1917.)

In the hope of obtaining a compound which would possess antineuritic properties Williams [1916] prepared and tested a series of substances. By adopting this line of research he hoped to throw some light on the chemical identity of the active principle which cures polyneuritis in birds and beri-beri in man. He confined himself to the study of pyridine derivatives mainly because the active fraction obtained from rice polishings is closely associated with nicotinic acid and under certain treatment gives off a pyridine-like odour, whilst the colour reactions given by antineuritic substances with phosphotungstic and phosphomolybdic acids respectively are also characteristic of the hydroxypyridines. He prepared and tested a large series of pyridine derivatives including nicotinic, cinchomeric, quinolinic, 6-hydroxynicotinic and citrazinic acids, glutazine, α-hydroxypyridine, 2 : 4 : 6-trihydroxypyridine (and its anhydride) and 2 : 3 : 4-trihydroxypyridine. The last three of the above Williams found to be definitely active but the activity disappeared after some time. As there was no evidence of decomposition, Williams ascribed the loss of the antineuritic properties to isomerisation. To substantiate his hypothesis he made a detailed study of one of the active substances—α-hydroxypyridine. He found that there were two crystalline modifications of this substance, needles and granules. By treating polyneuritic birds with the needle variety Williams managed to effect in every case a definite cure or improvement; the granules on the other hand were inactive. The absolute inactivity of the granular modification of α-hydroxypyridine induced him to put forward the further suggestion that the polyneuritic condition is brought about not by the lack of a particular isomer, since isomers are in equilibrium in solution and any biological preference for one

would disturb the equilibrium and thus effect the corresponding conversion, but by a want of a certain type of potential energy supplied by the process of isomerisation of certain substances. In a subsequent investigation Williams in collaboration with Seidell [1916] isolated from yeast an active substance which they considered to be identical with adenine and which lost its antineuritic activity on keeping. This loss of therapeutic potency the authors also ascribed to isomerisation.

If isomerisation is really responsible for the therapeutic activity of antineuritic principles it is of great scientific importance and interest and it was our object to study the matter in detail. We chose α-hydroxypyridine, with which Williams claims to have achieved some success, as our starting point. Although we could confirm most of the facts about the chemistry of that substance we were unable by its use to effect anything which in our opinion could be considered as a cure or even an improvement in the condition of the polyneuritic birds. We have taken great care in following Williams' method in the preparation of the active needles and no doubt exists in our minds that our preparations were identical with those described by Williams. We have also taken great pains in watching our birds and nothing of clinical importance could have missed our notice.

Experimental.

Like Williams we prepared the α-hydroxypyridine by distilling hydroxynicotinic acid according to the method of Pechmann and Baltzer [1891]. The latter was made from the methyl ester of coumalic acid by the method of Pechmann and Welsh [1884]. The coumalic acid was obtained from malic acid by the method described by Pechmann [1891]. Before distillation the hydroxynicotinic acid was decolorised with charcoal and purified by recrystallising it several times from hot 50 % acetic acid. It always gave the characteristic reactions and correct melting point. The α-hydroxypyridine obtained by distilling the hydroxynicotinic acid gave the correct nitrogen figures on determination by the Dumas method.

When hydroxynicotinic acid is distilled the α-hydroxypyridine comes over as syrup which on cooling crystallises in a mixture of needles and non-acicular crystals which Williams classified as granules. In Williams' opinion the amount of moisture present in the process of distillation is a determining factor as to the relative amounts of the two crystalline modifications formed. We found that by excluding moisture entirely needles only were formed.

We therefore heated the hydroxynicotinic acid and the distilling apparatus at 110° for several hours before distilling. The receiver was also fitted with a $CaCl_2$ tube in order to exclude all traces of moisture. From a recent publication of Williams [1917] which reached us only lately we find that he independently adopted a similar procedure and like ourselves was able to obtain needles only. On cooling, the needles crystallise out from the syrup and finally complete solidification occurs with formation of a dense mass. In parts of the apparatus where the layer of crystals is thin, well-defined needles are discernible even after complete cooling down. The solidified syrup did not show a definite melting point, but melted somewhere between 94°–98°. On keeping the open apparatus under ordinary laboratory atmospheric conditions a marked change could be observed in the needles in those parts of the distilling flask where their crystalline form was well defined after complete cooling. The needles after about 10 days broke up by transverse fissures and ultimately changed into non-acicular crystals of a granular form. On heating the cooled down syrup at a temperature of 120° for 2–3 hours and then allowing the molten mass to cool slowly in the absence of moisture dissolving it in hot dehydrated benzene and adding dehydrated ligroin to the cold solution a milky turbidity was produced and after a short time a deposit of acicular crystals was formed. Few, if any, granules were obtained by this precipitation, but a preponderance of granular crystals was obtained on concentrating the mother liquor. The needles thus obtained melted at 108°–110° (uncorrected), whilst the granules melted at 106°–108° (uncorrected). It is to be remarked that the configuration of the needles obtained by the benzene-ligroin recrystallisation differed in the different preparations obtained by that method. We did not however study the conditions governing these differential configurations. When the freshly distilled α-hydroxypyridine is dissolved in hot water and crystallised from it a mixture of needles and prisms is obtained. It is thus seen that there are at least two distinct crystalline modifications of α-hydroxypyridine, namely needles and non-acicular crystals. It will however be remembered that the melting points of the needles obtained by direct distillation of hydroxynicotinic acid and the needles obtained by the benzene-ligroin crystallisation method are not the same and therefore the two varieties of needles cannot be considered to be identical.

Polyneuritis was induced in our pigeons by forcible feeding with rice, the birds receiving daily 20 g. each morning and evening. The crops of the birds were examined daily and if any hardness was observed the feeding was suspended until the bird became normal again. The time taken by the

birds to develop polyneuritis varied from two to five weeks. All the injections were done intramuscularly in the breast and freshly prepared crops of crystals were always used unless otherwise mentioned. Following the practice of Williams the crystals were always dissolved in a convenient quantity of distilled water immediately before injection. The birds under treatment were kept under very close observation. Varying doses were tried. Needles obtained by direct distillation and needles from benzene and ligroin crystallisation as well as crystals obtained by crystallisation from hot water were tested. The following protocol describes eight experiments.

Expt. 1. 10 mg. of freshly distilled α-hydroxypyridine (needles) were dissolved in 2 cc. of distilled water and injected intramuscularly in pigeon 151 at 4 p.m. The bird showed at the time paralysis of the legs and retracted neck. At 8 p.m. its head was found to be markedly improved, and the condition of improvement was maintained at 11 p.m. Next morning the bird had a relapse. 20 mg. of needles obtained by distillation on the previous day were administered and as the bird showed no improvement a further 0·1 g. of the same substance was injected two hours later. The condition became worse and the bird died during the night.

Expt. 2. Pigeon 219 walked with great difficulty and was entirely unable to use its wings; head normal. It received 50 mg. of needles of freshly distilled α-hydroxypyridine at 12 noon. It was slightly worse at 11 p.m. the same evening, and worse again next morning. On the third day the bird showed a retracted neck. On the fourth day its condition was still worse and finally it was found dead on the morning of the fifth day.

Expt. 3. Pigeon 189 could not fly and walked with difficulty. It received 10 mg. of needles of freshly distilled α-hydroxypyridine at 12 noon. Next day there was no change in condition. On the third day the bird was decidedly worse. On the fourth day its condition was still worse, no retracted neck however was to be observed. It was found dead on the fifth morning.

Expt. 4. Pigeon 229 was paralysed and showed retracted neck. It received 50 mg. of needles obtained by recrystallising the α-hydroxypyridine from benzene with ligroin at 11.30 a.m. At 8 p.m. the same day its condition was worse and it died at 3 p.m. the following day.

Expt. 5. Pigeon 205 only showed incipient symptoms. It received 200 mg. of freshly distilled needles at 11.45 a.m. It became worse during the day and developed a retracted neck. Next day an improvement was observed but it became worse again in the course of the day and was found dead on the morning of the third day after treatment.

Expt. 6. Pigeon 230 showed paralysed limbs. It received 2 mg. of freshly distilled needles at 10 a.m. No change occurred in its condition during the day, but it became gradually but decidedly worse during the next three days and died on the fourth day.

Expt. 7. Pigeon 222 showed paralysed limbs. It received 50 mg. of α-hydroxypyridine recrystallised from hot water at 10.30 a.m. No change in condition occurred during that day. The next day the bird was worse and developed a retracted neck. It became gradually worse during the day and was found dead the next morning.

Expt. 8. Pigeon 149 showed paralysed limbs. It received 10 mg. of needles obtained by recrystallisation from benzene with ligroin at 6 p.m. No improvement was observed at 11 p.m. the same evening and the condition remained unchanged for some time. On the fourth day the bird was worse. 50 mg. of needles of the same crop were injected at 10.30 a.m. on that day. The bird died at 1 o'clock in the afternoon.

It is seen from the above experiments that no cure or improvement in the condition of the birds was effected by the treatment. Some birds lived several days after being treated, but they were types of cases, well known to those who work with polyneuritic birds, which would have behaved similarly if they had received no treatment at all. In many of the above experiments we started our treatment as soon as polyneuritic symptoms manifested themselves in order to give the drug a good chance to act. Some of these cases have turned out to be of the prolonged type where the onset is mild and gradual and lasts several days before terminating fatally, others have proved themselves to be of the intermittent type, in which the well-declared symptoms are strikingly relieved for a few hours before the second onset takes place; after the second attack the birds usually succumb. Expts. 2, 3, 6, and 8 are characteristic of the prolonged type. Expts. 1 and 5 represent the intermittent type. Special attention is to be paid to Expt. 5. That bird turned out to be of the intermittent type. It was treated when the symptoms were not more than of a premonitory character and the dose it received was very high, namely 200 mg. In spite of that, the first attack developed in the usual way and the bird succumbed as is common in cases of this type after the second onset within two days of the manifestation of the early symptoms. A special experiment showed that the injection of this amount of α-hydroxypyridine produced no toxic symptoms in a normal pigeon. In spite of the fact that we used both varieties of needles in various doses we are unable from our experiments

to attribute any antineuritic properties to the needle variety of *a*-hydroxypyridine.

Reference was made above to the work of Williams and Seidell [1916] in connection with the isomerisation of adenine. They obtained an active substance from autolysed yeast by adsorption which they considered to be adenine and which on keeping became inactive. The inactive adenine on being treated with sodium ethylate in a sealed tube at 100° for four hours, according to the observations of these workers became active. While we were engaged in repeating some of these experiments there appeared a paper by Voegtlin and White [1916] in which a variety of experiments with adenine, all of a negative nature, were described. We have therefore repeated only the crucial experiments of Williams and Seidell, and like Voegtlin and White were unable to confirm their results.

We prepared our adenine from a sample of pure adenine sulphate kindly supplied to us by Mr A. Chaston Chapman. The adenine sulphate which was recrystallised several times was obtained by Mr Chapman by hydrolysis from yeast nucleic acid. The adenine was precipitated from the sulphate by neutralising it exactly with ammonia and passing a stream of CO_2. The filtered residue was washed with water, alcohol and ether. The adenine gave Kossel's reaction: when heated quickly it melted at 343° (uncorrected) and yielded a picrate consisting of yellow silky needles which charred at 240°. In accordance with Williams and Seidell's directions 50 mg. of adenine were heated at 100° for five hours with 5 cc. of absolute alcohol in which 10 mg. of metallic sodium were dissolved. The orange coloured liquid was then evaporated to dryness on a water-bath and the residue dissolved in distilled water immediately before injection. The following protocol describes five experiments.

Expt. 9. Pigeon 256, which showed well-developed symptoms of polyneuritis with retracted neck, received 10 mg. of untreated adenine; it became worse next day and was found dead on the morning of the third day.

Expt. 10. Pigeon 187 showed paralysed limbs. It received at 4 p.m. the entire residue from 50 mg. of adenine treated as described above. Slight improvement was noticeable on the next day when it again received the residue from 50 mg. of adenine. No change occurred on the third and fourth days: it became worse on the fifth day and remained in the same state during the sixth. On the seventh day it became decidedly worse and was treated with the residue from 50 mg. of adenine. No improvement was observed and the bird died during the night.

Expt. 11. Pigeon 247 showed well-declared symptoms of polyneuritis with retracted neck. It received at 4 p.m. 10 mg. from residue obtained by treating 50 mg. of adenine. The bird was found dead next morning.

Expt. 12. Pigeon 198, which was paralysed but kept its head well forward, received 5 mg. of treated adenine. On the next day it was a little better and received another 5 mg. of the adenine preparation of the previous day in the morning and further 5 mg. of a fresh preparation in the afternoon. On the third day the condition was unchanged and the remaining portion of the 50 mg. of adenine, treated on the previous day, was administered at 10.30 a.m. The bird became worse and died at 2 p.m. the same day.

Expt. 13. Pigeon 217 showed incipient signs of polyneuritis. It received 50 mg. of adenine treated with sodium ethylate. It was better the next day, but had a relapse on the third day and received a further 50 mg. of treated adenine. No improvement ensued and the bird was found dead on the fourth morning.

The above experiments like those performed with α-hydroxypyridine must be regarded as negative in character. In Expt. 10 the bird remained alive for six days, but the condition of the bird during that time was such that nothing could be definitely ascribed to the action of the administered adenine. The results obtained in the remaining experiments are so distinctly negative that no comment is necessary.

Summary.

1. The authors can confirm the following facts observed by Williams about the nature of α-hydroxypyridine.

(*a*) α-Hydroxypyridine obtained by distilling hydroxynicotinic acid crystallises in needles and non-acicular crystals.

(*b*) On keeping the needles of α-hydroxypyridine, thus obtained, exposed under ordinary laboratory atmospheric conditions, they break up into granules in about 10–14 days.

(*c*) Needles of α-hydroxypyridine are also obtained by heating the freshly distilled substance for several hours at 120° and then adding ligroin to a solution of the cooled substance in benzene.

2. The authors find that the needles obtained from benzene-ligroin differ in their melting point from those obtained by direct distillation of α-hydroxypyridine.

3. Both varieties of needles of α-hydroxypyridine were injected into

polyneuritic pigeons but no cure or amelioration in the condition of the birds was observed. The observations of Williams were therefore not confirmed.

4. Crystals obtained by recrystallising α-hydroxypyridine from water proved also to be inactive.

5. Pure adenine, as well as adenine treated with sodium ethylate in a sealed tube for five hours at 100°, yielded negative results in agreement with Voegtlin and White but in disagreement with Williams and Seidell.

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XVIII. COLOUR CHANGES PRODUCED BY TWO GROUPS OF BACTERIA UPON CASEINOGEN AND CERTAIN AMINO-ACIDS.

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The reactions which are described in this paper were undertaken in the course of work which was carried out in an endeavour to discover the causes of discolouration in Stilton cheese. This discolouration which varies from pink to black through many shades of yellow and brown is very widespread, and is a source of serious loss to some Stilton cheese manufacturers. The particular line of work adopted was based on the facts that Beijerinck [1911] had shown that some organisms were capable of producing discolouration in a medium which he had prepared and which contained tyrosine, and that Golding [1912] had, more recently, shown that certain organisms which he had isolated from discoloured cheeses were also capable of producing discolouration on Beijerinck's medium. Sixty-two discoloured Stilton cheeses were procured from a factory, and organisms were isolated from them.

In the very early days of the work the material was plated upon Beijerinck's medium. Although some of the colonies gave well-marked discolouration it was found very difficult to recover the organisms from these colonies. It was thought, therefore, that Beijerinck's medium was too severe, and that there was at least a possibility that colour producing organisms existing in cheeses were being lost. The discoloured cheeses were therefore plated upon + 10, neutral and − 5 lemco agar, Beijerinck's medium and a cheese medium obtained by mixing 20 % cheese and 2 % agar, made neutral and − 5 to Eyre's standard. In this way it was hoped that the organisms of more vigorous growth which could act upon tyrosine would be found upon the Beijerinck plates but the others which were either of less vigorous growth or

required other fractions than tyrosine would be found either upon the agar or cheese media. The reaction was varied because it had appeared that this affected the capacity for colour production. All those colonies which showed colour change on any of these media, + 10, neutral and − 5 lemco agar, − 1 and − 3 Beijerinck's medium, or neutral and − 5 cheese agar, were reisolated and when pure strains had been obtained were once more tested upon the media mentioned before further work was undertaken.

Bacteriological examination of the pure strains which showed discolouration was then made, with the result that organisms belonging to many different groups were isolated, including *B. coli*, *B. proteus*, *Streptotricheae*, Moulds etc. The present paper deals with strains belonging to only two of these numerous groups, namely *B. proteus* and a group of bacilli (called Gram-negative alkaline), which gave a negative reaction with Gram's stain and produced an alkaline reaction on all the fourteen sugars, alcohols and glucosides into which they were inoculated.

Group 1. *B. proteus vulgaris.*

Thirty-six members of this group were isolated from seven cheeses. Twenty-four strains from these seven were examined bacteriologically and were all found to correspond one with another. From among these, ten from seven cheeses were arbitrarily selected, and were used for further inoculations. Seven strains belonging to this group were isolated from two out of thirty-six samples of milk. These all gave identical reactions. One strain from each sample was taken for further experiment. All the members of this group gave the following reactions. Upon agar plates a rapidly spreading growth occurred. When examined under the microscope their length was seen to vary from 1 to 3μ and a tendency to chain formation was observed. Sometimes very long forms up to 14μ without any apparent division into cells were present. They were Gram-negative and motile, liquefied gelatin, produced indole from peptone water, gave a negative Voges-Proskauer reaction, and produced acid and gas in glucose, saccharose and galactose. The production of acid and gas in salicin, glycerol and dextrin was irregular, while there was no gas production in lactose, mannitol, sorbitol, dulcitol, adonitol, inositol, inulin or raffinose. In litmus milk a soft bleached clot and whey, a bad smell and an alkaline reaction were produced. It was felt that the consistency of these reactions justified the assumption that all these strains were members of one group of organisms and that this group was *B. proteus vulgaris*. Since these organisms produced a reddish brown

colour upon neutral lemco agar and upon the cheese medium which had been made, it was felt that a study ought to be made of their reactions upon caseinogen and its decomposition products. The inoculations were made upon the following media. Caseinogen water, 0·2 % suspension of Griffin's "extra pure casein" in distilled water, the solid being distributed as evenly as possible by continuous shaking during the process of filling out. Tyrosine, tryptophan, alanine, cystine and histidine hydrochloride, 0·1 % solutions in distilled water. Histidine water 0·1 %, the hydrochloric acid being neutralised by the calculated quantity of sodium carbonate, and neutral alanine water 0·1 % in which the carboxyl group was neutralised by the calculated quantity of sodium carbonate. The agars were in all cases made like Beijerinck's medium but contained tyrosine, tryptophan, alanine, cystine and histidine hydrochloride, and in every case were made − 1 and − 3 to Eyre's standard.

The inoculations were all made by transference of a loopful of the culture from neutral lemco agar media. It was thought possible that if colour changes occurred they might be due to the action of the organisms upon traces of agar transferred with the culture. Control experiments in distilled water and saline did not lead to the belief that any serious source of error was introduced in this way. The further possibility that the transference of particles of the agar medium along with the cultures to the amino-acids may have stimulated their growth [Bainbridge, 1911], and thus enabled the organisms to produce the colour changes which were seen, has not been guarded against. All tubes after inoculation were incubated at 30° for one week, then kept in the dark at laboratory temperature. They were examined at the end of 48 hours, 7 days, 14 days, 1, 2 and 3 months and the colour changes produced were recorded. No attempt was made to demonstrate the presence or absence of growth in the tubes.

Tyrosine has long been known to be present in old cheeses, and, with the very kind assistance of Professor F. G. Hopkins, Cambridge, the presence of tryptophan and histidine (or its base) in old Stilton cheeses had been demonstrated. Cystine and alanine are straight chain compounds and were chosen for this work because it had been suggested that it would be impossible to obtain any colouration upon aliphatic substances.

Some striking results were obtained which are shown in Table I.

The results obtained were, on the whole, very consistent for the members of this group and were most striking on tryptophan, in which an amber colour was invariably produced. Upon histidine hydrochloride water there was no visible reaction, but in the neutralised medium a light pinkish brown

colour occurred. Upon tyrosine water there was practically no change, but upon tyrosine agar a discolouration was seen varying from very slight to claret. Upon the other media and the controls no discolouration was produced.

TABLE I.

No.	Strain	Histidine hydrochloride water	Histidine water	−1 Histidine agar	−3 Histidine agar	Cystine water	−1 Cystine agar	−3 Cystine agar	Tryptophan water	−1 Tryptophan agar	−3 Tryptophan agar
1	$C_{20}^{2}B_2$	–	L. P. B.	v. v. sl.	v. sl.	–	–	–	Amber	L. Amber	L. Amber
2	$C_{22}^{2}B$	–	L. P. B.	v. sl.	v. sl.	–	–	–	,,	,,	,,
3	$C_{22}^{9}B$	–	–	v. L. P.	v. L. P.	–	–	–	,,	Amber	Amber
4	$C_{26}^{4}A^{7}$	–	L. P. B.	v. L. P.	v. L. P.	–	–	–	,,	,,	,,
5	$C_{26}^{4}A^{8}$	–	L. P. B.	v. L. P.	v. L. P.	–	–	–	,,	,,	,,
6	$C_{26}^{4}B_1$	–	L. P. B.	v. L. P.	v. L. P.	–	–	–	,,	,,	,,
7	$C_{28}^{5}A$	–	L. P. B.	v. L. P.	v. L. P.	–	–	–	,,	,,	,,
8	$C_{29}^{9}A$	–	L. P. B.	–	v. sl.	–	–	–	,,	,,	,,
9	C_{33}^{3}	–	L. P. B.	+sl.	+sl.	–	–	–	,,	,,	,,
10	$C_{58}^{1}A$	–	v. L. P. B.	v. v. sl.	v. v. sl.	–	–	–	,,	,,	,,
11	$M_{20}^{1}C$	–	L. P. B.	v. L. P.	v. L. P.	–	–	–	,,	,,	,,
12	$M_{26}^{1}C$	–	L. P. B.	v. L. P.	v. L. P.	–	–	–	,,	,,	,,

No.	Strain	Alanine water	Neutral alanine water	−1 Alanine agar	−3 Alanine agar	Caseinogen water	Tyrosine water	−1 Tyrosine agar	−3 Tyrosine agar	Sterile water	Sterile saline
1	$C_{20}^{2}B_2$	–	–	–	–	–	v. sl.	+sl.	+sl.	–	–
2	$C_{22}^{2}B$	–	–	–	–	–	v. v. sl.	Claret	Claret	–	–
3	$C_{22}^{9}B$	v. v. sl.	–	–	–	–	–	+sl.	+sl.	–	–
4	$C_{26}^{4}A^{7}$	? +	–	–	–	–	–	+sl.	v. sl.	–	–
5	$C_{26}^{4}A^{8}$	? +	–	–	–	–	–	+sl.	v. sl.	–	–
6	$C_{26}^{4}B_1$	–	–	–	–	–	–	+sl.	+sl.	–	–
7	$C_{28}^{5}A$	–	–	–	–	–	–	+sl.	+sl.	–	–
8	$C_{29}^{9}A$	–	–	–	–	–	–	+sl.	+sl.	–	–
9	C_{33}^{3}	–	–	–	–	–	v. sl.	+sl.	+sl.	–	–
10	$C_{58}^{1}A$	–	–	–	–	–	+sl.	+sl.	+sl.	–	–
11	$M_{20}^{1}C$	–	–	–	–	–	?+	+sl.	+sl.	–	–
12	$M_{26}^{1}C$	–	–	–	–	–	–	sl. Claret	sl. Claret	–	–

L. P B. = Light Pinkish Brown. v. L. P. = Very Light Pink. sl. = slight.

GROUP 2. *Gram-negative, alkaline Bacilli.*

Eleven strains of this organism were isolated from four of the discoloured cheeses which had been obtained from the factory. Seven from four cheeses were studied in detail; twenty-six strains were isolated from twenty-one samples of milk which were being used in the factory. Seventeen from ten samples were examined in detail, and five from five samples were selected arbitrarily for further studies of colour changes.

These organisms all showed the following characteristics: a greenish yellow growth on neutralised potato and complete absence of growth on

lemco agar under anaerobic conditions. In litmus milk after fourteen days' incubation at 30° a flesh-coloured clot, whey, a brown ring at the surface and an alkaline reaction were found. When examined under the microscope they appeared as somewhat exaggerated *B. coli*; they were Gram-negative, motile, gave the indole reaction with peptone water, and a negative Voges-Proskauer reaction; they liquefied lemco gelatin with the production of a brownish colour when the medium was neutralised. Upon glucose, lactose, saccharose, galactose, raffinose, dulcitol, inositol, sorbitol, mannitol, adonitol, inulin, salicin, dextrin and glycerol they gave a consistently alkaline reaction. Fifty-four samples of water taken from the farms which were supplying milk to the factory were examined. Of these, eighteen showed organisms belonging to this group. Seventeen strains from seventeen samples have been further examined. Six of these strains, W_2^{E}, W_{17}^{1A}, W_{19}^{1B}, W_{26}^{2}, $W_{32}^{3B_1A}$ and $W_{50}^{1A^1}$, selected arbitrarily, showed all those reactions which have been already described for the cheese and milk strains. The remainder, $W_{14}^{1A^1}$, W_{21}^{1}, W_{25}^{3A}, W_{27}^{2}, W_{29}^{1}, W_{30}^{2B}, W_{31}^{1A}, W_{36}^{1C}, W_{42}^{3B}, $W_{45}^{1A_2}$ and W_{52}^{3A} were not tested upon galactose, raffinose, inositol, sorbitol, mannitol, adonitol, inulin, salicin, dextrin or glycerol, because Kahlbaum's preparations were being used and it was necessary to conserve them. In all other respects their reactions were identical with those which have been described for the other strains.

The twenty-nine strains which were selected comprised seven from four cheeses, five from five samples of milk and seventeen from seventeen samples of water. The colour changes resulting from the action of these organisms upon various media which have been already described under Group 1 are shown in Table II.

The results show that, when inoculated into histidine hydrochloride water, the reactions were either definitely negative or very doubtfully positive, and on only one occasion was a very light brown colour produced. When the hydrochloric acid of the histidine hydrochloride was neutralised, the colour was slightly more marked, though even then the maximum colour recorded is slight brown. Upon the histidine agar media the results were, for all practical purposes, negative. Negative results were also obtained with all the cystine media.

Upon the tryptophan water and agar media the results were very consistent, being much less marked upon the agar than upon the tryptophan water, upon which they showed a colour varying from slight yellow to a definite canary. Upon alanine again, the results on the agar media were

TABLE II.

No.	Strain	Histidine hydrochloride water	Histidine water	−1 Histidine agar	−3 Histidine agar	Cystine water	−1 Cystine agar	−3 Cystine agar	Tryptophan water	−1 Tryptophan agar	−3 Tryptophan agar	Alanine water
1	$C_{22}{}^{1}B$	–	v. sl. Y.	–	–	–	–	–	sl. Y.	+ v. sl.	+ v. sl.	sl. Y.
2	$C_{23}{}^{9}$	–	v. sl. Y.	–	–	–	–	–	sl. Y.	+ v. sl.	+ v. sl.	v. sl. Y.
3	$C_{26}{}^{5}C_{1}$	? +	sl. Y. B.	–	–	–	–	–	sl. Y.	+ v. sl.	+ v. sl.	sl. Y.
4	$C_{26}{}^{5}C_{2}$	? +	+ sl.	–	–	–	–	–	sl. Y.	+ v. sl.	+ v. sl.	v. sl. Y.
5	$C_{27}{}^{1}A$	+ sl.	+ sl.	–	–	–	–	–	Canary	+ sl.	+ sl.	v. sl. Y.
6	$C_{27}{}^{5}C_{4}$	? +	sl. B.	–	–	–	–	–	,,	+ sl.	+ sl.	v. sl. Y.
7	$C_{27}{}^{9}A$	–	? +	–	–	–	–	–	,,	+ v. sl.	+ v. sl.	v. sl. Y.
8	$M_{3}{}^{2}A$	? +	+ sl.	–	–	–	–	–	,,	+ sl.	+ sl.	sl. Y.
9	$M_{6}{}^{1}$	–	+ sl.	–	–	–	–	–	,,	+ sl.	+ sl.	v. sl. Y.
10	$M_{28}{}^{1}A$	–	+ v. sl.	–	–	–	–	–	v. sl. Y.	+ v. sl.	+ v. sl.	sl. Y.
11	$M_{29}{}^{3}A$	? +	+ sl.	–	–	–	–	–	Canary	+ v. sl.	+ v. sl.	sl. Y.
12	$M_{32}{}^{3}A$	+ v. sl.	+ v. sl.	–	–	–	–	–	,,	+ sl.	+ sl.	sl. Y.
13	$W_{2}E$	–	sl. B.	–	–	–	? +	? +	,,	+ v. sl.	+ v. sl.	sl. Y.
14	$W_{11}{}^{1}A_{1}$	–	+ sl.	–	–	–	–	–	L. Canary	+ v. sl.	+ sl.	sl. Y.
15	$W_{17}{}^{1}A$	–	? +	–	–	–	–	–	Canary	+ sl.	+ sl.	sl. Y.
16	$W_{19}{}^{1}B$	? +	? +	–	–	–	–	–	L. Canary	+ sl.	+ sl.	sl. Y.
17	$W_{21}{}^{1}$	–	+ sl.	–	–	–	–	–	,,	+ v. sl.	+ v. sl.	+ v. sl.
18	$W_{25}{}^{3}A$	v. L. B.	+ v. sl.	–	? +	–	–	–	,,	+ sl.	+ sl.	? +
19	$W_{26}{}^{2}$	? +	? +	–	–	–	? +	? +	Canary	+ sl.	+ sl.	sl. Y.
20	$W_{27}{}^{2}$	–	+ sl.	–	–	–	–	–	,,	+ sl.	+ sl.	sl. Y.
21	$W_{29}{}^{1}$	–	L. B.	–	–	–	–	–	L. Canary	+ v. sl.	+ v. sl.	sl. Y.
22	$W_{30}{}^{2}B$	? +	v. L. B.	–	–	–	–	–	,,	+ v. sl.	+ v. sl.	? +
23	$W_{31}{}^{1}A$	–	? +	–	–	–	–	–	,,	+ sl.	+ sl.	? +
24	$W_{32}{}^{3}B_{1}A$	+ v. v. sl.	+ v. sl.	–	–	–	–	–	,,	+ sl.	+ sl.	sl. Y.
25	$W_{36}{}^{1}C$	–	sl. B.	–	–	–	–	–	,,	+ sl.	+ sl.	? +
26	$W_{42}{}^{3}B$	–	v. L. B.	–	–	–	–	–	,,	+ sl.	+ sl.	sl. Y.
27	$W_{45}{}^{1}A_{2}$	–	+ sl.	–	–	–	–	–	,,	+ sl.	+ sl.	+ v. sl.
28	$W_{50}{}^{1}A_{1}$	? +	+ sl.	–	–	–	–	–	Canary	+ sl.	+ sl.	+ v. sl.
29	$W_{52}{}^{3}A$	–	+ sl.	–	–	–	–	–	,,	+ sl.	+ sl.	+ v. sl.

No.	Strain	Neutral alanine water	−1 Alanine agar	−3 Alanine agar	Caseinogen water	Tyrosine water	−1 Tyrosine agar	−3 Tyrosine agar	Sterile water	Sterile saline
1	$C_{22}{}^{1}B$	sl. Y.	+ v. v. sl.	+ v. v. sl.	Brown	Bl.	Bl.	Bl.	–	–
2	$C_{23}{}^{9}$	sl. Y.	? +	,,	,,	B.—Bl.	Bl.	Bl.	–	–
3	$C_{26}{}^{5}C_{1}$	sl. Y.	+ v. v. sl.	,,	,,	B.—Bl.	Bl.	Bl.	? +	? +
4	$C_{26}{}^{5}C_{2}$	sl. Y.	,,	,,	,,	B.—Bl.	Bl.	Bl.		
5	$C_{27}{}^{1}A$	sl. Y.	,,	,,	,,	B.—Bl.	Bl.	Bl.	? +	–
6	$C_{27}{}^{5}C_{4}$	+ sl.	,,	,,	,,	B.—Bl.	Bl.	Bl.		
7	$C_{27}{}^{9}A$	sl. Y.	,,	,,	,,	B.—Bl.	Bl.	Bl.	–	–
8	$M_{3}{}^{2}A$	sl. Y.	,,	,,	,,	B.—Bl.	Bl.	Bl.	–	–
9	$M_{6}{}^{1}$	v. sl. Y.	,,	,,	,,	D. B.	Bl.	Bl.		
10	$M_{28}{}^{1}A$	+ v. sl.	,,	,,	L. Brown	B.—Bl.	Bl.	Bl.	–	–
11	$M_{29}{}^{3}A$	sl. Y.	,,	,,	Brown	B.—Bl.	Bl.	Bl.		
12	$M_{32}{}^{3}A$	sl. Y.	,,	,,	,,	B.—Bl.	Bl.	Bl.		
13	$W_{2}E$	sl. Y.	,,	,,	v. L. Brown	B.	Bl.	Bl.		
14	$W_{11}{}^{1}A_{1}$	sl. Y.	,,	,,	Brown	B.—Bl.	B.—Bl.	B.—Bl.		
15	$W_{17}{}^{1}A$	v. sl. Y.	,,	,,	L. Brown	D. B.	Bl.	Bl.		
16	$W_{19}{}^{1}B$	v. sl. Y.	,,	,,	,,	B.—Bl.	Bl.	Bl.		
17	$W_{21}{}^{1}$	v. sl. Y.	,,	,,	,,	B.—Bl.	B.	Bl.		
18	$W_{25}{}^{3}A$	sl. Y.	,,	,,	Brown	B.—Bl.	B.—Bl.	Bl.		
19	$W_{26}{}^{2}$	sl. Y.	,,	,,	L. Brown	B.—Bl.	Bl.	Bl.		
20	$W_{27}{}^{2}$	sl. Y.	,,	,,	Brown	D. B.	B.	B.—Bl.		
21	$W_{29}{}^{1}$	sl. Y.	,,	,,	,,	B.—Bl.	B.—Bl.	Bl.	–	
22	$W_{30}{}^{2}B$	sl. Y.	,,	,,	,,	B.—Bl.	B.	B.—Bl.		
23	$W_{31}{}^{1}A$	sl. Y.	,,	,,	,,	B.—Bl.	L. B.	Bl.		
24	$W_{32}{}^{3}B_{1}A$	+ v. sl.	,,	,,	,,	B.—Bl.	B.—Bl.	Bl.	? +	
25	$W_{36}{}^{1}C$	? +	,,	,,	,,	B.—Bl.	B.—Bl.	B.—Bl.		
26	$W_{42}{}^{3}B$	sl. Y.	,,	,,	,,	B.—Bl.	B.—Bl.	Bl.		
27	$W_{45}{}^{1}A_{2}$	+ v. sl.	,,	,,	L. Brown	B.—Bl.	Bl.	Bl.		
28	$W_{50}{}^{1}A_{1}$	+ v. sl.	,,	,,	Brown	B.—Bl.	Bl.	B.—Bl.	? +	–
29	$W_{52}{}^{3}A$	v. sl. Y.	,,	,,	,,	B.—Bl.	Bl.	B.		

L. = Light. D. = Dark. sl. = Slight. Y. = Yellow. B. = Brown. Bl. = Black.

less marked than upon the water. The results upon alanine water and upon neutralised alanine water did not appear to differ greatly, the maximum effect obtained being a slight yellowish colour. Upon caseinogen water, these organisms again produced a definite brownish colour, which differentiated them completely from the *proteus* group, the members of which failed entirely to produce any change upon this medium. Upon the tyrosine media the colours varied from brown to black. The control tubes made by the inoculation of sterile saline and sterile water were, as a rule, quite negative, but occasionally showed a very doubtful colour change.

The maximum and minimum reactions for each group are shown in Table III.

TABLE III.

No.	Strain	Histidine hydrochloride water	Histidine water	−1 Histidine agar	−3 Histidine agar	Cystine water	−1 Cystine agar	−3 Cystine agar	Tryptophan water	−1 Tryptophan agar	−3 Tryptophan agar
1	*B. proteus vulgaris*	– to v. sl.	v. L. Y. to P. B.	– to L. P.	v. v. sl. to L. P.	– to +sl.	– to ?+	– to ?+	L. Amber to D. Amber	L. Amber to D. Amber	L. Amber to D. Amber
2	Gram-neg. alkaline	– to ?+	?+ to L. B.	– to v. sl.	– to +sl.	– to v. sl.	– to v. sl.	– to v. sl.	v. sl. Y. to Canary	v. sl. to +sl.	v. v. sl. to +sl.

No.	Strain	Alanine water	Neutral alanine water	−1 Alanine agar	−3 Alanine agar	Caseinogen water	Tyrosine water	−1 Tyrosine agar	−3 Tyrosine agar	Sterile water	Sterile saline
1	*B. proteus vulgaris*	– to v. v. s.	–	–	–	–	– to L. B.	+sl. to Claret	v. sl. to Claret	–	–
2	Gram-neg. alkaline	v. sl. to sl. Y.	– to sl. Y.	?+ to v. v. sl.	v. v. sl.	v. sl. to D. B.	B. to Bl.	L. B. to Bl.	B. to Bl.	– to ?+	– to ?+

B. = Brown. Bl. = Black. D. = Dark. L. = Light. P. = Pink. Y. = Yellow. sl. = Slight.

The most characteristic differences between the members of these groups under the particular conditions of experiment, are the production of a well-marked amber colour upon tryptophan and absence of reaction in caseinogen water by members of the *proteus* group, with the production of a colour which was never more than canary yellow upon tryptophan and of a definite brown colour in caseinogen water by members of the Gram-negative, alkaline group.

Summary.

A very large number of organisms belonging to many different groups have been isolated from discoloured Stilton cheeses, milk from which such cheeses were made and the water supplies of the farms from which the milk was obtained.

The bacteriological reactions of two of these groups have been studied.

Certain members of these two groups have been selected arbitrarily and their reactions upon caseinogen and various amino-acids resulting from its degradation have been investigated.

The results show that members of both groups are capable, under the conditions of experiment, of producing discolouration upon certain of the substances selected and that the two groups show very distinct differences in their capacity for producing colour upon the various media upon which they were tested.

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XIX. L'ACIDE LACTIQUE EST-IL UN PRODUIT INTERMEDIAIRE DE LA FERMENTATION ALCOOLIQUE?

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Slator [1906, 1907, 1908], Buchner et Meisenheimer [1909] ont démontré que l'acide lactique n'était pas fermentescible par la levure. Après la découverte de Wieland [1913] que celui se décompose facilement en présence du palladium, preparé d'après son excellente méthode, avec formation de CH_3COCO_2H, CO_2 et CH_3CO_2H, il fallait sans doute s'attendre à ce que le même phénomène se produise par la réductase de la levure en présence du bleu de méthylène. Ce point de vue a été appuyé expérimentalement par Palladin, Sabinin et Lovchinovskaya [1915] et Harden et Norris [1915]. De ma part j'ai démontré [1915][1] que l'acide lactique se décomposait dans ces conditions non seulement en solution neutre, mais aussi, d'une manière encore plus intense, en solution acide, de sorte que, contrairement à l'opinion de Palladin, il y aurait, sans intervention de l'eau, une déshydrogénation directe de CHOH par la réductase de la levure. En absence du bleu de méthylène l'acide lactique se décomposait sans formation de l'aldéhyde acétique, mais avec une formation d'acides, probablement de l'acide acétique en prépondérance; la formation de l'alcool était bien douteuse et même, s'il s'en formait une petite quantité, elle était 3 fois plus faible que celle de CO_2 dégagé [Lebedev, 1915, 1916, pp. 741–3], ce qui a été confirmé plus tard par Palladin et Sabinin [1916]. J'ai montré en outre que la vitesse de la décoloration du bleu de méthylène ne dépendait pas de la quantité de CH_3COH formé et que celle-ci ne faisait qu'une très faible partie de l'acide employé. Or, dans une expérience j'ai obtenu pour 500 mg. de $CH_3CHOHCO_2H$ neutralisé 20 mg. de CH_3COH et dans l'autre—pour 300 mg.—16·3 mg. de CH_3COH en solution acide et 15·9 en solution neutre. Il a fallu s'attendre à

[1] Ce mémoire a paru avant le mémoire de Harden et Norris que je viens de citer.

ce que, lorsque le bleu de méthylène se décolorait complètement, pour 1 mol. d'acide décomposé 1 mol. de CH_3COH se formât, mais on n'a trouvé que 1/3 de cette quantité. Je profite de l'occasion pour dire que les expériences de Lvoff, citées par Palladin, d'après lesquelles "in the process of glucose fermentation for each atom of hydrogen removed with the help of methylene blue, the production both of alcohol and of carbon dioxide is diminished by one molecule" [Palladin et Sabinin, 1916, p. 187], ne se laissent pas reproduire [Lebedev, 1915, pp. 317–8]. Il y a donc tout lieu à croire que Lvoff a obtenu ce résultat à cause de la méthode défectueuse dont il se servait.

Oppenheimer [1914, p. 60] a supposé que l'acide lactique pouvait se décomposer en présence de l'acide pyruvique en C_2H_6O et CO_2:

(1) $CH_3CHOHCO_2H - H_2 = CH_3COCO_2H$.

(2) $CH_3COCO_2H = CH_3COH + CO_2$.

(3) $CH_3COH + H_2 = CH_3CH_2OH$.

Il a constaté seulement la disparition d'une partie de l'acide lactique ajouté au suc de macération, mais il n'a pas dosé l'alcool, dont il supposait la formation en présence de l'acide pyruvique. Pour combler cette lacune, j'ai répété les expériences d'Oppenheimer, sans connaître celles de Palladin, en me servant, au lieu du suc, de la levure séchée à 35° et en dosant l'alcool par la méthode de Nicloux. Voici le résultat obtenu [Lebedev, 1916, pp. 742–3].

1.	Témoin ...	217 mg. CO_2	5.	$CH_3CHOHCO_2H$	287 mg. CO_2
2.	„	223 „	6.	„	287 „
3.	$CH_3COCOOH$	220 „	7.	$CH_3CHOHCO_2H + CH_3COCO_2H$	296 „
4.	„	228 „	8.	„	297 „

1, 2.	Témoin	436 mg. d'alcool
3, 4.	CH_3COCO_2H (20 mg.)	456 „ „
5, 6.	$CH_3CHOHCO_2H$ (432 mg.) ...	436 „ „
7, 8.	$CH_3CHOHCO_2H + CH_3COCO_2H$	476 „ „

Si l'on tient compte de ce que l'acide pyruvique s'est décomposé en alcool et CO_2, on obtient définitivement:

		CO_2	CH_3CH_2OH
I.	Témoin	440 mg.	436 mg.
II.	CH_3COCO_2H	438 „	446 „
III.	$CH_3CHOHCO_2H$	574 „	436 „
IV.	$CH_3CHOHCO_2H + CH_3COCO_2H$	583 „	466 „

Il s'en suit qu'en présence de l'aldéhyde acétique une partie de $CH_3CHOHCO_2H$ s'est oxydée probablement en CH_3COCO_2H, mais cette partie était tout à fait *insignifiante*. Si l'on calcule d'après la quantité de CO_2 dégagé, on trouve qu'il s'est décomposé 71 % d'acide lactique ajouté (chaque fiole contenait 10 g. de levure de Lebedev, 50 cc. d'eau et 0·2 cc.

de toluène); au contraire, d'après la quantité d'alcool on n'en trouve que 9 %. Le rapport $CO_2 : C_2H_6O$ au lieu d'être égal à 1 est égal à 8! Il est à noter qu'à la fin de l'expérience le liquide ne contenait que des traces de CH_3COH, parce que la réaction de Rimini était très faible. Palladin a trouvé que le rapport était égal à 6, mais il a commis une forte erreur de calcul. Le témoin de l'expérience 15 [Palladin et Sabinin, 1916, p. 194] a donné en 62 h. 400 mg. de CO_2 et 417 mg. d'alcool. En tenant compte de cela, on obtient pour les nos. 1, 2 et 3 par soustraction:

	Carbon dioxide		Alcohol	
1. Pyruvic acid	90	137	59	87
2. Lactic acid	47		28	
3. Lactic and pyruvic acids	295		97	

Or, $295 - 137 = 158$ mg. de CO_2 et $97 - 87 = 10$ mg. de C_2H_6O; on aura donc le rapport vrai $158 : 10 = 15{\cdot}8$! 10 mg. d'alcool ne font que 2 % de la quantité entière de celui-ci (514 mg.), c'est à dire qu'ils ne dépassent pas l'erreur de la méthode de Nicloux qui est de 5 % et que, par conséquent, *Palladin n'a pas démontré que l'acide lactique se décomposait en présence de* CH_3COCO_2H *en* CO_2 *et* C_2H_6O. Il est à remarquer en outre qu'il dosait l'alcool en présence d'une quantité considérable de CH_3COH, ce qui fait le résultat obtenu par lui plus incertain encore. Bien que mes propres expériences soient un peu plus favorables à la théorie d'Oppenheimer que celles de Palladin, il serait tout de même trop osé d'en conclure qu'une fermentation alcoolique de l'acide lactique eût lieu. Je puis répéter ici ce que j'ai dit il y a deux ans à propos des expériences de Palladin: "On a donc aucune raison pour regarder l'acide lactique comme un produit intermédiaire de la fermentation alcoolique" [Lebedev, 1915, p. 306].

D'après mes recherches [Lebedev, 1915, pp. 331–2] l'aldéhyde pyruvique est fermentescible par la levure et par le suc de macération, préparés d'après ma méthode, avec une production de C_2H_6O et de CO_2 en proportion presque égale à 1, si la concentration d'aldéhyde ne dépasse pas 1·5 %. En présence du bleu de méthylène *l'aldéhyde acétique* s'en forme, évidemment par les réactions suivantes:

$$1.\quad CH_3.CO.CH\begin{matrix}OH\\OH\end{matrix} - H_2 = CH_3COCOOH.$$

$$2.\quad CH_3COCOOH = CO_2 + CH_3COH.$$

Je ne décrirai ici que deux de mes expériences, les autres étant analogues [Lebedev, 1915].

Exp. 1. On a pris 2 fioles d'Erlenmeyer (A et B, à peu près 250 cc. chacune) et on les a chargées de 10 g. de levure de Lebedev + 0·25 g. de bleu de méthylène. A la fiole A on a ajouté 50 cc. de solution de CH_3COCOH à 1·3 %, à l'autre—50 cc. de solution de $CH_3CHOHCO_2H$ à 2 %. Après les avoir bien bouchées, on les a secoué à la machine pendant 17 h. et on a dosé l'aldéhyde acétique sous forme de *p*-nitrophenylhydrazone. Voici le résultat obtenu.

A. 65 mg. de CH_3COCOH ... 10·6 mg. de CH_3COH.
B. 1000 mg. „ $CH_3CHOHCO_2H$ ... 14·4 mg. „

Exp. 2. Mêmes conditions que dans l'expérience 1, mais on a dosé de l'aldéhyde après 9 h. La fiole A contenait 50 cc. de solution de CH_3COCOH à 2·6 %; la fiole B—50 cc. de solution de CH_3CHOH à 1 %.

A. 130 mg. de CH_3COCOH ... 13·5 mg. de CH_3COH.
B. 500 mg. „ $CH_3CHOHCO_2H$ ... 21·0 mg. „

Si l'on tient compte de la concentration de substances ajoutées, on constate que la formation de l'aldéhyde acétique est plus intense dans le cas de CH_3COCOH que de $CH_3CHOHCO_2H$. Etant donné que dans des processus biochimiques l'acide lactique est formé de l'aldéhyde pyruvique, on pourrait plutôt affirmer que c'est justement celui-ci qui soit un produit intermédiaire, néanmoins, bien que cette hypothèse soit bien séduisante, je ne le crois pas, parce que la fermentation de l'aldéhyde pyruvique est très lente et faible à cause de sa toxicité pour les diastases de la levure.

En connexion avec les faits relatés ici, je me permets d'attirer l'attention sur un autre mémoire [Lebedev, 1916], où j'ai montré que la levure de Lebedev décomposait l'acide malique en CO_2 et $CH_3CHOHCO_2H$ et qu'en présence du bleu de méthylène l'aldéhyde acétique en était formé. J'ai fait la supposition que cette réaction curieuse allait en trois phases:

1. $CO_2H.CHOH.CH_2.CO_2H = CO_2 + CH_3.CHOH.CO_2H$.
2. $CH_3.CHOH.CO_2H + R = CH_3.CO.CO_2H + RH_2$.
3. $CH_3.CO.CO_2H = CH_3.COH + CO_2$.

On pourrait faire une autre supposition, à savoir:

1. $CO_2H.CHOH.CH_2.CO_2H + R = CO_2H.CO.CH_2.CO_2H + RH_2$.
2. $CO_2H.CO.CH_2.CO_2H = 2CO_2 + CH_3.COH$.

Mais ce point de vue me semble moins plausible, parce que l'aldéhyde acétique se forme de l'acide malique plus lentement et en quantité plus petit que de l'acide lactique.

Le fait intéressant établi par Palladin [Palladin et Sabinin, 1916] que l'acide pyruvique en fermentant simultanément avec de l'acide lactique

dégageait sous l'influence de la levure plus de CO_2 que lorsqu'il fermentait seul, est tout à fait analogue à celui constaté par moi et Polonsky pendant la fermentation simultanée du sucre et de l'acide pyruvique. Nous avons dosé non seulement CO_2, mais aussi C_2H_6O et C_2H_4O. Voici le résultat d'une de nos expériences après la soustraction des quantités correspondantes des CO_2, C_2H_6O et C_2H_4O du témoin. La durée—25½ h. à 22°. CO_2 a été expulsé complètement par un courant d'air à la fin de l'expérience.

	CO_2	C_2H_6O	C_2H_4O
1. 400 mg. $C_{12}H_{22}O_{11}$	189 mg.	208 mg.	0·5 mg.
2. 100 mg. CH_3COCO_2H	25 „	8 „	0·7 „
3. 100 mg. $CH_3COCOOH$ + 100 mg. $C_{12}H_{22}O_{11}$	120 „	48 „	0·7 „
4. 100 mg. $C_{12}H_{22}O_{11}$	49 „	48 „	0·5 „
5. 100 mg. CH_3COCO_2H + 400 mg. $C_{12}H_{22}O_{11}$	243 „	208 „	0·9 „

On voit que pendant la fermentation de $C_3H_4O_3$ et du sucre (en concentrations égales) se forme presque la même quantité de C_2H_4O, au contraire, la quantité de C_2H_6O a été 6 fois et de CO_2 2 fois plus petite dans le cas de $C_3H_4O_3$, de sorte qu'il n'y avait aucun parallélisme entre les fermentations de ces deux substances. Prenons la somme des nos. 2 et 4 et 1 et 2 et comparons les aux nos. 3 et 5, nous obtiendrons les nombres pour la fermentation simultanée des deux substances et celle de chaque substance séparément.

	CO_2	C_2H_6O	C_2H_4O
2 et 4 (100 mg. $C_3H_4O_3$; 100 mg. $C_{12}H_{22}O_{11}$)	74 mg.	56 mg.	1·2 mg.
3 (fermentation simultanée)	120 „	48 „	0·7 „
1 et 2 (400 mg. $C_{12}H_{22}O_{11}$; 100 mg. $C_3H_4O_3$)	214 „	216 „	1·2 „
5 (fermentation simultanée)	243 „	208 „	0·9 „

Pendant la fermentation simultanée il s'est formé plus (+) ou moins (−) que séparément.

	CO_2	C_2H_6O	C_2H_4O
100 mg. $C_3H_4O_3$ + 100 mg. $C_{12}H_{22}O_{11}$	+46 mg.	−8 mg.	−0·5 mg.
100 mg. $C_3H_4O_3$ + 400 mg. $C_{12}H_{22}O_{11}$	+29 „	−8 „	−0·3 „

On peut en conclure que la fermentation du sucre et de $C_3H_4O_3$ va autrement, lorsque les deux substances se trouvent ensemble et que le travail de la carboxylase ne dépend pas de celui de la zymase. A ce propos je dois ajouter que la présence de $C_3H_4O_3$ n'influence pas d'une manière sensible l'autofermentation de la levure; ce fait a été aussi établi par moi et Polonsky. Il n'est pas facile pour le moment de résoudre la question aux dépens de quelle substance l'augmentation de la quantité de CO_2 a lieu. Il est possible qu'il y ait des substances qui suscitent ou favorisent la décomposition de l'acide pyruvique même, comme, par exemple, le sucre dans nos expériences ou l'acide lactique dans celles de Palladin

Palladin veut expliquer la différence énorme qui existe entre la fermentation du sucre et de l'acide lactique par une supposition que la levure vivante travaille autrement que la levure tuée. Sans dire de cela que la levure préparée d'après ma méthode bien qu'elle reste vivante [Lebedev, 1912, p. 36] décompose le sucre en présence et sans toluène absolument de la même manière, je n'indiquerai que sur le fait bien connu que la levure tuée (Acetondauerhefe, Hefanol et cet.) et vivante fermente le sucre avec production de CO_2 et de C_2H_6O en proportion $CO_2 : C_2H_6O$ qui est égale à unité.

Il ressort clairement de tout ce que je viens d'exposer que l'assertion de Palladin [Palladin et Sabinin, 1916, p. 195], "At any rate, our experiments give, firstly, a scheme which shows in which direction the work must be conducted, and secondly, prove that alcohol does not present the result of decomposition, but the product of reaction of acetaldehyde by removing the hydrogen from one of the intermediate products of decomposition of glucose," non seulement n'est pas démontrée par lui, mais qu'elle est en outre inexacte, parce que les mêmes idées ont été émises par Neuberg [Neuberg and Kerb, 1912, Neuberg, 1913, p. 13], moi [Lebedev and Griaznov, 1912, p. 3270; Lebedev, 1914] et Kostytschev [1912].

Palladin pense que la réaction de Cannizzaro n'a pas lieu dans la cellule de levure vivante, mais c'est le contraire qui a été montré par Kostytschev [1914]. J'ai démontré en collaboration avec Polonsky que l'aldéhyde acétique qui se formait pendant la fermentation des acides pyruvique et glycérique par la levure de Lebedev se transformait en partie d'après cette réaction en acide acétique et alcool. Cette transformation d'ailleurs ne devient considérable que si les acides sont employés sous forme de leurs sels alcalins.

Voici le résultat de 4 expériences pour $C_2H_4O_2$ et C_2H_4O.

	Acide acétique		Ald. acétique	
Solut. acide	33·3 mg.	33·0 mg.	10·4 mg.	10·0 mg.
Solut. neutre	40·8 „	47·4 „	0·8 „	2·2 „
Témoin	22·6 „	29·4 „	0·2 „	0·2 „

Ou après la soustraction des nombres correspondants au témoin (moyen):

Solut. acide	7·1 mg.	acide acét.	9·9 mg.
Solut. neutre	18·1 „		1·5 „

ald. acét.

Je donne encore le résultat de 5 expériences pour l'alcool et l'acide acétique (après la soustraction):

	Alcool en mg.		Acide acétique en mg.	
Sol. neutre	32; 34; 32	32·7	24·9; 18·2; 18·0	20·4
Sol. acide	16; 8	12	10·7; 3·6	7·2
		moyen		moyen

Nous trouvons donc de l'aldéhyde acétique sous forme de

	C_2H_6O	et	$C_2H_4O_2$
en milieu acide	8 mg.		3·2 mg.
„ neutre	31 mg.		13·6 mg.

On voit de ces chiffres qu'en milieu neutre *la réaction de Cannizzaro va parallèlement avec une réduction de l'aldéhyde acétique par la réductase de levure.* En milieu acide la réaction de Cannizzaro est de beaucoup plus faible.

Nous avons en outre constaté que, si l'on songe d'après la quantité de CO_2 dégagé, la décomposition de l'acide pyruvique en milieu acide est 2 fois plus forte que dans le milieu neutre[1], mais l'excédant de CO_2 n'est pas balancé par les produits nommés plus haut, c'est à dire C_2H_6O, C_2H_4O et $C_2H_4O_2$, de sorte qu'il faut admettre qu'une oxydation directe ou une formation des produits d'une condensation ait lieu. Voici le résultat de 3 expériences.

		m. neutre	m. acide		m. neutre	m. acide
1.	$C_2H_6O + C_2H_4O + C_2H_4O_2$	49·6 mg.	25·5 mg.	CO_2	43·0 mg.	51·0 mg.
2.	„ „ „	37·1 „	32·9 „	„	22·0 „	54·5 „
3.	„ „ „	34·5 „	30·9 „	„	51·0 „	77·0 „
		121·2 „	89·3 „		116 „	182·5 „

Le moyen de 3 expériences est alors:

Milieu neutre 40 mg. $[C_2H_6O + C_2H_4O + C_2H_4O_2]$ correspondant aux 38·7 mg. de CO_2.
„ acide 29·8 „ „ „ „ 60·8 „ „

Nous rappelons ici le fait bien connu que la fermentation du sucre se produit toujours en milieu acide (si l'on ne le fait pas artificiellement neutre ou alcalin); si l'acide pyruvique était un produit intermédiaire, la proportion $CO_2 : [C_2H_6O + C_2H_4O + C_2H_4O_2]$ devrait être au moins égale à 1, mais elle est, comme nous venons de le montrer, considérablement plus grande. C'est pourquoi on ne serait peut-être pas trop étonné, si un jour on trouvait que l'acide pyruvique ne soit pas un produit intermédiaire de la fermentation alcoolique.

Il y a quelques années j'ai fait une observation curieuse que l'acide glycérique se décomposait en présence de la levure de Lebedev d'après l'équation suivante:

$$CH_2OH.CHOH.CO_2H = C_2H_4O + CO_2 + H_2O.$$

Or, je puis à présent affirmer qu'il peut se former pendant la fermentation de cet acide plus d'aldéhyde acétique que dans le cas de l'acide pyruvique et que la décomposition de l'acide glycérique même en milieu acide suit de plus près l'équation donnée plus haut que celle de l'acide pyruvique. Je ferai connaître ici les chiffres moyens de deux expériences faites par moi et Polonsky.

[1] Il va de soi qu'à la fin de l'expérience quelques gouttes d'acide sulfurique ont été ajoutées.

Pour 25 cc. d'eau et 5 g. de levure on a pris 230 mg. d'acide glycérique et 250 mg. d'acide pyruvique. T. = 20°. Les fioles qui servaient au dosage de CO_2 ont été à la fin des expériences chauffées pendant une $\frac{1}{2}$ h. à 70°, après quoi on a fait passer pendant 1 h. un courant d'air à travers le liquide et la soupape de fermentation de Meissl. Les fioles qui servaient pour le dosage des $C_2H_4O_2$ et C_2H_4O ont été bien bouchées. On a dosé l'alcool par la méthode de Nicloux [1906] et le C_2H_4O par la méthode de Ripper [1900]. Voici le résultat obtenu :

	CO_2	C_2H_6O	C_2H_4O	$C_2H_4O_2$
1. Témoin	80	82·5	0·11	11·70
2. Ac. glycér.	124	105·0	10·44	12·15
3. Ac. pyruv.	154	116·0	4·35	15·15

Après la soustraction des chiffres-témoins on a calculé la quantité de C_2H_4O qui correspond à celle des C_2H_6O et C_2H_4O. On trouve par conséquent 32·2 mg. de C_2H_4O + 44 mg. de CO_2 pour l'acide glycérique et 38·8 mg. de C_2H_4O + 74 mg. de CO_2 pour l'acide pyruvique. (On obtient le même résultat en présence et absence de toluène.)

Ce fait dit en faveur de la supposition qu'à cause de la formation successive de l'acide pyruvique, celui-ci soit fermenté dès qu'il se forme, sans donner lieu à la production et l'accumulation des substances secondaires, mais il y a d'autres difficultés, dont j'ai parlé dans le travail cité plus haut qui ne se laissent pas expliquer si facilement et qui restent encore à surmonter pour croire que l'acide pyruvique soit vraiment un produit intermédiaire ; comme je viens de montrer dans le mémoire présent, les difficultés deviennent même énormes, si l'on veut admettre que l'acide lactique soit aussi un produit intermédiaire de la fermentation alcoolique, de sorte que je ne crois pas qu'on ne puisse jamais les surmonter.

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XX. CONTRIBUTIONS TO THE BIOCHEMISTRY OF PATHOGENIC ANAEROBES. II. THE ACID PRODUCTION OF *BACILLUS WELCHII* (*B. PERFRINGENS*) AND *BACILLUS SPOROGENES* (METCHNIKOFF).

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In a recent paper, one of us with Harris [Wolf and Harris, 1917] has shown that, in the fermentation with *Bacillus Welchii* (*B. perfringens*), all activity ceases when the reaction of the medium reaches a certain hydrogen ion concentration. The cessation of growth appeared to be connected with the production of acids. We were able to confirm the statements of previous observers, who have worked on acid production, that normal butyric acid was one of the constituents of the fermentation liquid, by isolating the acid in a fairly pure condition and identifying it. Other acids were also present.

With the *B. sporogenes* of Metchnikoff, there was also a large amount of acid formed when the organisms acted on various media. In milk it was difficult to say from what component of the medium the acid arose.

Owing to the difficulties in titrating a complex medium, containing buffer salts and amphoteric nitrogenous compounds, it was impossible to ascertain what were the amounts of acids that these bacteria produced.

As the production of acids by these two organisms has been advanced to account for certain clinical features in gas gangrene infections [Wright, 1917], it appeared advisable to devote some attention to an investigation of this property.

For some years the only method of estimating quantitatively the volatile acid production of micro-organisms has been that of Duclaux [1900]. Owing

to certain inherent difficulties in the method it has not been very largely used by biochemists. Recently there appeared an article by Dyer [1917], in which he described a new method of dealing with the volatile fatty acids in mixtures and suggested that it might be employed in the study of acid production in bacteriological media. Briefly stated, this method consists of a determination of the distilling rate in steam of an acid solution when the latter is maintained at constant volume. The distillate is collected in portions and titrated. The number of cc. of alkali required to neutralise each portion is noted and the percentage of acid calculated. The amount of acid in a given fraction, the "Distilling Constant" for the fraction, is plotted against the corresponding amount of the distillate on logarithmic coordinate paper. Pure acids are graphically represented as straight lines. With mixtures of acids, curves are obtained, the nature of which depends on the acid components.

With a mixture of any two volatile acids, the first part of the curve which is obtained occupies a position intermediate to the lines representing the distilling rate of the higher and lower boiling acids. As the higher boiling acid is removed, the curve gradually becomes parallel with the line representing the lower boiling acid. This serves to identify the lower acid with certainty. Dyer showed that it was possible to ascertain the amount of each acid present when the distilling rate of the mixture had been determined, and the acids present were known. He also gave a number of chemical tests to enable one to identify the acids present in the distillates.

The method appeared to present obvious advantages for the work in which we were engaged, and we therefore made a certain number of preliminary experiments before using it on fermentation mixtures.

Our apparatus differed in one essential from that of Dyer, in that the heating of both flasks was done by gas instead of electricity. By using von Babo's flask guards underneath the distilling and steam generating flasks, and especially by insulating heavily all connecting tubes throughout the apparatus, it was possible to regulate the heating flame, so that the volume of the acid mixture kept constant through a long distillation.

Our first experiments were made with pure acids. The acids chosen were those that are known to be present in fermentation liquors, and ranged from formic to caproic acids.

The distilling rates of these acids were then graphically represented on a logarithmic chart, and the lines which were obtained were used to interpret the results of other distillation experiments. The distilling rates found were very similar to those obtained by Dyer.

Dyer used his method to identify the lower boiling acid in any given mixture by means of the parallelism of the curve representing the last portion of his distillate to a known curve. He identified the higher boiling acid by means of chemical tests. Working on artificial mixtures of known acids, this is satisfactory. With an unknown mixture of several acids resulting from a fermentation experiment, this does not apply. We were therefore compelled to modify Dyer's method in a manner which made it much more tedious, but ultimately allowed us to arrive at more satisfactory results.

The reasons for making this change were based on the following considerations. When a mixture of two acids is distilled the nature of the curve obtained will depend on the distilling rate of each component. With a mixture containing an acid high in the series, for example, caproic acid, the first part of the curve will approximate to the straight line on the chart which represents caproic acid, and the degree of this approximation will depend on the relative amount of that acid present.

The final part of the curve will become parallel to the straight line on the chart which represents the lower acid.

If present in small amount the higher acid can be concentrated and partially separated from the lower acid by fractionation so that the curve obtained on redistillation will approximate so closely to the acid line for caproic acid that it can thereby be identified.

That is, we applied Dyer's method to determine the nature of fractionated portions of the fermentation liquid; and since the amount of acid in each of these portions was known, the calculation of the separate acids in the fermentation liquid could be made. Using caproic acid and butyric acid in a mixture, by means of this method of fractionation it was possible to obtain two portions of the mixture, one containing a relatively large amount of caproic acid and the other consisting chiefly of butyric acid. When distilled separately the curves obtained approximated to the lines for caproic acid and butyric acid on the chart.

When more than two acids are present the problem of identifying and estimating them becomes complicated. The difficulty is always with the acids of intermediate boiling point and appears in any method of separation of the acid series. In the Duclaux method this is emphasised in a recent criticism by Upson, Plum and Schott [1917]. In our opinion, Dyer's method affords no solution of this problem of dealing with the intermediate acids of a mixture. In conjunction with it we have had to resort to a process of fractionation which we describe below.

For example, in a mixture containing acetic, butyric, valeric and caproic acids, it is impossible without a number of refractionations, which is beyond the scope of ordinary technique, to separate these acids so that the colour tests for each will be distinctive. Moreover, the colour tests, which are undoubtedly helpful in determining what acids are present, are not specific; and unless the acid is present in sufficient quantity a positive result will not be obtained.

In making a quantitative analysis of a fermentation liquid we found it necessary to proceed in the following way:

(1) To distil a portion exhaustively in order to find the total amount of volatile acid present.

(2) To distil the fermented liquid as a simple acid mixture, according to Dyer's method, and graphically represent the distilling rate on a logarithmic chart. The curve obtained indicates the lowest acid and the probable nature of the highest acid in the liquid.

(3) To collect fractionated portions of a series of separate steam distillations conducted under conditions identical with the above. The total amount of acid in these portions can be calculated from the previously ascertained distilling rate of the mixture.

The application of chemical tests to the concentrated distillates of these fractions affords useful information at this stage.

(4) Finally, from consideration of the data so obtained to prepare an artificial mixture which will show a distilling rate sufficiently close to that of the fermentation liquid to warrant the assumption that they correspond in composition.

As will be seen from the above, the method is tedious in application, but it certainly gives important quantitative information regarding the acids produced by various organisms. At the same time it must be emphasised that the process above described will afford only approximate results.

After a large amount of preliminary work on mixtures of pure acids from which the limit of accuracy of the method was ascertained, we proceeded to examine some of the fermentation liquids yielded by *B. perfringens* and *B. sporogenes*.

The organisms were grown in vacuo in a Winchester quart bottle in various media.

The apparatus used for incubation was that employed by Wolf and Harris [1917]. Usually 1500 cc. of culture medium was employed, and samples taken from time to time. In all instances the production of gas was followed to correlate gas production with amount of acid produced.

Fermentation No. 1.

Bacillus sporogenes (*Metchnikoff*) *on milk.*

100 cc. of the sample of liquid were taken from the fermentation flask under aseptic precautions, placed in the distilling flask and rendered acid to Congo red paper by the addition of normal sulphuric acid.

The volume was then made up to 150 cc. and the flask connected to the condenser. The burner employed to heat the flask was placed in position, and at the first appearance of bubbles in the liquid, the gas jet was lowered so as to give a definite size of flame. The steam generating flask which contained water actively boiling was immediately connected to the distilling flask and the distillation begun. Fractions of 10 cc. were collected up to 50 cc. Then successively larger fractions of 50 cc., 100 cc., 200 cc., and 500 cc. were collected. It was usually found unnecessary to continue the distillation beyond 500 cc. in order to obtain sufficient data for the acid curve.

The total amount of acid present was obtained by exhaustively distilling another portion of the original liquid in a separate apparatus, and titrating the distillate with decinormal sodium hydrate. In all these experiments phenolphthalein was the indicator used.

Table I. *Fermentation at the end of* 117 *hours.*

100 cc. liquid = 47·6 cc. $N/10$ sodium hydrate.

Fraction of dist. in cc. ...	10	20	30	40	50	100	200	400	500
% acid in fraction ...	15·3	26·0	34·6	41·9	47·6	64·6	76·5	87·2	91·0

The figures in the lower column denoting the "Distilling Constants" were plotted on the chart against the corresponding fraction of the distillate.

From a consideration of the curve obtained (Fig. 1), it will be seen that the fermentation liquid contains a mixture of higher and lower volatile fatty acids, and that the curve tends to run parallel with the curve for acetic acid, after 75 % has distilled over.

The "distilling constant" for the first 10 cc. fraction (15·3) is very high when the presence of acetic acid in the mixture is taken into account, and caproic acid or higher acids were therefore suspected.

On applying Dyer's colour tests to the first portions of the distillate the presence of caproic acid was confirmed. The process of fractionating the fermentation liquid was therefore next employed in the following manner.

Fractional distillation.

100 cc. of the liquid were acidified with decinormal sulphuric acid and the volume made up to 150 cc. This was then steam distilled under the usual conditions of constant volume.

The first 25 cc. were collected and labelled "*A*." The intermediate portion distilling over between 25 cc. and 75 cc. labelled "*B*"; and the third portion distilling over between 75 cc. and 175 cc. labelled "*C*."

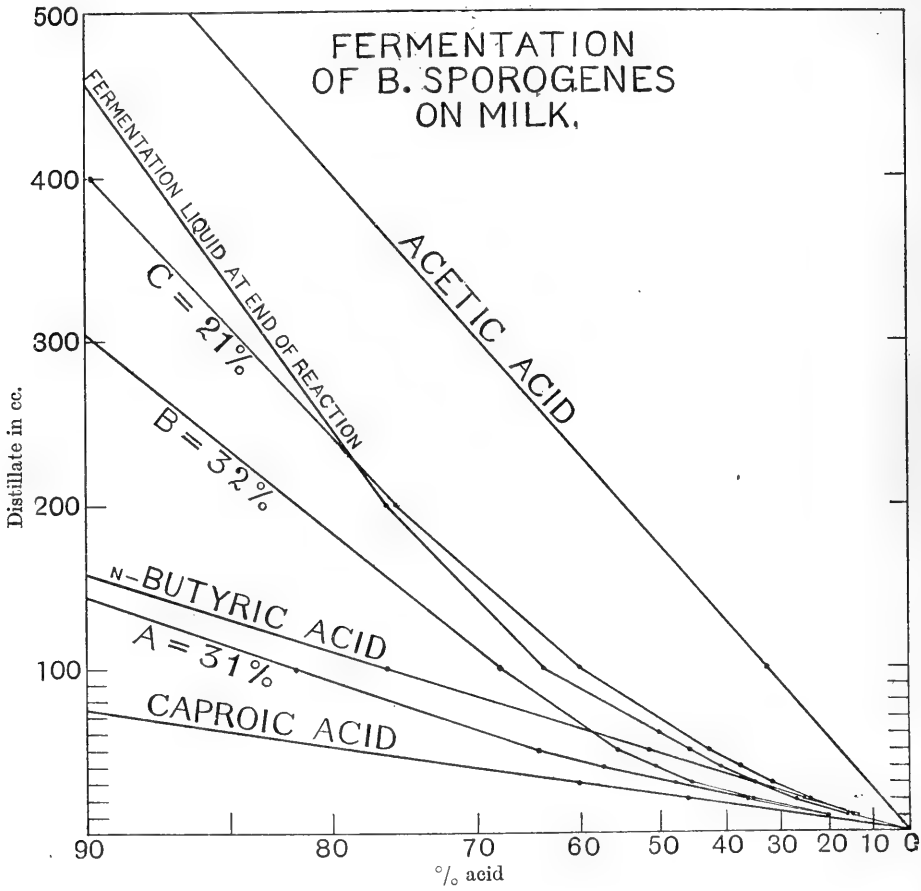

Fig. 1

This was repeated with other three portions of 100 cc. of the fermentation liquid. The four "*A*" fractions of 25 cc. were then mixed, an aliquot part drawn off and titrated; and the remainder placed in the distilling flask and the volume made up to 150 cc. This was steam distilled. The results were as follows:

TABLE II. "*A*" *portion* = 31 % *of total acid.*

Fraction in cc.	10	20	30	40	50	100	200	400
% acid in fraction	20·5	36·0	47·6	57·1	64·4	83·3	92·8	95·7

On examining this curve ("*A*" Fig. 1), it will be seen that it runs very nearly parallel with the line of *n*-butyric acid after 65 % of the total acid has distilled over.

The very high distilling constant for the first 10 cc. indicates that caproic acid was probably present, since it approximates to the "distilling constant" for that acid which is 22·6 %.

After concentrating the distillates caproic acid was definitely proved to be present by the colour tests. In this portion there must have been an almost complete absence of acids lower in the series than butyric acid, so that the 31 % of the acid of the fermentation liquid consisted almost entirely of acids high in the series and of these caproic and butyric acids were two.

The intermediate portion "*B*" was dealt with in a similar way, but since the mixed fraction constituting this portion amounted to 400 cc. it was evaporated on the water-bath after neutralisation with decinormal sodium hydrate and made up to 100 cc. exactly. 25 cc. of this solution were put in a separate apparatus, acidified with normal sulphuric acid to liberate the acids and exhaustively distilled. From the titration of this distillate the total amount of acid in the remaining 75 cc. was calculated.

The remaining 75 cc. were then acidified with normal sulphuric acid, made up to 150 cc. and steam distilled.

TABLE III. "*B*" *portion* = 32 % *of total acid.*

Fraction in cc.	10	20	30	40	50	100	200	400
% acid in fraction	20·4	35·6	45·0	50·4	55·4	68·0	81·7	93·0

The very high "distilling constant" (20·4) obtained for the first fraction indicates that caproic or higher acids were also present in this fraction. The curve ("*B*" Fig. 1) takes a very decided bend near the point when 55 % of the acids have distilled over indicating that these are of a mixed nature. With ferric chloride and ether a test corresponding to valeric acid was given, but the colour test for caproic acid was not positive.

This fraction, amounting to 32 % of the total acid of the fermentation liquid, consisted then of valeric acid as well as of lower acids. The amount of caproic acid, which was undoubtedly present, was evidently too small to give a positive colour test.

The third portion "*C*" was dealt with in exactly the same way as the

preceding. It corresponded to 21 % of the total acid in the fermentation liquid.

The results of the distillation were as follows:

TABLE IV. *"C" portion = 21 % of total acid.*

Fraction in cc.	10	20	30	40	50	100	200	400
% acid in fraction	14·9	24·0	31·6	37·3	42·5	59·9	76·0	89·8

When the distillates from this portion were concentrated and the ether-ferric chloride test applied none of the precipitated iron salt was found to be soluble in the ether solution, indicating the absence in quantity of acids higher than butyric in this portion. The last part of the curve ("*C*" Fig. 1) was almost parallel with the normal curve for acetic acid. Formic acid was never detected in any portion of the distillate.

This fraction, corresponding to 21 % of the total acid in the fermentation liquid, must have had butyric acid as its chief constituent along with a certain amount of acetic acid.

An analysis of the above results leads to the following conclusions:

(1) Caproic, valeric, butyric and acetic acids were present in the fermentation liquid since these have been demonstrated in the various fractions.

(2) From "*A*" portion (Table II), that 31 % of the acid of the fermentation liquid consisted almost wholly of butyric, valeric, caproic and higher acids.

(3) In the "*B*" portion (Table III), which corresponded to 32 % of the total acids in the fermentation liquid, about one-half consisted of butyric and valeric acids.

(4) Acetic acid was present in considerable quantity both in the "*B*" and "*C*" portion.

(5) Formic acid was absent.

It was impossible to estimate with any degree of accuracy the amount of these acids present. Nor could the presence or absence of propionic acid be definitely established either by colour tests or by consideration of the acid curves obtained.

The remaining quantity of acid which had not been dealt with as a fraction was equivalent to 16 % of the total acids in the fermentation liquid. It must have consisted chiefly of lower acids, probably acetic acid.

The problem then was to determine in what proportion the various acids were present. After some consideration it was decided that an attempt might be made to arrive at a solution by distilling artificial mixtures of caproic, valeric, butyric and acetic acids the result of which would give a close approximation to the figures obtained in distilling the fermentation liquid.

In making up such a mixture the calculation of the relative quantities of the components to be chosen was based on the rough estimate of the composition of the fermentation liquid which has just been made.

After several trials the following mixture was distilled and the results tabulated:

Acetic acid	25·2 %
Butyric ,,	17·2
Valeric ,,	16·2
Caproic ,,	41·4

TABLE V.

Fraction in cc.	10	20	30	40	50	100	200	400	500
% acid in fraction	14·9	25·6	34·1	41·4	47·6	65·2	77·5	87·7	90·0

The distilling rate of this mixture is an approximation to that of the fermentation liquid, as will be seen when the two tables are compared. It is clear however that the composition of the artificial mixture cannot be regarded as any more than this. No account has been taken of the presence or absence of propionic acid, or of small quantities of acid higher in the series than caproic acid; and it was found that the amount of intermediate acids could be varied considerably without appreciably changing the distilling rate of the mixture.

FERMENTATION No. 2.

B. perfringens *on milk.*

The fermentation liquid obtained by the growth of *B. perfringens* on milk under strict anaerobic conditions was subjected to a similar examination. As before, the sample was drawn off at the end of the fermentation when no further gas was evolved, and a quantity distilled exhaustively to determine the total amount of volatile acids present. 100 cc. of the liquid were then steam distilled under the usual conditions, the distilling rate of the acid mixture determined, and the results plotted on the logarithmic chart.

TABLE VI.

Fraction in cc.	10	20	30	40	50	100	200	400	500
% acid in fraction	8·4	15·8	22·3	28·2	33·3	52·3	71·3	86·6	91·3

From a consideration of the curve (Fig. 2) it will be seen that 52·3 % of the whole distills over in 100 cc., suggesting that butyric acid and some lower acids were present in the mixture. The presence of valeric acid and caproic acid could not be detected by colour tests when these were made, using the

first portion of the distillate, and these acids could only have been present in small quantities.

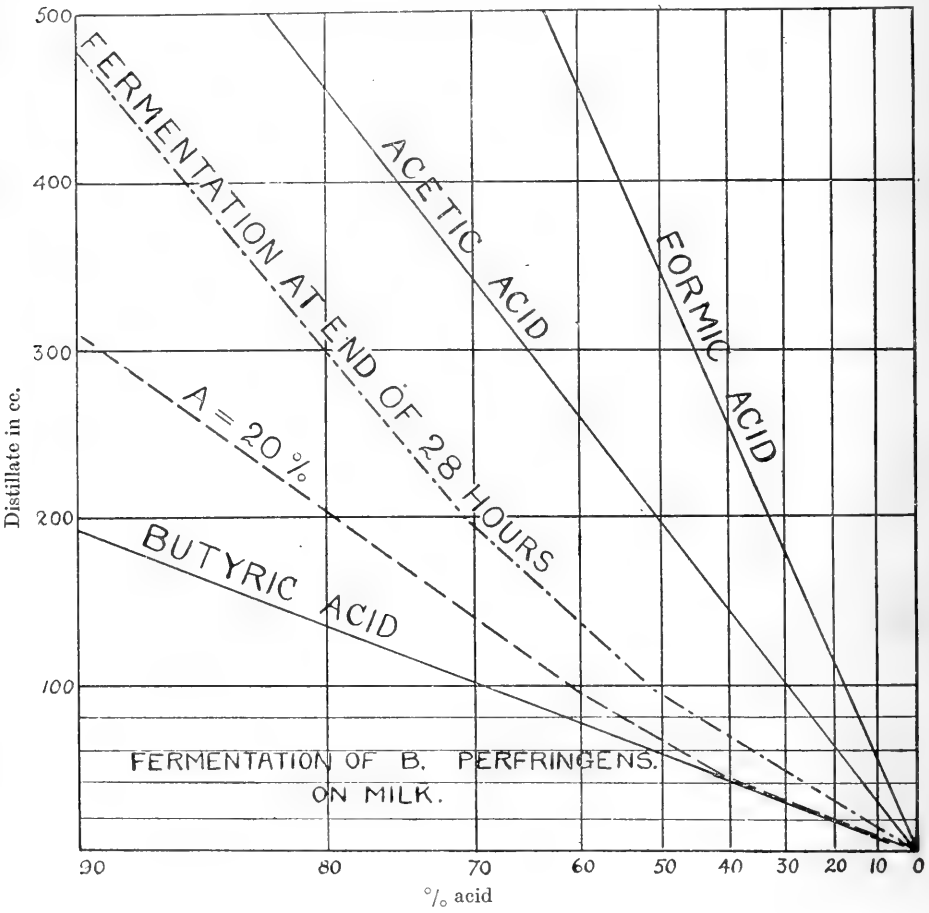

Fig. 2

Fractional distillation.

Three portions of 100 cc. each of the fermentation liquid were acidified with normal sulphuric acid and the volumes made up to 150 cc. and steam distilled. The first fractions of 25 cc. were collected and mixed. After titration of an aliquot part the remainder was steam distilled. The quantity of acid used represented 20 % of the total acid present in the fermentation liquid.

TABLE VII.

Fraction in cc.	10	20	30	40	50	100	200	400
% acid in fraction	12·3	21·7	29·6	36·8	43·3.	61·6	79·6	97·4

Acids higher in the series than butyric could not be detected by colour tests in this portion which probably consisted of butyric and lower acids. This is evident from the nature of the curve which finally becomes parallel to that of acetic acid. Also the "distilling constant" for the first 10 cc. (12·3) is considerably higher than that of propionic acid. Since valeric and higher acids are not present in any great quantity, butyric acid only could account for this fairly high figure. It was unnecessary to consider further fractionation since the assumption was that butyric and acetic acids constituted this mixture.

Assuming that the fermentation liquid consisted chiefly of butyric acid and acetic acid, it should be possible to find out what amount of each was present from its "distilling constant" by means of a simple calculation.

The "distilling constant" for 50 cc. of the fermentation liquid containing the mixed acids ... ... = 33·3 %

"Distilling constant" of butyric acid ... ... = 45·6

"Distilling constant" of acetic acid ... ... = 16·5

So that such a mixture would correspond approximately to 42 % acetic acid and 58 % butyric acid.

A mixture containing 40 % acetic acid and 60 % butyric acid was distilled under the usual conditions with the following results:

TABLE VIII. *Total acid* = 63·9 *cc.* *N*/10 *sodium hydrate.*

Fraction in cc.	10	20	30	40	50	100	200	400	500
% acid in fraction	8·6	15·8	22·4	28·5	33·8	53·5	73·9	89·0	92·5

When this table is compared with Table VI it will be seen that the fermentation liquid obtained from the action of *B. perfringens* on milk consisted approximately of the volatile fatty acids, butyric and acetic, in the proportions distilled.

It is of course open to objection that no account has been taken of the possible presence of propionic acid or of the acids higher in the series.

The colour test for propionic acid which is given by Dyer is perhaps the least definite of all; and since this acid occupies a position intermediate to butyric and acetic acids an attempt at isolation would entail a long and careful fractional distillation.

The artificial mixture of butyric and acetic acids can therefore only be regarded as an approximation to the composition of the fermentation liquid.

FERMENTATION No. 3.

B. sporogenes *on* 2 % *glucose peptone.*

100 cc. of the liquid at the end of fermentation was placed in the distilling flask, rendered acid to Congo red with normal sulphuric acid and the volume made up to 150 cc.

This was steam distilled under constant volume and the fractions collected and titrated as before.

The results given in Table IX were plotted on the chart. The total amount of acid present was obtained by distilling a separate 100 cc. exhaustively.

TABLE IX.

Fraction in cc.	10	20	30	40	50	100	200	400	500
% acid in fraction	7·2	13·2	18·7	23·8	28·5	44·9	65·5	86·1	92·4

From a consideration of the curve obtained it was seen that the final part tended to become parallel with the line for acetic acid. The "distilling constant" for the first 10 cc. fraction is 7·2, that for the first 10 cc. of a solution of pure propionic acid is 7·03. But since acetic acid is present in the mixture, the "distilling constant" of which is 3·5, an acid with a much higher distilling rate than propionic acid must be present.

Colour tests were applied to the collected distillates and also to fractions which contained the higher acids in greater proportion. In none of these was the presence of valeric acid or acids higher in the series shown.

The acid associated with acetic acid in the fermentation liquid must then have been butyric acid, the "distilling constant" for the first 10 cc. of which is 11·47 %.

In order to confirm the presence of this acid the fermentation liquid was subjected to a fractional distillation as before, the higher acids being obtained in more concentrated form. The following table shows the results obtained from the redistillation.

TABLE X. *Fraction* = 15 % *of total acid.*

Fraction in cc.	10	20	30	40	50	100	200	400	500
% acid in fraction	10·7	19·8	27·7	34·4	39·9	58·9	77·2	91·5	95·4

The first 10 cc. gives a "distilling constant" of 10·7 %. This indicates conclusively the presence of butyric acid in the fermentation liquid.

Assuming that butyric and acetic acids alone were present, a mixture containing these in pure form was made up and redistilled. The proportion in which they were mixed was obtained, as in the previous case, by a calcula-

tion based on the distilling rates of the fermentation liquid, pure acetic acid and pure butyric acid.

The "distilling constant" for the first 50 cc. of the fermentation liquid ... ... = 28·5 %

"Distilling constant" of butyric acid ... ... = 45·6

"Distilling constant" of acetic acid ... ... = 16·5

To obtain a mixture of butyric and acetic acids in which the first 50 cc. shall contain 28·5 % of the total acid, these acids must be present in the following proportion:

Butyric acid = 41 %

Acetic ,, = 59

Such a mixture when distilled gave the following results:

TABLE XI.

Fraction in cc.	10	20	30	40	50	100	200	400	500
% acid in fraction	7·0	13·3	18·9	23·8	28·3	45·5	64·6	84·6	90·9

Comparing this table with the results obtained from the distillation of the fermentation liquid shown in Table IX it will be observed that the latter must be similar in composition to the artificial mixture.

As before, no account has been taken of the presence or absence of propionic acid in the fermentation liquid, because it was impossible by colour tests or a consideration of the curve to determine its presence or absence. The close approximation of the curve of the above artificial mixture to that of the fermentation liquid cannot be taken as proof that the fermentation liquid contained no propionic acid. By the introduction of a certain quantity of propionic acid into a mixture of butyric and acetic acids a curve almost identical with that of acetic and butyric acids could be obtained.

The following example will illustrate this:

A mixture of butyric and acetic acids in almost equal parts was distilled under the usual conditions, and the distilling rate obtained.

The same mixture of these two acids was taken and 20 % of propionic acid added. This second combination was distilled under the same conditions as the first.

The results showing distilling rates of the two mixtures are tabulated for comparison.

TABLE XII.

Fraction in cc.	10	20	30	40	50	100	200	400	500
Butyric and acetic acid	8·0	14·9	21·1	26·6	31·7	51·2	73·4	91·0	95·0
Butyric and acetic acid with 20 % propionic	7·7	14·5	20·5	26·1	31·2	51·0	73·1	90·3	94·1

Here we have two acid mixtures differing widely in composition yet giving almost identical distilling rates.

This experiment could be repeated with a combination of any three other consecutive acids in the series, so as to demonstrate generally that the introduction of the "intermediate" acid in moderate amount has little effect on the distilling rates of the combination of the other two.

There appears to be no way out of this difficulty of dealing with the "intermediate" acid except by a long and tedious fractionation.

Fermentation No. 4.

B. perfringens *on* 2 % *glucose peptone.*

100 cc. of the sample at the end of fermentation was taken, acidified with normal sulphuric acid till acid to Congo red, the volume made up to 150 cc. and distilled as before.

The following results were obtained:

Table XIII.

Fraction in cc.	10	20	30	40	50	100	200	400	500
% acid in fraction	7·4	13·3	18·1	22·5	26·7	43·7	65·0	87·6	93·5

Comparing this with the results of the previous experiment, it will be seen that the volatile fatty acids produced by the action of *B. perfringens* and *B. sporogenes* on 2 % glucose peptone are almost identical. Also the total quantity of volatile acid formed by each organism was found to be the same. This fact is interesting when the great difference in the nature of the acid production by the growth of the organisms on milk is considered. Large quantities of valeric and caproic acids result from the action of *B. sporogenes* on the latter medium and this distinguishes this organism at once from *B. perfringens* which apparently produces very small amounts of these higher acids. A single distillation of a fermented milk would always serve to differentiate the two, but this would be impossible if the medium were glucose peptone.

On reviewing the above experiments we concluded that Dyer's method may be employed with limitations in the study of volatile acid production of fermented media.

It is impossible by a single distillation alone to determine from the distilling rates of the mixture the nature of the acids present. Partial isolation of the constituents is necessary to establish both the nature and the amount of each acid present. Dyer's colour tests are only of value when the acid exists in an almost pure state.

By employing the method of partial fractionation which has been described, an approximate analysis of the volatile acids present may be made. As we have shown, exact confirmation of such an analysis cannot be obtained by identifying the distilling rate of the fermentation liquid with that of an artificial mixture. As a means of obtaining comparative results the scheme of analysis which has been outlined above is decidedly useful.

When the distilling rates of a fairly large number of artificial mixtures have been graphically represented on a chart, the approximate composition of a fermented medium can at once be obtained simply by identifying the mixture with which its distilling rate coincides.

Non-volatile acid production.

The acidity of these fermentation liquors was found to be due partly to the formation of a relatively large quantity of non-volatile acids. The nature of these acids is not discussed in this paper. Their estimation was made solely to demonstrate that they cannot be neglected in any consideration of the effects of acid production. The following method was employed for their determination.

100 cc. of the fermentation liquid was acidified with normal sulphuric acid and exhaustively steam distilled. The distillate was titrated with decinormal sodium hydrate, using phenolphthalein as an indicator, and the total amount of volatile acids present ascertained.

The residue in the distilling flask, containing the non-volatile portion, was transferred to a liquid extraction apparatus and the acids dissolved out with ether. This operation was a continuous one, and extended over a period of forty-eight hours.

The ethereal solution was evaporated to dryness and the residue dissolved in water. The solution containing the acids was then filtered and titrated with decinormal sodium hydrate.

The following table shows the results obtained in the examination of the fermentation liquors which were produced by the action of *B. sporogenes* and *B. perfringens* on milk and 2 % glucose peptone.

Organism	Medium	Relative vol. acidity	Relative non-vol. acidity
B. sporogenes	Milk	69 %	31 %
	2 % glucose peptone	55	45
B. perfringens	Milk	68·5	31·5
	2 % glucose peptone	50	50

Summary.

The investigation of the acid production of our fermentations of *Bacillus perfringens* and *Bacillus sporogenes* shows that large quantities of volatile acids are produced. Of these butyric acid may be said to be a constant component, but it is worthy of note that in the action of *B. sporogenes* on milk, acids higher in the series than butyric are formed in considerable quantity. The action of *B. sporogenes* on milk results in the production of caproic and valeric acids. The presence of these higher acids is not however a specific quality of *B. sporogenes*, for the fermentation of glucose peptone by this organism produces only acetic and butyric acids. We have been unable to detect propionic acid in any of the fermentation liquids, but its presence is not excluded. Formic acid was not present.

In addition to volatile acids, there is a notable proportion of non-volatile acids, amounting roughly to 40 % of the total acid production. What the nature of these acids is has not yet been ascertained.

An experimental critique of the Dyer method of estimating volatile fatty acids has been made. This method, while perfectly satisfactory in the form stated by its author in dealing with a mixture of two volatile acids, the nature of which is known, fails when a mixture of unknown acids has to be analysed. The colour tests proposed by him are satisfactory when dealing with pure acids, but are not as positive as could be desired for the identification of an acid in the mixture. The separation of the acids is necessary before any reliance can be placed on these colour tests.

By refractionation clearer results are obtained; but when a mixture of more than two acids is dealt with, the components of which are consecutive in the acid series, the task of identifying and estimating them is a problem of extreme difficulty.

To what extent Dyer's method is applicable for this purpose has been indicated in the foregoing paper.

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XXI. CONTRIBUTIONS TO THE BIOCHEMISTRY OF PATHOGENIC ANAEROBES. III. THE EFFECT OF ACIDS ON THE GROWTH OF *BACILLUS WELCHII* (*B. PERFRINGENS*) AND *BACILLUS SPOROGENES* (METCHNIKOFF)

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In our first paper [1917] on the growth of pathogenic anaerobes we showed that growth of the bacillus of Welch ceased at a very definite hydrogen ion concentration when the organism was inoculated into milk. We also referred to the possibility of the inhibiting action of acids having a certain clinical interest in the treatment of infection by these bacteria.

The cessation of activity at a definite acidity is not a singular phenomenon. It is known that certain other bacteria, for example, the colon bacillus and *B. typhosus* and the allied *B. paratyphosus A* and *B*, cease growing at a fairly definite hydrogen ion concentration.

Clark and Lubs [1915, 1] found a certain correspondence in the final reaction and the type of colon bacillus with which they were working. Cole and Onslow [1916] claim to be able to differentiate the organisms of the typhosus group by the velocity with which they reach a certain acid reaction.

Previous to these observers, Brünn [1913] had shown that *B. coli* dies within twenty-four hours if exposed to an acid reaction of $P_H = 4{\cdot}7$ but not of $P_H = 5{\cdot}0$. Michaelis [1914] grew *B. coli* in lactose bouillon and found that the acidity always rose to but did not exceed $P_H = 5{\cdot}0$; but the amount of literature bearing on the subject of the effect of reaction on the growth of micro-organisms is not extensive. Very recently Clark and Lubs [1917] have

published a series of very interesting papers in which will be found many references to this important problem and which should be consulted by those interested in its history.

Attempts have been made by many investigators to estimate the effect of acid solutions on bacteria by simply adding definite amounts of various acids to media containing bacteria, or by treating the media with acids before inoculation. So far this method has yielded very little useful information, as the experimenters have for the most part neglected to take into account the influence of a complex medium containing many buffer substances—on the dissociation of the acids. Any investigation of the effect of acids on bacteria must be preceded by a study of the effects of the acids on the media which are being used.

So far as we are aware the only attempt to determine the effect of acids on culture media in altering their hydrogen ion concentration, where modern methods have been used, is that of Clark and Lubs [1915, 2]. Their conclusions regarding the futility of titrating media, as is done in most bacteriological laboratories, are of great importance and are fully borne out by the present work. The point at which their investigation touches our own is where they show that the effect of acids on media is not directly proportional to the quantity of acid added. In this connection it should be borne in mind that different acids affect media to different extents, depending on many factors. Amongst these one may mention the dilution, the degree of dissociation of the acids and the salts contained in the medium, and the combinations they may form with the complex substances of which bacteriological media are composed.

Clark and Lubs investigated the effect of adding lactic and hydrochloric acid to milk, peptone water, etc., and expressed the results by means of titration curves, in which the amounts of acid added were plotted against the absolute reactions resulting, as measured with the hydrogen electrode. These curves show clearly the buffer action of the media; how they absorb part of the hydrogen ions of the acid and cause the reaction to be far less acid than that which the same amount of acid would produce if added to a medium free from buffer substances. Thus, milk to which sufficient acid had been added to make the solution 0·023 *N* had a reaction of $P_H = 5{\cdot}4$, which is the reaction corresponding roughly to that of a solution of hydrochloric acid only 0·000004 *N*.

Recently Taylor [1917] has been working on the effect of acids on the growth of bacteria and claims to have obtained certain specific effects with

certain acids. He has, however, made no attempt to show what effect is due to the anions of the acid and what is due to that cation which is common to them all—namely the hydrogen ion.

This matter is of importance, for in the work of Wolf and Telfer [1917] a study has been made of the acids involved in the fermentation of *B. perfringens* and *B. sporogenes*. Should any of the acids which are normally found as the fermentation products of these organisms have a specific effect we should be inclined to their use in combating local infection. This specific action of acids cannot be excluded without experimental evidence to the contrary, for we know that a large number of anions have a very decided inhibitory effect on the growth of bacteria.

In the following experiments it will be shown that *B. perfringens* fails to grow in a glucose peptone medium containing sufficient hydrochloric acid to give the solution a reaction of $P_H = 4{\cdot}8$. This reaction corresponds to that of a solution of hydrochloric acid only 0·000016 *N* and is produced by adding to the medium containing buffer substances sufficient HCl to make it 0·02 *N* with regard to the acid itself. That this inhibitory effect is not due to the anion is proved by the fact that a 0·1 *N* solution of NaCl has no toxic action on bacteria, and may even have an accelerating effect on their growth.

On the other hand, acids like benzoic, salicylic or boric, distinctly inhibit bacterial growth, and this takes place whether they are present as free acids or as sodium salts.

We have therefore examined the effects of the more commonly occurring acids to see if any of them possess antiseptic properties outside of those conferred on them by their hydrogen ion.

The four main points to which attention has been directed in the present paper are the following:

1. What is the effect of equivalent amounts of acids of differing dissociation constants on the reaction of a standard medium?

2. Knowing this effect, what is the minimum amount of acid, and the corresponding hydrogen ion concentration, which exerts a lethal effect on the organisms chosen?

3. Is there in any of these acids a discoverable effect which cannot be set down to the hydrogen ion concentration?

4. Is the final hydrogen ion concentration, after the cessation of growth of the organisms, a so-called "physiological constant," or is it influenced by the initial reaction of the medium?

The question of the initial reaction of the medium is one of great interest

and may have an important bearing on the latent period of growth of these organisms. In a recent paper we showed that the latent period appears to have a connection with the amino-acid content of the medium, but there were many unexplained features, which we now think may have been due largely to the initial reaction of the medium. We found that the period of optimum growth on milk coincided with a very definite hydrogen ion concentration in the case of *B. perfringens* and suggested tentatively that this might be the most favourable reaction for growth. As the result of the present experiments we have been compelled to modify our views on this point.

If growth of *B. perfringens* and *B. sporogenes* ceases at a definite reaction, it is important to determine it, for in this knowledge we may have an additional weapon for the local treatment of infections of this type. As the hydrogen ion concentrations with which we are dealing are of the order of 1/50,000 *N*, it is obvious that one cannot employ highly dissociated acids of this strength, because the least trace of protein, amino-acid, or alkali from the walls of the containing vessel would at once transform this acid solution to a neutral one. It is necessary therefore to submit the organisms to these concentrations of hydrogen ions in media containing large concentrations of buffer substances, where the action of the glass or of the carbon dioxide of the air will have a much smaller or even negligible effect.

Experimental Methods.

Preparation of Media. Titration Curves.

When an acid is added to a medium such as glucose peptone water, the resulting reaction depends on several factors. Firstly, there is the buffer action of the solution itself, due to the presence of amino-acids, phosphates, etc. The degree of dissociation of the acid has also to be considered and that of the salts it may form. As a preliminary measure, therefore, we investigated the changes of reaction of the medium to which known amounts of various acids had been added.

The medium employed was a solution of peptone made by the tryptic digestion of caseinogen. This medium is quite definite in its properties and work that we have done has shown it to be very suitable for the development of *B. perfringens* and *B. sporogenes*, with or without the addition of glucose. As the presence of glucose causes a more vigorous fermentation with each organism, the solutions used in these experiments were prepared so as to contain 2 % of this sugar.

The effect of the following acids was tried: hydrochloric, lactic, formic, butyric, acetic, malic, and succinic[1].

For each acid a titration curve was drawn (Fig. 1), showing the relationship between the amount of acid contained in the medium and the absolute reaction resulting from its presence.

The following procedure—that for hydrochloric acid—was followed for each acid used, with slight modifications as to the amounts added. A double strength solution of glucose peptone water was prepared, containing 4 % of glucose and 4 % of peptone. Eight portions of 25 cc. of this were taken and pipetted into 50 cc. graduated measuring flasks, and the amounts of $N/10$ acid and water shown in Table I were added.

This gave eight lots of 2 % glucose peptone water, differing only in the amount of acid contained per 50 cc. Each lot was divided amongst five tubes containing Durham tubes, plugged and sterilised at 110° for forty-five minutes. The tubes were of hard glass and were specially selected so as to be uniform. Their dimensions were 1·5 cm. × 16·0 cm.

After sterilisation, the reaction of each lot of tubed medium was determined at 20° electrometrically, using the hydrogen electrode, the value of P_H being calculated from the E.M.F. obtained experimentally.

In later experiments only one tube from each lot was used for determining the reaction, as it was found that there was little or no difference between corresponding tubes. The remaining tubes were used for the fermentation experiments described later.

When the reactions had been measured they were plotted against the amount of acid added. The amount of acid was recorded as cc. of $N/10$ acid contained in 10 cc. of the 2 % broth. The results for hydrochloric acid and for the other acids employed are given in Table I and Fig. 1. It will be seen from the curves that the amounts of different acids required to produce the same reaction vary considerably. Highly dissociated acids, such as hydrochloric and lactic, produce a given reaction when added in much smaller quantities than weaker acids such as acetic and butyric. These latter acids, too, soon arrive at a value of P_H where large additions of acid cause only slight changes in the value of P_H; that is to say, they are very highly influenced by the action of the buffer, the peptone solution.

[1] An attempt was made to obtain a titration curve for trichloroacetic acid, and also to observe the effect of this acid on the growth of organisms. As might have been foreseen, however, the acid decomposed during the sterilisation, yielding chloroform and carbon dioxide. These latter passed off as vapour and the media remaining possessed the reaction of ordinary glucose peptone water to which no acid had been added. On inoculation with *B. perfringens* the whole of the tubes fermented vigorously.

TABLE I. *Data for Titration Curves.*

Hydrochloric Acid.

Tube No.	50 cc. of broth made up from 25 cc. double strength broth and: cc. of $N/10$ acid	50 cc. of broth made up from 25 cc. double strength broth and: water cc.	Amount of $N/10$ acid contained in 10 cc. of broth cc.	Reaction, P_H
1	0	25·0	0	6·63
2	3·0	22·0	0·6	6·03
3	4·5	20·5	0·9	5·78
4	6·0	19·0	1·2	5·52
5	7·5	17·5	1·5	5·30
6	9·0	16·0	1·8	4·99
7	10·5	14·5	2·1	4·75
8	12·0	13·0	2·4	4·60

Lactic Acid.

1	0	25·0	0	6·63
2	2·05	22·95	0·41	6·20
3	4·10	20·95	0·82	5·78
4	6·15	18·85	1·23	5·46
5	8·20	16·80	1·64	5·05
6	10·30	14·70	2·06	4·70
7	12·35	12·65	2·47	4·56
8	14·40	10·60	2·88	4·38
9	16·45	8·55	3·29	4·28

Formic Acid.

1	0	25·0	0	6·44
2	2·0	23·0	0·4	6·10
3	4·0	21·0	0·8	5·74
4	6·0	19·0	1·2	5·42
5	8·0	17·0	1·6	5·06
6	10·0	15·0	2·0	4·77
7	12·0	13·0	2·4	4·60
8	14·0	11·0	2·8	4·44
9	16·0	9·0	3·2	4·30

Malic Acid.

1	0	25·0	0	6·52
2	2·5	22·5	0·5	6·03
3	5·0	20·0	1·0	5·65
4	7·5	17·5	1·5	5·34
5	10·0	15·0	2·0	5·10
6	12·5	12·5	2·5	4·89
7	15·0	10·0	3·0	4·71
8	17·5	7·5	3·5	4·61

TABLE I. *Data for Titration Curves.—Continued.*

Acetic Acid.

Tube No.	50 c.c. of broth made up from 25 c.c. double strength broth and: cc. of $N/10$ acid	water c.c.	Amount of $N/10$ acid contained in 10 cc. of broth cc.	Reaction P_H
1	0	25·0	0	6·52
2	3·0	22·0	0·6	5·98
3	6·0	19·0	1·2	5·56
4	9·0	16·0	1·8	5·21
5	12·0	13·0	2·4	4·95
6	15·0	10·0	3·0	4·81
7	17·5	7·5	3·5	4·75
8	20·0	5·0	4·0	4·65
9	22·5	2·5	4·5	4·60

Butyric Acid.

1	0	25·0	0	6·63
2	2·5	22·5	0·5	6·20
3	5·0	20·0	1·0	5·80
4	7·5	17·5	1·5	5·47
5	10·0	15·0	2·0	5·26
6	12·5	12·5	2·5	5·02
7	15·0	10·0	3·0	4·90
8	17·5	7·5	3·5	4·80
9	20·0	5·0	4·0	4·76

Succinic Acid.

1	0	25·0	0	6·19
2	2·5	22·5	0·5	5·84
3	5·0	20·0	1·0	5·55
4	7·5	17·5	1·5	5·36
5	10·0	15·0	2·0	5·17
6	12·5	12·5	2·5	5·06
7	15·0	10·0	3·0	4·93
8	17·5	7·5	3·5	4·83

Reaction Series. Inoculation and Fermentation.

Following the methods outlined above, various lots of media at different reactions were prepared, using the above-mentioned acids. After sterilisation, one tube from each lot of medium was opened and its reaction determined. After a few preliminary experiments we were usually able to obtain media for each acid with reactions fairly evenly distributed between $P_H = 4·0$ and $P_H = 7·0$.

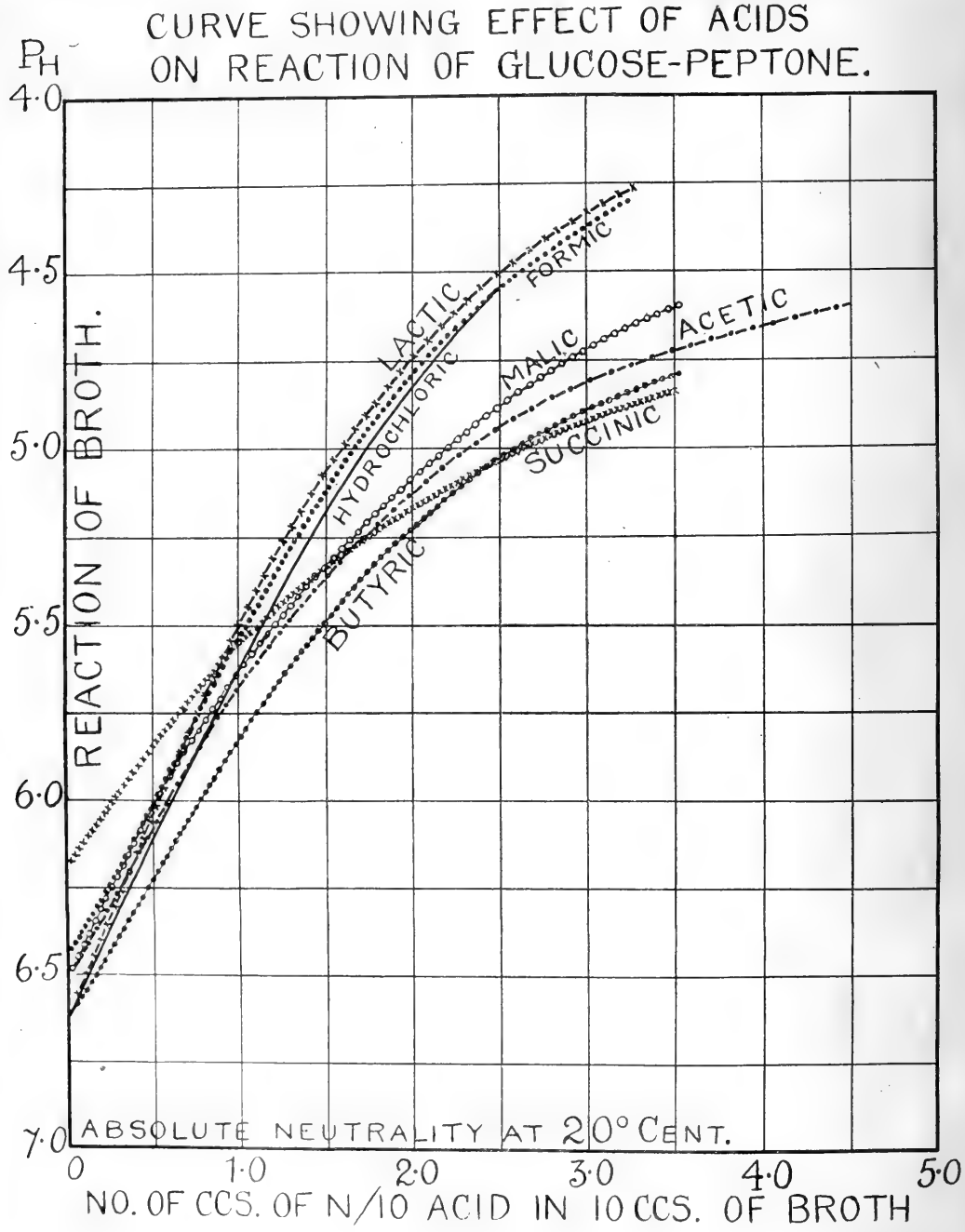

CURVE SHOWING EFFECT OF ACIDS
ON REACTION OF GLUCOSE-PEPTONE.
PH
4·0
4·5
5·0
5·5
6·0
6·5
7·0
REACTION OF BROTH.
LACTIC
FORMIC
HYDROCHLORIC
MALIC
ACETIC
SUCCINIC
BUTYRIC
ABSOLUTE NEUTRALITY AT 20° CENT.
0
1·0
2·0
3·0
4·0
5·0
NO. OF CCS. OF N/10 ACID IN 10 CCS. OF BROTH

Fig. 1

The remaining tubes were arranged into four similar "series," these latter consisting of one tube from each lot of medium. The series were called Series A, Series B, etc. The least acid member of the series was numbered 1, the others being numbered consecutively with increase of acidity.

The inoculation was made with a Pasteur pipette, each tube receiving three drops of the seed culture. As far as possible, a different culture was used for each series, so that representative results could be obtained. We were careful to use cultures, which, in our opinion, based on previous work, were vigorous.

After inoculation, the contents of each tube were covered aseptically with a layer of liquid paraffin previously sterilised, about 0·5 cm. in depth, to ensure anaerobic conditions. The necessity of this we have since shown to be open to serious doubt, but as our first experiments were conducted with the use of paraffin we continued to use it.

The inoculated tubes were now incubated at 37°. *Perfringens* cultures were allowed to remain in the incubator for three days before examination. It is advisable to give *B. sporogenes* four days to complete fermentation.

At the end of the incubation period, the series were examined and those tubes which had fermented were noted. Samples were then taken from each tube and the reaction of the fermented liquid determined, using the hydrogen electrode.

Reaction Resultants.

These final reactions proved to be of great interest and it will be well at this point to describe our method of plotting them and obtaining what we have named *reaction resultants*.

Fig. 2 shows the reaction resultant for *B. perfringens* grown on glucose peptone water acidified with varying amounts of hydrochloric acid.

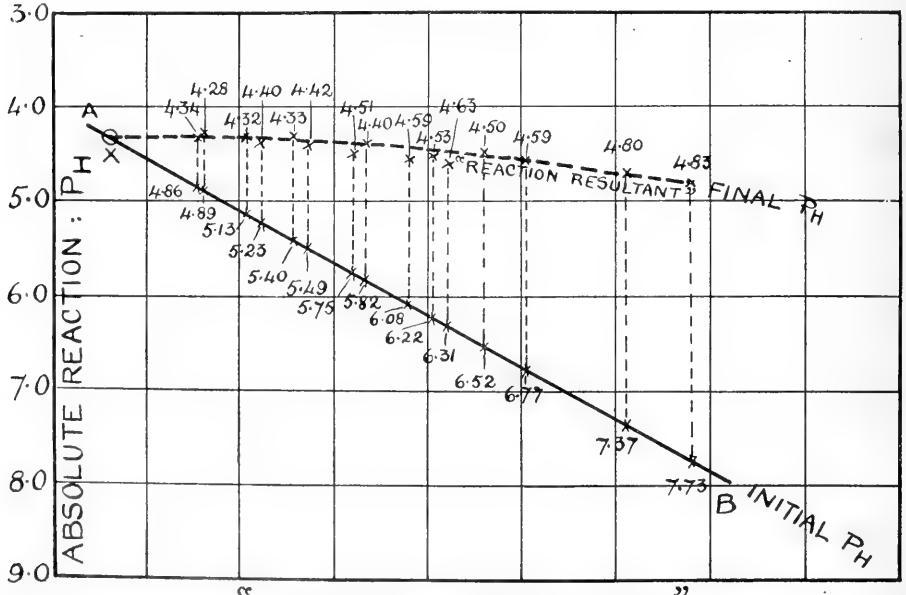
On ordinary squared paper a vertical line is taken for one axis and a scale of ordinates is chosen to represent the range of reactions over which we are working. Then at an angle of 120° to the vertical a line AB is drawn. This becomes the second axis. The only scale actually employed in plotting is the vertical one, as it will be seen from simple geometrical considerations that AB is conveniently divided into a scale by the parallel horizontal lines of the squared paper passing through the ordinates. On AB, the point corresponding to the initial reaction of the particular medium is marked off. Then, on a line through this point and parallel to the vertical axis, the point corresponding to the final reaction of the medium is marked. For each initial reaction marked on the line AB, a point for the final reaction is found in a similar manner, and

through the series of points thus obtained, a smoothed curve is drawn. This curve we have named the *reaction resultant.*

In employing this method of plotting results, what we have actually done is to make use of a pair of axes at an angle of 120° to each other, instead of being at right angles as axes usually are. The choice of the angle between the axes is only governed by considerations of general convenience. Where comparative results are required, the angle must necessarily be definitely fixed.

In Fig. 2, the reaction resultant is derived from large numbers of fermentations in which *B. perfringens* has acted on glucose peptone water containing various added amounts of hydrochloric acid. In the figure, only fifteen points are shown owing to lack of space, but we have many other confirmatory points from additional experiments.

It will be observed that the points recording the final values of P_H lie very close to the smooth curve that has been drawn to represent them, and establish quite definitely the form of this line.



"REACTION RESULTANT."
B. PERFRINGENS ON GLUCOSE-PEPTONE.
ACID REACTIONS PRODUCED BY HYDROCHLORIC ACID.

Fig. 2

Bacillus perfringens.

Data are given below for experiments carried out with *B. prefringens* on glucose peptone water in the presence of various acids.

The tables show the initial and final reactions of the media in which fermentation took place. In cases where the bacillus was able to ferment the medium a positive sign (+) is placed under the heading "Result"; where no fermentation took place there is a negative sign (−).

It was found that in tubes where there was no sign of fermentation—as evidenced by the absence of gas or turbidity in the medium—the reaction remained unchanged at the end of the incubation period.

The final reactions were plotted against the initial reactions by the method previously described. When two or three similar series of tubes were used, the mean value of the final P_H for each lot of medium was taken for the purpose of plotting the reaction resultant.

Special interest is attached to the particular value of initial P_H at which the bacillus loses its power to ferment, and this has been noted in the following experimental data.

It should be noted that the reactions of the media were determined at 20°, whereas the fermentations took place in the incubator at 37°. At the higher temperature, the media would have a higher acidity than at 20°. The difference is not considerable but cannot be overlooked.

We have not made experiments touching the change in reaction of the media with change in temperature, but if we may be allowed to use Michaelis' figures for sodium acetate—acetic acid mixtures which will probably serve the purpose, 0·8 millivolt may be subtracted for each degree above 20°.

This would give, for a rise of 17°, a decrease in E.M.F. of 13·6 mv. This correction on a P_H of 4·67 would give a final corrected value of $P_H = 4·45$.

Experiment No. 1 (Laboratory Experiment R. S. 1). Hydrochloric acid and sodium hydroxide.

In this preliminary experiment not only was hydrochloric acid added to the peptone water, but also sodium hydroxide to some of it in order to give media with a reaction above $P_H = 7·0$. There was every sign of caramelisation of these alkaline media during the sterilisation, and the experiments were discontinued[1].

Data (see also Fig. 3).

Series A. Inoculated with K 71 (thirteen hours old on milk).

[1] The effect of sterilisation on the reaction of media was investigated by the older methods by Hesse [1904]. He observed an increase in titratable acidity after heating. Clark and Lubs [1917] have investigated the change using the hydrogen electrode and have found an increase in P_H ranging from 0·01 with Witte's peptone to 0·44 with skimmed milk. The change is partly dependent on the carbohydrate content of the medium and the hydrogen ion concentration of the solution. By oxidation organic acids are formed. The caramelisation is due to melanines, formed by interaction of amino-acids and glucose [Maillard, 1913].

Series B. Inoculated with K 72 (thirteen hours old on milk).

The tubes were examined seventy-two hours after inoculation.

The most acid tube to ferment was No. 3, Series A, with value of $P_H = 4{\cdot}89$.

The next set of tubes, with $P_H = 4{\cdot}3$, failed to ferment.

TABLE II. (*Exp.* 1.)

No. of tube	Initial reaction, P_H	Series A		Series B	
		Result	Final reaction, P_H	Result	Final reaction, P_H
1	3·9	–	3·87	–	3·92
2	4·3	–	4·33	–	4·33
3	4·89	+	4·28	–	4·88
4	5·78	+	4·40	+	4·38
5	6·77	+	4·59	+	4·59
6	7·37	+	4·75	+	4·83
7	7·73	+	4·83	+	4·80
8	8·1	+	4·80	+	4·76

Experiment No. 2 (Laboratory Experiment R. S. 2). Hydrochloric acid.

Data (see also Fig. 3).

Series A. Inoculated with K 80 (fifteen hours old on milk).

Series B. Inoculated with K 78 (fifteen hours old on milk).

Series C. Inoculated with K 79 (fifteen hours old on milk).

The tubes were examined seventy-two hours after inoculation.

The most acid tube to ferment was No. 5, Series A, with value of $P_H = 4{\cdot}86$.

The next set of tubes, with $P_H = 4{\cdot}60$, failed to ferment.

TABLE III. (*Exp.* 2.)

No. of tube	Initial reaction, P_H	Series A		Series B		Series C	
		Result	Final reaction, P_H	Result	Final reaction, P_H	Result	Final reaction, P_H
1	6·22	+	4·54	+	4·52	+	4·53
2	5·82	+	4·40	+	4·44	+	4·37
3	5·40	+	4·33	+	4·33	+	4·35
4	5·13	+	4·34	+	4·32	–	...
5	4·86	+	4·34	–	...	–	...
6	4·60	–	...	–	...	–	...
7	4·36	–	...	–	...	–	...
8	4·19	–	...	–	...	–	...
9	3·78	–	...	–	...	–	...

Experiment No. 3 (Laboratory Experiment R. S. 5). Lactic acid.

Data (see also Fig. 3).

Series A. Inoculated with K 107 (twelve hours old on milk).

Series B. Inoculated with K 109 (twelve hours old on milk).

Series C. Inoculated with K 108 (twelve hours old on milk).

TABLE IV. (*Exp.* 3.)

No. of tube	Initial reaction, P_H	Series A		Series B		Series C	
		Result	Final reaction, P_H	Result	Final reaction, P_H	Result	Final reaction, P_H
1	6·19	+	4·60	+	4·57	+	4·58
2	5·46	+	4·49	+	4·51	+	4·49
3	5·15	+	4·51	+	4·51	+	4·51
4	4·85	+	4·80	−	...	−	...
5	4·61	−	...	−	...	−	...
6	4·42	−	...	−	...	−	...
7	4·26	−	...	−	...	−	...

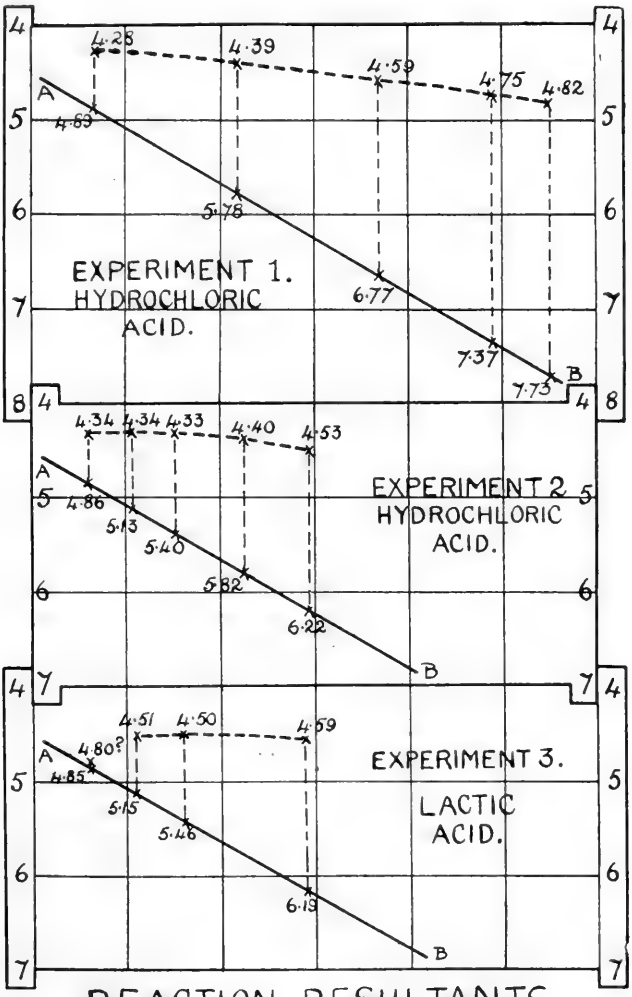


Fig. 3

The tubes were examined seventy-two hours after inoculation.

The most acid tube to ferment was No. 4, Series A, with value of $P_H = 4{\cdot}85$. The fermentation was only very slight.

The next set of tubes, with $P_H = 4{\cdot}61$, failed to ferment.

TABLE V. (*Exp.* 4.)

		Series A		Series B	
No. of tube	Initial reaction, P_H	Result	Final reaction, P_H	Result	Final reaction, P_H
1	6·44	+	4·50	+	4·50
2	6·10	+	4·39	+	4·39
3	5·74	+	4·39	+	4·41
4	5·42	+	4·40	+	4·44
5	5·06	+	4·60	?	4·81
6	4·77	–	...	–	...
7	4·60	–	...	–	...
8	4·44	–	...	–	...
9	4·30	–	...	–	...

Experiment No. 4 (Laboratory Experiment R. S. 12). Formic acid.

Data (see also Fig. 4).

Series A. Inoculated with K 127 (twenty-four hours old on cooked meat).

Series B. Inoculated with K 123 (ninety-six hours old on cooked meat).

The tubes were examined seventy-two hours after inoculation.

The most acid tubes to ferment were Nos. 5, Series A and B, with value of $P_H = 5{\cdot}06$. The fermentations were very slight in each case.

The next set of tubes, with $P_H = 4{\cdot}77$, failed to ferment.

TABLE VI. (*Exp.* 5.)

		Series A		Series B		Series C	
No. of tube	Initial reaction, P_H	Result	Final reaction, P_H	Result	Final reaction, P_H	Result	Final reaction, P_H
1	6·52	+	4·52	+	4·51	+	4·49
2	6·03	+	4·46	+	4·46	+	4·45
3	5·65	+	4·38	+	4·38	+	4·39
4	5·34	+	4·38	+	4·38	+	4·39
5	5·10	+	4·37	+	4·39	+	4·37
6	4·89	–	...	+	4·37	+	4·38
7	4·71	–	...	+	4·38	+	4·36
8	4·60	–	...	–	...	–	...

Experiment No. 5 (Laboratory Experiment R. S. 8). Malic acid.

Data (see also Fig. 4).

Series A. Inoculated with K 118 (thirteen hours old on milk).

Series B. Inoculated with K 119 (thirteen hours old on milk).

Series C. Inoculated with K 119 (thirteen hours old on milk).

The tubes were examined seventy-two hours after inoculation.

The most acid tubes to ferment were Nos. 7, Series B and C, with value of $P_H = 4{\cdot}71$.

The next set of tubes, with $P_H = 4{\cdot}60$, failed to ferment.

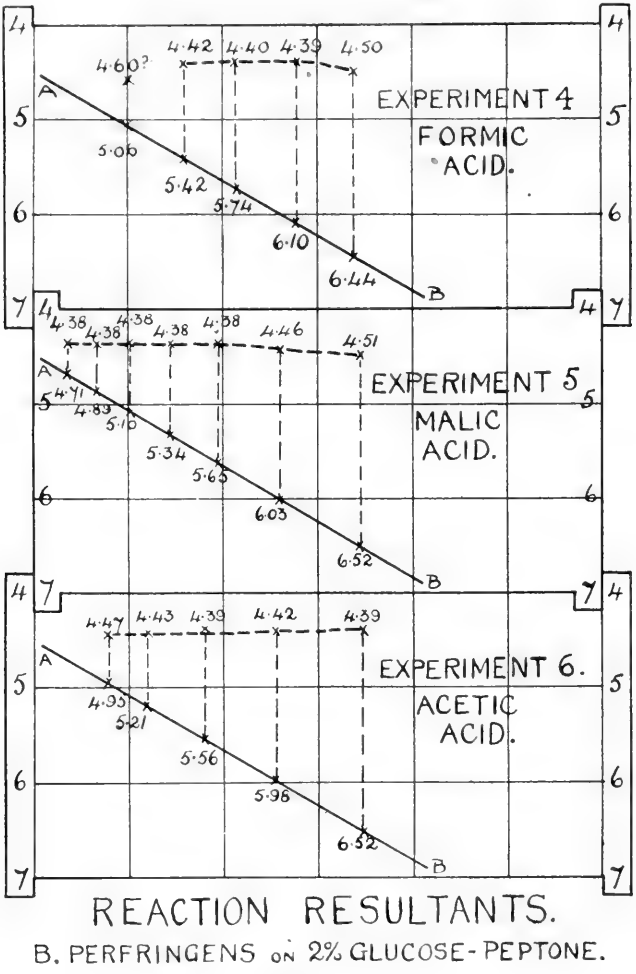


Fig. 4

Experiment No. 6 (Laboratory Experiment R. S. 13). Acetic acid.

Data (see also Fig. 4).

Series A. Inoculated with K 120 (twenty-four hours old on cooked meat).

Series B. Inoculated with K 121 (twenty-four hours old on cooked meat).

The tubes were examined seventy-two hours after inoculation.

The most acid tube to ferment was No. 5, Series B, with value of $P_H = 4{\cdot}95$. The next set of tubes, with $P_H = 4{\cdot}81$, failed to ferment.

TABLE VII. (*Exp.* 6.)

No. of tube	Initial reaction, P_H	Series A		Series B	
		Result	Final reaction, P_H	Result	Final reaction, P_H
1	6·52	+	4·38	+	4·41
2	5·98	+	4·43	+	4·41
3	5·56	+	4·39	+	4·39
4	5·21	+	4·43	+	4·44
5	4·95	–	...	+	4·47
6	4·81	–	...	–	...
7	4·75	–	...	–	...
8	4·65	–	...	–	...
9	4·60	–	...	–	...

Experiment No. 7 (Laboratory Experiment R. S. 4). Butyric acid.

Data (see also Fig. 5).

Series A. Inoculated with K 102 (fifteen hours old on milk).

Series B. Inoculated with K 103 (fifteen hours old on milk).

Series C. Inoculated with K 101 (fifteen hours old on milk).

The tubes were examined seventy-two hours after inoculation.

The most acid tubes to ferment were Nos. 5, Series A, B and C, with value of $P_H = 5{\cdot}03$.

The next set of tubes, with $P_H = 4{\cdot}96$, failed to ferment.

TABLE VIII. (*Exp.* 7.)

No. of tube	Initial reaction, P_H	Series A		Series B		Series C	
		Result	Final reaction, P_H	Result	Final reaction, P_H	Result	Final reaction, P_H
1	6·33	+	4·52	+	4·50	+	4·52
2	5·62	+	4·48	+	4·48	+	4·48
3	5·39	+	4·64	+	4·60	+	4·60
4	5·17	+	4·61	+	4·64	+	4·57
5	5·03	+	4·67	+	4·67	+	4·65
6	4·96	–	...	–	...	–	...
7	4·83	–	...	–	...	–	...
8	4·80	–	...	–	...	–	...
9	4·68	–	...	–	...	–	...

Experiment No. 8 (Laboratory Experiment R. S. 6). Succinic acid.

Data (see also Fig. 5).

Series A. Inoculated with K 113 (fourteen hours old on milk).

Series B. Inoculated with K 114 (fourteen hours old on milk).

Series C. Inoculated with K 115 (fourteen hours old on milk).

The tubes were examined seventy-two hours after inoculation.

TABLE IX. (*Exp.* 8.)

No. of tube	Initial reaction, P_H	Series A		Series B		Series C	
		Result	Final reaction, P_H	Result	Final reaction, P_H	Result	Final reaction, P_H
1	6·19	+	4·52	+	4·52	+	4·51
2	5·84	+	4·50	+	4·49	+	4·48
3	5·55	+	4·51	+	4·52	+	4·52
4	5·36	+	4·54	+	4·54	+	4·53
5	5·17	+	4·56	+	4·56	+	4·56
6	5·06	+	4·58	+	4·57	+	4·57
7	4·93	+	4·57	+	4·56	−	...
8	4·83	−	...	−	...	−	...

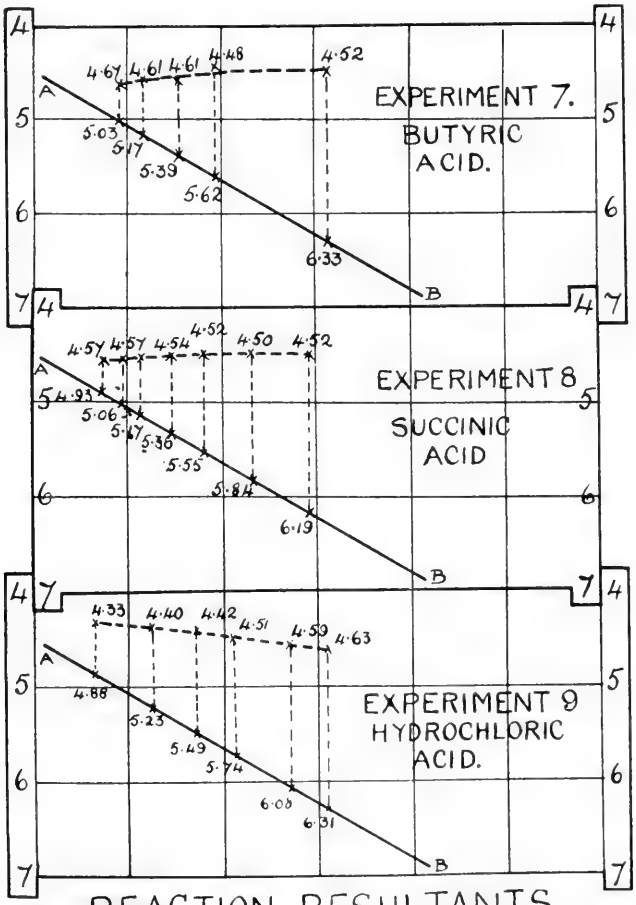


Fig. 5

The most acid tubes to ferment were Nos. 7, Series A and B, with value of $P_H = 4{\cdot}93$.

The next set of tubes, with $P_H = 4{\cdot}83$, failed to ferment.

One of the first things noticed in these experiments was that increasing the initial acidity of a medium had an effect on the latent period. Thus, when a series of tubes was inoculated with the same seed at the same time, the least acid member of the series was the first to ferment. The more acid the reaction of the medium the longer the latent period, showing that whatever may be the effect of the acidity on the subsequent fermentation it has a large influence in determining the latent period.

With *B. perfringens*, a glucose peptone solution begins to ferment in from four to five hours after inoculation when the reaction of the medium is from $P_H = 7{\cdot}0$ to $P_H = 6{\cdot}2$. As more acid media are employed, the latent period lengthens, until, for a medium with an initial reaction of 4·9, the latent period may last about forty-eight hours.

In reviewing the data given above it will be observed that the values of P_H at which the bacillus just manages to grow all lie close together in the neighbourhood of $P_H = 4{\cdot}8$. In the following table are given the most acid values at which growth takes place and those which just prevent it.

TABLE X.

Experiment No.	Acid	Most acid value of P_H which allows growth	Least acid value of P_H which inhibits growth	Mean value of P_H. Critical P_H
2	Hydrochloric	4·86	4·60	4·78
3	Lactic	4·85	4·61	4·68
4	Formic	5·06	4·77	4·91
5	Malic	4·71	4·60	4·66
6	Acetic	4·95	4·81	4·88
7	Butyric	5·03	4·96	4·99
8	Succinic	4·93	4·83	4·88

Mean value of critical $P_H = 4{\cdot}82$

With a large number of experiments it might be possible to find the exact value of P_H for each acid which would just permit growth, the slightest excess in acidity above this value preventing growth. In our experiments the differences between the "permitting reaction" and the "inhibiting reaction" are not great in any case, and probably the mean of the two values found experimentally is not far from this *critical* P_H. These mean values are given in the fifth column of the above table, and it will be observed that they are quite close together.

The following table shows the amount of each acid that must be present in the glucose peptone water to give these critical reactions. The amounts are determined from the titration curves (Fig. 1).

TABLE XI.

Acid	Critical P_H	cc. of $N/10$ acid contained in 10 cc. of broth	Acidity of medium in terms of N acid	Molar strength of medium in acid	G. of acid per litre of medium
Hydrochloric	4·78	2·05	0·0205 N	0·0205 M	0·748
Lactic	4·68	2·10	0·0210 N	0·0210 M	1·910
Formic	4·91	1·80	0·0180 N	0·0180 M	0·828
Malic	4·66	3·50	0·0350 N	0·0175 M	2·335
Acetic	4·88	2·70	0·0270 N	0·0270 M	1·620
Butyric	4·99	2·60	0·0260 N	0·0260 M	2·288
Succinic	4·88	3·20	0·0320 N	0·0160 M	1·888

The titration curves in Fig. 1 show the comparative strengths of the acids in presence of glucose peptone water. Thus, lactic, hydrochloric and formic acids behave as strong acids compared with acetic, butyric and succinic acids. It will be seen from the above table that the strong acids are more effective in inhibiting growth than the weaker acids when present in equivalent concentrations. Thus, when hydrochloric is present in sufficient quantity to make the medium 0·0205 N the critical reaction for *B. perfringens* is reached. On the other hand enough succinic acid must be present to give a 0·032 N solution before the critical reaction is attained.

These facts lead one to think that the dominant factor in the development of *B. perfringens* is not so much the amount of acid present as the *acidity* that the acid produces.

It seems that at about $P_H = 4·8$ there is a point where the reaction just permits the organism to develop. A slight excess in hydrogen ion concentration over this value prevents growth. Each acid may have some slight effect of its own, due to its molecular structure. Thus, malic acid is to some extent an anomaly, allowing growth to take place when the value of P_H is 4·71. Butyric acid on the other hand has a high value for its critical P_H (4·99), and seems to have a slight inhibiting effect in addition to that produced by its hydrogen ions.

On the whole it appears that the reaction due to the acid is more important than the actual amount or molecular quality of the acid in determining the growth or non-development of *B. perfringens*.

We now come to a consideration of the final reactions of the media after fermentation by *B. perfringens*. These final reactions are given in the tables and represented graphically in the reaction resultants.

These results show that the final reaction at which growth ceases is by no means a "physiological constant." On the contrary, it varies not only with the reaction of the medium before inoculation but also with the acid used to produce this particular reaction.

With hydrochloric acid (Fig. 2), when the initial reaction is $P_H = 6{\cdot}77$, growth stops at the point where the bacillus has produced sufficient acid to give the medium a reaction of $P_H = 4{\cdot}59$. For an initial reaction of $P_H = 4{\cdot}86$, however, the final reaction is $P_H = 4{\cdot}34$. For initial reactions between the two, final reactions are obtained which take up orderly positions between $P_H = 4{\cdot}59$ and $P_H = 4{\cdot}34$, and give the curve shown in the figure. There is therefore a very definite relation between the initial and final reactions.

Recently, in connection with *B. coli*, attempts at diagnosis have been made in which the final reaction of a particular medium when inoculated with the organism was determined. In view of the above results it would seem that great care must be exercised in using such a method. In order to use the final reaction of a fermentation as a criterion for a given organism, absolute uniformity of the medium is essential. This, applies especially to the hydrogen ion concentration, on which, as will be seen, the final reaction depends.

In examining the curves for the other acids used, one finds that the question of the final reaction becomes further complicated. Here, for the first time in these experiments, one sees signs of specificity in the action of the acids. The reaction resultants for hydrochloric, lactic and malic acids show that the final reactions of the media become definitely more acid as the initial hydrogen ion concentration increases. On the other hand, with butyric, succinic and acetic acids, the reverse rule holds good. The experiments appear to bring out the important point that all acids, in the presence of which *B. perfringens* can develop, probably give characteristic reaction resultants, as do the seven acids with which we have worked. Considerable interest is attached to the point X (Fig. 2), obtained by the intersection of the reaction resultant produced and the line AB. The reaction corresponding to this point might be considered theoretically as that which definitely stops the growth of *B. perfringens*. It should be remarked, however, that we have never been able to cause a medium having this reaction to ferment, no doubt on account of inhibiting influences other than the reaction. The value obtained at the point X differs for each individual acid. This, in itself, is an indication of specific action.

It is necessary to search closely for the factors responsible for these resultants. When *B. perfringens* ferments glucose peptone water, it forms,

as Wolf and Telfer [1917] have shown, a mixture of lactic acid, butyric acid and small amounts of other low members of the fatty acid series.

How far the *quality* of these acids is affected by the nature of the acid added to obtain the required initial reaction one is unable to say at present. The reaction resultants, however, will give a fair idea of the *quantity* of acids produced, provided one has some notion of the factors which influence them. To this end it will be necessary to take a specific example.

Let it be assumed that a series of flasks of glucose peptone water are prepared differing from one another in their initial hydrogen ion concentration, this effect being produced by the addition of increasing amounts of butyric acid to the medium. These flasks are then inoculated with an organism producing butyric acid exclusively. The quantity of butyric acid produced is also assumed to be independent of the initial reaction of the medium and is a constant amount. At the end of the fermentation the reaction resultants are plotted.

We have already determined the changes in reaction of glucose peptone caused by the addition of various amounts of butyric acid. These results can be expressed graphically in a titration curve, which will aid us to construct the reaction resultants for the above hypothetical fermentation. All that it is necessary to do is to select on the titration curve a number of points and consider the reactions for these points as the initial reactions of the media. To the quantity of acid corresponding to each point a definite amount of acid is added, and the reactions corresponding to the total amounts noted. These reactions will be the final reactions for the fermentation, and when plotted in the usual way against the initial reactions will give the reaction resultant. Thus, one can construct the reaction resultants for the production of any amount of acid providing one has a suitable titration curve. This has been done for four hypothetical fermentations (Fig. 6, I).

In the first case the fermentation is supposed to produce butyric acid equivalent to 3 cc. of $N/10$ acid per 10 cc. of broth; in the second, 5 cc.; in the third, 7 cc.; in the fourth, 9 cc. It is seen that the reaction resultants have a very definite form. They rise steadily from right to left on the paper, and the four curves tend to meet at a point on AB at which an addition of butyric acid will not make the reaction more acid. Different acids will, of course, give different curves, the general form of the reaction resultant, however, will be the same, providing the amount of acid produced does not vary. They will all rise from right to left of the paper towards the value of P_H where the acidity is not increased by further addition of the acid. It is clear also

that the resultant arising from the added acid must always approach the line *AB* tangentially, when a constant amount of acid is being formed.

Now let it be imagined that the accumulation of acids hinders the bacteria and causes their production of acid to fall off steadily in proportion to the concentration of acid present. The reaction resultants will then take the form

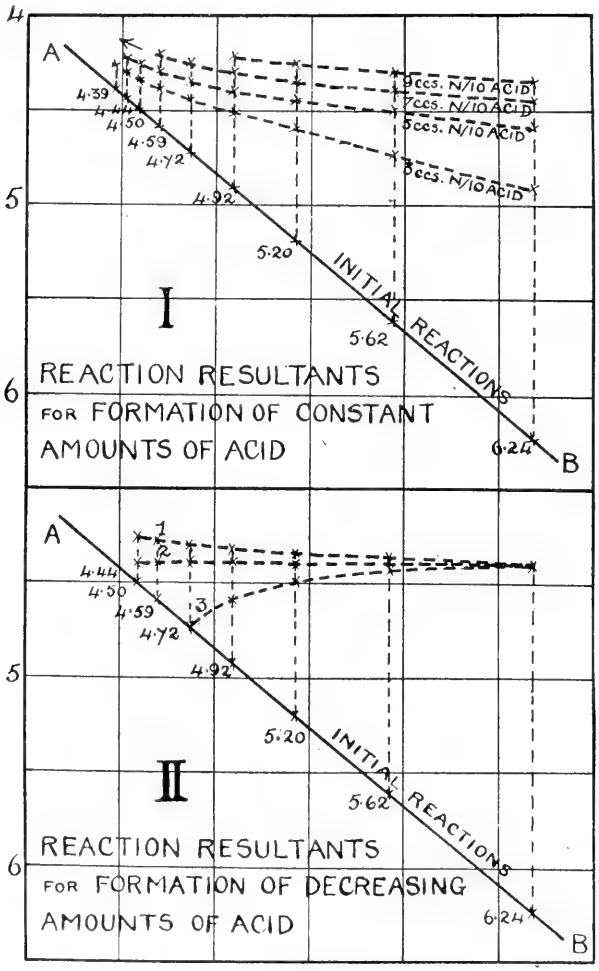


Fig. 6

shown in the second diagram (Fig. 6, II). We give the curves for three cases. In curve No. 1 the inhibiting influence is made slight, in No. 2 more marked, and in No. 3 greater still.

From a consideration of this imaginary fermentation one can now see how, from the reaction resultants, it can be estimated to what extent the acids have

affected fermentation. If the curve rises from right to left and tends to approach *AB* tangentially it means that the amount of acid produced is not falling off very greatly as the initial hydrogen ion concentration increases. If, on the other hand, the concentration of acids is causing a decrease in fermenting power, the reaction curves will tend to fall from right to left, according to the magnitude of the inhibiting influences. Where the bacteria cease activity as soon as a definite *concentration* of acid is reached, the reaction resultant will be a horizontal line (Fig. 6, II, 2).

From our experiments we have obtained various reaction resultants. The one most nearly approaching the case where the fermentation is not hindered by the accumulation of acids is with hydrochloric acid.

The following series of fermentations will show to what extent the foregoing considerations are borne out experimentally, and how far one may rely on conclusions as to acid formation and intensity of metabolism drawn from the reaction resultants.

Experiment No. 9 (Laboratory Fermentations Nos. 42–47).

Into each of six 500 cc. Kjeldahl flasks were placed 250 cc. of 2 % glucose peptone medium, as used in the foregoing reaction experiments. During preparation varying amounts of hydrochloric acid had been added to each lot of medium so that we had six broths exactly similar, except in so far as the amounts of contained acid, and consequently the reactions, were concerned. The flasks were plugged and sterilised in the ordinary way, afterwards being inoculated each with 1 cc. of the same culture of *B. perfringens*. The plugs were then pushed lower into the necks of the flasks and well fitting rubber stoppers, each provided with a short delivery tube, inserted. By means of a Fleuss pump the flasks were now evacuated by way of the delivery tubes, which were then closed off by means of a piece of pressure tubing and a screw clip. All joints were made tight with hot paraffin wax. The flasks were incubated for four days. At the end of this time the gas production was measured and a determination made of the amount of acid produced. To measure the gas production, the flasks were connected to an evacuated mercury manometer, the clip unscrewed, the pressure of the gas determined, and hence its volume calculated.

A sample of the gas was in each case analysed. From each flask also a portion of the liquid was taken and the final P_H measured electrometrically.

The amounts of volatile and fixed acids were determined by Captain Telfer, R.A.M.C., to whom our acknowledgements are due. The total volatile acids

were determined by exhaustive steam distillation and the fixed acids by ethereal extraction of the residue in a liquid extractor. As the final stages of the distillation are accompanied to some extent by volatilisation of the fixed acids, and as also the final traces of lower members of the fatty acid series are only removed with difficulty, we have, in tabulating our results, added together the amounts of volatile and fixed acids, and taken their sum to represent the total amount of acids produced.

TABLE XII. (*Exp.* 9.)

No. of Fermentation	Initial P_H	Gas production cc. per litre	CO_2 %	H_2 %	Final P_H	Acid production cc. of *N*/10 acid per litre of broth: Volatile acids cc.	Fixed acids cc.	Total acids cc.
F 42	6·31	1036	42·3	57·7	4·63	232	171	403
F 43	6·08	1096	40·5	59·5	4·59	259	196	455
F 44	5·74	1000	41·6	58·4	4·51	200	213	413
F 45	5·49	888	41·3	58·7	4·42	156	231	387
F 46	5·23	916	41·0	59·0	4·40	229	205	434
F 47	4·88	940	...	...	4·33	181	241	422

It will be seen from the above table that in the final reactions alone can we trace any sign of progressive relation between the final measurements on the different media. These final reactions have been plotted against the initial reactions (see Fig. 5), and give an ordinary reaction resultant such as has been found characteristic for hydrochloric acid.

The gas analyses indicate that the products of fermentation are very similar, differing as they do to only the smallest degree. One sees too that the final amounts of gas produced are so close that they may be considered equal, as may also the total amounts of acid. What differences we have obtained in the individual experiments may fairly be considered as due to experimental errors.

These results show that the fermentation of *B. perfringens* is very little affected by the addition of hydrochloric acid, up to a quantity sufficient to give the critical reaction.

We hope to carry out similar experiments with the other acids involved, but such experiments are long and laborious and for the moment one can obtain a large amount of information merely from the reaction resultants.

Lactic and malic acids, which act as strong acids, apparently play a less passive role than hydrochloric acid. The final products, judging from the resultants, fall off somewhat as the initial acidity rises. With the weaker acids, succinic and butyric (and it should be noted that the absolute strength

of these acids in the presence of peptone water can be accurately judged from the titration curves), there is a greater inhibiting effect shown. Acetic acid, which behaves more or less as an intermediate acid from the point of view of strength, has a corresponding intermediate resultant.

Formic acid behaves slightly anomalously, apparently having a rather larger inhibiting effect than acids of similar strength. The aldehyde grouping of the acid may be a partial explanation of this fact.

Generally speaking, then, we find little specificity of action in the acids as regards the initial P_H, but a greater difference in effect towards the final reaction. To what can this be ascribed? The best explanation seems to be that the inhibiting action of these acids, as shown by slackened fermentations, is due to an effect of *mass*. It should be remembered that we have been working throughout over the same range of initial reactions; also that to produce these initial reactions a larger amount of a weak acid is necessary than of a strong acid. Therefore, if we assume that all of these acids, considered merely as foreign substances, have an effect in concentrated solutions independent of their P_H but proportional to their mass, we can see why in the case where a weak acid is used to give the initial reaction the fermentations are partially inhibited. On the other hand, a strong acid, such as hydrochloric acid, only present in small molecular quantities, has little effect.

Summing up therefore these results, it would seem that the seven acids under consideration have a definite effect on *B. perfringens* proportional to the concentration of their common cation—hydrogen ion. Their anions, on the other hand, and the undissociated acids merely behave as foreign bodies, possessing a certain inhibiting effect proportional to their molecular concentration.

At some future date we hope to show the effect of adding various amounts of neutral salts of these acids to peptone water. We can obtain a constant initial reaction by adding hydrochloric acid which we have shown to have little effect on the fermentations. By this method we shall be able to estimate accurately the effect of the anions of the acids.

Bacillus sporogenes.

Experiments similar to those described above with *B. perfringens* were carried out with *B. sporogenes*. Glucose peptone water was again used as the fermentation medium, and the effect was tried of the following acids: hydrochloric, formic, lactic, acetic and butyric.

With the *B. sporogenes* the fermentation is accompanied by the formation

of a base, ammonia, which, although large amounts of acid are produced, causes the final reaction to be less acid than those resulting from *B. perfringens*.

The experimental details were similar. Readings were taken of the final reactions and the reaction resultants plotted.

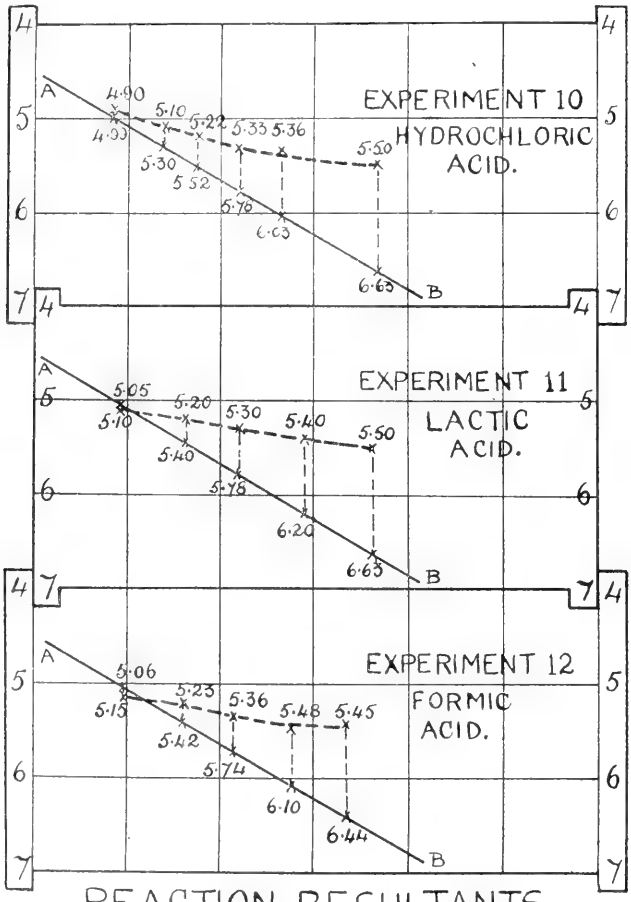


Fig. 7

B. sporogenes acts rather slower than *B. perfringens* and it was found necessary to continue the incubation for a fourth day, making ninety-six hours in all, before the final reactions were determined.

Experiment No. 10 (Laboratory Experiment R. S. 9). Hydrochloric acid.

Data (see also Fig. 7).

Series A. Inoculated with D 74 (forty-eight hours old on cooked meat).

Series B. Inoculated with D 75 (1) (forty-eight hours old on milk).

Series C. Inoculated with D 75 (2) (forty-eight hours old on milk).

The tubes were examined ninety-six hours after inoculation.

The most acid tubes to ferment were Nos. 6, Series B and C, with value of $P_H = 4{\cdot}99$.

The next set of tubes, with $P_H = 4{\cdot}75$, failed to ferment.

TABLE XIII. (*Exp.* 10.)

		Series A		Series B		Series C	
No. of tube	Initial reaction, P_H	Result	Final reaction, P_H	Result	Final reaction, P_H	Result	Final reaction, P_H
1	6·63	+	5·51	+	5·45	+	5·52
2	6·03	+	5·39	+	5·34	+	5·35
3	5·78	+	5·37	+	5·31	+	5·31
4	5·52	+	5·22	+	5·20	+	5·23
5	5·30	+	5·07	+	5·10	+	5·08
6	4·99	–	...	+	4·91	+	4·90
7	4·75	–	...	–	...	–	...
8	4·60	–	...	–	...	–	...

Experiment No. 11 (Laboratory Experiment R. S. 11). Lactic acid.

Data (see also Fig. 7).

Series A. Inoculated with D 74 (forty-eight hours on cooked meat).

Series B. Inoculated with D 75 (1) (forty-eight hours on milk).

Series C. Inoculated with D 75 (2) (forty-eight hours on milk).

The tubes were examined ninety-six hours after inoculation.

The most acid tube to ferment was No. 5, Series C, with value of $P_H = 5{\cdot}05$.

The next set of tubes, with $P_H = 4{\cdot}70$, failed to ferment.

TABLE XIV. (*Exp.* 11.)

		Series A		Series B		Series C	
No. of tube	Initial reaction, P_H	Result	Final reaction, P_H	Result	Final reaction, P_H	Result	Final reaction, P_H
1	6·63	+	5·51	+	5·45	+	5·52
2	6·20	+	5·49	+	5·34	+	5·37
3	5·78	+	5·32	+	5·25	+	lost
4	5·46	+	5·20	+	5·17	+	5·17
5	5·05	–	...	–	...	+	5·10
6	4·70	–	...	–	...	–	...
7	4·56	–	...	–	...	–	...
8	4·38	–	...	–	...	–	...
9	4·28	–	...	–	...	–	...

Experiment No. 12 (Laboratory Experiment R. S. 15). Formic acid.

Data (see also Fig. 7).

Series A. Inoculated with D 82 (ninety-six hours old on milk).

Series B. Inoculated with D 83 (ninety-six hours old on milk).

The tubes were examined ninety-six hours after inoculation.

The most acid tube to ferment was No. 5, Series B, with value of $P_H = 5{\cdot}06$.

The next set of tubes, with $P_H = 4{\cdot}77$, failed to ferment.

TABLE XV. (*Exp.* 12.)

		Series A		Series B	
No. of tube	Initial reaction, P_H	Result	Final reaction, P_H	Result	Final reaction, P_H
1	6·44	+	5·45	+	5·45
2	6·10	+	5·43	+	5·53
3	5·74	+	5·39	+	5·33
4	5·42	+	5·23	+	5·22
5	5·06	–	...	+	5·15
6	4·77	–	...	–	...
7	4·60	–	...	–	...
8	4·44	–	...	–	...

Experiment No. 13 (Laboratory Experiment R. S. 14). Acetic acid.

Data (see also Fig. 8).

Series A. Inoculated with D 81 (forty-eight hours old on milk).

Series B. Inoculated with D 80 (forty-eight hours old on milk).

The tubes were examined ninety-six hours after inoculation.

The most acid tubes to ferment were Nos. 4, Series A and B, with value of $P_H = 5{\cdot}21$.

The next set of tubes, with $P_H = 4{\cdot}95$, failed to ferment.

TABLE XVI. (*Exp.* 13.)

		Series A		Series B	
No. of tube	Initial reaction, P_H	Result	Final reaction, P_H	Result	Final reaction, P_H
1	6·52	+	5·39	+	5·40
2	5·98	+	5·37	+	5·35
3	5·56	+	5·30	+	5·29
4	5·21	+	5·29	+	5·10
5	4·95	–	...	–	...
6	4·81	–	...	–	...
7	4·75	–	...	–	...
8	4·65	–	...	–	...
9	4·60	–	...	–	...

Experiment No. 14 (Laboratory Experiment R. S. 10). Butyric acid.

Data (see also Fig. 8).

Series A. Inoculated with D 74 (forty-eight hours old on cooked meat).

Series B. Inoculated with D 75 (1) (forty-eight hours old on milk).

Series C. Inoculated with D 75 (2) (forty-eight hours old on milk).

The tubes were examined ninety-six hours after inoculation.

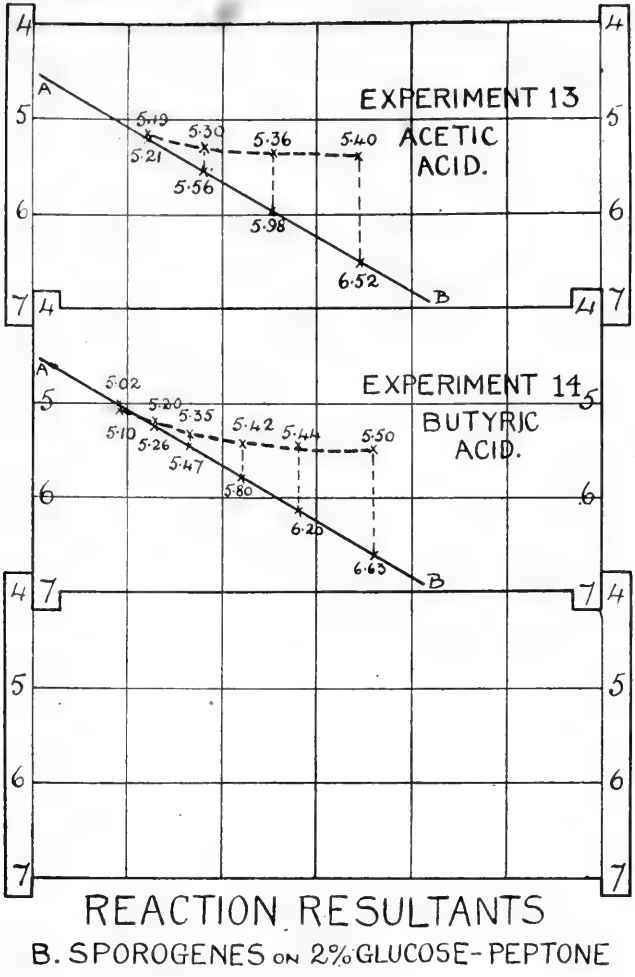


Fig. 8

TABLE XVII. (*Exp.* 14.)

No. of tube	Initial reaction, P_H	Series A		Series B		Series C	
		Result	Final reaction, P_H	Result	Final reaction, P_H	Result	Final reaction, P_H
1	6·63	+	5·51	+	5·45	+	5·52
2	6·20	+	5·46	+	5·37	+	lost
3	5·80	+	5·49	+	5·39	+	5·48
4	5·47	+	5·42	+	5·35	+	5·23
5	5·26	+	5·17	+	5·18	+	5·34
6	5·02	+	5·15	+	5·10	+	5·08
7	4·90	−	...	−	...	−	...
8	4·80	−	...	−	...	−	...
9	4·76	−	...	−	...	−	...

The most acid tubes to ferment were Nos. 6, Series A, B and C with value of $P_H = 5{\cdot}02$.

The next set of tubes, with $P_H = 4{\cdot}90$, did not ferment.

When inoculated into glucose peptone media at different reactions *B. sporogenes* behaves similarly to *B. perfringens*. In the first place the higher the acidity of the reaction the longer is the latent period before obvious signs of growth are seen. There also seems to be a fairly well defined critical reaction at which growth is just permitted, as can be seen from the table below.

TABLE XVIII.

Experiment No.	Acid	Most acid value of P_H which allows growth	Least acid value of P_H which inhibits growth	Mean value of P_H. Critical P_H
10	Hydrochloric	4·99	4·75	4·87
11	Lactic	5·05	4·70	4·87
12	Formic	5·06	4·77	4·91
13	Acetic	5·21	4·95	5·08
14	Butyric	5·02	4·90	4·96

Mean value of critical $P_H = 4{\cdot}94$

This critical reaction, $P_H = 4{\cdot}94$, is very close to the value obtained for *B. perfringens*. These two organisms, *B. sporogenes* and *B. perfringens*, are widely different in character and in chemical behaviour, yet they are both equally affected by the initial hydrogen ion concentration of the medium into which they are implanted.

When the final reactions of *B. sporogenes* are considered, the matter is found to be complicated by the formation of both acids and bases. In a previous paper we have shown that fermentation by *B. sporogenes* is accompanied by the production of large amounts of ammonia in addition to acids. This naturally affects the final reaction so that one is unable to draw such definite conclusions from the reaction resultants as could be done in the case of *B. perfringens*.

The reaction resultants, however, show a similar orderly relation between the initial and final reactions. This is the more surprising when one considers the complicated character of the fermentations. In spite of the formation of several distinct acids, large amounts of amino-acids and ammonia, all having a great influence on reaction either as sources of H-ions or OH-ions, or merely as buffers, this orderly reaction persists. Obviously the various phases of fermentation must be very definitely inter-related in order that this nicely balanced state of affairs should exist.

It will be observed that the reaction resultants in most cases cross the line *AB*, the hydroxyl ions of the ammonia actually causing a decrease in the acidity of the medium after a certain stage in the fermentation is reached. To this is no doubt due the ability of *B. sporogenes* to ferment for a very long period. The cessation of activity is probably caused almost entirely by the accumulation of the products of metabolism, and very little by the inhibiting influence of hydrogen ions.

Summary.

An investigation has been made on the effect of acids on the growth of *Bacillus perfringens* and *Bacillus sporogenes*. This was preceded by an examination of the behaviour of the acids used with nutrient media.

The addition of acids to liquids containing large amounts of buffer substances—to which category all efficient nutrient media belong—produces a complex effect on the true reaction of the media. This effect is partly determined by the type of acid employed and partly by the nature and content of buffer substances in the mixture. With the less highly dissociated acids a point is soon reached where successive amounts of added acid affect the reaction but slightly.

The action of acids on the growth of the bacteria in question can be divided into three parts.

The first effect noticed is on the latent period of growth. The more highly acid the medium, the greater time elapses before obvious signs of growth are observed.

The second effect is on the final reaction at which growth ceases. While the limits for the cessation of growth with all acids are comparatively narrow, the differences observed are distinct and obviously governed by definite laws. They are quite wide enough apart to negative the idea of a "physiological constant." The final reaction is undoubtedly dependent on the initial reaction. With a given acid, and varying initial concentrations, a series of final reactions is obtained which can be expressed by a curve. Each acid shows a curve which is individual and to this extent we may speak of specificity of acid effect. The difference between the acids is not large.

The third feature of the action of acids on these bacteria is the total inhibition of growth.

This varies for *Bacillus perfringens* between $P_H = 4{\cdot}99$ using butyric acid and $P_H = 4{\cdot}66$ using malic acid, and has an average value of $P_H = 4{\cdot}82$,

which we have called the critical reaction. This, for practical purposes, may be considered the inhibiting reaction.

It will be observed that for *B. perfringens* the hydrogen ion concentration for inhibition may be lower than that reached by the medium at the end of fermentation. That is to say, after overcoming the inhibiting influence of a certain hydrogen ion concentration (as shown by the lengthened latent period) the bacillus causes a rise in concentration of hydrogen ion by producing acids. During fermentation a secondary toxic effect is observed. This is due to the accumulation of metabolic products, and to the influence of the anions and the undissociated molecules of the acid initially present. Fermentation therefore ceases when different amounts of acid have been formed. The final hydrogen ion concentration also varies. When a highly dissociated acid has been used to produce the initial reaction, and the high concentration of hydrogen ion has been produced by the addition of a small quantity of acid, the acid and gas production is little affected. This is shown in Experiment 9, where hydrochloric acid was used for this purpose. With feebly dissociated acids, much larger amounts are necessary to produce the same hydrogen ion concentration, and the acid exerts an effect which is not only due to the cation. The *mass* of the acid molecule inhibits fermentation, and smaller amounts of acid fermentation products are formed. Hence the toxic action of a weak organic acid is twofold.

With *B. sporogenes,* inhibition takes place at an average value of $P_H = 4{\cdot}94$. This organism differs from *B. perfringens* in that the hydrogen ion concentration of the fermented medium may be lower than that of the medium before inoculation, although large amounts of acid have been formed. Obvious signs of growth may cease to be observed at a concentration of hydrogen ions which is less than the critical reaction. This is to be explained by the complex character of the katabolism of this organism whereby large amounts of basic and amphoteric substances are produced, and neutralise the acids, as well as acting as buffers.

Stoppage of growth is probably due almost entirely to the accumulation of metabolites. The tendency of *B. sporogenes* to produce a constant or even lower hydrogen ion concentration than that observed at the time of inoculation may account for the fact that fermentations with *B. sporogenes* may continue for very long periods.

In conclusion one may say that the fermentation of both these organisms is inhibited by a rise in hydrogen ion concentration. The acidity may merely delay growth, or it may stop it entirely. The effect of all the acids we have

used in the course of these experiments is alike. Specific qualities are almost entirely absent.

These experiments seem to show that by submitting the organisms involved in gas gangrene infections to solutions of a hydrogen ion concentration slightly above the critical point growth may be inhibited. They afford confirmation of the views expressed in our first paper that the treatment of local infections of this type by means of acid solutions, highly buffered, is worthy of trial.

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XXII. THE NITROGENOUS EXTRACTIVES OF TUMOURS.

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During the course of an investigation of the proteins of tumours, the results of which have already been reported by the author [1916], the protein-free aqueous extracts of a large number of tumours were obtained. In view of the scanty data recorded in the literature dealing with the extractive substances found in tumours, it was decided to make an examination of this material. The preparation of the extracts was carried out along the usual lines. The fresh tumour tissues in a finely minced condition were extracted with boiling distilled water, a trace of acetic acid being added to complete the removal of coagulable proteins. To the filtered extracts basic lead acetate was added until no further precipitation occurred, the resulting precipitate being filtered off, well washed, and the excess of lead removed from the filtrate by sulphuretted hydrogen.

The filtrate from the lead sulphide, freed from H_2S by passage of a rapid current of air, was acidified with sulphuric acid to the extent of 5 %, and the basic substances precipitated by the addition of a slight excess of a 30 % solution of phosphotungstic acid. After standing overnight in the refrigerator room the precipitate was filtered off and well washed with 5 % sulphuric acid. Each such precipitate was dried in a desiccator over sulphuric acid and stored in this form until sufficient material of each class had been collected for examination.

The filtrates from the phosphotungstic acid precipitations were quantitatively freed from the excess of the precipitating reagent and sulphuric acid by means of baryta, filtered and evaporated to small bulk by distillation *in vacuo* at 40°. In this concentrated form the fractions were stored at 0°, until sufficient had been collected.

The tumours examined were a number of human tumours of mammary origin, a lymphosarcoma, a large secondary deposit in the liver from a pancreatic carcinoma and a series of the avian neoplasm known as the Rous chicken sarcoma.

I. Extractive Nitrogen of the Rous Chicken Sarcoma.

By the method just described it was found possible during the course of several months to collect fractions from the aqueous extracts of 4320 g. of this tumour.

The tumours were grown by the inoculation of grafts of the tumour into the pectoral muscle of healthy, adult, pure bred, fowls of the Plymouth Rock strain. The resulting tumours grow rapidly, infiltrating and destroying the adjacent muscle tissue, so that after some three weeks' growth the tumour has usually attained the size of a hen's egg and weighs 100 g. or more. Under conditions which result in a very rapid rate of growth, such as implantation into young birds, the tumours usually undergo extensive central degeneration during the latter stages, and the cavity of the growth becomes filled with a thick, mucoid, blood-stained fluid.

I (*a*). *Phosphotungstate Precipitate.*

The combined precipitates of phosphotungstates from the extracts of the 4320 g. of tumour tissue weighed in the dry condition 225 g. After being ground to a fine state of division and passed through a fine mesh sieve, it was decomposed in aqueous suspension by means of a hot concentrated solution of baryta. The clear filtrate was quantitatively freed from the excess of barium by sulphuric acid and evaporated down by distillation under reduced temperature and pressure to a thick syrup. The residue was submitted to extraction with hot alcohol, thus removing 5·2 g. of a dirty white substance, insoluble in that solvent and mainly inorganic in nature. The alcohol was removed from the combined extracts by distillation and the residue taken up in a litre of distilled water. This solution was then fractionated by the use of silver nitrate and baryta into four fractions:

I (*a*) i. Purine fraction.
I (*a*) ii. Histidine fraction.
I (*a*) iii. Arginine fraction.
I (*a*) iv. Lysine fraction.

The last fraction consisted of the re-precipitated phosphotungstates after the separation of the first three fractions and the subsequent removal of the excess of silver and baryta.

I (*a*) i. *Purine Fraction.*

The purine silver compounds were decomposed in aqueous suspension by means of sulphuretted hydrogen gas and the silver-free filtrate concentrated down to dryness at reduced temperature and pressure.

The resulting dirty white residue was dissolved in a small volume of dilute hydrochloric acid, filtered and rendered alkaline with ammonia. On standing, a very small quantity of a dirty granular product separated out. Insufficient was obtained to permit of satisfactory purification and analysis, but its reactions, and the isolation of the crystals of the hydrochloride and phosphotungstate, were sufficient to identify the substance as guanine. After neutralisation of the filtrate and the addition of picric acid solution, a crop of crystals of adenine picrate was obtained. Recrystallised from water, 0·7 g. of the purified picrate was isolated (M.P. 273°). Analysis by means of the nitron method for the estimation of picric acid gave the figures:

0·0264 g. adenine picrate; 0·0372 g. nitron picrate; 59·5 % picric acid.

Calculated for $C_5H_5N_5$, $C_6H_3O_7N_3$, H_2O ... 60·0 % „ „

The filtrate from the adenine picrate, freed from the excess of picric acid by acidification with nitric acid and extraction with toluene, was evaporated almost to dryness, when typical crystals of hypoxanthine nitrate separated out. After recrystallisation from dilute nitric acid, 0·4 g. of the pure salt was obtained. No xanthine or other substance was isolated from this fraction.

I (*a*) ii. *Histidine Fraction.*

The silver compounds were decomposed in aqueous suspension by H_2S gas, and the neutralised, silver-free filtrate evaporated to dryness at reduced temperature and pressure. The small residue was extracted with hot alcohol, thus removing a small amount of insoluble inorganic salts, and the alcoholic extracts were freed from alcohol under reduced pressure. The residue gave positive reactions for histidine. Accordingly it was dissolved in 100 cc. of distilled water, and mercuric sulphate solution added until no further precipitation occurred. The small mercury precipitate was filtered off and decomposed in aqueous suspension by H_2S gas, the filtrate quantitatively freed from sulphuric acid and evaporated down on the water-bath with dilute hydrochloric acid. After standing for several days in the desiccator, a very small yield of the typical crystals of histidine hydrochloride was obtained, which, after one recrystallisation from dilute alcohol, melted at 251°. From the hydrochloride the picrolonate was prepared, twice recrystallised from hot

water, and analysed by the micro-Dumas method of Pregl, after being dried *in vacuo* at 110° for 2 hours (M.P. 224°).

3·57 mg.; 0·695 cc. N gas (19°, 766 mm.) 23·2 % N

Calculated for $C_6H_9O_2N_3$, $C_{10}H_8O_5N_4$ ... 23·45 % N

Although there was evidence of the presence of other substances in the histidine mother liquors, attempts to isolate these in a pure condition failed.

I (*a*) iii. *Arginine Fraction.*

The silver salts were decomposed in the usual manner in aqueous suspension with H_2S gas, and the silver-free filtrate evaporated down to small bulk at reduced temperature and pressure. No crystalline product could be isolated direct, but after the addition of a solution of picrolonic acid and on standing some days, a small amount of a micro-crystalline picrolonate was obtained. After recrystallisation from hot water 0·12 g. of the salt was yielded, which showed a melting point of 255° and gave, on analysis, figures which indicated that it was probably an impure preparation of arginine picrolonate. Attempts to purify the salt and analyse the purer product were unsuccessful.

The filtrate from this picrolonate was freed from the excess of picrolonic acid by extraction of the acidified solution with ether and concentrated down to a small bulk. On the addition of a saturated aqueous solution of picric acid a very small crop of a pale yellow crystalline picrate was deposited. After one recrystallisation from water the picrate melted at 214°, and was identified as creatinine picrate by the reactions of the hydrochloride prepared from the picrate. The occurrence of creatinine in this fraction is probably due to its being carried down during the precipitation of the silver compounds.

I (*a*) iv. *Lysine Fraction.*

The reprecipitated phosphotungstates comprising this fraction were decomposed in the usual manner by means of baryta, the excess of barium quantitatively removed, and the filtrate concentrated *in vacuo* at reduced temperature. Hot alcohol extraction of the resulting syrup left behind a considerable amount of insoluble inorganic salts. The hot alcohol extracts on cooling deposited a crop of a pure white crystalline substance, which was filtered off and recrystallised from hot alcohol. The purified substance separated out in clusters of needles and showed a melting point of 244°. Its reactions corresponded with those of carnosine, and its identity with that substance was established by the isolation of the characteristic copper salt, and the analysis of the free base.

0·0589 g. (dried 110° *in vacuo*); 10·20 cc. 0·1 *N* NH_3 ... 24·2 % N
Calculated for $C_9H_{14}O_3N_4$ ... ... 24·6 % N

After separation of the carnosine, it was found that the alcoholic filtrate gave a copious crystalline precipitate with alcoholic picric acid. The picrate after standing overnight at 0°, was filtered off and recrystallised from water, 16 g. of the pure compound being thus obtained. The product was readily identified as creatinine picrate, M.P. 213°.

0·0765 mg. of the picrate, dried at 110° for 2 hours *in vacuo*;
0·1205 mg. of nitron picrate ... ... 66·7 % picric acid.
Calculated for $C_4H_7ON_3$, $C_6H_3O_7N_3$ ... 67·0 % ,, ,,

The hydrochloride prepared from the picric acid salt melted at 264°, and gave all the reactions for creatinine hydrochloride.

No other substance was isolated from this fraction except a small yield of what was probably impure leucine.

I (*b*). *Phosphotungstic Acid Filtrate.*

The concentrated fractions, prepared as already described, had been stored in the refrigerator room and upon examination it was found that a considerable crystalline deposit had separated out. This consisted mainly of tyrosine, but typical crystals of leucine were also identified as being present. After recrystallisation 0·6 g. of fairly pure tyrosine was obtained and recognised by its reactions.

The filtrate from the tyrosine was concentrated to a thick syrup under reduced temperature and pressure, another small yield of tyrosine being obtained during the concentration, and taken up in hot alcohol.

The alcoholic extracts gave a heavy crystalline precipitate on the addition of alcoholic picric acid. The deep yellow picrate, recrystallised from water, showed an imperfect melting point at 236—250°, and was identified as creatinine potassium picrate.

0·0970 g.; 0·1731 g. of nitron picrate ... 75·5 % picric acid.
0·0953 g.; 0·1702 g. ,, ,, ... 75·6 % ,, ,,
Calculated for $C_4H_7ON_3$, K, $(C_6H_3O_7N_3)_2$ 75·3 % ,, ,,

Creatinine hydrochloride, with a melting point of 258°, was prepared from the double picrate.

Attempts to separate other substances in a pure condition from this fraction failed, although several impure products were isolated, of which one was apparently an impure specimen of leucine, whilst another was probably a still more impure preparation of glycine.

II. Extractive Nitrogen of Mammary Carcinomata.

The chief difficulty encountered in this investigation was the collection of sufficient material. Altogether, some 600 g. of mammary tumour tissue were available, but it was not all of the same type, as was the large amount of chicken tumour used in the first section of the work. The majority of the specimens—weighing together about 450 g.—were primary carcinomata of the mammary gland. Of these 75 % were of the slow growing scirrhus type, the remainder being more or less encephaloid in character. The remaining 150 g. of tissue examined was made up of carcinomatous deposits in the axillary glands, secondary to primary growths in the breasts, and one myxo-sarcoma of the same organ. The combined aqueous extracts of these tissues were treated by the process described in the first portion of this paper.

II (*a*) *Phosphotungstate Precipitate.*

The combined phosphotungstate precipitates weighed only 35 g. They were reduced to a fine state of division and decomposed in aqueous suspension by means of baryta. The neutralised filtrate was then sub-fractionated, as described in the first section of this paper (p. 247), into four fractions.

Owing to the fractions being so very small, the separation of substances in a pure condition from them was rendered exceedingly difficult.

From the purine fraction II (*a*) i, after an attempt at purification of the substances present by the copper bisulphite process, a very small yield of adenine picrate was obtained, M.P. 270°, but no other substance was isolated.

The second and third fractions, II (*a*), ii and iii, although worked up as carefully as possible, yielded no pure products. A substance giving a strong coloration with diazo-benzenesulphonic acid, and precipitated by mercuric sulphate, was detected in the histidine fraction; whilst from the arginine fraction a small yield of a very impure picrolonate was obtained, but in both cases the amount was insufficient to permit of identification.

From the lysine fraction, treated in a similar manner to the corresponding fraction from the Rous extract, a small yield of creatinine picrate was isolated. After purification 0·2 g. of the salt was obtained, M.P. 216°, from which a hydrochloride was prepared, showing the typical reactions and a M.P. of 261°. From the creatinine mother liquors a very small yield of a picrate melting at 180—190° was isolated, but the quantity was too small to permit of purification or identification.

II (*b*). *Phosphotungstic Acid Filtrates.*

From the phosphotungstic acid filtrates, decomposed and stored as already described, a small yield of tyrosine had separated out. This was removed, the filtrate evaporated to dryness and the residue taken up in alcohol. On the addition of alcoholic picric acid, and standing, a very small yield of creatinine picrate in a fairly pure condition was obtained. This was identified by its melting point, 214°, and by the reactions of the free base prepared from the picric acid salt. No other substance was isolated in a pure condition from this fraction.

III. Extractive Nitrogen of a Lymphosarcoma.

This tumour, weighing some 900 g., was an exceedingly rapidly growing sarcoma of the cervical glands occurring in a young man. The growth was firm and showed little evidence of necrotic change having occurred. The aqueous extract of 800 g. of the tumour was examined.

III (*a*). *Phosphotungstic Acid Precipitate.*

This precipitate weighed 29 g. in the dry state, and was decomposed in the usual manner. The resulting solution of bases was fractionated by the silver method.

III (*a*) i. *Purine Fraction.*

This was the largest silver fraction. It was decomposed and the purine bases reprecipitated by the copper bisulphite process. From the solution of the purine hydrochlorides, obtained by decomposition of the copper compounds, ammonia precipitated 0·02 g. guanine. This was identified by its reactions and the crystalline form of its hydrochloride. From the guanine filtrate picric acid precipitated a fairly heavy yield of adenine picrate. After one recrystallisation, 0·42 g. of the picrate, melting at 272°, was obtained. From the oxy-purine fraction a very small yield of impure xanthine was separated, but no evidence of the presence of hypoxanthine was obtained.

III (*a*) ii *and* iii. *Histidine and Arginine Fractions.*

These fractions were very small and no pure substance was isolated from them.

III (*a*) iv. *Lysine Fraction.*

This fraction was worked up in a similar manner to other lysine fractions, but only a small yield of creatinine in the form of its picrate (0·2 g., M.P. 214°) and traces of tyrosine and leucine were isolated.

III (*b*). *Phosphotungstic Acid Filtrate.*

From this fraction 0·12 g. tyrosine and 0·65 g. leucine were separated in a fairly pure condition.

IV. Extractive Nitrogen of Carcinoma of Pancreas.

The material for this examination was a mass of secondary deposits in the liver from a primary carcinoma of the pancreas. The primary growth weighed but 29 g., whilst nearly 5 kilos. of tumour tissue were obtained from the liver deposits. These growths had undergone somewhat extensive central necrosis, with the formation of caseous material. The aqueous protein-free extract of 3·5 kilos. of this material, excluding as far as possible the more extensively degenerated areas, was examined.

IV (*a*) i. *Purine Fraction.*

This fraction was fairly large and was submitted to an initial purification by the copper bisulphite process. Guanine was isolated in very small amount, 0·07 g. of the somewhat impure base being precipitated by ammonia. This was identified in the usual manner. From the guanine filtrate picric acid threw down a small precipitate of adenine picrate. After recrystallisation 0·25 g. of the purified salt was obtained (M.P. 275°).

After removal of the picric acid from the adenine filtrate the oxy-purines were reprecipitated by the copper bisulphite procedure.

From the decomposed copper compounds 0·03 g. of xanthine and 0·06 g. of hypoxanthine nitrate were isolated by the usual methods.

IV (*a*) ii *and* iii. *Histidine and Arginine Fraction.*

These fractions were very small in quantity and no substances were isolated in a pure condition from them. Indications of the presence of histidine were observed, but the amount present was too small to permit of its isolation or identification.

IV (*a*) iv. *Lysine Fraction.*

The reprecipitated phosphotungstates constituting this fraction weighed in the dry state 27 g. The solution of free bases obtained by decomposition of this precipitate gave upon concentration and treatment with picric acid solution a small yield of creatinine picrate. After one recrystallisation 0·37 g. of the pure picrate was obtained (M.P. 217°).

After freeing the solution from picric acid and further concentrating, a small yield (0·07 g.) of tyrosine was isolated in a fairly pure condition. Traces

of impure leucine were also separated from the residue, which, however, was largely inorganic in nature.

IV (*b*). *Phosphotungstic Acid Filtrate.*

Leucine (0·44 g.) and tyrosine (0·27 g.) were isolated from the concentrated filtrate of this fraction. From the mother liquors picric acid precipitated a further small yield of creatinine picrate (0·11 g. purified salt, M.P. 214°).

No other substance was isolated from this extract.

Discussion of Results.

The results of the four examinations recorded above agree with the scanty published data on tumour extractives. Saiki [1909] examined a mixed batch of tumours, consisting of human carcinomata from various sources, and weighing in all some 200 g. and found 0·001 % of purine nitrogen in the protein-free aqueous extracts. He was unable to isolate the individual purines owing to the small amount of material at his disposal. In another trial he found that the purine bases present in 570 g. of mixed cancerous tissue, mainly carcinomata of the ovary, were as follows: 0·1391 g. uric acid, 0·1866 g. adenine, 0·0190 g. hypoxanthine, and traces of guanine and xanthine. He suggests that these observations indicate the presence of guanase and xanthoxidase, and the absence of adenase in human tumours.

Wells and Long [1913] found that the purine content and purine enzymes of human tumours, both malignant and benign, resembled those of normal tissues, and that xanthoxidase was absent. They found it difficult to explain the high percentage of uric acid found by Saiki, since the latter author gives practically no details of the methods employed in the isolation or identification.

Saiki also records finding creatinine to the extent of 0·016 % in fresh cancer tissue, and very small amounts of creatine.

The presence of considerable amounts of carnosine and creatinine in the extract of the Rous chicken sarcoma may be attributed to some extent to the extensive degradation of muscle tissue consequent on the tumour invasion.

Autolysis is probably responsible for the presence of the amino-acids, for Beebe [1904] found leucine, tyrosine, glycine and tryptophan in tumour extracts.

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XXIII. A STUDY OF THE WATER-SOLUBLE ACCESSORY GROWTH PROMOTING SUBSTANCE IN YEAST. I.

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The researches of Stepp [1909, 1912, 1913], Hopkins [1912] and Funk [1913, 1, 2, 3] have demonstrated the importance of certain constituents of a normal dietary which for want of better names have been variously termed "vitamines," growth substances, and accessory growth hormones. It is now known that unless an adequate supply of these as yet unidentified substances is present in the food supply of an animal, serious consequences affecting the general nutrition of the subject will arise. Recently McCollum and Davis [1915, 1, 2, 3] have carried out some researches which have yielded results indicating that two distinct types of accessory substance exist. These by reason of their properties have been conveniently designated the "fat-soluble *A*" and the "water-soluble *B*." The view held by these authors that both substances are equally important components of a normal dietary has received experimental confirmation by Drummond [1916], and has been accepted by the majority of other investigators [Macallum, 1916; Osborne and Mendel, 1916]. Following up his studies along these lines McCollum, in collaboration with Kennedy [1916], has arrived at the conclusion that the production of polyneuritis in birds by exclusive rice feeding [Eykmann, 1897; Funk, 1913, 1, 2, 3], or exclusive feeding of a ration made up of purified foodstuffs [Funk, 1914, 1] is a specific result of the absence of the water-soluble factor *B*. In other words it is proposed to regard the so-called "antineuritic vitamine" as being identical with the water-soluble factor *B*. McCollum and Kennedy have based this suggestion upon the curative value possessed by many extracts, known to contain the water-soluble substance, when administered to birds which have developed the typical symptoms of polyneuritis gallinarum (Eykmann), as a result of feeding solely upon a diet lacking in that substance.

Our knowledge of the chemistry of these accessory substances or "vitamines" is as yet lamentably small, so that should further experimental evidence be forthcoming in support of the proposal advanced by McCollum and Kennedy it would mark a distinct advance in this field of research.

For some time past an investigation has been in progress in this laboratory aiming at the isolation and identification of the dietary factor *B*.

Viewed from this standpoint alone the results of the research have been disappointing, but on the other hand many observations have been made during its progress which help to throw a certain amount of light upon the nature of this elusive substance.

Taken collectively these observations tend to uphold the view advanced by McCollum and Kennedy, since it is found that the chemical properties of the water-soluble growth promoting factor *B* are, as far as they have been studied, very similar to those which have been described for the antineuritic principle. Ever since Eykmann demonstrated that the condition induced in fowls by a diet of polished rice was analogous to human beri-beri, and that there existed a substance in the layers of the rice grain removed during polishing which would cure the condition, numerous investigators have attempted to isolate the curative agent in a pure state.

From the mass of literature which records the results of these many attempts it is difficult to abstract that which may be regarded as sound. Various properties of the curative substance have been described at one time or another. Chamberlain and Vedder [1911] showed that it was soluble in alcohol and dialysable, whilst Cooper and Funk [1911] demonstrated that it would withstand drastic acid hydrolysis and that it was precipitated by phosphotungstic acid.

Since these facts seemed to point to the active substance being of the nature of a comparatively simply constituted nitrogenous base, several investigators attempted to isolate the pure substance along the lines usually adopted in such cases. Funk [1911] confirmed that the substance was precipitated by phosphotungstic acid, and made the further observation that upon sub-fractionation of the substances precipitated by this reagent by the silver process it was to be found in association with the pyrimidine bases. These results were repeated and confirmed by him [1912, 1], and upon these facts he based his reasons for assigning to the product the name "vitamine" [1912, 2]. At about the same time several isolations of the active substance were reported [Schaumann, 1914; Edie, Evans, Moore, Simpson and Webster, 1912; Susuki, Shinamura and Odake, 1912].

The last three claims, however, have now been shown to be unfounded [Funk, 1913, 1], whilst the crystalline derivatives isolated by Funk himself from the pyrimidine fraction of an alcoholic extract of rice polishings, and thought by him to be active [1912, 1], were shown to consist of impure specimens of nicotinic acid and betaïne [Drummond and Funk, 1914]. Pol [1917] has recently described the isolation of a crystalline acid from *Phaseolus radiatus* which he states has cured polyneuritis in birds and beri-beri in man. The details of his process have, unfortunately, not been available, but it is improbable that the crystalline substance he has isolated is the curative substance in a pure condition, because the author mentions that the preparation loses its activity as purification progresses. It is conceivably the same type of product as was isolated by Funk, where a definite crystalline body (nicotinic acid) in some cases contained the active substance as a contamination.

We are therefore practically without knowledge regarding the chemical nature of the antineuritic substance and cannot claim that much progress has been made in obtaining such information during the past few years.

With regard to the properties of the substance itself we are in possession of a number of scattered observations which have been made from time to time, and which generally speaking are in agreement.

The antineuritic substance is apparently only partially soluble in alcohol. This solubility is very small in absolute alcohol, but becomes greater as more dilute alcohol is used [Funk, 1912, 1; Cooper, 1913, 1; Vedder and Williams, 1913; Sullivan and Voegtlin, 1916].

It is usually described as being insoluble in ether, chloroform and other lipoid solvents [Cooper, 1913, 1]. It is adsorbed by animal charcoal [Chamberlain and Vedder, 1911; Cooper, 1913, 1] whilst a colloidal aluminium silicate (Lloyd's reagent) has recently been used for quantitatively adsorbing the antineuritic substance [Seidell, 1916; Eddy, 1916].

Most authors agree that the substance is unstable under certain conditions to alkali. Cooper [1913, 1] reported its destruction by that agency, and this observation has been confirmed [Chamberlain, Vedder and Williams, 1912; Fraser and Stanton, 1915; Funk, 1915]. On the other hand Williams and Seidell [1916], are inclined to doubt whether cold alkali does exert an adverse influence upon the substance, whilst Steenbock [1917] definitely states that only hot alkali has such an effect.

With regard to the effect of acids, most authors agree that it is stable even when they are applied hot and in a relatively concentrated condition. Cooper

and Funk [1911] found the curative value of yeast extracts unaffected by hydrolysis for twenty-four hours with 20 % sulphuric acid, and this stability to acid is confirmed in papers by Funk [1911], Vedder and Williams [1913], Williams and Seidell [1916], and Steenbock [1917]. The degree of thermostability possessed by the substance has been noted by several investigators who appear to agree that temperatures below 100° do not exert a deleterious effect [Cooper and Funk, 1911; Steenbock, 1917]. Higher temperatures, however, are usually considered to injure its activity.

The value of yeast as an antineuritic has been recorded upon several occasions [Cooper and Funk, 1911; Funk, 1912, 1; Cooper, 1913, 2; Schaumann, 1914; Willcox, 1916; Seidell, 1916].

As has already been remarked the first named authors showed that acid hydrolysis failed to lower the antineuritic value of yeast, whilst Cooper [1914] has shown that autolysed yeast is as active as was the original yeast.

Barsickow [1913] found yeast itself to be curative, but that heated yeast and "cerolin"[1] (a yeast extract) possessed no such value. He believed that the curative action was due to the action of nuclein or certain salts.

Funk [1913, 1] investigated the curative value of yeast- and thymus-nucleic acids and some of their degradation products when administered to polyneuritic pigeons, and found that certain of these substances exerted a beneficial influence upon the progress of the disease. Some of the many attempts to isolate the active substance from yeast preparations have already been alluded to. More recent ones are those described by Seidell [1916], and Williams and Seidell [1916].

Attention must now be directed to a consideration of the recorded properties of the water-soluble growth promoting accessory factor. That yeast possessed a rich supply of a growth promoting factor was noted by Hopkins [1912] and by Funk [1913, 2; 1914, 2]. The latter author has since then made several attempts to isolate the substance along lines such as were employed by him in his efforts to isolate the antineuritic substance from the same source.

These attempts have failed to achieve their object, but his results show that the active substance possesses several properties, also possessed by what he terms the "anti-beri-beri vitamine." Funk and Macallum [1915] confirmed the presence of the growth promoting substance in dried brewer's yeast, and in a later paper [1916] they described its presence in autolysed yeast and recorded that it is precipitated by phosphotungstic acid. Attempts at further fractionation of the resulting precipitate failed, for no growth

promoting powers were possessed by the purine or pyrimidine fractions obtained from it. Funk [1915, 1916] has stated that the growth promoting accessory from yeast forms a phosphotungstate insoluble in acetone.

With regard to the solubility of the growth promoting accessory *B* it is known that it is extractable by alcohol [Stepp, 1909; Hopkins, 1912; and others]. It is stable to temperatures below 100° [Drummond, 1916], but is apparently to some extent adversely affected by autoclaving at higher temperatures [Hogan, 1917]. From this brief summary, which is scarcely a complete review of the literature describing the two substances, it will be seen that many of the properties by which the antineuritic substance is described are also possessed by the water-soluble growth accessory factor.

As has already been remarked McCollum and Kennedy have suggested the identity of the two substances [1916], and the same view is apparently held by Macallum [1916].

The experiments which are reported in this paper entirely support this view and make it almost certain that the two substances are either identical or are individually closely related members of the same class of substance.

Experimental[1].

For the purposes of this research young rats from five to six weeks old and weighing approximately 50 g. were utilised. They were drawn from healthy pure-bred stock reared under careful observation and belonged to the black variety of *Mus norvegicus* [Donaldson, 1915]. This particular strain has been found eminently satisfactory for the studies in growth and nutrition which are being made in this laboratory.

The experimental animals were kept in wooden boxes with sawdust covered floors and provided with warm enclosed sleeping quarters.

They were fed twice daily, unused food from the previous meal being removed from the cage upon each occasion. The artificial dietaries were made up into the convenient form of firm balls weighing approximately 5 g. This forms a rough and ready method of estimating the food consumption of the animals. Fresh drinking water is at all times an essential to the health and welfare of the rat, and was in these experiments renewed at least twice daily, the earthenware drinking pans being scalded out on each occasion.

A basal artificial diet was employed which contained all the necessary

[1] I wish to express my gratitude to Mr A. Chaston Chapman for his kindness in supplying me with the highly pure yeast-nucleic acid used in this work.

components of a normal diet with the exception of the water-soluble accessory substance. This diet was made up as follows:

Caseinogen	18 parts.	Salt mixture	5 parts
Starch	40 „	Sucrose	17 „
Agar	5 „	Butter-fat	15 „

The various components were carefully purified before use by extractions designed to remove all trace of the water-soluble growth factor. The composition of the salt mixture together with full details of the preparation and purification of the various components of the basal diet are described in a recent communication by Halliburton and Drummond [1917].

That this diet was for all intents and purposes free from the water-soluble accessory may be seen from Chart I, which may be compared with Charts II and III in which are represented the growth curves of rats fed upon a diet such as the above to which had been added the missing accessory substance in the form of yeast preparations. The animals fed upon these diets showed normal growth and reproduction upon the completed ration.

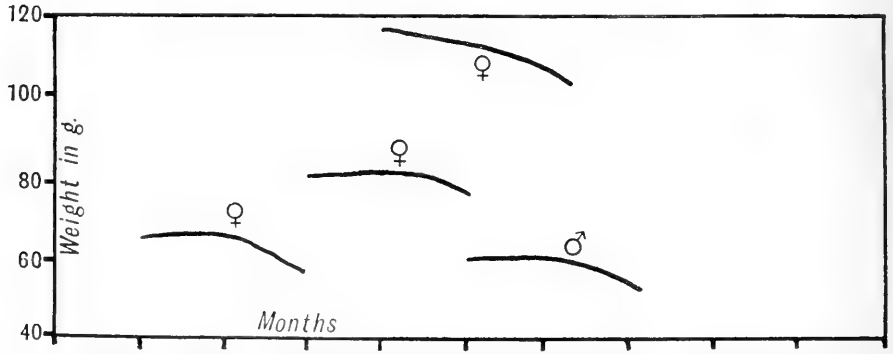

Chart I. Growth curves of rats fed upon basal diet deficient in water-soluble growth promoting accessory factor *B*.

1. *Presence of the water-soluble growth promoting accessory substance in dried yeast and commercial yeast extract.*

Chart II shows the influence upon the growth of young rats exerted by the addition of 3–12 % of dried yeast powder to the basal diet. The rats were able to complete their life cycle without any difficulty, and the animals reared solely upon this diet appeared as healthy and as resistant to disease as those which had enjoyed a normal mixed ration.

Chart III indicates that "marmite," a commercial yeast extract, is rich in the water-soluble accessory factor. The addition of 6 % of this preparation to the basal diet satisfactorily makes good the inadequacy it previously

possessed. Smaller additions of "marmite" were less efficient in this respect, whilst greater percentages were apparently not well tolerated by the animals. These results confirm the findings of the authors that have been referred to with regard to the presence of the water-soluble accessory factor in yeast and its aqueous extract.

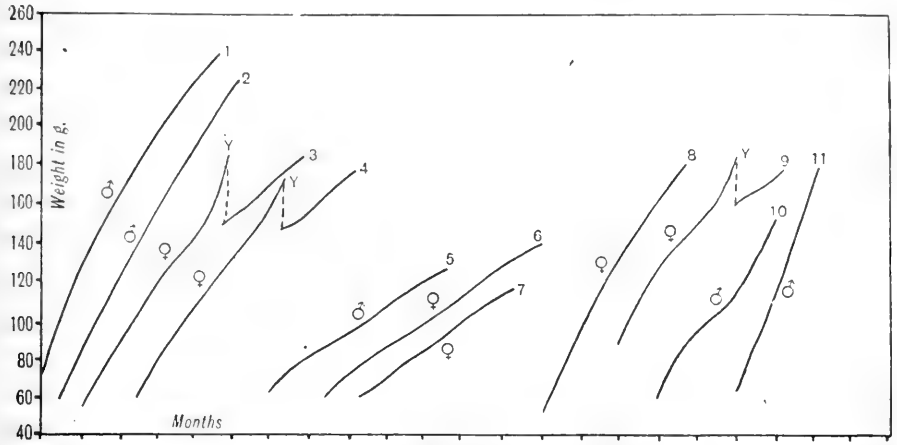

Chart II. Curves 1–4. Growth of rats on basal diet + 6 % dried yeast. Curves 5–7. Growth of rats on basal diet + 3 % dried yeast. Curves 8–11. Growth of rats on basal diet +12 % dried yeast. Y indicates birth of young. These were all reared satisfactorily by the mothers.

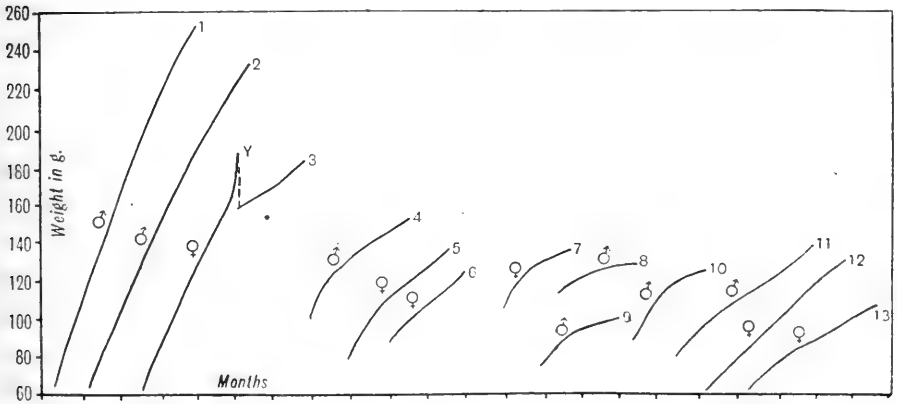

Chart III. Curves 1–3. Growth of rats on basal diet + 6 % "marmite." Curves 4–6. Growth of rats on basal diet + 3 % "marmite." Curves 7–10. Growth of rats on basal diet + 1 % "marmite." Curves 11–13. Growth of rats on basal diet + 12 % "marmite." Y indicates birth of young. All were reared satisfactorily by the mother.

2. *Solubility of the water-soluble accessory substance in alcohol and ether.*

The accessory factor is not removed from dried yeast by extraction with absolute alcohol, so that it appears that the substance is insoluble in pure

alcohol (Chart IV). The dried yeast for this experiment was extracted with several changes of boiling alcohol under reflux. Similarly from the same chart it can also be seen that prolonged ether extraction removes little or none of the active substance.

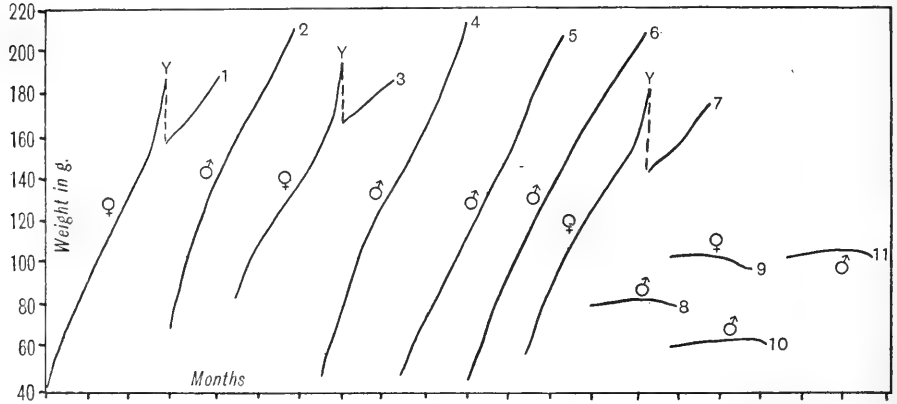

Chart IV. Curves 1–4. Growth of rats fed on basal diet + 6 % alcohol-extracted dried yeast. Curves 5–7. Basal diet + 6% ether extracted yeast. Curves 8–11. Basal diet + alcohol extract equivalent to 6 % of dried yeast. Y marks birth of young.

It may be mentioned here that dried yeast contains but negligible traces of the fat-soluble growth promoting accessory substance.

Extraction of "marmite" by grinding up with absolute alcohol and repeatedly shaking removes but a small proportion of the accessory factor (Chart V), but when extracted with a more dilute alcohol (70 %) the proportion that goes into solution is greatly increased.

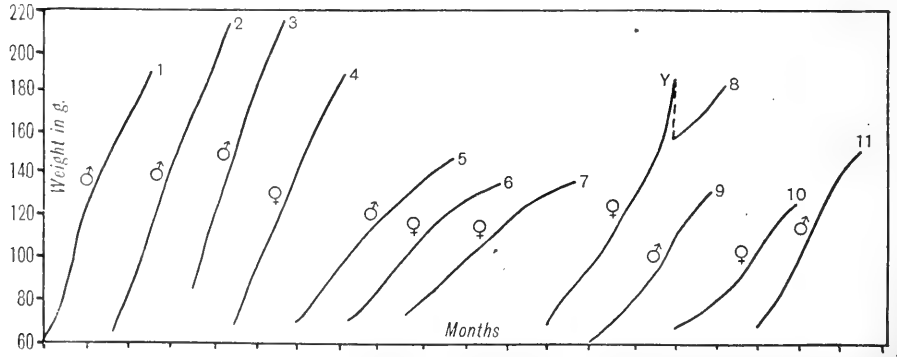

Chart V. Curves 1–4. Growth of rats fed on basal diet + 6 % alcohol-extracted "marmite." Curves 5–7. Ditto on basal diet + 6 % "marmite" extracted by 70 % alcohol. Curves 8–11. Ditto fed on basal diet + alcohol extract equivalent to 6 % "marmite." Y indicates birth of young. All were reared.

3. *Dialysis of yeast extract.*

A strong solution of "marmite" in distilled water was dialysed against running distilled water for forty-eight hours. The combined dialysates were evaporated to small bulk *in vacuo* at low temperature and tested for the presence of the accessory substance under consideration. Chart VI shows that the substance is dialysable through parchment paper, although it was not completely removed from the "marmite" in the time during which the dialysis was carried out. This dialysate contained large amounts of inorganic salts chiefly potassium phosphate and sodium chloride and considerable amounts of leucine and adenine.

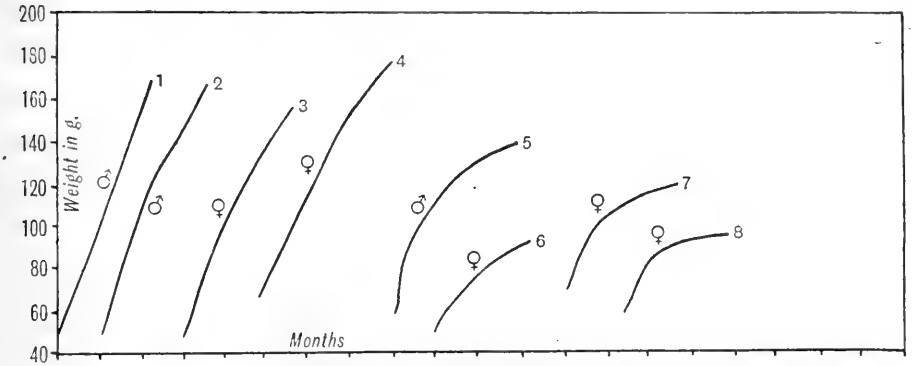

Chart VI. Curves 1–4. Rats fed upon basal diet + dialysate representing 6 % "marmite.' Curves 5–8. Rats fed upon basal diet + 6 % residue remaining after dialysis.

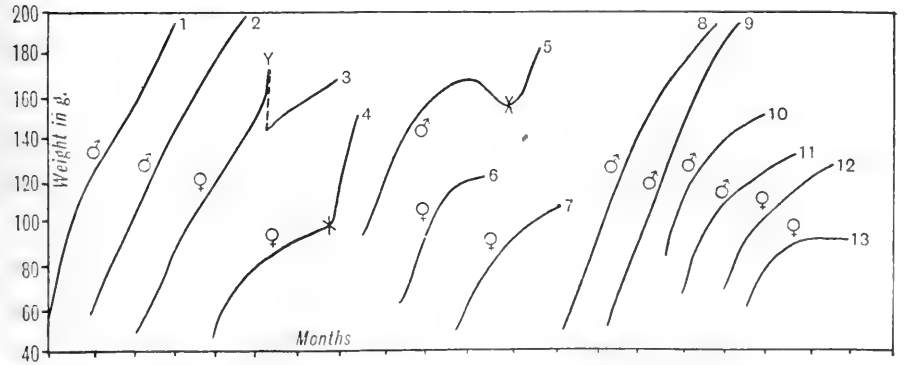

Chart VII. Curves 1–4. Basal diet + 6 % dried yeast heated to 100° for 30 mins. Curves 5–7. Basal diet + 6 % dried yeast autoclaved at 120° for 30 mins. Curves 8 and 9. Basal diet + 6 % "marmite" heated at 100° for 30 mins. Curves 10–13. Basal diet + 6 % "marmite" autoclaved at 120° for 30 mins. Y indicates birth of young. × indicates the time at which the addition of 6 % "marmite" was made to the diet.

4. *Effect of temperature upon the water-soluble growth promoting accessory substance.*

Chart VII demonstrates the growth promoting value of yeast and yeast extracts that have either been subjected to a temperature of 100° or have been autoclaved at 120° for thirty minutes.

The activity of these preparations was practically unaffected by the first treatment, whilst injury but not complete destruction resulted in the second case.

5. *Influence of acids.*

Chart VIII indicates the behaviour of the accessory factor in the presence of acids. One sample of "marmite" was subjected to the influence of dilute hydrochloric acid (1 %) at boiling point for twelve hours, the resulting fluid being carefully neutralised and concentrated to the original bulk. This preparation was not as valuable in promoting growth when added to the basal diet as was a corresponding amount of the untreated marmite, but the activity was not greatly diminished.

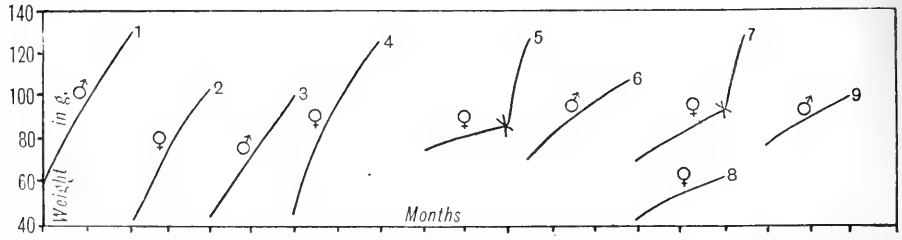

Chart VIII. Curves 1–4. Basal diet + 6 % "marmite" after dilute hydrochloric acid treatment. Curves 5–9. Basal diet + 6 % "marmite" after sulphuric acid hydrolysis. × indicates the time at which the addition of 6% "marmite" to the diet was made.

A second quantity of "marmite" was subjected to hydrolysis with 20 % sulphuric acid at boiling point for ten hours. The sulphuric acid was then quantitatively removed from the diluted liquid by means of baryta, and the neutral filtrate, together with the washings of the bulky precipitate, was evaporated to a thick syrup at a low temperature under diminished pressure.

This preparation possessed a greatly decreased power to promote growth in the experimental animals. In view of the observations which are given later in this paper, this result is regarded as being due not so much to an actual destruction of the accessory substance as to loss occurring by adsorption on the bulky precipitate of barium sulphate and pigmented matter.

6. *Influence of alkali.*

Chart IX displays the growth curves of rats fed upon the basal diet together with additions of treated yeast preparations submitted to various treatments with alkali. One set of curves demonstrates that treatment of "marmite" for twenty-four hours at room temperature with 5 % sodium hydroxide results in very little depreciation in its growth promoting value.

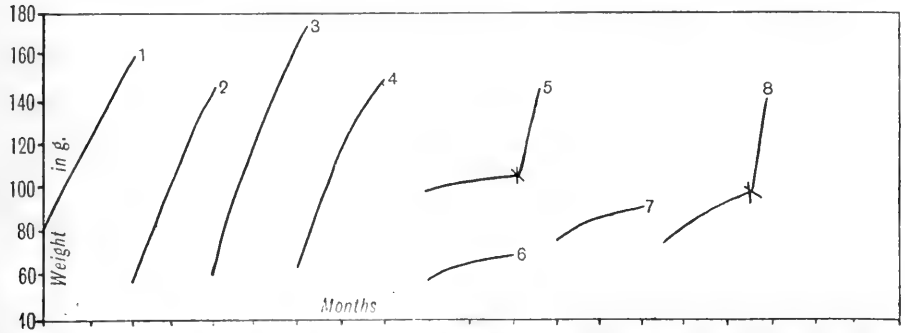

Chart IX. Curves 1–4. Basal diet + 6 % "marmite" after treatment with alkali at room temperature. Curves 5–8. Basal diet + 6 % "marmite" after hot alkali treatment. × indicates the time at which the addition of 6 % "marmite" to the diet was made.

On the other hand treatment with hot 5 % sodium hydroxide for five hours tends to destroy its activity. In both these cases after treatment with alkali the fluid was neutralised and carefully evaporated *in vacuo* at low temperature to its original bulk.

Attempted separation of the active substance.

1. *Precipitation from extracts by phosphotungstic acid.*

A preparation consisting of the concentrated dialysate from 200 g. of commercial yeast extract, prepared as already described, was diluted to a volume of 2000 cc., acidified to the extent of 5 % with sulphuric acid, and precipitated by the addition of a 30 % aqueous solution of phosphotungstic acid. The flocculent, buff-coloured precipitate was allowed to stand overnight at ice-room temperature and filtered off next morning, the precipitate being thoroughly washed with 5 % sulphuric acid. When dry the precipitated phosphotungstates weighed 87 g. Since the majority of such salts contain phosphotungstic acid to the average extent of 85 % of their weight, this yield represents some 12 g. of substances removed from the extract. The precipitated phosphotungstates were decomposed by treatment with

hydrochloric acid and extraction with the amyl alcohol-ether mixture recommended by van Slyke [1915]. Very incomplete decomposition was effected by this method, for a bulky flocculent precipitate, probably representing precipitated pigment, resisted decomposition. The aqueous fraction was separated after repeated extraction, filtered, neutralised and evaporated to small bulk at reduced temperature and pressure.

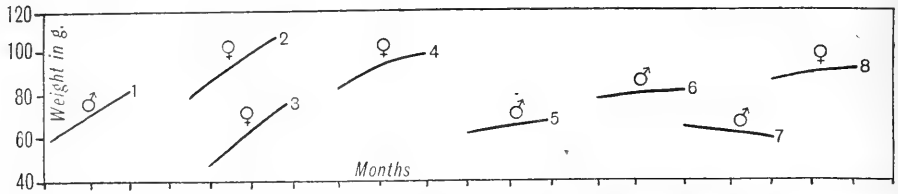

Chart X. Curves 1–4. Basal diet + phosphotungstic acid precipitate fraction corresponding to 18 % "marmite." Curves 5–8. Basal diet + phosphotungstic acid filtrate fraction corresponding to 18 % "marmite."

Chart X demonstrates that this fraction possessed a small growth promoting activity when administered in doses representing 6–18 g. of the original "marmite." Great loss had, however, occurred during the process. The filtrate from the phosphotungstic acid precipitation was freed from sulphuric acid and the excess of the precipitant by means of baryta and after concentration tested for activity. None however was observed (Chart X).

Whether the active substance is completely precipitated by phosphotungstic acid is uncertain, for any which was unprecipitated would be adsorbed and lost during the removal of the sulphuric and phosphotungstic acids by baryta, owing to the very bulky precipitate which results. Great loss also occurred without a doubt due to the adsorption of the active body by the flocculent residue of undecomposed phosphotungstates which has been already noted.

A further preparation of the phosphotungstates from 200 g. of "marmite" was made, and weighed in the dry condition 90 g. This was treated when quite dry by shaking up with repeated changes of acetone [Funk, 1916]. A large proportion (69 g.) of the precipitate remained undissolved. It would seem probable from studies upon the solubilities of the phosphotungstates in acetone (not yet published), that the inference to be drawn from this fact is that the chief extractives of yeast precipitated by phosphotungstic acid are purine bases or the inorganic bases potassium and ammonium. Both fractions of the phosphotungstic acid precipitate were freed from acetone, decomposed by the amyl alcohol-ether process and worked up for inclusion in the diets

as in the former case. A large proportion (40 %) of the acetone-insoluble phosphotungstate was not decomposed by this process, and again formed a flocculent precipitate which much interfered with the separation.

The curves shown in Chart XI demonstrate that the water-soluble growth accessory factor is mainly present in the acetone-insoluble fraction of the phosphotungstic acid precipitate, although great loss has occurred during the separation.

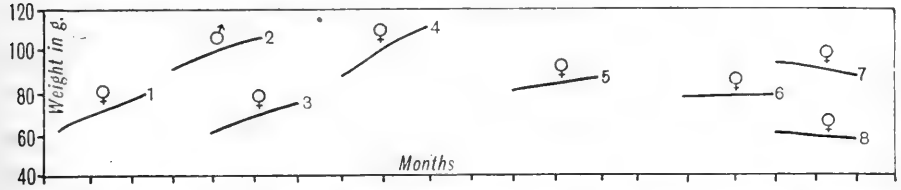

Chart XI. Curves 1–4. Basal diet + acetone-insoluble phosphotungstic acid precipitate fraction corresponding to 10 % "marmite." Curves 5–8. Basal diet + acetone-soluble phosphotungstic acid precipitate fraction corresponding to 10 % "marmite."

2. *Basic lead acetate precipitation of yeast extracts.*

A 10 % solution of "marmite" gave an exceedingly bulky flocculent precipitate upon the addition of basic lead acetate solution. After removing this precipitate and subjecting it to a thorough washing, both precipitate and filtrate were decomposed by sulphuretted hydrogen gas, the lead-free solutions being neutralised and concentrated at low temperature and diminished pressure.

Little growth promoting power was observed in either fraction (Chart XII), it being considered probable that the heavy and flocculent nature of the lead sulphide precipitates had resulted in practically complete adsorption and loss of the active substance.

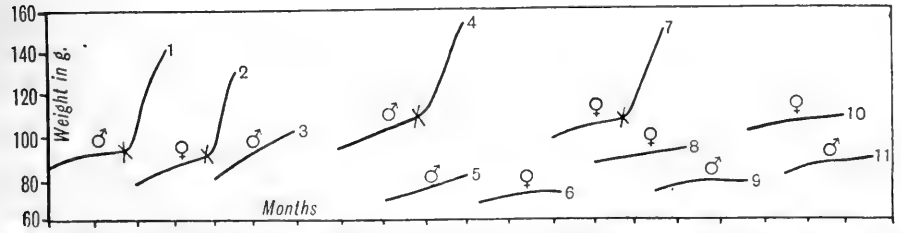

Chart XII. Curves 1–3. Basal diet + lead acetate filtrate fraction corresponding to 6 % "marmite." Curves 4–6. Basal diet + lead acetate precipitate fraction corresponding to 6 % "marmite." Curves 7–9. Basal diet + lead acetate filtrate fraction representing 6 % dialysed "marmite." Curves 10 and 11. Basal diet + lead acetate precipitate fraction representing 6 % dialysed "marmite." × indicates the time at which the addition of 6 % "marmite" to the diet was made.

A sample of the dialysate from the "marmite" was precipitated in a similar manner with basic lead acetate and little evidence of the presence of the active substance in either fraction was obtained, and again great loss had occurred.

3. *Silver nitrate precipitation.*

To the forty-eight hours' dialysate from 250 g. yeast extract silver nitrate was added until no further precipitation occurred. The silver-free filtrate after neutralisation and concentration showed little or no evidence of the presence of the growth accessory substance under investigation (Chart XIII).

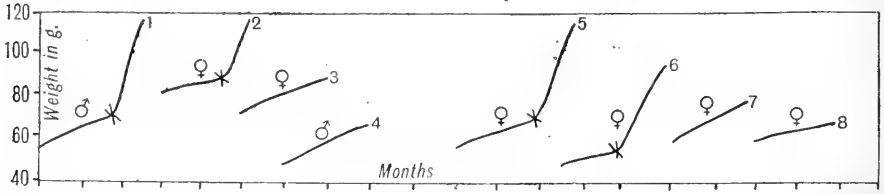

Chart XIII. Curves 1–4. Basal diet + purine fraction from dialysed "marmite" corresponding to 6 % "marmite." Curves 5–8. Basal diet + corresponding pyrimidine fraction. × indicates the time at which the addition of 6% "marmite" to the diet was made.

The silver precipitate was decomposed by sulphuretted hydrogen gas and the filtrate concentrated with care after neutralisation. Small traces of the active substance appeared to be present. Chemical examination of the fraction demonstrated that the majority of the nitrogen was present in the form of adenine.

4. *Relation of adenine to the water-soluble accessory substance.*

Adenine itself was found to possess no growth promoting properties when added to a diet deficient in the water-soluble factor *B* (Chart XIV). This is of interest in view of the fact that the recent work of Williams and Seidell [1916] has not received confirmation [Voegtlin and White, 1916; Steenbock, 1917; Harden and Zilva, 1917]. Williams and Seidell attributed an anti-neuritic action to a supposed isomeric form of adenine. Funk [1913, 1] found a distinct improvement in the condition of polyneuritic pigeons following the administration of thymus-nucleic acid, guanosin, adenosin and cytidine, and slightly less satisfactory results when certain purine and pyrimidine bases were given.

The influence upon growth of pure yeast-nucleic acid and of a nucleic acid prepared from the spleen in a case of spleenic leucaemia was therefore studied, but neither preparation in any way improved the growth value of the basal diet (Chart XIV).

Commercial meat extract ("Lemco") does not show evidence of the presence of the water-soluble accessory factor (Chart XV), so that the identity of the latter substance with any of the meat bases is rendered doubtful. Commercial meat extracts have been found to possess no power to improve the condition of polyneuritic birds [Chick and Hume, 1917].

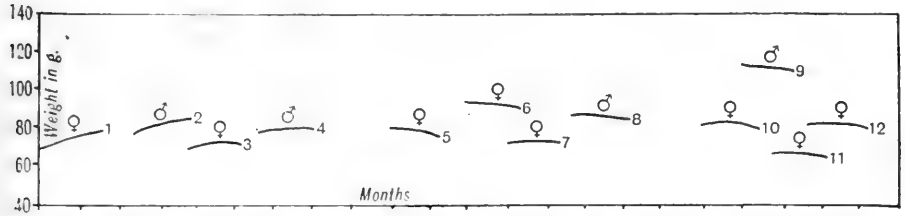

Chart XIV. Curves 1–4. Basal diet + 1 % yeast nucleic acid. Curves 5–8. Basal diet + 1 % spleen nucleic acid. Curves 9 and 10. Basal diet + 0·5 % adenine. Curves 11 and 12. Basal diet + 0·2 % adenine.

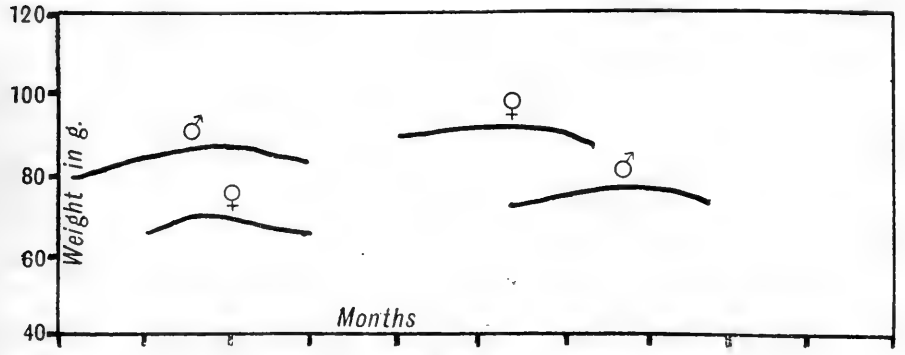

Chart XV. Basal diet +6 % "Lemco."

Discussion.

The results of this investigation, in so far as they are complete, tend to support the view held by McCollum and Kennedy that it is a deficiency of the water-soluble accessory substance in the dietary of birds that is responsible for the incidence of the typical symptoms which are held to represent a pathological condition analogous to beri-beri in man.

Attempts have been made along many lines to isolate the accessory substance from yeast extracts. All of them have however failed to achieve their object, and the only useful result of the investigation which has as yet been obtained has been that a knowledge of a few of the properties of the elusive substance has been gained.

Many workers have lamented the difficulties which are encountered in research in this particular field, and have drawn attention to the way in which the active substances disappear as the fractionation proceeds.

The usual explanation of this has been in the past to regard the substance as unstable. Thus Funk [1913, 3] believed that he had found evidence of the frequent occurrence of nicotinic acid in fractions which had once possessed, but had later lost, curative power, and on these grounds he was inclined to regard that acid as a derivation product of the active "vitamine." Similarly more recent writers have described the existence of isomeric modifications of adenine [Williams and Seidell, 1916], and of certain betaïnes [Williams, 1917], which possess curative properties. This latter work has not, however, received the support of other workers [Voegtlin and White, 1916; Harden and Zilva, 1917], and therefore stands in need of confirmation before it can be accepted.

It seems far more probable that the correct explanation of the loss which occurs during fractionation of extracts containing the antineuritic substance or the water-soluble growth promoting substance is to be found in the readiness with which these substances, whether identical with one another or not, are adsorbed from solution by precipitates.

As has already been mentioned this property was observed several years ago by Chamberlain and Vedder [1911], and Cooper [1913, 1], whilst certain modern work confirms the observation. Thus Seidell [1916] has based a process for separating the substance from autolysed yeast upon its adsorption by fullers' earth, and Voegtlin and White [1916] record that mastic emulsion and colloidal arsenious sulphide will carry down the antineuritic principle in the adsorbed condition.

Further proof that this is probably the explanation of the great loss which is so often encountered in the isolation of the substance is to be found in the fact, that whenever in the course of the fractionation recourse to precipitation is made, the precipitate, particularly if bulky and flocculent in nature, will usually carry down the majority, if not all, of the active substance from solution.

It is naturally a very difficult matter to trace the growth promoting principle during fractionation, because minute traces of that substance do not always demonstrate their presence by causing an appreciable increase in the body weight of the experimental animal, whereas much smaller quantities of such extracts are often sufficient to cause a decided improvement in the condition of a polyneuritic pigeon. Unfortunately neither the experimental results recorded in this paper nor this discussion can throw much light upon

the chemical identity of the class of substance under consideration. One point remains clear, namely, that there is now less evidence than ever to support the continued use of the word "vitamine" coined by Funk. Williams and Seidell [1916] have quite recently supported the adoption of the term, but McCollum and Kennedy [1916] have clearly pointed out that the arguments against its inclusion in biochemical nomenclature far outweigh those advanced by the supporters of Funk, and will continue to do so until our knowledge of the chemical nature of these substances justifies the adoption of the appellation.

Summary.

1. Experimental evidence is given which demonstrates that certain of the properties possessed by the antineuritic substance and the water-soluble accessory growth factor *B*, are very similar.

2. This fact supports the view that the two substances are identical.

3. The repeated failures to isolate substances of this class in the pure condition are attributed more to the fact that they are readily removed from solution in the adsorbed condition by precipitates, than to their reputed instability.

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XXIV. THE REDUCTION OF PHENYLETHYLAMINE.

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(*Received August 31st, 1917.*)

The purpose primarily in view was to reduce several compounds closely allied to tyrosine by that method in which the reduction is brought about by shaking the compound in question with hydrogen and a catalyser in the shape of finely divided platinum or palladium. The catalysers were prepared by reducing solutions of the respective chlorides by means of formaldehyde and alkali [Willstätter and Hatt, 1912][1]. A point to be noted is that in all instances mentioned in the course of this paper where reduction did not take place, the catalyser employed was subsequently tested and found to be active. This goes to prove that the negative results in these instances are to be attributed to inherent reasons, rather than to inactivity of the catalyser. The test applied was to shake castor-oil in ethereal solution with hydrogen and the catalyser to be tested. In every case reduction proceeded very rapidly, the white, solid product of reduction simultaneously falling out of solution. Generally ten minutes sufficed for the complete reduction of 5 g. of the oil.

Previous to being subjected to reduction, the hydrochloride of phenylethylamine was purified and tested. This sample of the base had been prepared from phenylalanine by splitting off carbon dioxide. Recrystallised from alcohol, the hydrochloride was found to melt at 215°, thus proving its purity. The compound decolorised permanganate solution instantly. Its chloroplatinate (rhombs and flakes from water) was found to decompose at approximately 250°. The properties of the free base were likewise found to agree with the properties of phenylethylamine in all respects.

Of this hydrochloride, 0·6 g. was reduced by shaking at ordinary temperature in aqueous solution with hydrogen and a catalyser in the shape of finely divided platinum. The absorption of hydrogen proceeded but very slowly, so that the 253 cc. theoretically requisite for complete reduction had been absorbed only at the end of about 1·5 hours. Almost precisely at this point, the absorption stopped entirely. This is a decided proof that no leakage,

[1] Cf. H. Meyer's *Determination of Radicals in Carbon Compounds* (3*d.* original edition) for a detailed description of the method and its application.

but rather solely absorption had been taking place. Another 1·35 g. sample of the same hydrochloride was reduced in the same way. In this case also, the absorption stopped as soon as the requisite volume of hydrogen (570 cc.) had been absorbed. After filtering off the platinum, the solutions were evaporated, thus yielding the *hydrochloride* of the reduction-product in the shape of a white, crystalline mass. This compound was found to be readily soluble in water. It was purified by dissolving it in alcohol, and reprecipitating by means of ether. It melts at 252°, *i.e.* approximately 40° higher than the hydrochloride of unreduced phenylethylamine. The compound no longer decolorises permanganate solution—an additional sign that complete reduction has taken place. Millon's reagent causes a white precipitate which however readily redissolves when gently heated.

The chloroplatinate. With platinic chloride, a voluminous precipitate resulted. This salt was always obtained finely crystalline on recrystallising it from water. It decomposes and chars at 255–257°, previously becoming somewhat darker at 220°. While the chloroplatinate was being decomposed by magnesium, during the chlorine determination, a very strong basic odour became apparent.

0·1362 g. vac.-dry; 0·0000 g. H_2O; 0·0399 g. Pt; 0·1751 g. AgCl.
0·1363 g. vac.-dry; 0·0000 g. H_2O; 0·0398 g. Pt.

	Found		Calculated for hexahydrophenylethylamine chloroplatinate $(C_8H_{17}NHCl)_2PtCl_4$
Pt	29·29 %	29·20 %	29·40 %
Cl	31·80		32·04

The aurichloride. Recrystallised from water containing a few drops of hydrochloric acid, this salt takes the shape of large, very thin, striated, glistening flakes. It was dried in a vacuum desiccator. It melts somewhat indistinctly at about 135–138°.

0·1275 g.; 0·0000 g. H_2O; 0·0538 g. Au
0·1421 g.; 0·0000 g. H_2O; 0·0599 g. Au
Found 42·23 % 42·19 % Au
Calculated for $C_8H_{18}N$, $AuCl_4$: 42·23 % Au

The picrate. Recrystallised from water, this at times formed prismatic needles, and at other times, transparent, glistening flakes. It was found to melt at 155–156°.

This examination of the reduction-product fully suffices to establish its identity as hexahydrophenylethylamine, for it and its derivatives agree in every respect with the data upon cyclohexylethylamine [Wallach, 1908], as obtained by the reduction of cyclohexylacetonitrile.

An additional attempt to reduce phenylethylamine was undertaken. In contradistinction to that of the two reductions just mentioned, the sample of base now employed represented the purchased synthetic product, in the shape of the hydrochloride. An attempt to reduce 1 g. of this by means of the afore-mentioned method showed that the compound would absorb no hydrogen whatsoever. Before repeating the attempt, the compound in question was thoroughly examined, in order to confirm its identity with phenylethylamine.

The hydrochloride crystallised from alcohol in thin flakes, melting at 214–216°, thus agreeing perfectly with the previous sample. The substance however decolorised permanganate solution but very slowly; a dilute solution requiring several hours. The base, liberated by alkali, represented an oil with an odour much like that of methylamine. The base quickly absorbs carbon dioxide from the air, thus producing a carbonate which forms thin flakes. The base dissolves in ether and in alcohol. The chloroplatinate, recrystallised from water, forms small, faintly yellow flakes, generally lozenge-shaped, joined and superimposed to form spear-like structures. The compound becomes darker at 210°, black at 220°, and then melts at about 255°. It is practically insoluble in alcohol, sparingly soluble in cold water, more readily soluble in hot water. As regards the chloroplatinate, it will be noted that Bernthsen (working with the synthetic base) found it to be more readily soluble in alcohol than in water, whereas Schulze and Barbieri (working with the base from phenylalanine) found it to be far more readily soluble in water. In addition a few minor discrepancies were evidently noted to exist between the bases mentioned. The picrate crystallises from water or alcohol, either in small plates or large, thick prisms, M.P. 169°. The aurichloride is readily formed upon gently heating the mixture of the components. Recrystallised from water acidulated with hydrochloric acid, it forms very thin, shapeless, faintly yellow flakes, M.P. 98–99° (air-dried). Millon's reagent and mercuric chloride form white precipitates with the compound.

According to this examination—as becomes apparent upon consulting the properties quoted for phenylethylamine and its salts—the base in question agrees with phenylethylamine in all respects, and is accordingly identical with it. Practically the sole difference noted between the synthetic base and that obtained from phenylalanine was the slightly differing behaviour towards permanganate solution. In judging this point, it is well to recall that Bernthsen, as opposed to Schulze and Barbieri (as has been pointed out), noted a difference between natural and synthetic phenylethylamine; this

slight difference being evident in the solubility of the respective chloroplatinates. Two additional attempts to reduce the present sample of phenylethylamine were made according to the afore-mentioned method of reduction, and with freshly prepared platinum and palladium catalysers. In neither case was any hydrogen whatsoever absorbed by the compound. For the time being, no explanation can be ventured for this apparent difference between the two samples of phenylethylamine here subjected to reduction.

Attempted reduction of tyrosine. The tyrosine employed was obtained by hydrolysis of Italian silk, by means of sulphuric acid. The purified product, M.P. 301–302°, was subjected to reduction by the above-mentioned method. Six attempts were made under various conditions and with various active catalysers, but in no case was any hydrogen whatsoever absorbed. In working up the tyrosine from these attempts, a small amount of phenylalanine was isolated in the shape of its copper-salt. Precipitated from alcohol by means of water, this salt formed pale blue, minute flakes.

0·1578 g. (air-dry); 0·0129 g. H_2O; 0·0297 g. CuO
0·1357 g. (air-dry); 0·0109 g. H_2O; 0·0256 g. CuO

	Cu	H_2O
Calculated for $(C_9H_{10}O_2N)_2Cu$, $2H_2O$:	14·86 %	8·41 %
Found:	15·02	8·18
	15·08	8·04

The amino-acid itself, upon being subjected to an extensive examination, proved itself absolutely identical with *dl*-phenylalanine [Abderhalden, 1911].

An additional attempt was made to reduce pure isolated phenylalanine (obtained from cheese) by this method of reduction, but the result was again negative.

Attempted reduction of p-*hydroxyphenylethylamine.* The *p*-hydroxyphenylethylamine here subjected to treatment by the above method, had been prepared from tyrosine by carefully heating small amounts in a partial vacuum, in order to split off the carbon dioxide. The purified hydrochloride of this base melted at 266–268° [cf. Takaoki Sazaki, 1914, who obtained a hydrochloride of M.P. 268–269°], its picrate at 200°, and all the tests applied agreed with the properties of *p*-hydroxyphenylethylamine. Several attempts to reduce this compound were undertaken, but in no case could reduction be effected.

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XXV. FURTHER OBSERVATIONS ON THE INFLUENCE OF PHENOL AND OF CRESYLIC ACID ON THE CONCENTRATION OF ANTITOXIC SERA BY THE BANZHAF (1913) PROCESS.

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It has been already pointed out [Homer, 1917] that in the Banzhaf [1913] process for the concentration of antitoxic sera the difficulties encountered in filtration can be obviated by (1) an adjustment of the reaction of the sera, and (2) by the addition of phenol, cresylic acid or "trikresol" to the ammonium sulphate mixtures previous to their being heated.

The first of these methods of procedure requires more care and attention than can always be afforded in a laboratory where concentrations are carried out on a commercial scale. The second is much simpler and gives good results as may be seen from the details of the further experimental work which has been carried out in this connexion.

Phenol and its homologues not only increase the heat denaturation of the serum proteins but also so influence the precipitability of the particles of heat denaturated protein that they are retained with the First Fraction precipitate. This phenomenon, presumably brought about by virtue of the effect of the above mentioned substances on the surface tension of the protein particles in colloidal solution, leads to the production of a final product which is clear and which filters readily through filter candles.

Experience has shown us that, in order to obtain the desired results, the percentage of these substances added to the serum, must fall within certain limits. Should the amount not reach the lower limit then the end products are more unsatisfactory than if no phenol, etc., had been added. Should the upper limit be exceeded then some of the antitoxin is transferred to the First Fraction Precipitate from which it cannot be recovered by extraction with 30 % of saturation with ammonium sulphate; for this purpose prolonged extraction with brine is necessary.

Experimental concentrations illustrating this point, each with not less than one litre of plasma, were carried out as follows:

To oxalated plasma were added 2 % of solid sodium chloride and one-fifth of its volume of water[1]. To the diluted plasma were then added varying amounts of phenol or of cresylic acid followed by the volume of a saturated solution of ammonium sulphate necessary to bring the ammonium sulphate content of the mixture to 30 % of saturation.

The phenol or cresylic acid-serum-ammonium sulphate mixtures were distributed in stoppered glass jars and placed in a bath containing water at a temperature of from 62 to 64°. The temperature of the serum mixtures gradually rose to 60°. As soon as this was reached the jars were taken from the bath and allowed to cool to 45° and filtered (First Fraction Precipitate).

The precipitates were washed with a solution containing ammonium sulphate to the extent of 30 % of saturation. The filtered washings were added to the main bulk of the filtrates which were then brought up to 50 % of saturation with ammonium sulphate. The ensuing precipitate (Second Fraction Precipitate) was filtered, pressed and dialysed in the usual way. To the residue from dialysis was added 1 % of sodium chloride and 0·35 % of cresylic acid.

A comparison was then made between the various end products thus obtained as regards their clearness and their tendency to become cloudy on standing. At the same time data were furnished as to the percentage removal of the total serum proteins and also as to the percentage of the total antitoxic units of the original serum appearing in the end product in those cases in which the higher percentages of phenol and cresylic acid had been used. The antitoxic content of the end products, where lower percentages of these substances had been used, were not estimated in the published series of experiments owing to our shortage of experimental animals. From other work we have satisfied ourselves that in the latter cases there is no appreciable loss of antitoxin during the process of concentration.

The results which have been embodied in the accompanying table (p. 281) indicate that:

[1] Banzhaf recommends that the plasma be diluted with one half its volume of water previous to the addition of the ammonium sulphate. However, in our routine work, we no longer consider the dilution of the plasma of any advantage, for it is our experience that the heat denaturation and precipitation of the serum proteins is a factor of the reaction and not of the dilution of the sera.

In my experimental work the dilution of the plasma with one-fifth its volume of water is a matter of personal convenience for the reading of the scale of the Zeiss Refractometer.

(1) Cresylic acid has a greater effect on the heat denaturation and precipitation of the heat denaturated proteins than has phenol. Moreover, the effect of the former takes place at a lower temperature than that of the latter.

The end products obtained from concentrations in which 0·30 % of cresylic acid had been added to the plasma were more clear and filtered better than when 0·50 % of phenol had been used.

(2) The addition of progressively increasing amounts of phenol or of cresylic acid to samples of the same sera previous to the heating of the serum mixtures was followed by a correspondingly increased percentage removal of the serum proteins of the original plasma.

(3) For the production of clear and readily filterable end products which would remain clear after filtration it was found necessary to add a minimum of 0·30 % of cresylic acid to the diluted plasma.

With a smaller amount of cresylic acid it was found that the end products were cloudy and far more unsatisfactory than if the Banzhaf process had been carried out without the addition of this substance.

(4) While the percentage of cresylic acid may be increased to 0·35 %, it is inadvisable to go beyond this limit, as the ensuing increased denaturation induces a transference of antitoxin to the First Fraction Precipitate from which it can only be recovered by long extraction with brine.

(5) The degree of concentration is considerably increased by the addition of 0·30 to 0·35 % of cresylic acid to the plasma previous to concentration.

Thus, whilst by the Banzhaf (1913) technique during the process of concentration, there is usually a 4 to 5 times increase in potency per cc., with the above modification the potency per cc. is increased 7 to 8·5 times[1].

In view of the above experimental data the concentration of routine batches of 50 and 100 litres of sera was conducted in the same way and similar results ensued. The protocol of the concentration of a 50 litre batch of antitetanic serum is given below.

T. R. 65.

Volume of oxalated plasma taken	50 litres
Volume of water added	10 ,,
Weight of NaCl added	1200 g.
Volume of cresylic acid shaken with 1 litre of water and added to the diluted plasma	180 cc.
Volume of a saturated solution of ammonium sulphate added	25,700 cc.

[1] The potency can be still further increased (viz. to the order of 10 times) by making the precipitation limit for the Second Fraction Precipitate 45 per cent. of saturation with ammonium sulphate instead of 50 per cent. as hitherto used [Homer, 1917].

Temperature to which the cresylic-plasma-ammonium sulphate mixture was just heated ... ... 60°

Percentage increased precipitation of the serum proteins induced during the heating of the mixtures to 60° 28

Original Plasma	Final Product
Volume, 50 litres	Volume, 6·5 litres
Potency per cc., 200 (slightly +)[1]	Potency per cc., 1650
Protein content, 8·26 %	Protein content, 18·6 %

We thus see that, in the above concentration:

(1) there has been a removal of 70 % of the total proteins of the sera taken for concentration,

(2) the loss of antitoxic units during the process has been negligible,

(3) the potency of the serum has been increased eight times.

The final product was clear and limpid. On dilution with saline it showed no trace of the presence of a colloidal suspension of euglobulin or of heat denaturated protein. Its filtration through filter candles proceeded with ease and the filtered product showed no signs of becoming cloudy on standing.

From these results it will be seen that, by the addition of 0·30 to 0·35 % of cresylic acid to the diluted plasma before concentration by the Banzhaf method, the degree of concentration and the percentage removal of the serum proteins thereby induced are of the same order as those obtained by the method recently published from these laboratories [Homer, 1916]. Such a simple modification of the technique of the former process entailing the production of a highly concentrated product will probably be welcomed by those laboratories in which my double heating process has not yet been adopted.

Summary.

The difficulties encountered in the Banzhaf (1913) technique and the tendency of the end products to become cloudy can be obviated by the addition of 2 % of sodium chloride and of 0·30 to 0·35 % of cresylic acid to the plasma previous to the heating of the plasma-ammonium sulphate mixtures.

There will not be undue loss of antitoxin in presence of the specified amounts of cresylic acid provided that:

(*a*) the addition of 2 % of sodium chloride be made a matter of routine,

[1] The experimental guinea-pig to which 100 M.L.D.'s of U.S.A. Standard Tetanus Toxin and 1/2000 cc. of the above plasma had been given died within 120 to 136 hours after the injection.

A comparison between the end products obtained from the concentration of antitoxic sera by the Banzhaf (1913) *Process and by a modification of the same process in which to the plasma diluted with one-fifth its volume of water have been added varying amounts of phenol and of cresylic acid previous to the heating of the serum mixtures.*

Experimental No.	Percentage of protein in the original plasma	Percentage of phenol or of cresylic acid added to the *diluted* plasma	Percentage increase in the precipitation of the soluble proteins during the raising of the temperature of the phenol or cresylic acid-serum-ammonium sulphate mixtures to 56°	Percentage increase in the precipitation of the soluble proteins during the raising of the temperature of the phenol or cresylic acid-serum-ammonium sulphate mixtures to 60°	Percentage of the total proteins of the original plasma appearing in the final product	Percentage of the total antitoxic units of the original plasma appearing in the final product	Increased potency per cc. over that of the original plasma	Appearance of the final product standing in bulk in the cold room within (*a*) 24 hours, and (*b*) three months of being taken from the dialysing bags: (*a*) after 24 hours	Appearance of the final product standing in bulk in the cold room within (*a*) 24 hours, and (*b*) three months of being taken from the dialysing bags: (*b*) after 3 months	The effect of dilution of the end product with twice its volume of saline
Antidiphtheritic serum										
49 *a*	7·81	0·00 phenol	2·1	5·2	64·5	—	—	slightly turbid	turbid	opalescent
49	7·81	0·35 ,,	11·0	17·0	45·0	>84*	4·5	,, ,,	,,	slightly opalescent
50	7·81	0·50 ,,	15·5	20·8	40·0	>83*	5·5	very slightly turbid	slightly turbid	very slightly opalescent
51	7·81	0·35 cresylic acid	24·6	26·0	30·0	82	7·5	clear	clear	clear
Antitetanic serum										
75 *a*	6·49	0·00 cresylic acid	—	2·1	65·0	—	—	slightly turbid	turbid	opalescent
75	6·49	0·15 ,, ,,	—	15·0	45·0	—	—	,, ,,	,,	,,
80	6·49	0·20 ,, ,,	—	17·0	36·7	—	—	,, ,,	,,	slightly opalescent
81	6·49	0·25 ,, ,,	—	22·5	35·0	88·0	6·5	,, ,,	slightly turbid	,, ,,
82	6·49	0·30 ,, ,,	—	25·0	28·1	88·0	8·5	clear	clear	clear
Antidiphtheritic serum										
85	7·61	0·00 cresylic acid	—	3·0	63·1	—	—	clear	turbid	opalescent
86	7·61	0·25 ,, ,,	—	26·0	42·9	—	—	turbid	,,	,,
87	7·61	0·30 ,, ,,	—	30·0	37·1	—	—	clear	clear	clear

* Not tested further on account of the shortage of experimental guinea pigs. For the same reason tests were not carried out on 49 *a*, 75 *a*, 75, 80, 85, 86, and 87 as we have (in other work) satisfied ourselves that with as much as 0·5 and 0·35 % of cresylic acid there is no undue loss of antitoxin provided that the temperature of the serum mixture be not raised beyond 60°.

(*b*) the mixtures be neither heated beyond 60° nor allowed to remain at this temperature for more than two or three minutes.

The addition of the cresylic acid has considerably enhanced the degree of concentration that can be obtained by the Banzhaf method. It has also increased the percentage removal of the serum proteins.

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XXVI. A NOTE ON THE USE OF INDICATORS FOR THE COLORIMETRIC DETERMINATION OF THE HYDROGEN ION CONCENTRATION OF SERA.

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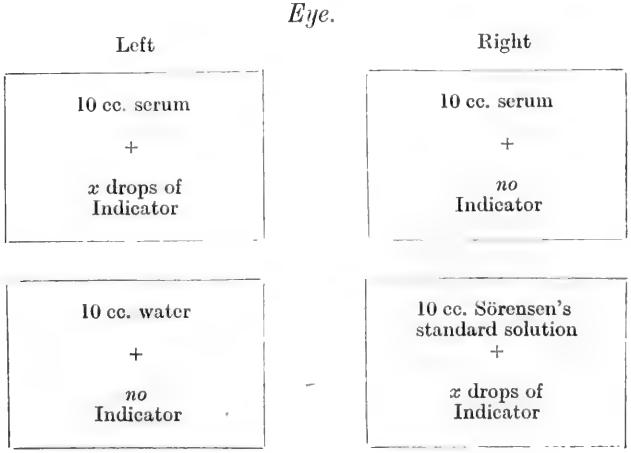
(*Received Aug. 27th, 1917.*)

During the progress of a series of investigations on the concentration of antitoxic sera it was found necessary to adjust the reaction of the sera to certain fixed values in order to get concordant and consistent results. In the experimental work, owing to lack of data with regard to the reliability of indicators in the presence of the serum proteins, the hydrogen ion concentration of the sera was determined by the electrical method. Since the latter method cannot be conveniently used in a general routine laboratory, experimental work was undertaken to ascertain whether determinations of sufficient accuracy for practical purposes could be made by the colorimetric method.

In this connexion to several samples of different sera were added varying quantities of acid or of alkali. The hydrogen ion concentration [H·] of each of the samples was estimated by the electrical method and the values thus found were compared with those obtained by the colorimetric method using the stated indicators and Sörensen's standard solutions in a Walpole's colorimeter, the colour system being as follows:



Source of Light.

In the colorimetric determination of the values for the [H·] care must be taken to use a constant amount of the specific indicator. It is a matter of experience that, both with the standard solutions and with the test sera, the intensity of the colour reaction at a definite hydrogen ion concentration can be varied by alterations in the amount of indicator added, so that, even with indicators which may prove sufficiently reliable for use with sera, unless the amount of indicator be kept constant, neither consistent nor comparable results will be forthcoming.

For my purpose litmus, methyl violet, methyl orange and Henderson and Forbes' indicator were useless with serum as I was unable to match the two colour systems. In these cases the colour reaction between the serum and each of the above-mentioned indicators seemed to me to be different from that between the indicator and the standard solutions.

With *p-nitrophenol* and *tropaeolin OOO.* it was found that, although it was possible to match the two colour systems, the colour changes were neither sufficiently sharp nor in sufficient contrast to the colour of the serum itself for me to obtain satisfactory readings.

With *phenolphthalein* the colorimetric determinations gave values not sufficiently near those obtained by the electrical method to be of any service even for approximate values for routine work. Thus, the colour reaction between the indicator and serum corresponding to that with the standard solutions at P_H 8·25 was not evidenced until the true reaction of the serum had been brought to values of the order of P_H 10·5.

On the other hand, contrary to expectation, *neutral red* proved sufficiently reliable for routine determinations with sera. It was found that with the addition of five drops (Pasteur capillary pipette) of a 0·05 % solution of this indicator in alcohol to 10 cc. of the Sörensen's standard solutions the useful range was between P_H 5·6 and 7·8. The same amount of indicator was then added to 10 cc. of samples of the different sera and the colour reaction thus produced was matched with the colour scheme obtained by viewing the standard solutions and indicator through serum in the colorimeter: in serum the useful range was between P_H 5·9 and 7·6. The results thus obtained for the values of the [H·] of the sera together with those obtained by the electrical method have been embodied in Table I.

TABLE I.

A comparison between the values obtained for the hydrogen ion concentration (P_H) of various sera by the electrical method and by the colorimetric method, using neutral red as the indicator.

[To 10 cc. of the test solutions were added five drops of a 0·05 % solution of neutral red in alcohol.]

Plasma No.	Percentage of protein in the sample of plasma taken	P_H of the plasma determined by the colorimetric method	P_H of the plasma determined by the electrical method
1	6·73	7·6	7·7
1 *a*	4·92	7·14	7·14
2	6·16	6·98	7·02
2 *a*	4·62	between 6·98 and 6·81	6·82
2 *b*	4·62	6·60	6·40
3	6·16	5·91	6·29
4	8·12	7·35	7·46
4 *a*	6·09	between 7·64 and 7·35	7·50
4 *b*	4·06	7·34	7·40
5	6·09	5·91	6·02
5 *a*	4·06	5·91	6·02
6	6·56	7·34	7·40
6 *a*	6·56	5·90	5·92
6 *b*	4·92	6·97	6·98
6 *c*	4·92	6·60	6·40

A study of Table I shows that the [H·] of sera can be determined between P_H 5·9 and 7·6 with sufficient accuracy for practical purposes by the colorimetric method using neutral red as indicator.

The studies of Sörensen and Palitzch [1910] have shown the value of *α-naphtholphthalein* as an indicator. It was tried with sera as its useful range, using three drops of a 0·10 % solution with the standard solutions, was from P_H 7·6 to 8·5.

From the data given in Table II it will be seen that the determinations by the electrical method are consistently higher than those by the colorimetric method using this indicator. Thus, the slightest tinge of green colorimetrically corresponding to that for P_H 7·6 with the standard solutions does not show until the true value for P_H is of the order of 8·5. However, with this indicator the "buffer" action of the proteins is not sufficiently marked to be too serious a drawback to its use for routine work. Thus, it was found that, if to 10 cc. of the serum containing three drops of the solution of α-naphtholphthalein

ammonia was added until the appearance of the faintest green tinge in the serum was detected, the reaction of the serum thus treated was approximately P_H 8·5.

TABLE II.

A comparison between the values for the P_H *of various sera obtained by the electrical method and by the colorimetric method, using α-naphtholphthalein as the indicator.*

[To 10 cc. of the test solutions were added three drops of a 0·10 % solution of the indicator in alcohol.]

Plasma No.	Percentage of protein in the plasma	P_H determined by the colorimetric method	P_H determined by the electrical method
7	6·73	7·64	8·29
7 *a*	4·92	7·64	8·04
8	8·12	7·86	8·65
8 *a*	6·09	7·64	8·44
8 *b*	4·06	7·64	8·55
8 *c*	2·03	7·65	8·60
9	6·56	7·64	8·27
9 *a*	4·92	7·64	8·48
9 *b*	4·92	8·04	8·71
10	6·16	7·64	8·52
10 *a*	4·62	7·64	8·45
10 *b*	4·62	7·86	8·25

For routine work in which a value of about P_H 8·0 was desired it was found that the following procedure would approximately give the desired result: To an aliquot part of the serum was added the necessary volume (x cc.) of a standard solution of ammonia to bring the $[H^{\cdot}]$ to the value P_H 7·6, using neutral red as the indicator. To a similar volume of serum was added the necessary volume (y cc.) of the ammonia solution to bring the $[H^{\cdot}]$ to the value P_H 8·5 (faint green tinge with α-naphtholphthalein).

The addition of $\frac{x+y}{2}$ cc. of the standard solution of ammonia to the stated volume of serum brought the $[H^{\cdot}]$ approximately to the value of P_H 8·0. This is a purely empirical arrangement which has given results of sufficient accuracy for routine work for the reason that, while an excess of alkali is undesirable, slight variations in the reaction round about P_H 8·0 are not followed by the marked variations in the heat denaturation of the serum proteins so characteristic of sera more acid than P_H 5·5.

It was next desired to find reliable indicators for the determination of the $[H^{\cdot}]$ of sera more acid than could be tested by neutral red. For this purpose,

owing to the unreliability of litmus, methyl orange, methyl red, Henderson and Forbes' indicator, and also to my inability to match the colour reaction with *p*-nitrophenol, resource was had to the sulphonephthalein indicators, the use of which has been so strongly advocated by Mansfield Clark and Lubs [1915]. The brilliancy of the colour reactions of these substances suggested that besides possibly furnishing indicators for the desired acid ranges they might also replace the indicators already in use on the alkaline side.

In this connexion, the following indicators in 0·05 % alcoholic solution, were used.

	Useful range P_H
Thymol-sulphonephthalein*	8·0–9·6
o-Cresol-sulphonephthalein*	7·2–8·8
Phenol-sulphonephthalein	6·8–8·4
Dibromothymol-sulphonephthalein*	6·0–7·6
Dibromo-*o*-cresol-sulphonephthalein*	5·2–6·8
Methyl red	4·4–6·0
Tetrabromophenol-sulphonephthalein	3·0–4·6

* Samples of these indicators were very kindly sent to me by Dr Mansfield Clark as some delay was experienced in obtaining the chemicals necessary for their preparation.

The useful range of each indicator was tested against Sörensen's standard solutions which were also checked by the electrical method. A comparison was then made between the values of the [H·] of the various sera obtained by the colorimetric method using the above mentioned indicators and also by the electrical method. The data with regard to these observations have been embodied in Table III.

Thymol-sulphonephthalein was found to be unreliable with serum. Thus, the colour reaction corresponding to P_H 8·3 with the standard solutions did not appear in the serum until the value P_H 10·3 had been reached.

The colour changes with *o-cresol-sulphonephthalein* in serum could not be detected by me with sufficient ease for its adoption in routine work.

The colour reactions of *phenol-sulphonephthalein* and of *bromothymol-sulphonephthalein* in serum could be readily matched throughout their useful ranges with the standard solutions, but, the values for the [H·] of the sera thus determined were consistently lower than the true values determined by the electrical method. As it was found that, in sera containing from 4 to 10 % of protein, these deviations from the true values were constant for the same worker, use was made of these indicators for the determination of the [H·] of sera between the ranges P_H 6·8 and 9·0 as follows.

To the test serum were added six drops of a 0·05 % solution of the indicator. The colour reaction was then matched against standard solutions containing

TABLE III.

A comparison between the values obtained for the P_H of various sera by the electrical method and by the colorimetric method using the indicators of the sulphonephthalein series.

Plasma No.	Percentage of protein in the plasma	Indicator used	P_H of the plasma determined by the colorimetric method	P_H of the plasma determined by the electric method
11	8·12	Thymol-sulphonephthalein	8·03	10·34
12	6·16	,, ,,	8·03	10·21
13	6·56	,, ,,	8·03	10·33
14	4·40	,, ,,	8·03	10·55
15	6·73	Phenol-sulphonephthalein	6·81	7·65
15	6·73	,, ,,	7·14	8·00
15	6·73	,, ,,	7·34	8·25
15	6·73	,, ,,	7·64	8·55
15	6·73	,, ,,	7·86	8·70
15	6·73	,, ,,	8·03	8·97
16	4·92	,, ,,	7·64	8·43
17	6·16	,, ,,	7·64	8·46
18	8·12	,, ,,	7·64	8·25
19	6·09	,, ,,	7·86	8·70
20	4·06	,, ,,	7·44	8·27
20 *a*	4·06	Bromothymol-sulphonephthalein	6·23	6·86
21	6·09	,, ,,	6·46	7·31
22	4·92	,, ,,	6·46	7·58
23	4·40	,, ,,	6·46	7·38
24	8·12	,, ,,	6·81	7·86
24	8·12	,, ,,	7·10	8·10
24	8·12	,, ,,	7·30	8·66
25	6·97	,, ,,	7·30	8·60
26	9·90	,, ,,	7·30	8·70
27	6·73	Methyl red	4·98	4·00
28	4·92	,,	6·00	5·24
29	8·12	,,	5·60	4·80
30	8·12	Bromo-*o*-cresol-sulphonephthalein	5·2	5·14
31	6·09	,, ,,	5·2	5·06
32	6·56	,, ,,	5·2	5·14
33	4·40	,, ,,	5·2	5·23
34	6·73	,, ,,	5·4	5·50
35	7·56	,, ,,	5·4	5·58
36	8·12	,, ,,	5·4	5·65
37	6·56	,, ,,	5·4	5·32
38	6·73	Bromophenol-sulphonephthalein	3·0	2·90
39	8·12	,, ,,	3·0	2·87

a corresponding amount of indicator, the colour produced with the latter being viewed through serum. The value thus obtained was transmuted to the true value according to the following table:

Phenol-sulphonephthalein		Bromothymol-sulphonephthalein	
Value for P_H found by the colorimetric method	True value for P_H of the serum (approximately)	Value for P_H found by the colorimetric method	True value for P_H of the serum (approximately)
6·81	7·65	6·23	6·86
7·14	8·00	6·46	7·40
7·34	8·25	6·81	7·80
7·64	8·55	7·10	8·10
7·86	8·70	7·30	8·60
8·03	8·95		

For the colorimetric work with serum, I found it more easy to match the yellowish green to blue colour reactions of bromothymol-sulphonephthalein than the orange to reddish purple colour reactions of phenol-sulphonephthalein. For this reason in routine work the reaction of sera was approximately adjusted to the value P_H 8·0 by the addition of ammonia until the colour reaction of bromothymol-sulphonephthalein with the serum matched that with a standard solution at P_H 7·1, the latter being viewed through serum in the colorimeter. However, although fairly consistent results were obtained, I found that I was not always as successful with bromothymol-sulphonephthalein as with α-naphtholphthalein, the sensitiveness of my colour vision to the delicate colour changes of the former being very much influenced by personal fatigue.

With *dibromo*-o-*cresol-sulphonephthalein* and serum it was found somewhat difficult to match the various shades of purple through the ranges of the indicator. However, in practice, it was an easy matter to bring the serum to the degree of acidity at which the indicator showed a slight greenish tinge, *i.e.* just before the acid limit of the useful range of the indicator was reached. The reaction of the sera so adjusted was found to be approximately P_H 5·5.

If acid were added to serum containing bromo-*o*-cresol-sulphonephthalein until the final slight green tinge had just disappeared it was found that approximately the value P_H 5·2 had been reached.

For the adjustment of the sera between the values P_H 5·5 and P_H 4·5 the following arbitrary method was adopted which gave results of sufficient accuracy for routine work, provided the colorimetric determinations were made by the same observer.

Sera containing from 5 to 8 % of protein were brought to P_H 5·5 (circ.) by the addition of the requisite amount (x cc.) of acetic acid to a stated volume of serum containing a fixed amount of indicator until only the faintest tinge of green was detected. Since the colour of serum is affected by its acidity or alkalinity, the comparison in all cases was made with the same volume of serum containing no indicator but to which had been added

a corresponding amount of acetic acid. It was found that the further addition of acetic acid to the serum beyond this point gave results of the following order:

Volume of N acetic acid (or equivalent) added to 100 cc. of the serum	P_H of the acidified serum (approximately)
x cc.	5·5
$x+0·5$ cc.	5·4
$x+1·0$	5·3
$x+1·5$	5·2
$x+2·0$	5·1
$x+2·5$	5·05
$x+3·0$	4·9
$x+4·0$	4·7
$x+5·0$	4·6

The value of such an arbitrary method depends on the acuteness of the colour vision of the individual and, for this reason, in order to obviate the personal error in obtaining the determination of the initial value depending on the colour tint, which, for me, approximated to the value P_H 5·5, it must be urged that each investigator should work out his own table.

Methyl red proved to be unsatisfactory with serum, as the colorimetric determinations gave results not sufficiently near those obtained by the electrical method to justify its use in routine work, slight changes in the reaction of sera on the acid side of P_H 5·5 leading to marked variations in the heat denaturation and precipitation of the serum proteins.

Difficulty was also experienced in matching the purple colour of *bromophenol-sulphonephthalein* in serum. It was however found that, with the sera taken, the faint green tinge just before the useful range of the indicator on the acid side was passed (*i.e.* at P_H 3·0) could be regulated with approximate accuracy.

During the course of the investigation it was found that dilution of the serum was followed by sharper changes in the colour reactions throughout the useful ranges of all the above-mentioned indicators. But, even where the dilution had been of the order of 1 in 4, there was no appreciable variation in the values obtained for P_H by the colorimetric method.

Summary.

During the course of the investigation it has been demonstrated that the hydrogen ion concentration of sera can be determined, with sufficient accuracy for routine work, by the colorimetric method with the use of certain indicators.

It was my experience that:

1. Neutral red gave reliable results with serum throughout the ranges P_H 5·9 to 7·6.

2. The results obtained with α-naphtholphthalein were consistently higher than the true values. Thus, the colorimetric determination of the values for P_H 7·6 was not given until the reaction of the sera had been adjusted to the value P_H 8·5.

3. By a combination of the use of neutral red and of α-naphtholphthalein the adjustment of routine batches of sera to P_H 8·0 can be arranged with sufficient ease for practical purposes.

4. Of the indicators of the sulphonephthalein series thymol-sulphonephthalein and *o*-cresol-sulphonephthalein proved unsatisfactory with serum. Phenol-sulphonephthalein and bromothymol-sulphonephthalein gave results which were consistently higher than the true values. Bromo-*o*-cresol-sulphonephthalein could be used with ease for P_H 5·4 and 5·2 while bromophenol-sulphonephthalein was satisfactory for values about P_H 3·0.

5. By a purely arbitrary arrangement based on my own observations, I was able to adjust the reaction of sera approximately to values between P_H 7·6 and 8·95 with the use of phenol-sulphonephthalein; to values between P_H 6·8 and 8·6 with the use of bromothymol-sulphonephthalein and to values between P_H 5·5 and 4·5 with the use of bromo-*o*-cresol-sulphonephthalein.

6. In view of the personal error involved in the determination of colour reactions with sera it is advisable that each worker using the colorimetric method should ascertain the degree of approximation of his own values to the true values determined by the electrical method.

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XXVII. ON THE INFLUENCE OF THE HEAT DENATURATION OF PSEUDOGLOBULIN AND ALBUMIN ON THE NATURE OF THE PROTEINS APPEARING IN CONCENTRATED ANTITOXIC SERA.

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In previous communications [Homer, 1916, 1917, 1, 2] it has been demonstrated that, as a result of the heating of serum, considerable changes are effected in the precipitability of the serum proteins by 30 % of saturation with ammonium sulphate, the extent of the changes being a function of the reaction of the serum.

The practical application of these results to the concentration of antitoxic sera has led to the preparation of end products (the residue from the dialysis of the protein precipitated between 30 and 50 % of saturation with ammonium sulphate) containing from 20 to 30 % only of the total proteins of the original serum. These results show an improvement on those obtained by the methods hitherto published.

Recent work, however, has shown that the final products obtained by methods involving a heat denaturation of the serum proteins contain albumin as well as pseudoglobulin and, moreover, that the relative amount of albumin increases with the extent of the heat denaturation of the proteins.

The presence of albumin in the final product is to be avoided for two reasons; the one that, since the antitoxin is associated with the pseudoglobulin and not the albumin constituent of the serum proteins, the presence of albumin in the final product must lower the degree of concentration; the other that the inclusion of albumin may be undesirable from the clinical point of view [Dale and Hartley, 1916].

Before attempting to ascertain whether an alteration of the precipitation limits for the Second Fraction precipitate would obviate the precipitation of heat denaturated albumin with the pseudoglobulin, it was necessary to furnish

experimental evidence with regard not only to the extent to which the denaturation of the serum proteins was affected by the time of heating but also to the extent to which the denaturation of the individual serum proteins was affected by the reaction of the serum.

Owing to the necessity for the immediate practical application of the results of the investigation to routine work, the experiments have, for the present, been confined to a study of the behaviour of pseudoglobulin and of albumin in this latter respect.

A. The influence of the time of heating of sera on the extent of the heat denaturation of the serum proteins.

During the progress of the experimental work involved in the study of the influence of the reaction of sera on the extent of the heat denaturation of the serum proteins it was noticed that, with alkaline sera, the changes were a gradual function of the time of heating while, with acid sera, the changes were complete within a comparatively short time.

In order to furnish detailed evidence on this point the following experiments were undertaken. Antitoxic serum to which had been added 2 % of sodium chloride was diluted with one-third its volume of water. To separate volumes, each of 100 cc. of the diluted plasma, were added varying amounts of acid and of alkali and the hydrogen ion concentration [H·] of the various experimental liquids was measured by the electrical method. The stoppered bottles containing the experimental liquids were then placed in a water bath at 57·5° and were heated for several hours at that temperature.

Samples of the experimental sera, previous to their being heated, were made 30 % of saturation with ammonium sulphate and filtered. The protein content of the filtrates was measured by means of the Zeiss refractometer. Similar measurements were made with samples of the heated liquids withdrawn from time to time from the various bottles of sera in the bath. From the data thus obtained was calculated the percentage increase in the precipitability of the serum proteins after the heating of the sera for the stated periods of time.

The results have been incorporated in Table I and indicate that:

(1) During the heating of alkaline sera the extent of the heat denaturation gradually increases with the prolongation of the time of heating.

The maximum value is obtained after four hours' heating at 57·5°.

(2) In the acid sera the extent of the heat denaturation has practically reached its maximum value after one hour at the specified temperature.

(3) The nature of the changes involved in the alkaline sera is different from that in the acid sera, for at no time during the heating of the alkaline sera does acidification of the clear heated liquids to a value P_H 5·0 produce the precipitate of denaturated protein so characteristic of heated acid sera.

During the course of the work it has also been observed that both with acid and alkaline sera the maximum effects are produced:

(*a*) more quickly if the temperature to which the serum is heated be raised beyond 57·5°, and

(*b*) more slowly if the sera be heated at temperatures lower than 57·5°.

TABLE I. *The influence of the time of heating the sera at 57·5° on the extent of the heat denaturation of the serum proteins.*

[Protein content of the plasma = 7·15 %.]

Volume of acid or of alkali added to 100 cc. of the diluted plasma	P_H of the diluted plasma measured by the electrical method	Percentage increase in the precipitability of the serum proteins by 30 % of saturation with ammonium sulphate after heating the serum at the specified temperature for a period of				
		1 hr	2 hrs	3 hrs	4 hrs	6 hrs
4 cc. *N* NH_3	9·4	23·1	37·2	—	44·0	45·0
2	9·1	23·0	32·0	—	40·0	40·0
1	8·8	22·2	30·0	—	40·0	40·0
0·6	8·2	17·7	27·0	—	34·6	35·6
0·5 cc. *N* HAc	7·4	12·0	17·0	—	22·0	22·6
0·75	7·1	9·0	11·0	—	16·0	16·8
2·5	5·9	13·0	12·9	13·0	—	—
3·0	5·7	13·0	16·6	16·6	—	—
3·5	5·5	15·0	16·0	16·0	—	16·0
4·0	5·3	20·0	23·5	24·2	—	—
4·5	5·1	30·0	32·8	33·0	—	33·0
5·0	5·0	34·8	40·0	40·0	—	—
6·0	4·8	60·9	67·5	67·0	—	—
6·5	4·7	64·6	79·8	79·8	—	80·0

These results will be of service to those engaged in the concentration of antitoxic sera. They show that, in order to get the maximum effect, in the case of sera more alkaline than P_H 7·4 the time of the preliminary prolonged heating of the serum at 57·5° must not be less than four and need not be greater than six hours after the liquid has reached the specified temperature. In the case of acid sera the full effect is produced within from one to two hours of the temperature reaching 57·5°.

To what extent variations in the temperature at which the serum is heated affect the order of the maximum value for the heat denaturation of the serum proteins at a particular [$H^·$] of the serum is under investigation.

B. The effect of changes in [H·] on the denaturation of pseudoglobulin induced during the heating of its solutions at a temperature of 57·5° for a period of six hours.

A solution of pseudoglobulin was prepared as follows:

To four litres of antidiphtheritic serum were added two litres of water and six litres of a saturated solution of ammonium sulphate. The liquid was allowed to stand for one hour and was then filtered. The precipitate was washed with four litres of a half-saturated solution of ammonium sulphate and, after being thoroughly drained, was thrown into four litres of a saturated solution of salt containing an excess of solid salt. The salted liquid was allowed to stand for two days, care being taken to macerate the precipitate thoroughly. The liquid was then filtered and to the filtrate was added 0·25 % of glacial acetic acid. The ensuing precipitate was filtered, pressed and, after the addition of 3 % of solid crystallised sodium carbonate, was dialysed. The residue from dialysis was diluted until the protein content was of the order of 8 %, and 1·5 % of solid salt was then added.

Aliquot parts of the solution were measured into stoppered glass bottles; varying amounts of acid and of alkali were added and the [H·] of each of the liquids was determined by the electrical method. The bottles containing the liquids were then heated in a water bath at a temperature of 57·5° for a period of six hours. The protein content of the unheated liquids and also of the filtrates from samples of the unheated liquids brought up to 30 % of saturation with ammonium sulphate were measured by means of the Zeiss immersion refractometer. The values thus obtained were compared with the corresponding values for the heated liquids. From the data thus furnished were calculated the percentage precipitation of pseudoglobulin itself and also the percentage increased precipitability of the pseudoglobulin by 30 % of saturation with ammonium sulphate induced during the heating of the pseudoglobulin solution. The results which have been incorporated in Table II (p. 297) have also been plotted in Figure 1, Curves 1 and 2.

The appearance of the heated liquids was characteristic throughout the ranges of acidity and alkalinity investigated. On the alkaline side the liquids were clear or were characterised by an opalescence. On the acid side the liquids were converted during the heating into thin suspensions, the semi-solid consistency of which became more marked as the acidity increased.

From a study of the data given in Table II, Column *A*, it will be seen that the conversion of soluble pseudoglobulin into insoluble protein was of the

order of 5 % or less in the liquids of which the reaction lay between the ranges P_H 5·5 and 9·7. On the acid side of P_H 5·5 there was a marked precipitation of insoluble protein; thus, at P_H 5·34 it was of the order of 17·9 % while at P_H 4·6 it was of the order of 78 %.

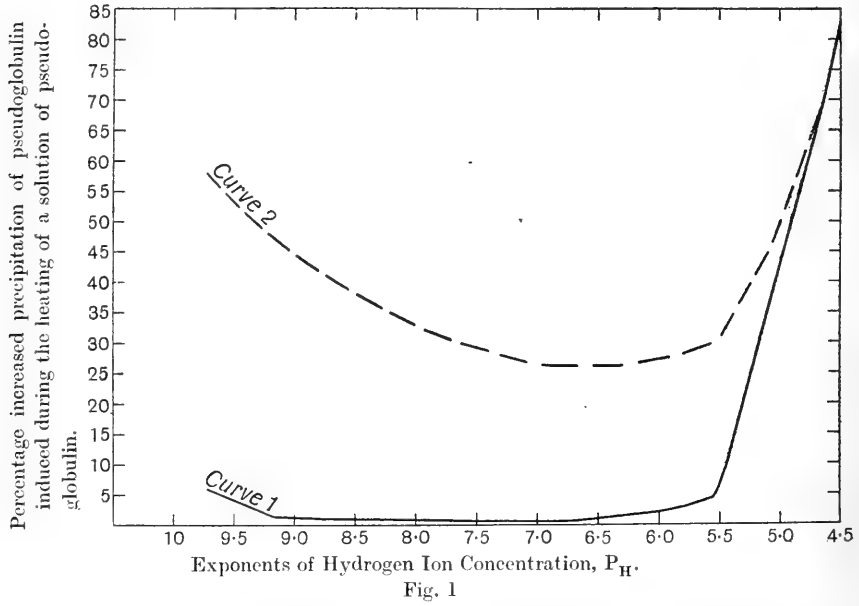

Fig. 1

Curve 1 ——— represents the influence of changes of reaction on the conversion of soluble pseudoglobulin into an insoluble condition during the heating of solutions of pseudoglobulin at 57·5° for 6 hours.

Curve 2 — – – represents the accompanying percentage increased precipitability of the heat-denaturated pseudoglobulin.

The data given in Column *B*, Table II show that there was, throughout the whole range investigated, a marked increase in the precipitability of the serum proteins by 30 % of saturation with ammonium sulphate: the extent of the increase was a function of the reaction of the liquid.

The increased precipitability was least between the range P_H 5·5 and 8·0. On either side of these limits the rate of increase was appreciable: the gradient for the increase being more gradual on the alkaline than on the acid side. As the degree of alkalinity decreased from P_H 9·7 to 8·0 the increased precipitability decreased from 57·6 to 32·6 %; between P_H 8·0 and 5·5 it varied from 25 to 30 %, while between P_H 5·3 and 4·6 it rapidly rose from 40·0 to 75·5 %.

TABLE II. *Showing the influence of the hydrogen ion concentration on the denaturation of pseudoglobulin induced during the prolonged heating of a 7·95 % solution of pseudoglobulin at a temperature of 57·5° for 6 hours.*

Volume of normal acid or alkali added to 100 cc. of the pseudoglobulin solution	P_H of the solutions measured by the electrical method	Appearance of the heated liquids	*A* Percentage conversion of soluble into insoluble protein during the heating of the pseudoglobulin solutions	*B* Percentage increase in the precipitability of the pseudoglobulin by 30 % of saturation with ammonium sulphate
5·0 cc. *N* NH_3	9·72	clear	5·5	57·6
4·0	9·51	,, ...	5·5	57·0
3·0	9·40	,, ...	4·2	50·4
2·0	9·30	,, ...	1·3	44·0
1·50	9·03	very slightly opalescent ...	1·3	43·7
1·00	8·63	slightly opalescent	1·3	42·4
0·50	7·97	,, ,, ...	0	32·6
0·25	7·40	opalescent	0	29·2
0·00	6·81	very thin emulsion	0	25·6
0·50 cc. *N* HAc	6·43	thin emulsion, filtrate opalescent	1·3	26·3
1·00	5·99	thin emulsion, filtrate slightly opalescent	4·2	27·6
1·60	5·34	thick emulsion, filtrate clear	17·9	40·0
2·50	5·06	,, ,, ,,	43·0	48·2
3·30	4·80	,, ,, ,,	60·9	58·9
4·25	4·64	almost solid, filtrate clear ...	66·0	64·2
5·0	4·60	,, ,, ,, ...	78·0	75·5

C. THE EFFECT OF CHANGES IN [H·] ON THE HEAT DENATURATION OF ALBUMIN INDUCED DURING THE HEATING OF ITS SOLUTIONS AT 57·5° FOR A PERIOD OF SIX HOURS.

The solution of albumin was prepared as follows:

The filtrate from the precipitated globulins (ante, p. 295) was saturated with ammonium sulphate and the ensuing precipitate was filtered off. The precipitate was washed from the filter paper into three litres of a half-saturated solution of ammonium sulphate. The liquid after standing for twenty-four hours was filtered, and the filtrate was saturated with ammonium sulphate and allowed to stand for two days at room temperature. The precipitated albumin was filtered off, thoroughly drained, pressed and dialysed until the residue in the bags was practically free from sulphate. The residue from dialysis was diluted until the protein content was of the order of 6 % and 1·5 % of solid sodium chloride was then added.

To aliquot parts of the above solution of albumin were added varying amounts of acid and alkali so as to bring the [H·] of the series of experimental liquids within the ranges P_H 9·7 and 4·5; the [H·] of each of the solutions being measured by the electrical method previous to the liquids being heated at a temperature of 57·5° for a period of six hours.

The protein content of each of the above liquids and of the filtrates from

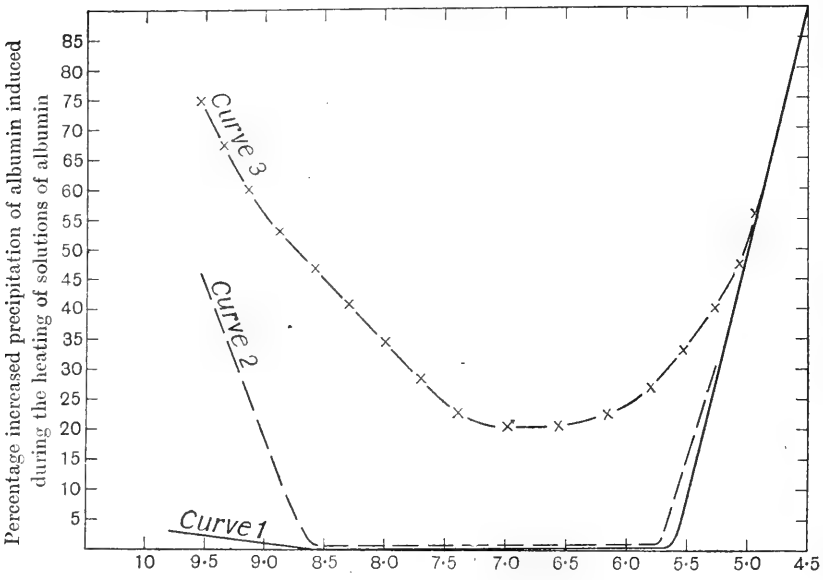

Fig. 2

Curve 1 ——— represents the influence of changes of reaction on the conversion of soluble albumin into an insoluble condition during the heating of solutions of albumin to 57·5° C. for 6 hours.

Curve 2 – – – represents the accompanying percentage increased precipitability of the heat denaturated albumin by 30 % of saturation with ammonium sulphate.

Curve 3 — × — × — represents the accompanying percentage increased precipitability of the heat denaturated albumin by 50 % of saturation with ammonium sulphate.

them made 30 % and also 50 % of saturation with ammonium sulphate were estimated before and after their having been heated.

The data thus obtained have been incorporated in Table III and have been plotted in Curves 1, 2 and 3 in Figure 2.

From a study of these data it will be seen that, as in the case of solutions of pseudoglobulin, the heated liquids on the alkaline side were either clear or else showed only a slight opalescence, whereas on the acid side the liquids were converted into thin suspensions the consistency of which became more markedly solid as the degree of acidity increased.

From the results given in Column *A* of Table III it will be seen that the actual conversion of soluble albumin into an insoluble condition was less than 5 % between the ranges P_H 9·5 and 5·5. On the acid side of P_H 5·5 it rapidly rose from 6·0 % at P_H 5·5 to 57·6 % at P_H 4·87 and to 100 % at P_H 4·40.

From the results given in Column *B* of the same table it will be seen that the precipitability of albumin by 30 % of saturation with ammonium sulphate is not appreciably affected during the heating of solutions of which the

TABLE III. *Showing the influence of the hydrogen ion concentration on the denaturation of albumin induced during the prolonged heating of a 5·20 % solution of albumin at a temperature of 57·5° for a period of 6 hours.*

Volume of normal acid or alkali added to 100 cc. of the solution of albumin	P_H of the solutions measured by the electrical method	Appearance of the heated liquids	Column *A* Percentage conversion of soluble into insoluble protein during the heating of the albumin solutions	Column *B* Percentage increase of the precipitability of the albumin by 30 % of saturation with ammonium sulphate	Column *C* Percentage increase of the precipitability of the albumin by 50 % of saturation with ammonium sulphate
4·3 cc. *N* NH_3	9·53	slightly cloudy	2·2	46·0	75·0
3·0	9·11	,, ,, ...	1·0	36·7	58·0
2·2	8·70	clear	0·0	8·0	50·0
1·00	8·41	,, ...	0·0	0	44·0
0·50	7·81	,, ...	0·3	0	31·5
0·25	7·40	,, ...	0	0	22·7
0·00	6·90	,, ...	0	0	21·3
0·70 cc. *N* HAc	6·27	slightly opalescent, filtrate opalescent	0	0	26·1
1·40	5·53	cloudy, filtrate opalescent ...	6·0	14·2	34·2
2·20	5·18	thin emulsion, filtrate clear ...	30·0	36·7	44·0
3·0	4·87	thick ,, ,, ...	57·6	55·0	57·0
6·0	4·52	,, ,, ,, ...	89·0	Too thick to be measured in a pipette	Too thick to be measured in a pipette
8·5	4·40	almost solid, filtrate clear ...	100		
6·0 cc. *N* H_2SO_4	3·40*	,, ,, ,, ...	100		

* Opalescent after the addition of acid.

reaction falls between the limits P_H 8·4 and 5·5. On either side of these limits there is a rapid rise in the precipitability of the albumin.

In Column *C* have been given the data with regard to the increased precipitability of the solution of albumin by 50 % of saturation with ammonium sulphate. It will be seen that the increased precipitability of the albumin is a function of the [H·] of the solutions. Thus, as the [H·] increased from P_H 9·5 to 7·8 so the increased precipitability decreased from 75 % to 31 %; between the values of P_H 7·8 and 5·5 it varied from 20 to 30 % while on the acid side of P_H 5·5 the percentage increased precipitability was practically equivalent to the percentage conversion of soluble into insoluble protein.

The results recorded above for pseudoglobulin and for albumin respectively show certain similarities with those previously obtained from corresponding experiments with antitoxic sera [Homer, 1917, 1]. Thus it was noticed that:

(1) The extent of the heat denaturation of the individual proteins, pseudoglobulin and albumin, is a function of the [H·] of the solutions.

(2) There is coincidence between the curves representing the conversion of soluble into insoluble protein during the heating of whole serum, of solutions of albumin and of pseudoglobulin respectively.

(3) The curve for the increased precipitability of albumin by 50 % of saturation with ammonium sulphate has a configuration very similar to those for the increased precipitability of pseudoglobulin and of the proteins of whole serum respectively by 30 % of saturation with ammonium sulphate.

(4) The values for the increased precipitability of the serum proteins by 30 % of saturation with ammonium sulphate are somewhat lower than those for solutions of pseudoglobulin at the same [H·]. This is probably due, partly to the diluent effect of the accompanying albumin in whole serum and partly to the "buffer" action of the latter.

(5) In all cases the minimum change takes place between the ranges P_H 5·5 and 8·0 and there is coincidence of the curves on the acid side of P_H 5·5.

(6) The changes taking place during the heating of alkaline sera and of alkaline solutions of pseudoglobulin and of albumin are not of the same type as those induced in the acid solutions, for acidification of the heated alkaline liquids to P_H 5·0 does not produce the thin suspension so characteristic of the heated acid liquids.

(7) The increased precipitability of the heated alkaline sera and of alkaline solutions of albumin and of pseudoglobulin only attains its maximum value after several hours' heating. On the other hand the changes in the acid liquids are practically complete within one hour's heating at the specified temperature.

From the work of Chick and Martin [1910, 1911, 1912, 1, 2] it was anticipated that while a certain similarity would exist between the configuration of the curves for the heat denaturation of pseudoglobulin and of albumin respectively at hydrogen ion concentrations on either side of their isoelectric points, there would be a shift in the position of corresponding portions of the curves due to the fact that the isoelectric point of pseudoglobulin is at P_H 5·52 while that of albumin is at P_H 4·70. Curiously enough not only was the region of least change the same in both cases, viz., from P_H 5·5 to 8·0, but there was absolute coincidence of the curves on the acid side of P_H 5·5.

These phenomena present points of interest in connexion with the views held both as to the justification for regarding the protein constituents of serum as well defined entities and as to the possible conversion of pseudo- into euglobulin and of albumin into pseudoglobulin.

The results of an investigation of the nature of the heat denaturated proteins obtained from albumin and pseudoglobulin will be dealt with in a later paper.

D. The precipitation of heat denaturated pseudoglobulin and heat denaturated albumin with the First and Second Fraction precipitates.

From a consideration of the above results it is obvious, that in sera which have been subjected to prolonged heating the First and Second Fraction precipitates respectively comprise the various proteins in proportions which are somewhat different from those in the corresponding precipitates in the unheated serum.

An investigation was therefore undertaken in order to furnish data with regard to the composition of the First and Second Fraction precipitates in heat denaturated sera throughout the ranges of acidity and alkalinity previously considered above in sections A, B and C.

The wider aspect of the problem has not yet been completed but, in view of the need for the immediate application of my results to routine concentration work, data are being published with regard to the precipitability of heat denaturated pseudoglobulin and albumin by ammonium sulphate from solutions the reaction of which had been adjusted to that of the routine batches of sera taken for concentration. The application of those results to the routine work has led to the production of end products in which the proportion of albumin has been lessened while the degree of concentration has been increased.

Solutions of pseudoglobulin and of albumin (prepared as in B and C), previous to their being heated to a temperature of 57·5° for six hours, were adjusted to about P_H 8·0. Samples of the heated and of the unheated liquids were made varying percentages of saturation with ammonium sulphate and were then filtered, the protein content of the filtrates being estimated by means of a Zeiss refractometer. From the data thus obtained was calculated the percentage conversion of pseudoglobulin and of albumin respectively from the emulsoid to the suspensoid condition induced by the addition of the various amounts of the electrolyte to the heated and to the unheated solutions.

The results obtained with pseudoglobulin have been embodied in Table IV. They show that in the unheated solutions the extent of the precipitation of pseudoglobulin increases with the concentration of ammonium sulphate, the change being complete at about 50 % of saturation with the electrolyte. In the heated solutions the precipitability of the protein by concentrations of ammonium sulphate beyond 20 % of saturation is considerably enhanced, the precipitation being practically complete at 42·5 % of saturation.

TABLE IV. *Showing the extent to which the precipitability of pseudoglobulin by ammonium sulphate is affected by the prolonged heating of its solution at 57·5° for 6 hours.*

[Percentage of pseudoglobulin in original solution = 7·10.]

P_H of the solution = 8·0.

Percentage of saturation with ammonium sulphate	Residual percentage of pseudoglobulin in solution in the filtrates from the ammonium-sulphate-pseudoglobulin mixtures		Percentage precipitation of pseudoglobulin by the ammonium sulphate in the	
	(*a*) before heating	(*b*) after heating	(*a*) unheated liquids	(*b*) heated liquids
20	6·92	6·77	2·5	4·6
25	6·77	5·65	4·6	20·5
28	6·67	4·56	6·0	35·9
30	6·04	3·77	15·0	47·0
33	3·86	2·76	45·5	61·2
37·5	1·65	0·75	79·5	89·5
42·0	1·14	0·19	84·0	97·5
44·5	0·54	0·03	92·5	99·5
47·5	0·26	0·00	96·5	100·0
50·0	0·07	0·00	99·0	100·0

In the case of albumin (Table V) similar results were obtained. Thus, in the unheated liquids at 44 % of saturation with ammonium sulphate about 6 % of the total albumin was converted from the emulsoid to the suspensoid state: at 50 % of saturation with the electrolyte the conversion was of the order of 10 %; beyond this point the precipitation of albumin gradually increased, the precipitation being complete at about 75 % of saturation. In the heated solutions the precipitation of albumin at concentrations of ammonium sulphate greater than 40 % of saturation was considerably increased. Thus, at 42 % of saturation the precipitation was of the order of 29 % of the total albumin; at 44 % of saturation it increased to 33, while at 50 % of saturation the precipitation of albumin rose to the order of 52 %.

E. THE PRACTICAL APPLICATION OF THE ABOVE RESULTS TO THE ROUTINE CONCENTRATION OF ANTITOXIC SERA.

While recognising that the values recorded in Tables IV and V for the percentage precipitation of the individual proteins from their respective solutions may be somewhat higher than when they are associated together in serum, it seemed justifiable to conclude that, in the routine work, by lowering the concentration of ammonium sulphate for the precipitation of the Second Fraction from 50 to 44 % of saturation, there would still be complete precipitation of the pseudoglobulin and antitoxin while the proportion of albumin precipitated at this stage would be appreciably lessened.

TABLE V. *Showing the extent to which the precipitability of albumin by ammonium sulphate is affected by the prolonged heating of its solution at 57·5° for 6 hours.*

[Percentage of albumin in the original solution = 6·27.]

P_H of the solution = 8·24.

Percentage of saturation with ammonium sulphate	Residual percentage of albumin in solution in the filtrates from the ammonium-sulphate-albumin mixtures		Percentage precipitation of albumin by the ammonium sulphate in the	
	(*a*) before heating	(*b*) after heating	(*a*) unheated liquids	(*b*) heated liquids
20	6·27	6·27	0	0
25	6·27	6·27	0	0
28	6·05	6·14	3	2
30	6·14	6·02	2	4
33	6·05	5·83	3	7·1
37·5	5·95	5·61	5	10·6
42	5·92	4·46	5·5	29·0
44·5	5·84	4·20	6·7	32·9
47·5	5·77	3·86	8·0	38·4
50·0	5·64	2·98	10·0	52·5
56·5	4·76	1·10	23·7	82·5
62·5	3·45	0·81	45·0	87·0
66·5	0·73	0·15	88·5	97·5
70·0	0·18	0·00	97·0	100·0

In order to test this point concentrations were carried out with antidiphtheritic and with antitetanic sera. In both cases a denaturation of the serum proteins to the extent of about 30 % was induced during the initial heating of the serum. Aliquot parts of the filtrates from the First Fraction precipitates were respectively brought up to 44, 47 and 50 % of saturation with ammonium sulphate and the ensuing precipitates were filtered, pressed and dialysed in the usual way.

TABLE VI. *Showing the increase in the degree of concentration of antitoxic sera induced by lowering the precipitation limit for the Second Fraction precipitate.*

Precipitation limits for the Second Fraction precipitates (percentage of saturation with ammonium sulphate)	Percentage of the total antitoxic units appearing in the Second Fraction precipitates	Percentage removal of the total proteins of the original serum	Increased potency per cc.	Units of antitoxin per g. of protein in the final product
Antitetanic plasma*				
30–44	95	69	10 times	6,250
30–50	95	64	8 „	5,250
Antidiphtheritic plasma*				
30–44	92	72·5	9·5 „	18,750
30–47	96	67	8·0 „	16,500
30–50	96	66	7·5 „	15,000

* Comparable results were obtained irrespective of whether the heat denaturation had been induced by the prolonged heating of serum at 57·5° or by the shortened heating process after the addition of cresylic acid to the serum.

A comparison was then made between the removal of the serum proteins, the increased potency and the relative recovery of antitoxin in the several experiments (Table VI).

From these results it is obvious that for the complete precipitation of the antitoxin and the pseudoglobulin from sera in which a heat denaturation of about 30 % has been induced, the concentration of ammonium sulphate need not be greater than 44 % of saturation.

The adoption of 44 % of saturation with ammonium sulphate in preference to 50 % hitherto recommended presents the following advantages:

(1) The precipitation of heat denaturated albumin with the Second Fraction precipitate is appreciably lessened, thereby ensuring in the end product:

(*a*) a further reduction in the percentage of the total proteins of the original serum without transference of antitoxin to the First Fraction precipitate, and

(*b*) a corresponding increase in the degree of concentration.

(2) The colour of the end products is much less pronounced than when the precipitation limit of 50 % of saturation is used. The extra amount of albumin precipitated between 44 and 50 % of saturation adsorbs an appreciable amount of the colouring matter of serum.

(3) The smaller volume of saturated solution of ammonium sulphate thus required for the precipitation of the Second Fraction precipitate entails a slight reduction of labour and expense.

From the above observations it is clear that, if the precipitation limits for the Second Fraction precipitate be altered so as to exclude the precipitation of heat denaturated albumin, the maximum load of antitoxin which can be carried by one gram of protein in the final product will be somewhat higher than the values previously found [Homer, 1917, 2]. Experimental work in this connexion is in progress and will be published later.

Summary.

During the course of the investigation it has been shown that:

1. The heat denaturation of the serum proteins in alkaline sera is a function of the duration of the time of heating. The maximum effect is produced after four hours' heating at 57·5°.

2. In acid sera the change is complete within one hour's heating at the specified temperature.

3. The heat denaturation of pseudoglobulin and of albumin is a function of the [H·] of their solutions.

4. There is a marked similarity between the curves for the conversion of soluble into insoluble protein during the heating of serum, of solutions of albumin, and of pseudoglobulin throughout the ranges P_H 4·6 to 8·0.

5. The curves for the increased precipitability of the serum proteins and of pseudoglobulin by 30 % of saturation with ammonium sulphate and for the increased precipitability of albumin by 50 % of saturation with ammonium sulphate show a minimum amount of change through the region P_H 5·5 to 8·0 while on the acid side of P_H 5·5 there is absolute coincidence of the curves.

6. In heat denaturated sera the protein precipitated between 30 and 50 % of saturation with ammonium sulphate contains a mixture of pseudoglobulin, antitoxin and heat denaturated albumin.

7. The precipitation of pseudoglobulin from solutions of which the reaction, previous to the liquids being heated, had been adjusted approximately to P_H 8·0 was complete between 42 and 44 % of saturation with ammonium sulphate.

8. The precipitation of albumin from solutions of which the reaction, previous to the liquids being heated, had been adjusted approximately to P_H 8·0 gradually rose from 29 to 52 % between 42 and 50 % of saturation with ammonium sulphate.

9. The practical application of the above observations to the routine concentration of sera has shown us that :

(*a*) The preliminary prolonged heating of sera of which the reaction is on the alkaline side of P_H 7·0 must be for not less than 4 hours and not more than 5 hours at 57·5°.

(*b*) In methods involving a heat denaturation of the serum proteins, the amount of heat denaturated albumin appearing in the final product can be appreciably lessened by including in the Second Fraction precipitate the protein precipitated between 30 and 44 % of saturation with ammonium sulphate instead of between 30 and 50 % of saturation as hitherto employed.

(*c*) This decreased precipitation of albumin entails the production of end products showing a less pronounced colour, a greater removal of the total proteins of the original serum and a degree of concentration considerably greater than if the higher precipitation limit had been used.

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XXVIII. THE FORMATION OF CREATINE. EFFECTS ON THE EXCRETION OF CREATINE IN THE BIRD PRODUCED BY PARAFORMALDEHYDE AND HEXAMETHYLENE-TETRAMINE GIVEN SEPARATELY AND COMBINED WITH ARGININE CARBONATE AND OTHER SUBSTANCES.

By WILLIAM HENRY THOMPSON.

(With an Addendum by Emil Alphonse Werner.)

(*Received August 21st, 1917.*)

In a series of investigations extending over the past five years, the results of which have been recently published [Thompson, 1917, 1], it has been shown that when arginine is administered to animals a portion of its guanidine nucleus is methylated and either excreted in the urine, or stored in the muscles as creatine, or both. The proportion methylated was greater when the substance was given by hypodermic injection than when added to the food. It was also increased by combining with the arginine (particularly if hypodermically injected) substances containing a methyl group such as methyl citrate and methyl benzoate [Thompson, 1917, 2].

When given with food to dogs on non-meat diet, 2·5 % of methylated guanidine nucleus appeared in the urine: in the case of dogs on meat diet 14·6 %. This latter figure was however admittedly open to question owing to the difficulty of ensuring a constant amount of creatine in the food.

If given hypodermically, dogs on non-meat diet excreted 4·5 % of the guanidine nucleus in the urine, while one dog on a meat diet excreted 4·58 %.

In birds the output of methylated guanidine nucleus when arginine was given with the food, amounted to 1·1 %, if given by hypodermic injection to 2·5 %, of that administered.

Experiments to test the effect of intravenous injection of arginine on the creatine content of rabbit's muscle, showed that there was invariably an increase, which corresponded on the average to a storage in the muscles of

14·5 % of the methylated guanidine nucleus, the variations lying between 8 % and 25 %. At the same time there was an output of the methylated nucleus in the urine secreted in the first three hours after the injection amounting to 0·1 % of the quantity injected.

For the dog the average percentage partition of arginine nitrogen in the urine (*a*) when given with food, (*b*) when given by hypodermic injection was as follows:

	Total nitrogen recovered	Urea nitrogen	NH_3 nitrogen	Amino-acid N	N as total creatinine	N not recovered in the period
(*a*) Given with food (5 expts.)	56·5 %	34·7 %	13·7 %	2·33 %	3·47 %	43·5 %
(*b*) Injected	67·87	35·4	4·05	4·7	4·12	32·13

These results amplify and confirm those of previous experiments [Thompson, 1905, 1906].

The main conclusion indicated by these facts is that when a complex substance such as arginine is introduced into the animal body—more especially after reaching the tissues—it is attacked in various ways and dealt with by different agencies.

Many attempts were made to influence the degree of methylation of the guanidine nucleus but in the majority of cases with little obvious effect, so far as the excretion of total creatinine in the urine could be taken as an index. Various circumstances however pointed to the possibility that the presence of formaldehyde in the tissues and the extent to which it is produced, affect the results. Formaldehyde has long been known to be produced by the tissues in plant metabolism. It is also probable that this accounts for the formation of methylated compounds such as betaïne, etc., in many vegetables. Betaïne has also been shown to be present in the muscle of invertebrate animals whereas creatine is absent. This has been established by Kutscher [1914] for the crab and by D. Wright Wilson [1914, 1, 2] for the scallop and periwinkle. On passing to a stage higher in the animal scale, namely to the lamprey, the latter author was able to isolate both betaïne and creatine from the extracts of its muscle. Arginine was also found by these observers to be present in invertebrate muscle. The tissues of invertebrates thus resemble those of plants in the absence of creatine and the presence of betaïne. Further the tissues of lower animals contrast with those of the higher in the presence of betaïne and the predominance of simple amino-acids in the former, whereas in the latter there is usually an excess of diamino-acids and complex methylated basic extractives such as creatine, carnitine, oblitine, etc.

It seemed therefore probable that formaldehyde, which appears undoubtedly to be produced in the tissues of invertebrates is also formed to some extent in those of higher animals. The presence of formic acid as an excretory product in the urine also lends support to this hypothesis.

Reasoning from these considerations and assuming that under normal conditions the production of formaldehyde in the tissues of higher animals is small, it was inferred that the introduction of formaldehyde combined with arginine would probably result in an increased degree of methylation of its guanidine nucleus, as shown by an increased formation and excretion of creatine.

Preliminary observations were made on two birds to test this hypothesis by noting the effects on the excretion of creatine in the urine. It did not seem advisable however to use formaldehyde as such, owing to its violent action on tissues, either for feeding or hypodermic injection. At the suggestion of my friend Dr Emil Werner, Professor of Applied Chemistry in Trinity College, Dublin (whom I consulted on the possible chemical reactions involved) paraformaldehyde was used instead. This in solution is slowly converted into formaldehyde, and proved very suitable for the experiments. The following is a summary of the results.

TABLE I. *The effects on the excretion of creatine of paraformaldehyde and arginine given with food and injected.*

Animal used	No. of days and quantity given	Creatinine (total) excreted per day in mg. (*a*) before	(*b*) during	(*c*) after	Effect on weight of animal in g.
Duck 8	2 days, arg. 1 g., paraformald. 0·3 g., with food	6·72	15·09	8·21	+ 7
„ 8	1 day, same quantities, with food	8·21	8·00	8·16	+40
„ 9	2 days, same	21·61	34·12	31·10	+20
„ 9	1 day, same quantities, injected	8·99	50·13 1st day 70·92 2nd „ 21·90 3rd „	8·27	+16

Only in two of the observations in which paraformaldehyde was given with food were effects seen on the excretion of total creatinine in the urine. It is to be remarked however that the third case was a "repeat" observation and in such cases as previously pointed out there is usually a decline or actual reversal of the effect on the excretion in the urine. In the two cases where an effect was seen, the increase of total creatinine corresponded to a methylation of 2·2 % of the guanidine nucleus given, being double the amount

observed as stated in previous experiments with birds when arginine alone was given with the food.

Turning now to the experiment in which paraformaldehyde and arginine were given by subcutaneous injection it will be seen that the effects were very pronounced, much more so than in any of the previous experiments with birds. Only one injection was made, the effects of which lasted for three days. During this period there was an average daily excretion of 47·65 mg. as compared with the normal daily average of 8·99 mg. That is to say in the three days there was an excess of total creatinine in the urine amounting to 115·98 mg. over the normal, which represents an excretion in methylated condition of 24·2 % of the guanidine nucleus given. It may be recalled that the injection of arginine alone in previous experiments led to a corresponding excretion of 2·5 % of its guanidine nucleus. It cannot however be taken as definitely proven that the whole of the methylated guanidine nucleus excreted in the recent experiments in excess of the normal, was actually derived from the arginine injected. The results given in the following table seem to indicate the contrary since the injection of paraformaldehyde alone was followed also by a great increase in the excretion of total creatinine.

TABLE II. *The effects on the excretion of creatine of paraformaldehyde alone, given with the food and injected.*

Animal used	No. of days and quantity given	Creatinine (total) excreted per day in mg. (a) before	(b) during	(c) after	Effect on weight of animal in g.
Duck 8	2 days, 0·3 g., with food	8·03	9·92	6·72	+25
„ 9	2 days, same	13·74	12·55	15·75	−50
„ 8	1 day, 0·3 g., injected	11·79	79·36 1st day 63·42 2nd „	10·31	nil

It will be seen that in the two observations in which paraformaldehyde was given with the food there was no appreciable effect produced on the excretion of creatinine, while in the third in which the substance was injected there was a marked increase which lasted for two days. During this period the excess excretion of total creatinine over the normal, amounted to 109·2 mg. This excess cannot however be strictly compared with the corresponding excess seen after the injection of paraformaldehyde and arginine combined, since the two observations were made on different birds. Further experiments are needed to settle beyond doubt whether or not the simultaneous injection of arginine with paraformaldehyde produces a decidedly greater effect on output of total creatinine than the latter alone.

One thing however seems unquestionably to be established, namely that formaldehyde is in part at all events disposed of in the tissues by combination with a guanidine nucleus to form creatine. This is confirmed also by results obtained from the injection of urotropine (hexamethylene-tetramine), a substance which is known to liberate formaldehyde in the tissues. Thus

$$N_4(CH_2)_6 + 6H_2O = 6CH_2O + 4NH_3.$$

Three observations were made on the same bird, one in which urotropine and arginine were given with the food, a second in which urotropine alone was similarly given, and a third in which the last-named substance was given by subcutaneous injection. The results are shown in Table III. On examining them it will be seen that in the observations in which the substances were added to the food, urotropine alone produced no appreciable effect; whereas when arginine was given along with it the output of creatinine was more than doubled. When the same quantity of urotropine was *injected* but without arginine, the effect which followed lasted three days and even then had not

TABLE III. *The effects on the excretion of creatine of urotropine (hexamethylene-tetramine) and arginine given with food, and of urotropine alone given with food and injected.*

Animal used	No. of days and quantities given	Creatinine (total) excreted per day in mg. (a) before	(b) during	(c) after	Effect on weight of animal in g.
Duck 9	1 day, urotropine 0·3 g., with food	8·27	10·07	—	− 5
„ 9	1 day, arg. 1 g., urotropine 0·3 g., with food	8·27	18·99	9·83	+10
„ 9	1 day, urotropine 0·3 g., injected ...	9·83	13·11 1st day 24·13 2nd „ 20·68 3rd „	12·27	−20

fully subsided. During this period an excess of 28·43 mg. of creatine over and above the normal (9·83 mg. per day) was excreted. That is to say on the average the output was doubled for three days following a single injection. If the whole of the formaldehyde theoretically liberated from the quantity of hexamethylene-tetramine injected were disposed of by combination with a guanidine nucleus and forthwith excreted, the output of creatine should have been much greater, that is approximately equal to the output after a corresponding injection of paraformaldehyde. Further experiments are needed before an explanation of the difference in the two cases can be assigned.

Three observations were also made on the effects of combining paraformaldehyde with substances other than arginine. The substances used

were glycine, sarcosine and guanidine carbonate, and in all cases they were given with the food. The results are shown in Table IV. On examining them it will be seen that in all cases there was an increased output of total creatinine during the feeding period. The effects are small in comparison with some of those considered in this paper but by no means negligible, the increase in each case being approximately 60 % over the normal.

TABLE IV. *The effects on the excretion of creatine of paraformaldehyde given in the food with other substances than arginine.*

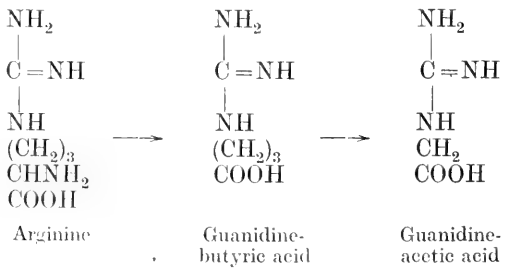
Animal used	No. of days and quantities given	Creatinine (total) excreted per day in mg. (*a*) before	(*b*) during	(*c*) after	Effect on weight of animal in g.
Duck 8	2 days, glycine 1 g., paraformald. 0·3 g. ...	6·94	11·40	—	+20
,, 8	1 day, sarcosine 1·3 g., paraformald. 0·3 g....	6·94	11·79	—	+30
,, 8	2 days, guanidine carb. 0·25 and 0·3 g., paraformald. 0·25 g.	4·67	7·06	6·94	−20

Observations were also made in each case on the output of preformed creatinine but in no case was the effect appreciable.

DISCUSSION.

It remains to suggest a chemical hypothesis by which the foregoing facts might be explained.

It has been pointed out by Neubauer [1910], Dakin [1912, p. 62] and others that in accordance with typical oxidation processes known to occur in the animal body guanidine-acetic acid (glycocyamine) can readily be derived from arginine, the stages being as follows:



Arginine — Guanidine-butyric acid — Guanidine-acetic acid

It has also been conclusively proved [Jaffé, 1906; Dorner, 1907; Thompson, 1917, 1] that the last-named body is methylated in the tissues with the production of creatine, which is partly stored in the muscles and partly excreted in the urine.

No suggestion has been offered as to how this methylation occurs, but it is not improbable that in the production of creatine from guanidine-acetic acid the methylation arises from the action of formaldehyde. The reaction may provisionally be represented in the following way:

(1) $HN:C\begin{matrix}NH_2\\ NHCH_2COOH\end{matrix} + CH_2O = HN:C\begin{matrix}N:CH_2\\ NHCH_2COOH\end{matrix} + H_2O$

(2) $HN:C\begin{matrix}N:CH_2\\ NHCH_2COOH\end{matrix} + CH_2O + H_2O = HN:C\begin{matrix}NHCH_3\\ NHCH_2COOH\end{matrix} + HCOOH$

By migration of the CH_3 group which is more likely to occur in the animal body than in vitro, the isomer of creatine,

$$HN:C\begin{matrix}NHCH_3\\ NHCH_2COOH\end{matrix}$$

would become

$$HN:C\begin{matrix}NH_2\\ N(CH_3)\,CH_2COOH\end{matrix}$$

This hypothesis has the merit that the results obtained from the injection of arginine and paraformaldehyde both singly and combined could be explained on similar lines. It is improbable however that the methylation of the arginine would be deferred till, by the process of oxidation, it had reached the stage of glycocyamine. It is more likely that methylation and oxidation go hand in hand, the end results being the production of creatine, ammonia, carbon dioxide and water, the major part of the NH_3 and CO_2 appearing in the urine as urea.

Before considering this suggestion further it is necessary to refer to Sörensen's results [1908] on the interaction of formaldehyde and amino-compounds. With neither arginine (free base) nor with guanidine was acidity immediately developed when titration with neutral formaldehyde was carried out. Acidity was however obtained when he used arginine hydrochloride. Sörensen concluded from these results that the amino-group of the arginine reacts as usual with formaldehyde but that the guanidine group on the contrary is unable to fix the aldehyde.

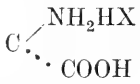
I am indebted to my colleague, Prof. E. Werner, for a critical discussion of Sörensen's results and conclusions in which he states that the results are exactly what he would have predicted but that the inferences are not what he would have drawn. Nor would the acidity in the case of arginine hydrochloride be entirely due to a liberation of HCl but to the production also of formic acid. Werner holds, and I think rightly, that when CH_2O is added

to any compound containing NH_2 in such a complex as occurs in all the amino-acids the result may be expressed in general terms as follows:

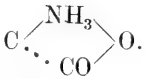
(1) $C...NH_2 + CH_2O = C...N : CH_2 + H_2O$,

(2) $C...N : CH_2 + CH_2O + H_2O = C...NH . CH_3 + HCOOH$.

In the case of guanidine a methylguanidine[1] is formed which neutralises the formic acid as fast as it is produced, hence the neutrality observed by Sörensen. In the case of arginine free base the guanido-group is methylated and neutral methylated arginine formate is produced. If the arginine be present as a stable salt, this salt is formed by the acid (say HCl) being attached to the guanido-group



while on the "cyclic" or "internal ammonium salt" theory of the structure of amino-acids there would be mutual neutralisation of the NH_2 and COOH end groups as here indicated:



Hence the formic acid produced would remain free, the guanido-group being neutralised by the HCl. This explanation implies that in all cases where acidity is developed by titrating a neutral amino-acid compound with neutral formaldehyde, formic acid is set free, at all events, to some extent. (See Addendum, p. 318.)

Applying these considerations to the interaction of arginine and formaldehyde in the tissues the stages would conceivably be those already suggested (p. 312).

The arginine would thus by the processes of methylation and oxidation pass through the stages of (1) methylene-guanido-butyric acid, (2) methylene-guanido-acetic acid, (3) to an isomer of creatine which by migration of the methyl group becomes ordinary creatine.

The scheme at all events provides a working hypothesis, which will I believe, in the main if not in all its details, prove to be correct. I had hoped to find time before publishing this preliminary account to test on the animal two of the points which could readily be confirmed or the reverse, namely (1) whether the combination of formaldehyde with glycocyamine would increase the methylation of the latter, (2) whether formic acid appears or is increased in the urine as a result of the injections. Unfortunately, however, although I have

[1] A methylene-guanidine formed in accordance with equation (1) would no doubt be itself a strong base and hence no acidity would be developed. In a lesser degree this would also hold for arginine (free base).

waited a year in the hope of finding the necessary time this has not proved possible and the prospect is so distant that I am constrained to publish the investigation in its present incomplete condition. Obviously much has yet to be done for which it is hoped that opportunity will ultimately present itself.

In conclusion I have to express my indebtedness to Dr Werner for his kind criticism and valuable suggestions.

Summary.

1. The administration of arginine and paraformaldehyde with food to ducks was followed by an increase in the excretion of creatine which corresponded to a methylation of 2·2 % of the guanidine nucleus contained in the arginine. Previous experiments with arginine alone, given in the same way led to a corresponding excretion of 1·1 % of the guanidine nucleus. Paraformaldehyde alone produced no effect.

2. Arginine and paraformaldehyde given to the same birds by subcutaneous injection led to an excess excretion of creatine which represented a methylation of 24·2 % of the guanidine nucleus of the arginine. In previous experiments with arginine alone the corresponding amount methylated and excreted was 2·5 % of the guanidine nucleus. Paraformaldehyde injected alone also caused a large excess in the excretion of creatine. It is concluded therefore that formaldehyde is disposed of in animal tissues, in part at least, by combination with a guanidine nucleus to form creatine.

3. Arginine and hexamethylene-tetramine similarly given with food to one of the ducks increased the output of creatine in the urine to double the normal. Hexamethylene-tetramine alone had no effect.

4. Hexamethylene-tetramine given by subcutaneous injection to the same bird, doubled the output of creatine for three days after a single injection. The whole of the formaldehyde theoretically obtainable and presumably liberated in the tissues from the quantity of hexamethylene-tetramine used, cannot have been disposed of by combination with a guanidine nucleus or if so, the creatine formed was not forthwith excreted in the urine.

5. Paraformaldehyde given in the food along with glycine, sarcosine, and guanidine carbonate respectively increased the excretion of creatine in the urine by about 60 % in each case.

6. No appreciable effect was produced by any of the substances on the excretion of preformed creatinine.

7. A chemical explanation is offered to account for the rôle played by formaldehyde in the methylation of the part of the guanidine nucleus of arginine converted into creatine in the animal body.

PROTOCOLS.

Duck VIII. In each case the days marked with letters are those on which the effects appeared, that is to say, the administration of the substance was begun a day earlier than that marked. Lines of space across the page are introduced to mark off the periods.

Date 1916	Preformed creatinine per day in mg.	Total creatinine per day in mg.	Total nitrogen per day in mg.	Weight kilos
June 16	4·24	8·17	0·698	1·495
,, 17	4·11	5·85	0·669	1·495
,, 18	4·16	10·06	0·734	1·540
,, 19 (*a*)	4·17	**12·47**	0·718	1·560
,, 20 (*a*)	2·81	**7·37**	0·561	1·565
,, 21	2·41	7·31	0·581	1·540
,, 22	2·01	6·14	0·664	1·550
,, 23 (*b*)	2·91	**15·33**	0·485	1·560
,, 24 (*b*)	2·66	**14·87**	0·595	1·545
,, 25	3·85	9·14	0·598	1·520
,, 26	2·82	7·28	0·660	1·540
,, 27 (*c*)	2·62	**8·00**	0·559	1·580
,, 28	3·36	8·16	0·428	1·560
,, 29	2·73	4·67	0·625	1·565
,, 30 (*d*)	3·13	**7·77**	0·556	1·550
July 1 (*d*)	2·79	**6·35**	0·599	1·545
,, 2	2·42	6·94	0·609	1·580
,, 3 (*e*)	4·33	**10·82**	0·819	1·590
,, 4 (*e*)	3·98	**11·98**	0·859	1·600
,, 5 (*f*)	4·06	**11·79**	0·777	1·630
,, 6 (*g*)	4·01	**79·36**	0·985	1·630
,, 7	4·06	**63·42**	0·708	1·630
,, 8	3·71	13·12	0·736	1·625
,, 9	3·66	7·50	0·681	1·630

(*a*) Paraformaldehyde 0·3 g. with food.
(*b*) Paraformaldehyde 0·3 g., arginine carb. 1 g. with food.
(*c*) Paraformaldehyde 0·5 g., arginine carb. 1 g. with food.
(*d*) Paraformaldehyde and guanidine carbonate with food, first day 0·25 g. of each, second day 0·25 g. of paraformaldehyde and 0·3 g. guanidine carbonate.
(*e*) Paraformaldehyde 0·3 g., glycine 1 g. with food.
(*f*) Paraformaldehyde 0·3 g., sarcosine 1·3 g. with food.
(*g*) Paraformaldehyde 0·3 g., injected.

Duck IX.

Date 1916	Preformed creatinine per day in mg.	Total creatinine per day in mg.	Total nitrogen per day in g.	Weight kilos
June 10	2·53	13·33	0·387	1·770
„ 11	3·14	14·16	0·386	1·750
„ 12 (*a*)	3·57	12·35	0·422	1·770
„ 13 (*a*)	3·49	12·74	0·444	1·740
„ 14	2·97	13·16	0·374	1·720
„ 15	4·03	17·38	0·355	1·725
„ 16	4·08	18·75	0·402	1·725
„ 17	4·25	20·83	0·315	1·720
„ 18	3·82	22·39	0·300	1·700
„ 19 (*b*)	3·83	**28·43**	0·567	1·710
„ 20 (*b*)	2·71	**39·80**	0·453	1·720
„ 21	4·49	**40·78**	0·517	1·690
„ 22	4·44	**21·43**	0·471	1·720
„ 23	2·30	7·78	0·429	1·730
„ 24	3·61	8·05	0·297	1·725
„ 25	3·37	9·93	0·318	1·705
„ 26 (*c*)	4·16	**50·13**	0·644	1·750
„ 27	3·75	**70·92**	0·506	1·725
„ 28	3·25	**21·90**	0·446	1·740
„ 29	3·26	8·11	0·402	1·750
„ 30	3·97	8·43	0·346	1·760
July 1 (*d*)	3·45	10·58	0·475	1·750
„ 2 (*d*)	4·19	9·55	0·470	1·750
„ 3 (*e*)	5·70	**15·69**	0·678	1·700
„ 4 (*e*)	4·39	**22·30**	0·625	1·760
„ 5	3·76	9·83	0·315	1·750
„ 6 (*f*)	5·13	**13·11**	0·567	1·730
„ 7	4·69	**24·13**	0·645	1·700
„ 8	4·12	**20·68**	0·465	1·710
„ 9	—	12·06	0·449	1·710
„ 10	4·29	12·48	0·649	1·710

(*a*) Paraformaldehyde 0·3 g. with food.
(*b*) Paraformaldehyde 0·3 g., arginine carb. 1 g. with food.
(*c*) Paraformaldehyde 0·3 g., arginine carb. 1 g. injected.
(*d*) Hexamethylene-tetramine 0·3 g. with food.
(*e*) Hexamethylene-tetramine 0·3 g., arginine carb. 1 g. with food.
(*f*) Hexamethylene-tetramine 0·3 g. injected.

ADDENDUM.

By Emil Alphonse Werner.

It may be well to point out the following facts, in connexion with equations (1) and (2) given by Prof. Thompson (p. 313), which briefly represent the mechanism of the methylation of amino-compounds by formaldehyde in accordance with the theory recently put forward by the writer [Werner, 1917].

Whilst equation (1) expresses the main change upon which Sörensen's titration method is based; three conditions are necessary for the promotion and completion of equation (2). These are, (*a*) rise of temperature, (*b*) the presence of formaldehyde in excess, and (*c*) the production of a methylene-amino-compound of the type $\vdots C \,.\, N : CH_2$, more or less prone to reduction. It is obvious that in Sörensen's method, condition (*b*) is always fulfilled, whilst (*c*) will depend upon the nature of the amino-compound under investigation.

It will be seen therefore, that it is not suggested by the theory in question, that equation (2) is completed under the conditions employed by Sörensen, but that it may proceed simultaneously with equation (1) in certain cases seems highly probable.

So far as the interaction of formaldehyde and arginine with the ultimate formation of creatine is concerned, it would appear from a purely chemical point of view, that the scheme put forward by Prof. Thompson gives a very plausible explanation of the series of changes.

It may be fairly assumed that in an animal body the conditions would be favourable for the promotion of equation (2).

In conclusion, it may be pointed out that the velocity of reaction (2) is enhanced by another condition, namely the removal of formic acid as it is produced, either by neutralisation, or by its oxidation to carbon dioxide, and water.

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XXIX. THE PREPARATION OF PLANT NUCLEIC ACIDS.

By GEORGE CLARKE and SAMUEL BARNETT SCHRYVER.

(*From the Department of Plant Physiology, Imperial College of Science and Technology, London.*)

(*Received August 10th*, 1917.)

The methods described in the literature for the preparation of plant nucleic acids involve the extraction and separation of nucleoproteins containing varying amounts of phosphorus, and subsequent digestion with pepsin hydrochloric acid mixture until a product giving no biuret reaction and containing 8–9 % phosphorus is obtained. Several fermentations and treatment with picric acid are required to obtain a biuret-free substance. The methods are somewhat cumbersome and necessitate the manipulation of large volumes of liquid.

Nucleic acid of plant origin was required in some quantity for biological experiments and the following work was undertaken with the object of finding out if its preparation could be simplified and a biuret-free product obtained without peptic digestion.

Preliminary experiments were made with the raw material used—wheat embryos and dried yeast—by quantitative extraction with water and with solutions of sodium acetate or sodium chloride and afterwards with very dilute caustic potash. The proportion of the total phosphorus extracted by the various solutions was thus ascertained and a rough indication obtained of the form in which this element existed.

The results are given below.

Wheat embryo (1·13 % *total phosphorus*).

Percentage of the total phosphorus extracted by	10 % sodium acetate	= 63·7
,, ,, ,, ,,	$N/10$ caustic potash	= 32·5
	Total	= 96·2
Percentage of the total phosphorus extracted by	10 % sodium chloride	= 85·2
,, ,, ,, ,,	$N/20$ caustic potash	= 12·4
	Total	= 97·6
,, ,, ,, ,,	Water	= 75·7

Wheat embryo (another sample) (0·78 % *total phosphorus*).

Percentage of the total phosphorus extracted by 10 % sodium chloride = 80·4

Dried, alcohol-extracted yeast (2·33 % *total phosphorus*).

Percentage of the total phosphorus extracted by 10 % sodium chloride = 57·2

In the above experiments the extraction was carried out at a temperature 60–80°, and in cases where a second extraction of the same material was made the residue was thoroughly washed with water on the centrifuge.

A very large proportion of the total phosphorus of wheat embryos is soluble in a 10 % solution of sodium chloride and if this extraction is followed by extraction with very dilute alkali, practically the whole of the phosphorus is removed. Some of the phosphorus thus extracted is combined in the form of nucleic acid and can be separated by the addition of hydrochloric acid to the NaCl solution, the nucleic acid being combined as a salt with varying amounts of protein.

The remainder of the phosphorus is present as phytin ($C_{12}H_{22}O_{44}P_{10}Ca_7Mg$) and can be isolated in this form after the separation of nucleic acid by the methods already described by one of the authors [Clarke, 1914].

From dried yeast less of the phosphorus is extracted with sodium chloride solution, although a somewhat larger yield of nucleic acid is obtained from this material than from wheat embryos. A considerable proportion of the phosphoric acid appears to exist in dried yeast in an uncombined form.

As pointed out by Osborne and Harris, nucleic acid exists in plant tissues combined with varying amounts of proteins in the form of salts, the number of which known to exist in plants is exceedingly large. These salts, which contain in many cases only a small amount of phosphorus, retain their protein characters and the phosphorus was formerly regarded as part of the protein molecule.

During the course of the present investigation in which untreated raw material was extracted with 10 % sodium chloride solution and the clear extract treated with hydrochloric acid, substances were obtained in which the phosphorus varied from an inconsiderable trace to 3 %, and which all contained large amounts of protein and gave a well defined biuret reaction.

The problem of obtaining protein-free nucleic acid by direct extraction therefore, resolved itself into devising some means of so altering the protein complex that it was no longer extracted with sodium chloride solution. It was found that boiling the material used with a large excess of 95 % ethyl

alcohol for two hours and subsequently extracting the dried material with warm 10 % sodium chloride solution gave a clear extract from which by the addition of hydrochloric acid a characteristic precipitate of nucleic acid could be obtained that quickly settled and could be ground to a fine powder. This substance gave no biuret reaction and contained in the case of yeast 7·79 % phosphorus, and in the case of wheat embryo 4·6 % phosphorus. The wheat embryo employed was mixed with a considerable amount of endosperm and the crude nucleic acid obtained from this source was mixed with starch. This could be removed by suitable treatment during the subsequent purification but the presence of gelatinised starch in the extract was very inconvenient and caused a loss of a certain amount of nucleic acid.

In later preparations from wheat embryo the starch was gelatinised by placing the alcohol-extracted material in boiling water and heating. The starch was then fermented with taka-diastase in the presence of toluene until the solution no longer gave the starch reaction with iodine. Salt was added in sufficient quantity to give a 10 % solution and the extraction and separation of the nucleic acid continued in the usual way. This method gives extracts that can be easily filtered and a starch-free product.

Preparation of nucleic acid from yeast.

30 lbs. of freshly pressed yeast were treated with a large excess of 95 % alcohol, allowed to stand 24 hours, filtered on a Buchner funnel and dried in air. The air-dried material, in successive batches of 1000 g. was boiled for two hours with 95 % alcohol, filtered, pressed and dried in a current of air at 37°. It was then ground to a fine powder in a laboratory mill. 1000 g. of finely ground material, prepared in the above manner was extracted with 10 litres of 10 % sodium chloride at a temperature 60–80°. The extraction was continued with frequent stirring and intermittent heating to 60–80° for 4–5 days. The residue was separated by pouring through muslin and pressed, the turbid extract being filtered clear through paper. 90 cc. hydrochloric acid (1 pt. conc. HCl: 1 pt. water) were then added to the clear extract and the solution vigorously stirred. A characteristic precipitate of nucleic acid separated, quickly settling to a hard cake at the bottom of the vessel. After standing for two hours the liquid was syphoned off and the crude nucleic acid was washed with 50 % alcohol till free from chlorine, left standing over night in 95 % alcohol and finally washed with absolute alcohol and ether. The yield was 15 g. In other experiments the yields varied from 1·4–1·6 % of the dry alcohol-extracted yeast. The crude nucleic acid thus obtained

was a light brown powder and contained 7·8 % phosphorus. It gave no biuret reaction and had the characteristic properties of nucleic acid.

The crude substance was best purified by a method described by Osborne and Harris [1902] by solution in warm 10 % sodium acetate solution and precipitation with excess of hydrochloric acid. 20 g. of the crude acid were dissolved in 500 cc. 10 % sodium acetate by warming for a short time on a water-bath. A small amount of insoluble matter was separated by centrifuging and a clear solution obtained, to which 100 cc. alcohol and excess of hydrochloric acid were added. The nucleic acid separated out as a white mass which settled quickly. It was well washed with 50 % alcohol, 95 % alcohol and ether, and finally dried in a vacuum. It was a perfectly white powder. There is no loss in this method of purification which gives a much whiter product than purification from dilute potash.

The pure nucleic acid thus prepared gives no biuret reaction. Dried at 110° in air it gave the following results on analysis:

0·5626 g.; 0·189 g. $Mg_2P_2O_7$	P = 9·3 %
0·3905 g.; 45·9 cc. $N/10$ H_2SO_4	N = 16·4
Calculated for $C_{38}H_{50}O_{29}N_{15}P_4$	P = 9·5
	N = 16·1

5 g. of the above preparation were suspended in 200 cc. water and 100 cc. of a 0·1 % solution of caustic potash added. A clear solution was obtained from which the nucleic acid was precipitated by hydrochloric acid (20 cc. $N/2$ HCl). After the nucleic acid had separated, an equal volume of 95 % alcohol was added to the solution and the pure nucleic acid separated and washed with alcohol and ether in the usual way. Dried at 110° in air it gave on analysis the following results:

0·359 g.; 0·124 g. $Mg_2P_2O_7$	P = 9·6 %
0·2903 g.; 34·7 cc. $N/10$ H_2SO_4	N = 16·5

These results are in good agreement with the formula proposed by Levene [1909] for yeast-nucleic acid.

5 g. of pure nucleic acid were heated with 20 cc. of 10 % sulphuric acid solution on a boiling water-bath for two hours and strong ammonia in slight excess was added to the dark coloured solution. Guanine separated out on cooling as a dark coloured powder. It was purified by dissolving in dilute sulphuric acid, clearing with a little animal charcoal, and reprecipitating with strong ammonia. It gave a characteristic salt with hydrochloric acid and the usual reaction for guanine.

Preparation of nucleic acid from wheat embryos.

1000 g. of fresh wheat embryos (containing 0·78 % P) were boiled for two hours with 3 litres of 95 % alcohol, pressed free from the solvent and dried in air. The dry material was extracted with 10 litres of 10 % sodium chloride at 60–80° for five days[1]. The extract, which was somewhat viscid owing to the presence of gelatinised starch, was separated from the residue of wheat embryos by filtering through muslin and washing the latter with water, and was then centrifuged as clear as possible. 100 cc. hydrochloric acid (1 pt. conc. HCl : 1 pt. water) were added. Crude nucleic acid separated out and was washed with alcohol and ether in the usual way. Yield = 14 g. of a white powder containing a large amount of starchy impurity. It gave no biuret reaction and contained 4·6 % phosphorus which corresponded to approximately 50 % nucleic acid. In subsequent experiments yields varying from 1·1–1·4 % dry material were obtained.

50 g. of the crude nucleic acid (P = 4·6 %) was dissolved in 1000 cc. of a 10 % sodium acetate solution by gently warming on a water-bath. A large amount of starchy impurity remained undissolved and was easily separated by centrifuging. Nucleic acid was precipitated from the sodium acetate solution by the addition of excess of hydrochloric acid. The precipitated acid was washed with 50 % alcohol, 95 % alcohol and finally with ether. The yield of purified nucleic acid was 22·5 g.

5 g. of the nucleic acid thus purified was dissolved in 400 cc. 0·1 % caustic potash solution. A clear solution was obtained from which the nucleic acid was precipitated by adding 5 cc. of strong hydrochloric acid and an equal volume of 95 % alcohol. The precipitated acid was washed with alcohol and ether in the usual way and dried in a vacuum.

Dried at 110° it gave on analysis the following results:

0·3916 g.; 0·116 g. $Mg_2P_2O_7$ P = 8·3 %

0·329 g.; 45·2 cc. $N/10$ H_2SO_4 N = 16·1

It has been mentioned that loss of nucleic acid and considerable inconvenience was caused in this method of preparation by the presence of gelatinised starch in the extract. In subsequent preparations this difficulty was overcome by digesting the starch with taka-diastase before extracting the nucleic acid with sodium chloride solution.

1500 g. of wheat embryo (P = 1·13 %) were treated in the usual manner with boiling alcohol and dried in air. The air-dried material was then added

[1] The mixture was not kept constantly at 60–80°, but was heated up every day to this temperature and allowed to cool slowly.

in small portions at a time to 10 litres of boiling water and heating continued for two hours in a boiling water-bath. A thick paste was formed. This was transferred to two large flasks, cooled to 40° and a solution of taka-diastase containing 2·5 g. was added to each flask. The fermenting liquid was covered with toluene and the fermentation continued until no starch reaction with iodine was given. The liquid was then heated to 100° cooled to 60° and sufficient sodium chloride added to make a 10 % solution. The extraction with salt solution was continued for four days at 60–80° (intermittent heating). A perfectly clear extract was obtained by filtering through paper pulp and on pressing the residue practically the whole of the extract could be recovered. Nucleic acid separates from the clear extract in the usual characteristic way. Yield = 7 g.

The crude product was purified by dissolving in 400 cc. 0·1 % caustic potash solution, precipitating with hydrochloric acid, washing with alcohol and ether and drying in air. Dried at 110° it gave on analysis the following results:

0·3717 g.; 0·119 g. $Mg_2P_2O_7$ P = 8·9 %
0·3997 g.; 46·6 cc. $N/10$ H_2SO_4 N = 16·3

The authors desire to express their thanks to Mr A. E. Humpheries, Cox's Lock, Weybridge, and to Mr Julian Baker, F.I.C., Stag Brewery, Pimlico, for kindly supplying the raw material used in this investigation. They are also indebted to Messrs Parke, Davis and Co. for the supply of taka-diastase.

Summary.

General methods have been worked out for preparing protein-free nucleic acid from plant tissues without peptic digestion by first boiling the material used with alcohol and then extracting the alcohol-treated material with warm 10 % sodium chloride solution. Protein-free nucleic acid is precipitated from the sodium chloride extract on the addition of hydrochloric acid.

In the case of material containing starch, such as wheat embryos obtained in the modern processes of milling, the starch was removed by fermentation with diastase before extracting with sodium chloride solution. In this way extracts were obtained which could easily be filtered.

The analytical results obtained were in good agreement with the formula $C_{38}H_{50}O_{29}N_{15}P_4$ suggested by Levene for plant nucleic acid.

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XXX. A COMPARATIVE STUDY OF TUMOUR AND NORMAL TISSUE GROWTH.

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(*Received September 12th, 1917.*)

Introduction.

From early times the profound influence exerted upon the nutrition of an animal by the composition of its diet has been recognised, so that it is not surprising that a vast amount of research has been carried out upon the subject.

Whilst much of this research has furnished results of great value, it is as a result of the investigations of the last decade in particular that the theoretical basis of the science of nutrition has become so firmly established.

These results have permitted a far clearer definition of the many factors which control the nutritive value of a foodstuff than it was ever possible to deduce from calculations respecting the calorific value and nitrogen content.

Particular interest lies in the determination of the dietary factors which influence the growth and development of the young organism. Many of these factors are now recognised to such an extent that by an experimental application of them the growth of young animals may be controlled. This control may be so complete as to permit the total inhibition of the growth processes for a considerable period of time, without the subject suffering any apparent ill-health.

It is here that such results may be viewed in the light of cancer therapy, since the question arises as to whether dietary measures which inhibit the growth of the young cells of the animal organism may not be with justification applied as a means of arresting growth in tumour cells.

Tumours are frequently described as autonomous, in that their growth is self-regulated without regard for the laws governing the nutritive condition of the host. On the other hand there are facts, such as the relatively slow

growth of tumours in old or emaciated hosts, which lend support to the opposite view.

It is not easy to trace opinions, based upon clinical observations, which concern this subject, since they are scattered widely apart throughout the literature upon cancer.

Before proceeding to discuss the laws of the growth of tumours, it is necessary that the known facts concerning their metabolism be considered. Unfortunately, comparatively little is known with regard to the metabolic processes of the cancer cell. From what is known, it appears highly probable that there is a close similarity between the chemical processes occurring in the tumour cell and those which occur in the normal cell of similar type. It is unnecessary to give in detail an account of the experimental results which support this view, but Wells [1914] who has reviewed the whole subject very completely, expresses the opinion that little has been detected which indicates any important deviation of the chemical processes of tumours from those of normal cells of similar origin.

As this opinion has received the support of the majority of the experimental investigations since carried out, it will be seen that it is justifiable to apply factors which are known to influence the growth of normal cells in an attempt to influence in a similar manner the growth of tumour cells.

It is now necessary to review briefly what is known of these factors which may so profoundly influence the nutritional condition of the animal organism.

It is recognised as necessary that the diet of an animal shall satisfy certain requirements before it can be regarded as adequate to supply the demands of the organism throughout its life cycle.

As far as these requirements have been determined they are as follows:

1. The calorific value of the diet must be sufficient to supply the necessary potential energy.

2. Sufficient nitrogen must be supplied in a form suitable for the building up or repair of tissue.

3. This nitrogen must be supplied in a form which will ensure an adequate supply of certain amino-acids which the animal organism is unable to synthesise for its own use.

4. The diet must contain an adequate supply of inorganic salts capable of satisfying the mineral requirements of the animal.

5. Certain substances, probably two in number, the nature of which is at present unknown, but which have been provisionally termed "accessory growth promoting substances," must be present in a sufficient amount.

With regard to the first two of these requirements little need be said, for until recently they, together with the fourth stipulation, constituted the basis for the standardisation of the nutritive value of foodstuffs. The value and importance of the other two factors has, however, but recently been appreciated. Prior to 1911, repeated attempts had been made to rear and maintain animals upon dietaries composed entirely of chemically pure food units, but although every care was taken to ensure that the diets used contained satisfactory proportions of protein, fat, carbohydrates and mineral salts, yet such experiments met with repeated failure. It was Osborne and Mendel [1911] who first described an extensive research conducted along these lines which was attended with considerable success.

The results obtained by these two authors were of far-reaching importance and opened up a new field of research on protein metabolism.

Earlier studies than these had, however, determined the importance of the quality of the protein fraction of a diet, for Willcocks and Hopkins [1906] demonstrated the inferior nutritive value of the protein zein, which is totally deficient in the amino-acid tryptophan. Osborne and Mendel followed up their investigations along the lines they had previously adopted, and not only confirmed the indispensability of tryptophan [1912, 1] but also pointed out how the amino-acid deficiencies of such a protein as gliadin may influence the nutrition of an animal [1912, 2, 3; 1913]. Much of this earlier work has now received ample confirmation which, together with the results obtained by an extension of the study, have been the cause of very material alterations in the theory of protein metabolism.

That the nutritive value of a protein may be largely determined by its amino-acid content is now so fully appreciated as to have passed the stage when it was regarded as of academical interest alone, and to have become of great value when applied to certain problems of animal husbandry.

Not only may the nutritive value of protein be greatly influenced by the absence of certain amino-acids, but it may be limited by a low content of one such indispensable unit. The case of gliadin, which possesses a low nutritive efficiency as a result of a low lysine content has been referred to above, whilst a very striking example has been given more recently by Osborne and Mendel [1915, 1].

When the diet of a growing rat contains 18 % of protein in the form of caseinogen or lactalbumin, and is at the same time satisfactory with regard to the other requirements, a normal rate of growth is accomplished. Upon reduction of the plane of protein intake, it is found that a diet containing

9 % of caseinogen is of a lower food value to the growing rat than one containing the same percentage of lactalbumin. The explanation of this was found to lie in the fact that caseinogen has a low content of cystine, an amino-acid which the organism of the rat is unable to synthesise.

At the lower plane of intake, the caseinogen diet contained insufficient protein to supply the cystine requirements of the young rat, whereas no such limiting factor was in operation in the case of the lactalbumin diet. That this explanation was the correct one was proved by the addition of the pure amino-acid cystine to the deficient caseinogen diet, whereupon its nutritive value was at once raised to that possessed by the corresponding lactalbumin ration.

Such experiments as these have led to the establishment of an entirely new standard by which the food value of the proteins is judged [Osborne and Mendel, 1916, 1].

The necessity of ensuring an adequate supply of what are termed the "accessory growth promoting substances" in the diet, particularly that of a growing animal, is but now being fully recognised. That such substances existed and played important rôles in animal nutrition was indicated by the researches of Stepp [1909, 1912, 1913], Hopkins [1912] and Funk [1913, 1]. Their existence has been doubted by other investigators, amongst whom may be mentioned Abderhalden [1913, 1] and Röhmann [1916], but it is definitely established that in these cases the authors were not utilising food mixtures of sufficient purity. These substances have received many appellations and are frequently spoken of as "vitamines."

At first one of these substances was found in association with certain naturally occurring fats, such as butter fat and cod liver oil, by Osborne and Mendel [1912, 1] and almost simultaneously by McCollum and Davis [1913].

Later it was demonstrated by McCollum and Davis [1915, 1, 2, 3] that a second accessory exists in the aqueous extracts of certain foodstuffs, particularly seeds.

These authors have shown clearly the importance of these two unidentified substances which they have provisionally designated *the fat-soluble A* and *the water-soluble B*. Both substances are at present unidentified chemically, and indeed very little definite knowledge of their properties is possessed. It appears highly probable that the second substance is identical with the so-called "anti-beriberi vitamine," studied extensively by Funk, since they possess closely similar properties, Drummond [1917]. The need of the animal organism for an adequate supply of both these factors is now admitted by the majority of investigators in this field of research.

Having now very briefly considered the nature of the requirements which a dietary must satisfy before it can be considered adequate to supply the nutritive demands of the animal organism, the recorded experiments upon the influence of diet upon tumour growth will be considered in the light of this knowledge.

Haaland [1907] recorded that mice fed upon a diet of bread, oats, hempseed and milk were less resistant to inoculation with Ehrlich's mouse sarcoma than when fed upon bread and oats alone. This observation received support from Stahr [1908], who attributed a different degree of susceptibility to tumour inoculation exhibited by mice from Berlin and those from Düsseldorf to an influence exerted by differences in their diets. A somewhat similar conclusion was reached by Jensen [1909]. In all these cases, however, the explanation given by the authors was that which seemed best to account for the results, but no experimental proof was given which excluded the possible disturbing influence of other factors.

It was Moreschi [1909] who first attempted a study of the influence of diet upon tumour growth. He found that by underfeeding mice the growth of inoculated grafts of tumour tissue, as yet unvascularised, was retarded. Rous [1911, 1] made a similar study to this, but also examined the influence of such dietary measures applied after the tumour grafts had become established in the host. He obtained the interesting result that whereas a tumour may not take well when implanted into an underfed animal, yet the same tumour may show no retardation in growth as a result of dietary restriction applied after vascularisation of the graft.

Sweet, Corson-White and Saxon [1913] investigated the influence upon tumour growth exerted by a diet in which the protein was supplied in form of gluten. Such a diet had been found by Osborne and Mendel [1912, 1] to permit maintenance, but not growth, in rats owing to its low content of lysine.

Sweet, Corson-White and Saxon aimed at determining whether a cancer could grow in a body rendered incapable of normal cell-growth. They found that the number of successful inoculations of grafts of the Flexner-Jobling rat carcinoma was smaller in the animals which had been fed upon the gluten diet than in the normally fed controls. At the same time the grafts which succeeded in becoming established in the specially fed animals exhibited a slower rate of development and more frequently suffered retrogressive change than in the control series.

They were therefore led to suggest that tumour cells and somatic cells agree with respect to their laws of growth.

Van Alstyne and Beebe [1913] did not agree with the conclusion reached by Sweet, Corson-White and Saxon. They themselves examined the influence of carbohydrates upon tumour growth in rats, and found that a greater degree of success in tumour implantation was obtained in the animals which received a diet containing carbohydrate, as compared with those which received a carbohydrate-free diet.

It is unfortunate that these authors should have chosen lactose as the carbohydrate with which to work, for it has been pointed out that lactose is frequently contaminated with traces of nitrogenous impurities. The possible influence of these impurities upon the course of such experiments as were carried out by van Alstyne and Beebe has been pointed out by Funk [1914, 1] and by Sweet, Corson-White and Saxon [1915].

In spite of this, Woglom [1915] challenged the conclusion of van Alstyne and Beebe, since he found no effect upon tumour growth following the administration of lactose to the hosts. What was probably the correct explanation of these two diverse results was given by Drummond [1916, 1] who pointed out that lactose, unless carefully purified, usually carries traces of the water-soluble accessory factor, *B*. It was probably this factor which influenced tumour growth in the experiments of van Alstyne and Beebe, whereas no such influence would be exerted in Woglom's experiments since he added the lactose to a basal diet already rich in accessory substances.

Rous [1914] followed up his earlier studies and extended his investigations to the more practical determination of the influence of diet upon established tumours in mice. Rous appreciated the doubtful practical value of results such as those obtained by Sweet and his co-workers, and by van Alstyne and Beebe. Commenting upon the lower rate of tumour development obtained in the hosts which had been subjected to dietary measures prior to inoculation, he says, "Unfortunately it is not certain whether the results of these investigations are to be attributed to a specific lack in the foods employed or to the circumstance that the diet of the specially fed hosts differed from that of the animal furnishing the tumour transplanted to them." He therefore conducted a most important investigation in which he studied the effects of dietary restrictions upon spontaneous tumours in mice.

By underfeeding mice upon a gluten ration, similar in composition to that employed by Sweet, for several days prior to operation, the development of recurrences, following incomplete removal of the primary tumour, was in many cases considerably delayed. If, however, the special diet was employed after operation this delay was not brought about. Unoperated spontaneous

tumours seemed unaffected by rigorous dietary restriction and no cures were obtained in any dieted animal.

In the same paper he gives evidence which shows that the reactionary processes of the organism are considerably weakened by the malnutrition which results from the dieting. It therefore appears probable that this fact may furnish the explanation of the lower percentage of successful inoculations in poorly nourished hosts reported by Sweet, and by van Alstyne and Beebe.

Reviewing his work Rous [1915] has remarked, "Special experiments have shown that our results are to be attributed solely to the underfeeding and resultant loss of body weight, and not to the character of the food. The treatment was drastic. The best results were obtained with animals losing weight rapidly at the time of operation." In view of these results he does not feel justified in advocating the employment of underfeeding as a palliative treatment of cancer.

Following a somewhat different line of investigation Funk [1914, 1, 2] has made a study of the influence of what he terms "vitamines" upon the growth of a transplantable sarcoma of the fowl. Unfortunately his results must be regarded as inconclusive, chiefly as there is considerable doubt as to whether, under the conditions employed for the experiments, the chicken is a satisfactory subject for such studies [Drummond, 1916, 2].

Benedict and Rahe [1917] have recently investigated the influence of the "vitamines," or accessory growth factors, upon tumour growth. They employed dietaries similar in composition to those which have been used by Funk and Macallum [1915, 1916, 1, 2], and determined that tumour cells had no power to synthesise the accessory substances when these were absent from the diet of the host, but that at the same time a certain amount of tumour growth occurred under these circumstances, at the expense of the tissues of the animal.

From this brief summary of the more important work which has been carried out upon this subject, it will be realised how little is known regarding the influence of diet upon tumour growth. This is largely due to the disconnected character of the researches, and to the difficulty of co-ordinating the results they have yielded.

Few of these investigations can be said to have a close bearing upon the cancer problem, for the determination of the fact that underfeeding an animal will render it less susceptible to tumour inoculation scarcely applies to any phase of the disease in man. A low state of nutrition, whether induced by

poverty in the diet or by an intercurrent disease, is known to have an unfavourable influence upon tumour implantation.

Theoretically, all such studies as these should be carried out upon animals bearing spontaneous tumours, for they alone are to be regarded as the equivalent of the cancer patient. Rous has carried out a study upon such animals, which has already been referred to, but it is seldom that the opportunity of working with large numbers of these subjects presents itself.

The nearest approach to this ideal condition of cancer research is to study the implanted tumour after it has become established in the body of the host. It must be admitted that even this substitute for the cancerous animal falls regrettably short of what is desired, but it is at present the best available.

The present investigation was designed to study the influences exerted upon tumour growth by the following dietary factors.

(*a*) The plane of protein intake of the host.

(*b*) The character of the protein constituents of the diet of the host.

(*c*) The amino-acid content of the diet.

(*d*) The so-called accessory factors, the *fat-soluble A* and the *water-soluble B*.

Experimental.

Methods.

Rats were chosen as the subjects for this experimental research on account of their great suitability for such work. The great majority of the animals employed were of the albino variety of *Mus norvegicus*, but a few of the black hooded variety of the same animal were also used.

A large number of the experimental animals were those bred from the healthy laboratory stock, whilst others were purchased from reliable breeders.

All stock rats were kept under the closest observation during the period before they were used for experimental purposes. This provided the opportunity of weeding out unsuitable specimens. The general health of the stock was, however, so excellent that the number of rejected animals was very low indeed. As soon as batches of the selected animals had reached a suitable size and weight they were inoculated with the tumour under investigation and returned to the pens.

They were again closely observed during the initial stages of the development of the inoculated graft, until a number of them showed well established and actively growing tumours. These animals were then placed in special boxes and used for dietary experiments. The boxes used for these

experiments were shallow wooden ones with wire-covered tops and sawdust-sprinkled floors. At one end was placed an enclosed sleeping-box of a size proportionate to the number of occupants. All these boxes were cleaned out at least every three days, whilst occasionally the inmates were transferred to new and thoroughly cleansed quarters.

The experimental rats were fed every morning and evening with fresh supplies of the various food preparations. These latter were prepared by thoroughly incorporating the ingredients in a mechanical mixer and adding sufficient water to form the whole into a very stiff paste. This was then made up into small hard balls weighing approximately 5 grams apiece, in which form it was supplied to the animals. By this means it was possible to make a rough estimate of the twenty-four hours' food consumption. Small quantities of the diets were prepared at one time and stored at the temperature of the refrigerator room (1·1°), as the superior value of freshly prepared food mixtures was found to repay the trouble which the more frequent preparations entailed.

All experimental animals were subjected to the closest observation. Body weights were recorded every four days and tumours were charted once a week, whilst at the same time occasional observations on the body temperature were registered.

The tumours utilised for this work were two rat sarcomas which are designated for laboratory purposes, *A* and *S*.

The former was used in some of the earlier studies, but its employment was discontinued owing to certain characteristics it possessed which rendered it less suitable for the purpose than the *S* tumour.

The *A* tumour is a round celled sarcoma. Grafts inoculated into suitable rats usually develop satisfactorily in from 60—80 % of the cases. The tumour grafts generally show a rapid rate of growth, but the resulting tumours are particularly prone to early and extensive necrotic changes. It is frequently found that after a week or two's growth the whole central portion of the tumour is so extensively degenerated that there remains but a thin shell of growing tissue surrounding a mass of necrotic cell debris.

It was owing to this characteristic that the use of this particular tumour was discontinued and the *S* tumour adopted. The latter tumour is a spindle-celled sarcoma showing a high degree of virulence. Inoculated grafts in suitable hosts usually develop satisfactorily in 85—100 % of the cases. It exhibits a slightly slower rate of growth on the average than does the *A* tumour, but it is not nearly so prone to degenerative changes. The *S* tumour

remains firm until a relatively late stage in its development, and when necrotic changes in the centre of the growth do occur they do not extend rapidly.

Neither tumour is accompanied during its growth by microscopic evidence of the formation of metastases when the grafts are subcutaneous.

The inoculation of the rats was carried out by implanting small pieces of tumour tissue, of approximately equal size, subcutaneously in the neighbourhood of the right axilla by the trochar method. The question of the importance of what is termed the effective initial dose [Bashford, Murray, Haaland and Bowen, 1908] was considered before this method of inoculation was decided upon.

Several methods have been elaborated which aim at ensuring a closer approximation to uniform dosage at inoculation, but it is uncertain, having due regard to the more or less uncontrollable influences exerted by those factors which determine the susceptibility of the host, whether these methods are successful in attaining their object. In view of this uncertainty, the method of inoculating small pieces of tissue of an approximately equal size was considered sufficiently accurate for the purposes of this research. No animal which failed to show a satisfactory rate of tumour development during the period of observation following the inoculation was used for feeding experiments. All animals at the conclusion of the experiments were subjected to a post-mortem examination, at which pieces of the tumours and certain organs were removed and fixed in Zenker's fluid, for subsequent microscopical examination.

The mortality amongst the experimental animals from intercurrent disease was exceedingly low, only one or two cases occurring throughout the investigation.

The preparation of the individual components of the various dietaries was carried out by the processes described below.

Caseinogen was prepared from diluted skimmed milk by acidification as described by Osborne and Mendel [1911]. The crude protein was twice reprecipitated from solution in dilute alkali by the same method, and was then extracted by being shaken vigorously in repeated changes of distilled water, the first two changes containing a trace of acetic acid. The resulting product was dehydrated and repeatedly extracted with alcohol and ether at room temperature.

Lactalbumin was prepared by heat-coagulation of the proteins present in the faintly acidified filtrate from the precipitated caseinogen obtained in the last preparation. It was purified by several extractions with boiling

distilled water and subsequent treatment with changes of boiling alcohol and ether.

Edestin, zein and gliadin were prepared from hempseed, crushed maize, and wheat respectively by the processes described by Osborne [1910]. They were in each case subjected to careful purification, as directed by this author, to ensure preparations of a high degree of purity.

Ovalbumin was prepared from egg-white according to the process of Hopkins and Pinkus [1900]. It was a crystalline product of considerable purity.

Gelatin was that supplied by Coignet Père et Fils et Cie and bore the mark "Gold label, extra." It gave at the most only a faint coloration with Millon's reagent.

Starch was purchased as pure wheaten starch. It was submitted to two extractions with boiling alcohol before being included in the dietaries.

It is not out of place to discuss at this juncture the process of alcohol extraction which is somewhat commonly used to render foodstuffs free from traces of the accessory factors. Stepp [1909, 1912] found that alcohol extraction removed indispensable substances from natural foodstuffs, and it has been used extensively for this purpose by Funk [1914, 1] and others. From recent work, however, it appears that in the absence of water absolute alcohol will not dissolve the water-soluble accessory substance [Osborne and Mendel, 1917; Drummond, 1917]. It is therefore possible that alcohol extraction does not remove traces of this substance when the foodstuff is extracted in the dry state.

This point was, however, only determined after the present investigation was completed.

Agar was included in the majority of rations to provide an indigestible intestinal ballast or "roughage." It was purchased in the powder form and was subjected to the somewhat empirical "purification" by hot alcohol extraction.

Sucrose was not subjected to any further purification.

Lactose was added to some dietaries, not only to serve as a source of carbohydrate, but also as a means of adding a certain amount of the water-soluble growth accessory factor, which it usually contains [McCollum and Davis, 1915, 1; Drummond, 1916, 1]. In some instances the lactose used was a crude preparation, very rich in this unidentified dietary factor, which was very kindly supplied by the courtesy of "Casein, Ltd." It was a light sandy coloured crystalline powder representing the first crystalline fractions obtained in the commercial preparation of lactose.

The nitrogen content of specimens of lactose is to a large extent a measure of their impurity. A large number of samples of this sugar were analysed for nitrogen. Preparations sold as pure lactose were found to contain from 0 to 0·02 %. The nitrogen-free specimens were usually preparations consisting of large crystals, and were found to be uncontaminated by the presence of the water-soluble growth factor.

The crude lactose referred to above was found to contain 0·19 % nitrogen.

"*Protein-free milk*" was used as a source of lactose, inorganic salts and the water-soluble growth promoting factor in some rations. It was prepared as described by Osborne and Mendel [1911], and was a sandy coloured crystalline powder having a nitrogen content varying between 0·57—0·67 %.

The use of "protein-free milk" in artificial rations has not escaped criticism upon the grounds of its indefinite composition [Funk, 1914, 1; Funk and Macallum, 1914; McCollum and Davis, 1915, 4]. Whilst there is undoubtedly some justification for these criticisms, it must be recognised that the use of this product has greatly assisted the elucidation of many important points concerning the nutritive value of the proteins [Osborne and Mendel, 1916, 1, 2].

Lard was used as a source of fat when it was desired that the diet should be free from the fat-soluble *A* accessory substance, since it has been repeatedly shown that lard does not contain this substance.

Butter fat was used as a source both of fat and of the fat-soluble accessory factor. It was prepared by centrifugalisation of butter at a temperature just above its melting point, and separation of the clear fatty layer. Determinations of nitrogen by the Kjeldahl process indicated that the butter fat was nitrogen-free. There is, however, some uncertainty whether this fat does not usually carry traces of nitrogen and phosphorus [Funk and Macallum, 1914; McCollum and Davis, 1914; Osborne and Wakeman, 1915].

"*Yeast Preparation*" was used in a number of dietaries as a means of ensuring an adequate supply of the water-soluble growth accessory. The preparation was made by evaporating down to a thick syrup the aqueous dialysate of a commercial yeast extract (marmite), at low temperature and reduced pressure.

The resulting pale brown syrup is very rich in the water-soluble accessory substance. It also contains relatively large amounts of adenine, leucine, sodium chloride and potassium phosphate [Drummond, 1917].

Amino-acids were prepared by the usual methods from various protein hydrolysates. Tryptophan was obtained from the tryptic digest of caseinogen.

Histidine from hydrolysed ox-blood, lysine and arginine from hydrolysed gelatin, tyrosine from silk waste, and cystine from hair. In the case of the last two preparations the amino-acids tyrosine and cystine were freed from traces of each other by the phosphotungstic acid process described by Plimmer [1913].

Hydrolysed Meat-Protein. Fresh lean beef was minced and extracted three times with boiling water, which was slightly acidified with acetic acid at boiling point to complete the coagulation of proteins. The residue of tissue protein was well pressed out in a meat press and both dehydrated and extracted by treatment in a continuous extraction apparatus firstly with alcohol and subsequently with ether.

The resulting product, after being ground and passed through a fine mesh sieve, was submitted to hydrolysis with boiling 15 % sulphuric acid for 48 hours. From the resulting deep brown coloured fluid sulphuric acid was removed quantitatively by the careful addition of boiling saturated barium hydroxide solution. The bulky precipitate of barium sulphate, contaminated with much pigmented matter, was extracted by boiling with several changes of distilled water. The combined washings were united with the main filtrate and the whole was evaporated down until a thick syrup was formed. Extracted wheaten starch was then added until a stiff paste was obtained, which was spread out in a thin layer over heated glass plates and dried until it could be powdered and passed through a fine meshed sieve.

This powder possessed a pale brown colour and contained approximately 7 % of nitrogen. To render this product nutritively the equivalent of the original protein it was necessary to replace the destroyed tryptophan. This was done by adding 0·7 % of the pure amino-acid prior to the incorporation with starch.

Hydrolysed Meat-Protein, Monamino-acid fraction. Beef protein was submitted to acid hydrolysis as described in the preceding preparation. To the neutral filtrate after removal of the sulphuric acid was added sufficient of a 20 % solution of sulphuric acid to render the whole about 5 % in strength. A 30 % solution of phosphotungstic acid was then added until no further precipitation occurred. The heavy precipitate was allowed to stand for 24 hours at 2°, being then filtered off and well washed with an ice-cold 5 % solution of sulphuric acid. The filtrate and washings were quantitatively freed from the excess of sulphuric acid and the precipitant by the careful addition of baryta, evaporated down and incorporated with starch as described in the previous preparation.

The final product was a pale sandy coloured powder containing approximately 5 % of nitrogen.

"*Erepton.*" I was fortunate in being able to secure several hundred grams of a commercial preparation of hydrolysed meat-protein, placed on the market by Meister, Lucius and Brüning.

The product was labelled "Erepton. Vollständig abgebautes Fleischeiweiss, nach Prof. Dr Abderhalden." It consisted of a brown, granular, slightly hygroscopic mass and contained from 12·5—12·8 % of nitrogen. As far as could be ascertained the degradation of the protein had been completed. I have to thank Mr E. Scholl, of John George Haller and Co., for his great kindness in placing this preparation at my disposal.

Salt mixture. Inorganic salts were supplied in some of the rations in the form of a salt mixture possessing a composition identical with one of those used by McCollum in his extensive researches. It was made up as follows:

NaCl	1·73 g.	$CaH_4(PO_4)_2$, $2H_2O$	5·40 g.
$MgSO_4$ (anhydrous)	2·66 g.	Calcium lactate	13·00 g.
NaH_2PO_4, H_2O	3·47 g.	Ferric lactate	1·18 g.
K_2HPO_4	9·54 g.		

Traces of iodine were supplied in the drinking water once a week, as recommended by McCollum.

Alcoholic Extract of Dried Milk. This preparation has been extensively used by Hopkins and his co-workers [Ackroyd and Hopkins, 1916] as a means of adding to the dietary a sufficient amount of accessory substances.

The use of this preparation during this work has not been found as satisfactory for this purpose as the employment of the combination of butter-fat and yeast preparation. This is probably explainable by the fact that the water-soluble substance is insoluble in absolute alcohol, and unless the milk powder contains sufficient moisture, the extraction of this substance is liable to be incomplete. The results of the series of experiments carried out during this investigation will now be considered in detail.

The influence of the level of protein intake of the host upon normal and tumour growth.

Much important work has been carried out recently upon the influence which the protein content of the diet may exert upon the nutrition of the animal. The papers by Osborne and Mendel [1912, 2; 1915, 1, 2; 1916, 1], and Janney [1915] contain results of importance dealing with this subject.

From these results it is now known that a reduction in the level at which protein is furnished in the diet of a young animal will sooner or later induce a more or less complete inhibition of the growth processes of that animal. This will occur either when the nitrogen intake falls below the level required

	Days 14	21	28	35	42	Remarks	Weight of tumour g.	Weight of host - tumour g	Change in weight of host g	Change in weight of host per 1 g tumour
♂						Tumour solid and rapidly growing	25	126	+30	
♂						Slight central necrosis of tumour	27	140	+37	
♂						—	15	132	+39	
♂						Tumour had ulcerated and undergone central necrosis	19	120	+24	
♀						—	22	130	+36	
♀						Tumour retrogressed	–	152	+44	
						Average	21·6	–	+35	+1·62

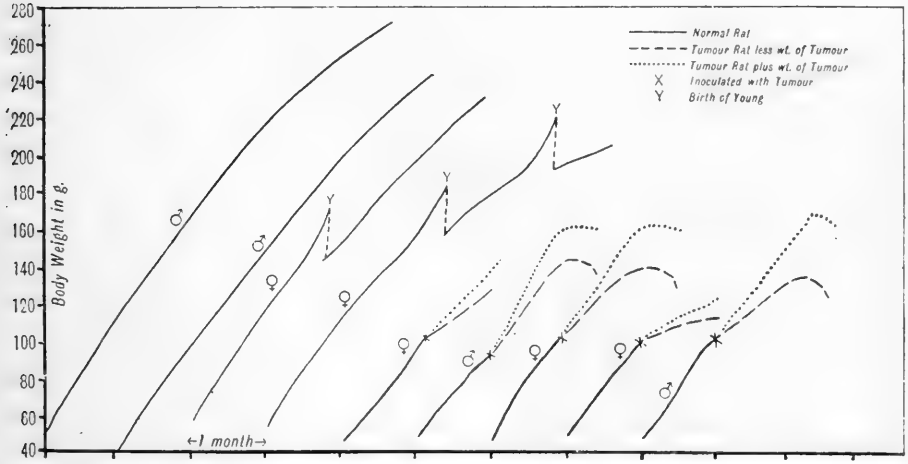


Chart 1. Tumour development, and growth curves of normal and tumour-bearing rats upon a normal diet of bread, oats, corn, greenstuffs and meat. Tumour *S*/113*a*.

to supply the demands of the organism, or when the lowered protein intake brings about a deficiency in the supply of one or more amino-acids which are indispensable to the animal. Examples of how this may occur have been given in the introduction to this paper. A large number of proteins, par-

ticularly those of vegetable origin, possess amino-acid contents which limit their nutritive value to no inconsiderable extent [Mendel, 1915].

Chart 1 serves as a control to a number of the experimental results of this work, for it illustrates the growth of young rats, the growth of tumour-bearing rats and the development of the tumours (*S*) they carry, upon a normal mixed diet of bread, seeds and greenstuffs, with occasional additions of meat or milk. The normal growth of the *A* sarcoma is given in Chart 2.

Rat	Days 12	19	26	33	40	Remarks	Weight of tumour g	Weight of rat–tumour g	Change in weight of rat g	Change in weight of host per 1 g tumour
♀						Tumour ulcerated and very necrotic.	37·0	41·0	−9·0	
♀						ditto	18·0	62·0	+1·0	
♀						ditto	17·0	73·0	−6·0	
♂						ditto	15·0	87·0	+7·0	
						Average	21·7	—	−1·8	−0·08

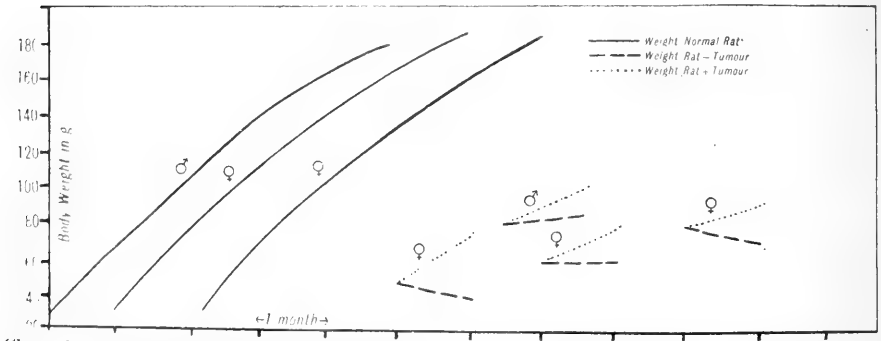


Chart 2. Tumour development, and growth curves of normal and tumour bearing rats upon complete artificial diet. Tumour *A*/30*d*. Diet: Caseinogen, 18 %, butter-fat, 20 %, "Protein-free milk," 30 %, Agar, 5 %, Starch, 27 %.

Chart 3 indicates the results which were obtained by feeding a "complete" artificial dietary composed as given below:

Caseinogen	18 %	Salt mixture	5 %
Starch	48 %	"Yeast preparation"	6 %
Agar	3 %	Butter-fat	20 %

It will be seen from Chart 3 that this diet is adequate to supply the nutritive requirements of the rat throughout its life cycle.

Tumour development proceeds at an equally normal rate in hosts fed upon this dietary, and is not accompanied by a loss in weight of the animal itself, until secondary disturbances, such as an ulceration of the tumour with resulting sepsis, set in.

Rat	Days 12	19	26	33	40	Remarks	Weight of tumour g.	Weight of rat − tumour g	Change in weight of rat g	Change in weight of rat per 1 g tumour
♀						Tumour firm and solid	10·7	114·3	+17·3	
♂						Slight central necrosis	25·5	140·5	+40·5	
♀						Animal died 17.4.17 Tumour firm	6·7·	141·3	+55·3	
♀						Central necrosis	24·0	146·0	+55·0	
♀						—	16·0	127·0	+42·0	
♂						Tumour retrogressed	—	—	—	
						Average	16·6	—	+42·0	+2·5

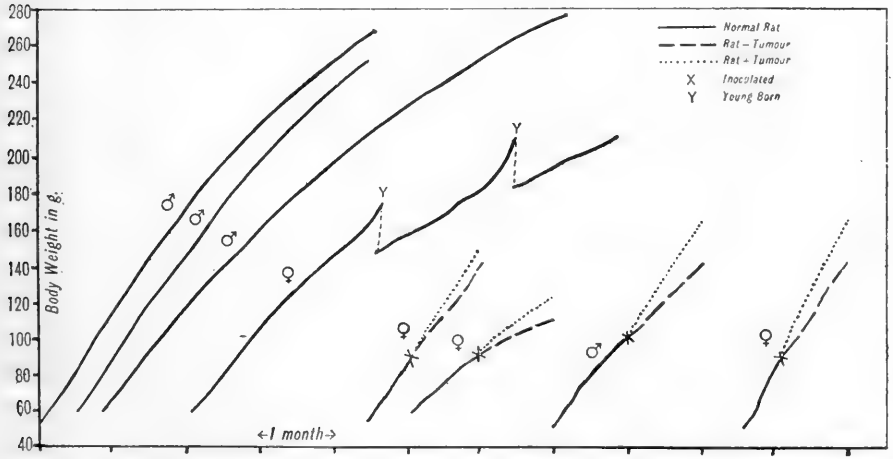


Chart 3. Tumour development, and growth curves of normal and tumour-bearing rats upon a complete artificial dietary. Tumour *S*/120*d*. Diet: Caseinogen, 18 %, Starch, 40 %. Agar, 3%, Salt Mixture, 5 %, "Yeast preparation" 6 %, butter-fat, 20 %.

As has already been stated, Osborne and Mendel [1915, 1] found that when the caseinogen content of such a diet as the above was lowered, a stage

was eventually reached where the low cystine content of the caseinogen became a factor limiting the growth processes of the animal. Accordingly the effect of a similar reduction in the protein content upon the growth of tumours was investigated. Two diets were compounded containing respectively 10 and 6 % of caseinogen, but in other respects possessing a composition similar to the diet described above. The results obtained in feeding these rations to the normal young rats support the work of Osborne and Mendel, although growth inhibition appears to have become marked at an earlier stage in the reduction of the protein level (Charts 4 and 5).

Rat	Days 12	19	26	33	40	Remarks	Weight of tumour g.	Weight of rat – tumour g.	Change in weight of rat g.	Change in weight of rat per 1 g. tumour
♂						Much central necrosis of tumour	27·0	53·0	−23·0	
♀					–	Animal died 7. 1. 17. Tumour very necrotic and ulcerated.	14·0	63·0	−15·0	
♀						Firm tumour	8·5	60·5	−11·5	
♂						ditto	5·5	112·5	−17·5	
♀					–	Died 29. 12. 16. Tumour firm.	6·0	65·0	−15·0	
♂					–	Tumour retrogressed	–	–	–	
						Average	12·2	–	−16·4	−1·35

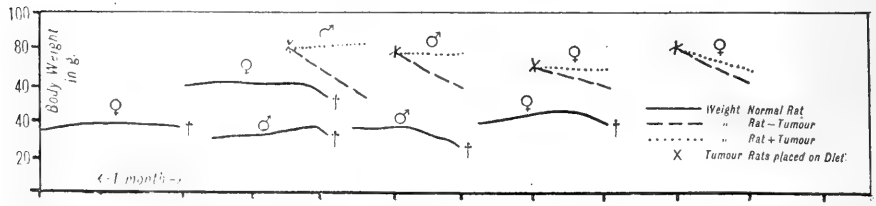


Chart 4. Tumour development, and growth curves of normal and tumour-bearing rats upon low protein diet. Tumour *S*/117*c*. Diet: Caseinogen, 6 %, Starch, 48 %, Agar, 3 %, Salt mixture, 5 %, "Yeast preparation" 6 %, Butter-fat, 20 %, Sucrose, 12 %.

The results yielded by the tumour-bearing animals may be conveniently summarised in tabular form.

TABLE 1.

% of caseinogen in diet	Average weight of tumours, g.	Average change in weight of host, g.	Average change in weight of host per 1 g. tumour
18	16·6	+42·0	+2·50
10	16·0	−19·4	−1·21
6	12·2	−16·4	−1·35

No evidence of tumour retardation is apparent in the case of the animals fed upon the 10 % caseinogen diet, but tumour growth is in these cases accompanied by a somewhat serious drop in the body-weight of the host. Where the diet contained 6 % of the protein there is a small but appreciable decrease in the size of tumour growth, also accompanied by a drop in the body-weight of the host. It will be noticed that the curves representing the weight of the

Rat	Days 12	19	26	33	40	Remarks	Weight of tumour g.	Weight of rat − tumour g.	Change in weight of rat g.	Change in weight of rat per 1 g. tumour
♀						Slight central necrosis	290	460	−260	
♀						Tumour firm	250	530	−170	
♀						ditto	140	860	−110	
♀					–	Animal died 29. 12. 16. Tumour firm	85	515	−245	
♀							35	965	−185	
♂						Tumour retrogressed	–	–	–	
						Average	160	–	−194	−1·21

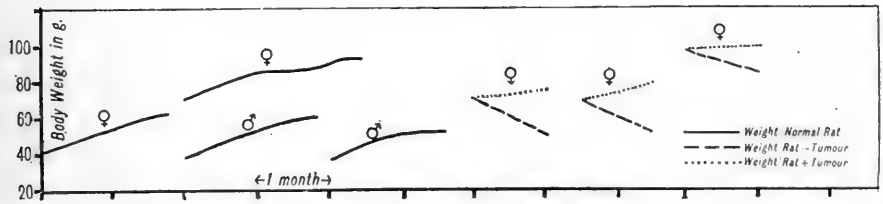


Chart 5. Tumour development, and growth curves of normal and tumour-bearing rats upon low protein diet. Tumour *S*/117c. Diet as in Chart 4, but containing 10 % of Caseinogen.

host plus tumour run roughly parallel to those which indicate the growth of the normal animal upon the same diet, whilst the weight of the host alone shows a gradual fall. This makes it probable not only that the food requirements of the tumour are satisfied before those of the host, but also, that if necessary the tissues of the host are drawn upon to supply deficiencies in the former.

Chart 6 illustrates the results of feeding a diet containing a low level of

protein to normal and tumour-bearing animals. The diet was composed as given below:

Dried whole-milk powder	15 %	Salt mixture	3 %
Starch	49 %	Butter-fat	10 %
Agar	3 %	Sucrose	20 %

Rat	Days 12	19	26	33	40	Remarks	Weight of tumour g.	Weight of rat–tumour g.	Change in weight of rat g.	Change in weight of rat per 1 g tumour
♂						Tumour ulcerated and very necrotic,	25·0	55·0	−24·0	
♂						Tumour very necrotic	20·0	82·0	−29·0	
♀						Ulcerated and necrotic	15·0	35·0	−29·0	
♂						ditto	16·0	74·0	−41·0	
♂						Tumour much degenerated	11·0	69·0	−22·0	
♀							3·0	59·0	−29·0	
						Average	15·0	–	−29·0	−1·94

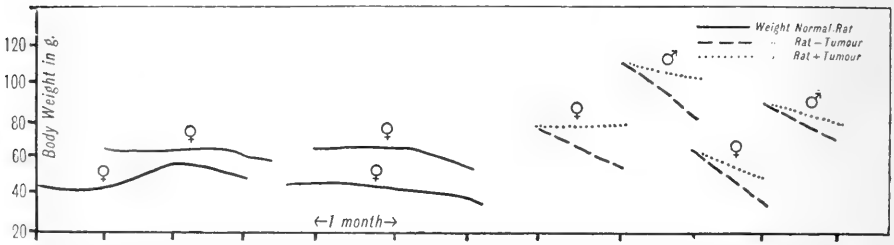


Chart 6. Tumour development, and growth curves of normal and tumour-bearing rats upon low protein level diet. Tumour *A*/24*a*. Diet: Dried milk, 15 %, Starch, 49 %, Agar, 3 %, Salt mixture, 4 %, Butter-fat, 10 %, Sucrose, 20 %.

Calculating from the known composition of the dried milk powder, this diet contained approximately 5 % of milk protein. Normal young rats fed upon this diet remain in good health, but show no change in body-weight for several months. The nitrogen balance and body temperature remain normal during this period of growth inhibition. Tumour growth in rats fed upon this same diet is however a little retarded, as is shown by the average weight of the tumours (15 g.) compared with the average weight (21 g.) grown on the normal dietary. A severe loss of body-weight upon the part of the host on the low protein diet may be observed in these cases also.

The influence of the character of the protein present in the diet upon normal and tumour growth.

Any consideration of the results of the preceding section of this paper cannot be dissociated from a study of the results given in this one, since they are so closely interrelated.

Rat	Days 12	19	26	33	40	Remarks	Weight of tumour g.	Weight of rat – tumour g.	Change in weight of rat g.	Change in weight of rat per 1 g. tumour
♀						Tumour very necrotic and ulcerated.	31·0	59·0	−1·0	
♀						Tumour necrotic	12·0	68·0	+14·0	
♀						ditto	9·0	83·0	+13·0	
♀						ditto	10·0	90·0	−9·0	
						Average	15·5	—	+4·2	+0·27

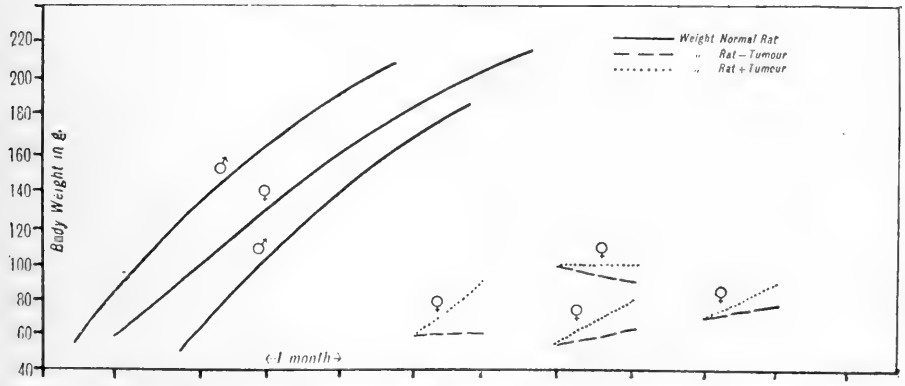


Chart 7. Tumour development, and growth curves of normal and tumour-bearing rats upon Edestin diet. Tumour *A*/30*d*. Diet as in Chart 2, but Edestin replacing Caseinogen.

The relative nutritive values of the proteins have already received attention in the introductory section, so that the results which are illustrated in Charts 2 and 7—11 may be considered forthwith.

The diets used in this series of experiments were similar in composition to those employed by Osborne and Mendel [1911].

Purified protein	18 %	Agar	5 %
Butter-fat	20 %	Starch	27 %
"Protein-free milk"	30 %		

Rat	Days 12	19	26	33	40	Remarks	Weight of tumour g	Weight of rat – tumour g	Change in weight of rat g	Change in weight of rat per 1 g. tumour
♂						Tumour very necrotic and ulcerated.	29·0	83·0	−9·0	
♀						ditto	19·0	77·0	+15·0	
♀					–	ditto	9·0	49·0	−11·0	
♀						Tumour necrotic	4·0	86·0	+26·0	
						Average	15·2	–	+5·2	+0·34

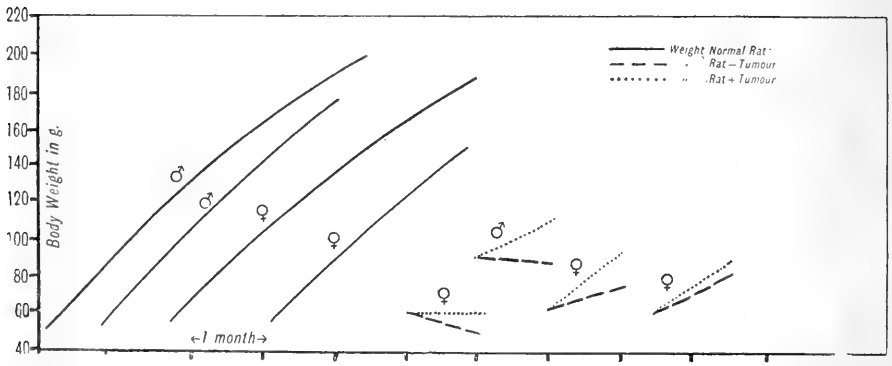


Chart 8. Tumour development, and growth curves of normal and tumour-bearing rats upon Lactalbumin diet. Tumour *A*/30*d*. Diet contains 18 % Lactalbumin.

The following proteins were investigated: caseinogen, lactalbumin, ovovitellin, edestin, gliadin, and zein.

The influence of these diets upon tumour growth may be summarised in the following table:

TABLE 2.

Protein in diet	Growth of normal animal on diet	Average weight of tumour, g.	Average change in weight of host, g.	Average change in weight of host per 1 g. tumour in four weeks
Caseinogen	normal	21·7	− 1·8	−0·08
Edestin	normal	15·5	+ 4·2	+0·27
Lactalbumin	normal	15·2	+ 5·2	+0·34
Ovovitellin	normal	17·0	+ 5·7	+0·34
Gliadin	cessation of growth and maintenance at constant weight	21·1	− 9·9	−0·47
Zein	rapid decline in body-weight ...	17·5	−21·2	−1·20

Rat	Days 12 19 26 33 40	Remarks	Weight of tumour g.	Weight of rat − tumour g.	Change in weight of host g.	Change in weight of rat per 1 g tumour
♂		Tumour ulcerated and necrotic.	20·0	720	+11·0	
♀		Tumour extensively necrotic	22·0	780	+9·0	
♀		Tumour very necrotic	16·0	850	−5·0	
♂		Slight central necrosis	10·0	900	+8·0	
		Average	17·0	—	+5·7	+0·34

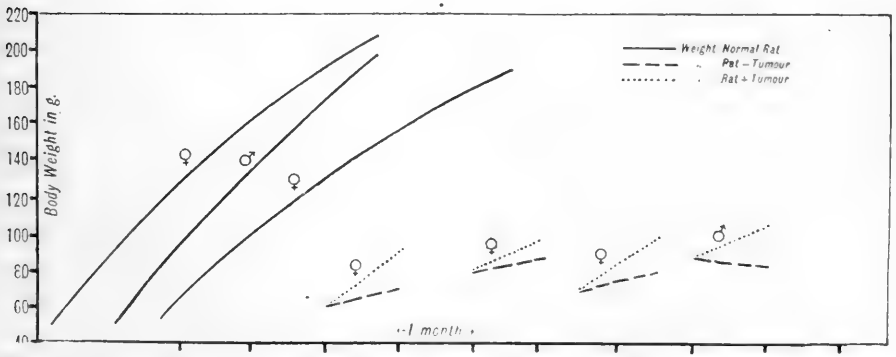


Chart 9. Tumour development, and growth curves of normal and tumour-bearing rats upon Ovo-vitellin diet. Tumour *A*/30*d*. Diet contains 18 % Ovo-vitellin.

For comparative purposes these results are valuable in that they indicate that tumour growth may be uninfluenced by dietary restrictions which

adversely affect the growth of the host. Individually, however, the results are unsatisfactory because of the irregularity in the changes in the body-weight of the rats on the first four dietaries. Thus there is no apparent explanation of the decrease of body-weight shown by the tumour-bearing rats fed upon the caseinogen diet. The only suggestion which can be advanced to explain this is that the *A* tumour was used throughout this experiment. As has already been mentioned this tumour tends to show an early

Rat	Days	Remarks	Weight of tumour g	Weight of rat – tumour g	Change in weight of rat g	Change in weight of host per 1 g tumour
♀		Tumour extensively necrotic.	42·0	48 0	−10 0	
♀		Tumour very necrotic and ulcerated.	28 0	32 0	−19 0	
♀		Tumour firm, slight central necrosis	10 0	60 0	−12 0	
♀		Small fibrous growth	4·5	61 5	+1 5	
♀		Tumour retrogressed	–	–	–	
		Average	21 1	–	−9 9	−0·47

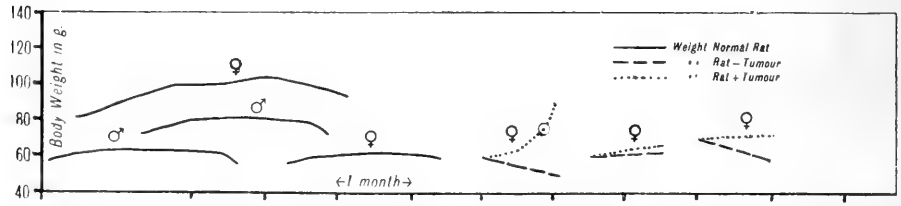
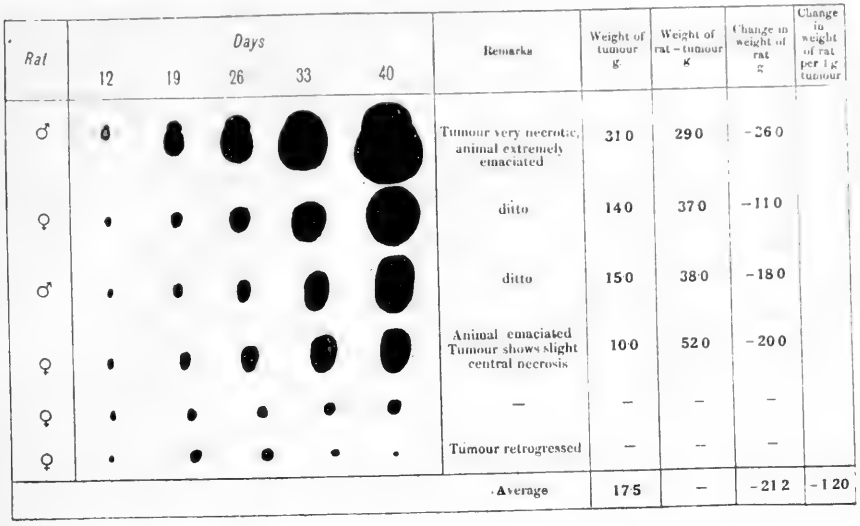


Chart 10. Tumour development and growth curves of normal and tumour-bearing rats upon Gliadin diet. Tumour *A*/30*d*. Diet contains 18 % Gliadin.

and extensive central necrosis, so that the possible deleterious influence upon the nutrition of the host caused by the absorption of the products arising from the degenerating tissue must be considered. Whatever may be the explanation of this point, it does not modify the importance of the observation that tumour growth may be practically normal in hosts whose growth is inhibited by the nature of their diet. The results obtained upon the gliadin

ration are of interest in view of the large amount of work which has been done upon the nutritive value of that protein.

Osborne and Mendel [1914, 1; 1916, 2] found that gliadin was adequate to supply the amino-acid requirements for maintenance in the rat, but that the addition of some 2 % of the missing unit, lysine, was necessary before the requirements for growth were satisfied.



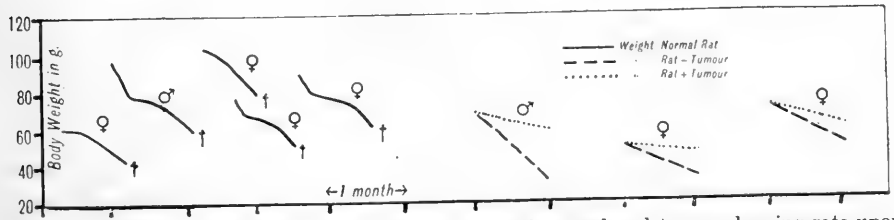


Chart 11. Tumour development, and growth curves of normal and tumour-bearing rats upon Zein diet. Tumour *A*/30*d*. Diet contains 18 % Zein.

It was the earlier results of Osborne and Mendel on this subject [1912, 1] that stimulated the investigations of Sweet, Corson-White and Saxon [1913], to which reference has already been made. The view which regards lysine as of great importance in growth receives support from the work of Buckner, Nollau and Kastle [1916] who worked upon the growth of chickens, and also from similar studies of Osborne and Mendel [1916, 4]. Other workers however do not attach as great an importance to the rôle of this amino-acid in nutrition. Thus McCollum, Simmonds and Pitz [1917] have shown that the

addition of gelatin, which possesses a high lysine content, does not improve the nutritive value of the lysine-poor proteins of the maize kernel, whilst Geiling [1917] does not regard lysine as necessary for the maintenance of the adult mouse. The true value of lysine in the nutrition of the organism is therefore at present uncertain.

Equal interest is to be attached to the results illustrated in Chart 11, where the influence of the well-known amino-acid deficiencies of the protein zein upon normal and tumour growth are indicated. The low nutritive value of this protein was shown by Willcocks and Hopkins [1906] to be largely due to the absence of tryptophan from its molecule. Their work has been repeatedly confirmed, and the serious nature of a tryptophan deficiency is now universally admitted. As is to be seen from Chart 11, young rats rapidly decline in body-weight and die upon a zein ration such as was used in this experiment.

It was, therefore, somewhat unexpected when no inconsiderable amount of tumour development was observed in rats fed upon the same ration. In the light of the results of some later experiments with zein dietaries, attention must be given to one of the criticisms which have been employed in deprecating the use of "protein-free milk," namely that the traces of milk protein which that product contains may possibly supply traces of the missing amino-acids, which may be of welcome assistance to the animal in its endeavour to overcome the serious deficiencies of its diet. Whether this will to some extent explain the result of this feeding experiment is uncertain, but it is apparent from a consideration of the experiment about to be described that a tryptophan deficiency, in the absence of any uncertain factor such as "protein-free milk," may cause retardation of tumour growth. Chart 12 illustrates the results which were obtained with a ration composed as given below:

Zein	18 %	Butter-fat	10 %
Lactose	30 %	Salt mixture	5 %
Starch	40 %		

Alcohol extract of 20 g. milk powder.

The summarised results of the experiments in this series are given in Table 3.

TABLE 3.

Diet	Average weight of tumour, g.	Average change in weight of host, g.	Average change in weight of host per 1 g. tumour in four weeks
18 % caseinogen	12·0	+19·9	+1·66
18 % zein	4·1	−21·1	−5·15
18 % zein + 0·5 % tryptophan ...	9·2	− 9·8	−1·06

As will be seen from the third series in this table, the influence of the addition of the missing amino-acid was investigated (Chart 13). In both the normal and tumour-bearing rats this addition markedly improved the nutritive value of the ration. Upon the zein diet there was a distinct retardation, but not a total inhibition, of tumour growth which was accompanied by a very rapid fall in body-weight of the host.

Rat	Days 12	19	26	33	40	Remarks	Weight of tumour g	Weight of rat – tumour g	Change in weight of rat g.	Change in weight of host per 1 g tumour
♀						Tumour fibrous, but well vascularised	6·0	74·0	−13·0	
♂					–	Animal very thin.	5·0	53·0	−27·0	
♀						Tumour slow growing. Animal very thin.	2·5	58·5	−24·5	
♀					–	–	3·0	45·0	−20·0	
♂					–	Tumour retrogressed	–	–	–	
♂			–	–	–	ditto	–	–	–	
						Average	4·1	–	−21·1	−5·15

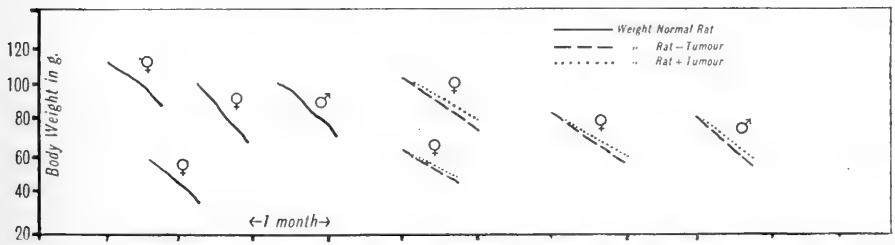


Chart 12. Tumour development, and growth curves of normal and tumour-bearing rats upon Zein diet. Tumour *S*/116*b*. Diet: Zein, 18 %, Crude lactose, 30 %, Starch, 40 %, Butter-fat, 10 %, Salt mixture, 5 % + Alcoholic extract 20 g. dried milk.

The addition of tryptophan to the extent of 0·5 % of the diet both assisted the growth of the tumour and lessened the sacrifice of the tissues of the host. It should be remarked that the consumption of the dietaries was satisfactory, except that of the zein ration during the fourth week, which declined considerably.

The low nutritive value possessed by gelatin has been recognised for many years. It has been shown that gelatin cannot serve to build up new tissue: whilst Kauffmann [1905] demonstrated that when the amino-acids

tyrosine, cystine and tryptophan are added to gelatin, and the deficiencies of the latter thereby to a large extent made good, nitrogen equilibrium can be established.

His work has received confirmation from that of Rona and Müller [1906] and Abderhalden [1912]. More recently the subject has been carefully investigated by Totani [1916], who found that the addition of tryptophan to gelatin greatly raised its nutritive value. The effect of adding the missing tyrosine was hardly appreciable; so that the author was led to suggest that the animal organism may have the power of synthesising the benzene nucleus.

Rat	Days 12	19	26	33	40	Remarks	Weight of tumour g.	Weight of rat + tumour g.	Change in weight of rat g.	Change in weight of rat per 1 g. tumour
♂	•	•	●	●	●	Tumour slightly necrotic. Animal thin.	14·0	78·0	−9·0	
♀	•	•	●	●	●	ditto	14·0	69·0	−12·0	
♀	•	•	•	•	•	ditto	7·0	92·0	−8·0	
♂	•	•	•	•	•	Tumour firm and fibrous. Animal thin.	2·0	90·0	−10·0	
♀	•	•	•	—	—	Tumour retrogressed	—	—	—	
						Average	9·2	—	−9·8	−1·06

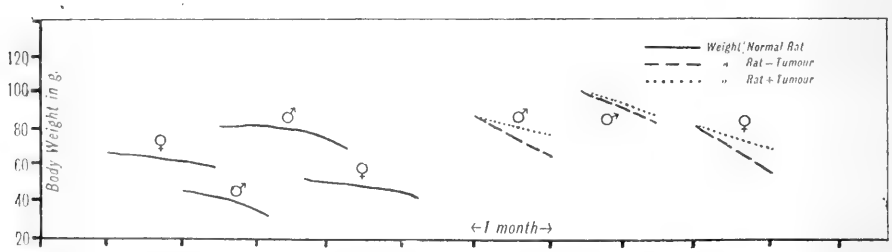


Chart 13. Tumour development, and growth curves of normal and tumour-bearing rats upon Zein diet (Chart 12) + 0·5 % tryptophan. Tumour *S*/116*b*.

The investigation of the influence of the amino-acid deficiencies of gelatin upon tumour growth, which are described in this paper, was carried out before the appearance of Totani's communication, so that his observation upon the more complete assimilation of gelatin administered in the hydrolysed

form was not applied. The gelatin was included in the diets in these experiments in the powder form.

The average results obtained in the four batches of experimental animals are tabulated in Table 4, which has been compiled from the data contained in Charts 14, 15 and 16.

Rat	Days 12	19	26	33	Remarks	Weight of tumour g	Weight of rat – tumour g	Change in weight of rat g.	Change in weight of rat per 1 g. tumour
♂					Rat very emaciated. Tumour firm, well vascularised and actively growing.	12·0	60·0	−40·0	
♂					ditto	10·5	74·5	−29·5	
♂					ditto	5·0	82·0	−28·0	
♀					ditto	4·0	73·0	−27·0	
♀					Tumour fibrous and slow growing. Rat very thin	2·5	65·5	−22·5	
					Average	6·8	—	−29·4	−4·3

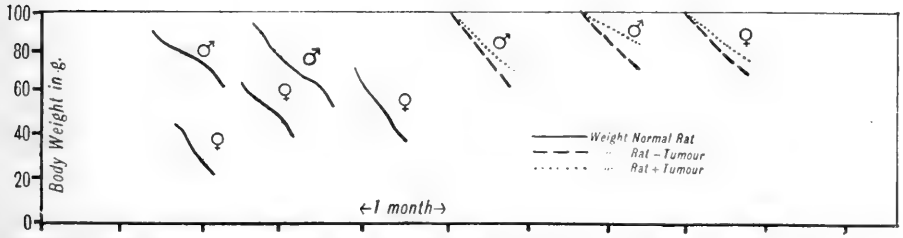


Chart 14. Tumour development, and growth curves of normal and tumour-bearing rats upon Gelatin diet. Tumour *S*/118*a*. Diet: Gelatin, 18 %, Starch, 52 %, Lard, 20 %, Agar, 5 %, Salt mixture, 5 % + Alcoholic extract 20 g. dried milk.

TABLE 4.

Diet	Average weight of tumour, g.	Average change in weight of host, g.	Average change in weight of host per 1 g. of tumour
18 % caseinogen	14·2	+19·0	+1·34
18 % gelatin	6·8	−29·4	−4·30
18 % gelatin + 0·5 % tryptophan	12·7	−19·3	−1·52
18 % gelatin + 0·5 % tryptophan + 1 % tyrosine	13·2	−16·5	−1·25

The influence of these dietaries upon the growth of the normal rats supports the work of Totani, in that the addition of tryptophan greatly raises the nutritive value of the gelatin diet, whereas the further addition of tyrosine has little if any such effect.

The retarding influence upon tumour growth which is exerted by the amino-acid deficiencies of the gelatin ration is well marked. As in other similar cases this is accompanied by a severe drop in the body-weight of the host.

Rat	Days 12	19	26	33	Remarks	Weight of tumour g.	Weight of rat – tumour g.	Change in weight of rat g.	Change in weight of host per 1 g. tumour
♀					Rat thin * Tumour shows slight central necrosis	18·5	71·5	−20·5	
♂					ditto	15·0	85·0	−28·0	
♂					ditto	14·0	94·0	−16·0	
♂					ditto	11·0	65·0	−25·0	
♀						5·0	83·0	−7·0	
					Average	12·7	—	−19·3	−1·52

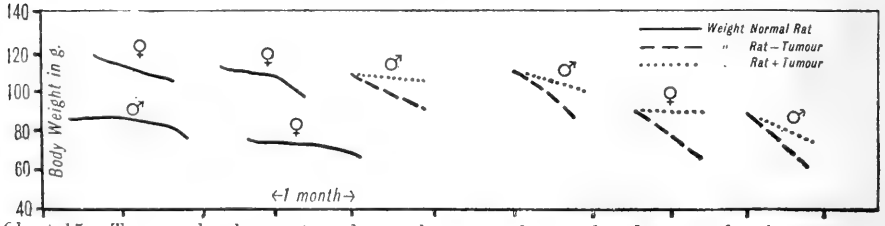


Chart 15. Tumour development, and growth curves of normal and tumour-bearing rats upon Gelatin diet, described in Chart 14, + 0·5 % tryptophan. Tumour *S*/118*a*.

When tryptophan has been added to the diet, tumour growth becomes normal, but there is a slight loss of weight upon the part of the host. The further addition of the missing tyrosine cannot be said appreciably to influence either tumour growth, or the sacrifice of the tissues of the animal.

The gelatin dietary to which the missing tryptophan and tyrosine have been added possesses a much lower nutritive value than the "normal"

caseinogen ration. This is probably to a large extent due to the defective assimilation of the solid gelatin, which has been observed by Totani. This imperfect utilisation does not apparently adversely affect tumour growth when tryptophan is added, but is reflected in the lowered nutrition of the host.

Rat	Days 12	19	26	33	Remarks	Weight of tumour g.	Weight of rat − tumour g.	Change in weight of rat g.	Change in weight of rat per 1 g tumour
♂	•	●	●	●	Tumour shows slight central necrosis	17·0	79·0	−22·0	
♂	•	●	●	●	ditto	16·0	83·0	−17·0	
♀	•	●	●	●	ditto	11·0	75·0	−12·0	
♀	•	●	●	–	Animal died, and partially devoured 20.1.17	9·0	75·0	−15·0	
♀	•	•	•	•	Tumour fibrous, with small blood supply	–	–	–	
					Average	13·2	–	−16·5	−1·25

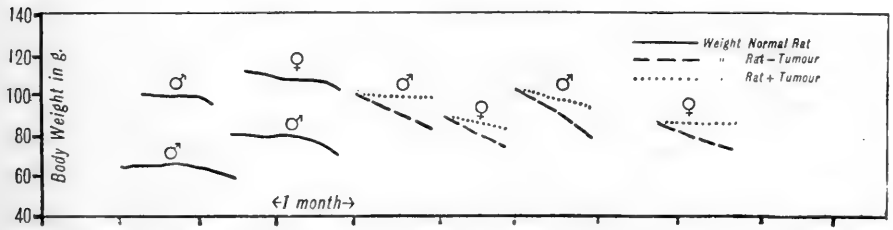


Chart 16. Tumour development, and growth curves of normal and tumour-bearing rats upon Gelatin diet + 0·5 % tryptophan + 2·0 % tyrosine. Tumour *S*/118*a*.

The effect upon normal and tumour growth of replacing the protein of a diet by amino-acid mixtures.

It is mainly due to the extensive researches of Abderhalden that it is now proved that an animal can be maintained, and even show an increase of body-weight, when it is fed upon a diet in which protein has been entirely replaced by an amino-acid mixture prepared from a completely hydrolysed protein. Of outstanding importance in this respect are the classical experi-

ments made by him, in which he accomplished the successful nutrition of dogs over long periods of time upon such diets [1913, 2; 1915].

He also found the same amino-acid preparation adequate to supply the nitrogen requirements of rats.

Abderhalden has extended his researches so as to ascertain the effect which the removal of certain amino-acids from his protein digest has upon the nutrition of the animal [1915].

By removing tryptophan and tyrosine the nutritive value of the hydrolysate was greatly lowered, becoming totally insufficient to maintain the body-weight of the animals. Restoration of the missing amino-acids was immediately effective in correcting this deficiency.

The removal of lysine was followed by a negative nitrogen balance, as was also the administration of a preparation of hydrolysed gliadin. The addition of the missing lysine did not in these two cases completely restore the food value of the rations. Other experiments dealing with the relative importance of histidine, arginine, and cystine gave somewhat indefinite results; although the evidence in the last case tended to show that cystine is an indispensable component of the protein molecule. Equally interesting results have been obtained by Hopkins and his co-workers in this country [Hopkins, 1916].

Ackroyd and Hopkins [1916] have again confirmed the absolute indispensability of tryptophan, and have also obtained most interesting and important results bearing upon the physiological value of the amino-acids arginine and histidine. They have presented evidence which indicates that these two substances are interconvertible in the animal organism and that an adequate supply of at least one of them in the diet is necessary for satisfactory nutrition. From their results it would also appear that these amino-acids can furnish the starting point of purine synthesis in the animal body.

Geiling [1917] has recently carried out an investigation the results of which have once again demonstrated the importance of the so-called diamino-acids in the processes of nutrition and growth.

This worker regards tryptophan and cystine as indispensable dietary units, and he adds confirmation of the relationship between arginine and histidine, described by Ackroyd and Hopkins. He does not regard lysine as of special importance.

Attempts have been made to effect the nutrition of animals upon diets in which protein has been replaced by artificial mixtures of amino-acids in

the pure state. Abderhalden [1912] encountered great experimental difficulties in attempting this, but was successful in maintaining a dog for eight days at constant body-weight upon a diet containing all the common amino-acids excepting oxyproline. The nutrition of the animal was far less satisfactory than when "erepton" a completely hydrolysed meat-protein was employed.

Rat	Days 12	19	26	33	40	Remarks	Weight of tumour g.	Weight of rat–tumour g.	Change in weight of rat g.	Change in weight of host per 1 g. tumour
♂						Tumour extensively necrosed and ulcerated. Animal thin.	32·0	134·0	+20·0	
♂						ditto.	27·0	99·0	−18·0	
♂						Tumour very necrotic	12·0	114·0	+14·0	
♀						Tumour small and fibrous	–	–	–	
						Average	23·6	–	+5·3	+0·22

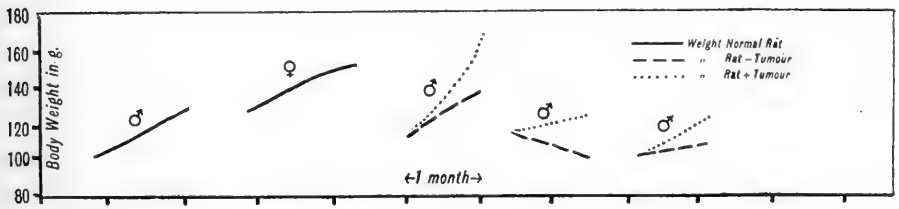


Chart 17. Tumour development, and growth curves of normal and tumour-bearing rats upon "Erepton" diet. Tumour *S*/121*d*.

Osborne and Mendel [1916, 2] record a failure to maintain rats upon a non-protein diet containing tryptophan, cystine, histidine, tyrosine, phenylalanine, proline and ammonium salts. Mitchell [1916] working with mice, has to some extent succeeded in surmounting the great difficulties which impede experimentation along these lines. His results support the indispensability of tryptophan, and indicate that the benzene nucleus may be omitted from the diet without adversely affecting nutrition.

It will be seen from a summary of these results that there is general agreement in regarding tryptophan, cystine and arginine or histidine as indispensable dietary components, but that opinion is divided regarding the value of lysine and the amino-acids containing the benzene nucleus.

The application of these results to the determination of the influence of individual amino-acids upon tumour growth was carried out in a similar manner to that employed in the study of the nutrition of the normal animal by Abderhalden and by Hopkins. Two amino-acid mixtures were prepared

Rat	Days 12	19	26	33	40	Remarks	Weight of tumour g.	Weight of rat−tumour g.	Change in weight of host g.	Change in weight of host per 1 g. tumour
♂						Tumour somewhat firm and fibrous. Animal very emaciated	8·5	67·5	−35·5	
♀						ditto	8·0	56·0	−18·0	
♂					—	Tumour well vascularised, firm and slow growing. Animal very emaciated	8·0	67·0	−33·0	
♂					—	ditto	3·5	46·5	−15·5	
♀				—	—	Killed and devoured 28.12.16	—	—	—	
						Average	7·0	—	−25·5	−3·6

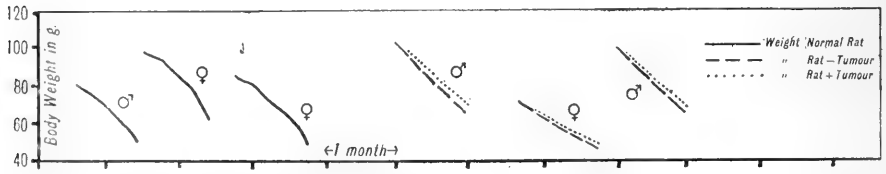


Chart 18. Tumour development, and growth curves of normal and tumour-bearing rats upon acid-hydrolysed meat-protein diet. Tumour *S*/117*c*.

from hydrolysed muscle protein, the one containing all the amino-acids excepting tryptophan, whilst from the other all the amino-acids which are precipitated by phosphotungstic acid had been removed. A commercial sample of completely hydrolysed meat-protein ("erepton") containing tryptophan, and prepared according to the method of Abderhalden, served as a control to these preparations.

The general composition of the rations employed is given below.

"Erepton" diet		Hydrolysed meat protein diet		"Monamino-acid fraction" diet	
"Erepton" (12·5 % N)	20 %	Acid-hydrolysed muscle preparation (7 % N)	40 %	"Monamino-acid fraction" preparation (5 % N)	50 %
Starch	40	Starch	20	Starch	25
Sucrose	15	Sucrose	15	Salt mixture	5
Salt mixture	5	Salt mixture	5	Agar	5
Agar	5	Agar	5	Butter-fat	15 +
Butter-fat	15 +	Butter-fat	15 +	Alcoholic extract of 20 g. dried milk	
Alcoholic extract of 20 g. dried milk		Alcoholic extract of 20 g. dried milk			

Rat	Days 12	19	26	33	40	Remarks	Weight of tumour g.	Weight of rat – tumour g.	Change in weight of rat g.	Change in weight of rat per 1 g tumour
♀						Tumour necrotic and ulcerated. Animal thin.	19·0	55·0	−23·0	
♀						Tumour firm Animal thin.	16·0	62·0	−28·0	
♀						ditto	16·0	72·0	−25·0	
♀					–	ditto	10·0	57·0	−14·0	
♂					–	ditto	9·0	80·0	−10·0	
♀						ditto	3·0	77·0	−7·0	
						Average	12·0	–	−17·8	−1·52

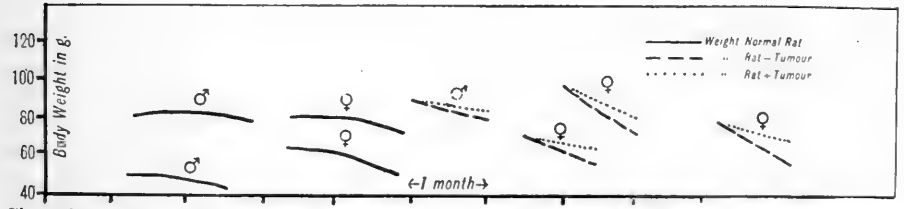


Chart 19. Tumour development, and growth curves of normal and tumour-bearing rats upon acid-hydrolysed meat-protein diet + 0·5 % tryptophan. Tumour *S*/117*c*.

Amino-acid additions were made, where indicated, in the following amounts:

Tryptophan	0·5 %	of total diet
Cystine	0·5	,, ,,
Lysine dihydrochloride (in neutral solution)	2·0	,, ,,
Arginine nitrate ,, ,,	2·0	,, ,,
Histidine hydrochloride ,, ,,	1·5	,, ,,
Tyrosine	2·0	,, ,,

Throughout the course of this series of experiments some difficulty was encountered in getting the rats to consume adequate amounts of the diets. This was particularly marked in the case of rations to which had been added the various pure amino-acids. Many attempts were made to render the diets more palatable without materially altering their composition but without much success.

The average results of this series of experiments are summarised in Table 5.

Rat	Days 12	19	26	33	40	Remarks	Weight of tumour g.	Weight of rat – tumour g.	Change in weight of rat g.	Change in weight of rat per 1 g. tumour
♀						Tumour firm. Animal very emaciated	15·0	53·0	−50·0	
♀						Slight central necrosis of tumour	13·0	48·0	−48·0	
♀						Tumour retrogressing.	—	—	—	
						Average	14·0	—	−49·0	−3·5

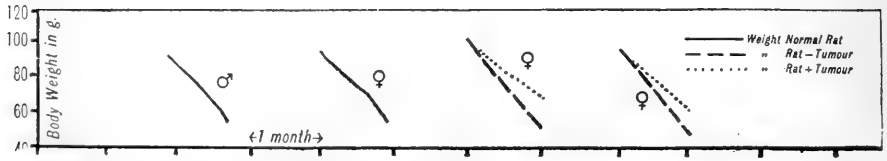


Chart 20. Tumour development, and growth curves of normal and tumour-bearing rats upon "monamino-acid fraction" diet. Tumour *S*/120*d*.

TABLE 5.

Diet	Average weight of tumour, g.	Average change in weight of host, g.	Average change in weight of host per 1 g. tumour
"Erepton"	23·6	+ 5·3	+0·22
Acid-hydrolysed protein	7·0	−25·5	−3·50
Acid-hydrolysed protein + 0·5 % tryptophan	12·0	−17·8	−1·52
"Monamino-acid fraction" + lysine, arginine, histidine, cystine and tryptophan	17·0	+1·50	+0·89
"Monamino-acid fraction" + lysine, arginine, histidine, and tryptophan	16·3	−10·3	−0·63
"Monamino-acid fraction" + lysine, arginine and histidine	15·0	−33·0	−2·20
"Monamino-acid fraction" + arginine and histidine ...	21·0	−39·0	−1·90
"Monamino-acid fraction" + tryptophan	19·5	−29·0	−1·49
"Monamino-acid fraction"	14·0	−49·0	−3·50

These results are illustrated more fully in Charts 17 to 25. It is unfortunate that the great labour involved in the preparation of the materials for this work should have necessitated a limitation of the number of experimental animals. This fact naturally greatly reduces the value of the average figures given in the preceding table, but it does not lessen the importance of observations that tumour growth was possible upon certain diets. The normal rats fed upon the "erepton" diet exhibited a sub-normal rate of growth, which may be attributable in part to the low food consumption of the animals. Tumour growth was quite normal in hosts fed upon the same ration, and was not accompanied by a loss of tissue upon the part of the animal (Chart 17).

Rat	Days 12	19	26	33	40	Remarks	Weight of tumour g.	Weight of rat – tumour g	Change in weight of rat g	Change in weight of rat per 1 g tumour
♀						Tumour somewhat necrotic and ulcerated	22	63	−39	
♂						Tumour firm	17	73	−19	
						Average	19·5	—	−29	−1·49

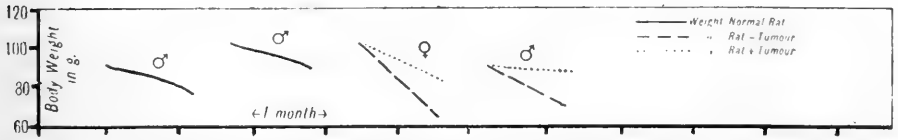


Chart 21. Tumour development, and growth curves of normal and tumour-bearing rats upon "monamino-acid fraction" diet + 0·5 % tryptophan. Tumour *S*/120*d*.

The diet prepared with the acid-hydrolysed meat-protein plus tryptophan should on theoretical grounds possess a nutritive value almost equal to that of "erepton." As a matter of fact it was found to possess a somewhat lower food value for normal rats, whilst tumour growth appeared somewhat retarded (Chart 19).

The adverse effects upon the nutrition of the animal which follow the withdrawal of tryptophan from the diet are illustrated in Chart 18. Normal animals quickly declined in weight and succumbed on this inadequate diet, whilst the tumour rats showed an appreciable inhibition of tumour growth, accompanied by a rapid fall in the body-weight of the host.

The low nutritive value possessed by an amino-acid mixture which is deficient in the so-called diamino-acids is illustrated in Chart 20. This ration was totally inadequate for the nutrition of normal rats, but the same restrictions imposed upon the tumour-bearing animals brought about comparatively little retardation of tumour growth.

As has already been remarked, it is not possible to place much reliance in the data given in Table 5, as in many cases they represent the average figures for a very small number of subjects.

28

Rat	Days 12	19	26	33	40	Remarks	Weight of tumour g.	Weight of rat − tumour g.	Change in weight of rat g.	Change in weight of rat per 1 g. tumour
♀						Tumour shows slight central necrosis Animal very thin	30	69	−49	
♀						ditto	12	75	−29	
						Average	21	—	−39	−1·9

29

♀						Tumour firm. Animal very thin.	15	75	−32	
♂						ditto	15	72	−34	
						Average	15		−33	−2·2

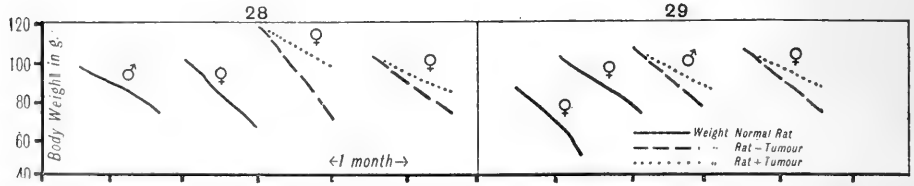


Chart 22. Tumour development, and growth curves of normal and tumour-bearing rats upon "monamino-acid fraction" diet, + 2 % arginine nitrate + 1·5 % histidine hydrochloride. Tumour *S*/120*d*.

Chart 23. Tumour development, and growth curves of normal and tumour-bearing rats upon "monamino-acid fraction" diet + 2 % arginine nitrate + 1·5 % histidine hydrochloride + 2 % lysine dihydrochloride. Tumour *S*/120*d*.

From the other Charts, 21—25, may be seen the influence exerted upon the nutrition of the experimental animals by additions of the various missing amino-acids.

The outstanding feature of this series of experiments is the demonstration

of the ability of the rat sarcoma to grow with comparatively little retardation in a host subjected to most drastic dietary restrictions.

Since it is apparent that the cells of these tumours can obtain considerable supplies of food units necessary for growth, when these are not present in the diet of the host, it becomes of importance to determine from what sources they are procured by the tumour.

30

Rat	Days 12	19	26	33	40	Remarks	Weight of tumour g	Weight of rat – tumour g	Change in weight of rat g	Change in weight of host per 1 g. tumour
♂	•	•	•	•	•	Tumour much necrosed and ulcerated	24	96	−14	
♀	•	•	•	•	•	Tumour firm, shows slight central necrosis	14	96	+1	
♀	•	•	•	•	•	ditto	11	90	−18	
						Average	16·3	—	−10·3	−0·63

31

♀	•	•	•	•	•	Tumour firm, slight central necrosis	20	90	+1	
♀	•	•	•	•	•	ditto	14	99	+2	
						Average	17	—	+1·5	+0·89

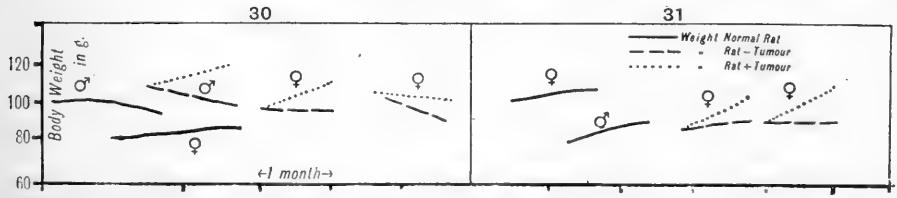


Chart 24. Tumour development, and growth curves of normal and tumour-bearing rats upon "monamino-acid fraction" diet, + 2 % arginine nitrate + 1·5 % histidine hydrochloride + 2 % lysine + 0·5 % tryptophan. Tumour *S*/120*d*.

Chart 25. Tumour development, and growth curves of normal and tumour-bearing rats upon "monamino-acid fraction" diet, + 2 % arginine nitrate + 1·5 % histidine hydrochloride + 2 % lysine dihydrochloride + 0·5 % tyrosine + 0·5 % cystine + 0·5 % tryptophan. Tumour *S*/120*d*.

Tumour cells require a comparatively large supply of the diamino-acids for their growth; since it has been shown by the analysis of a large number of tumour proteins that they contain a high percentage of these substances [Drummond, 1916, 3]. There is no evidence which indicates that tumour

cells differ from normal tissue cells in possessing the power to synthesise important cell units, such as the diamino-acids.

The question therefore arose as to whether the composition of the tumour proteins was in any way influenced by restrictions in the amino-acid intake of the host. Accordingly a number of the tumours grown in hosts fed upon certain of the diets employed in the last series of experiments were submitted to analysis in order to determine the amino-acid distribution of their proteins.

The preparation of the tumour material for analysis and the details of the latter were as described in a previous communication [Drummond, 1916, 3]. The partition of the various units constituting the diamino-acid fraction was not studied, in view of the fact that there appears to be some doubt as to the accuracy of the methods usually employed for that purpose.

A number of analyses were also carried out upon the proteins of the tissues of the host. Unfortunately the small amount of material furnished by individual organs prevented separate analyses being made of these. A representative sample of the tissues of the animal was therefore analysed. To ensure uniformity, the sample was composed, in each case, of the heart, lungs, liver, spleen, one kidney, equal sized strips of skeletal muscle, and one testicle or the ovary.

TABLE 6.

Partition of nitrogen in proteins of rat sarcoma S, and in the proteins of the tissues of hosts bearing these tumours.

Nature of diet of tumour-bearing rat	Protein analysed	No. of tissue	% N in dry tissue	Monamino-acid filtrate: % Amide N	% Humin N	% Monamino-acid N	% Nonamino-acid N	% Diamino-acid N	Total % N recovered
Normal diet	Organs	1	12·80	6·27	4·61	50·56	6·53	32·23	100·20
	Tumour	2	13·83	5·25	3·16	50·80	5·40	34·42	99·03
"Erepton" diet	Organs	3	12·62	5·51	5·66	55·93	4·77	30·97	100·64
	Tumour	4	13·55	4·42	3·59	51·77	6·73	32·42	98·93
Hydrolysed meat protein diet (tryptophan absent)	Organs	5	12·78	4·83	4·87	51·90	4·60	32·20	98·40
	Tumour	6	13·81	4·69	4·25	52·68	6·12	31·72	99·36
Monamino-acid diet	Organs	7	12·37	4·93	4·73	52·60	3·95	32·72	98·96
	Tumour	8	13·84	5·02	3·98	52·40	6·50	32·00	99·10

In the animals which had suffered a severe loss of body-weight upon certain of the diets, the post-mortem examination showed great wasting of the skeletal muscles. Blood examination of such animals carried out some days before death frequently revealed a state of anaemia. The number of animals under investigation was unfortunately too small to permit of a comparison being made of the weights of the various organs in these cases with the standard

data given by Donaldson [1915]. No apparent wasting was noticeable upon examination of the heart, spleen or kidneys, but the liver in some cases appeared smaller than normal.

Table 6 contains the results obtained from the examination of the tumour and tissue proteins referred to above. A study of this table indicates, as far as it goes, that the composition of both the tumour proteins and the proteins of the tissues of the host is unaffected as a result of deficiencies in the amino-acid content of the diets.

This chemical evidence is supported by the histological examination of the tumours and organs removed from the tumour-bearing rats. No appreciable differences could be detected between the microscopical appearance of sections of the tumours grown in animals fed upon the diets showing serious amino-acid deficiencies, and those removed from normally fed animals. Even the tumours removed from the rats fed upon the amino-acid diet deficient in histidine, arginine, cystine, lysine and tryptophan presented an apparently normal microscopical appearance.

The influence of the accessory growth factors "fat-soluble A" and "water-soluble B," upon tumour growth in rats.

The importance of these two unidentified dietary factors in the nutrition of the growing animal has received attention in the introduction to this paper. To investigate the possible influence which they might exert upon tumour growth the following four rations were prepared. Diet *A* contained both factors, and being in other respects adequate, may be considered a "complete" diet. Diet *B* lacked the "fat-soluble *A*" factor, but in other respects possessed a similar composition to *A*. Diet *C* was deficient only in the "water-soluble *B*" substance, whilst Diet *D* contained neither accessory factor.

The composition of the dietaries was as given below.

	A	*B*	*C*	*D*
Caseinogen	18	18	18	18
Starch	48	48	54	54
Agar	3	3	3	3
Salt mixture	5	5	5	5
"Yeast preparation"	6	6	—	—
Butter-fat	20	—	20	—
Lard	—	20	—	20

The results which were obtained are recorded in Charts 2, 26, 27 and 28 and are summarised in Table 7. Charts 29–31 refer to a similar experiment dealing with the fat-soluble *A*.

TABLE 7.

Diet	Deficiency in diet	Average weight of tumour, g.	Average change in weight of host, g.	Average change in weight of host per 1 g. tumour
A	Nil	16·6	+42·0	+2·5
B	Fat-soluble *A*	16·3	+12·9	+0·9
C	Water-soluble *B*	13·5	− 3·5	−0·3
D	Fat-soluble *A* and water-soluble *B*	12·7	− 5·0	−0·4

Rat	Days 12	19	26	33	40	Remarks	Weight of tumour g.	Weight of rat − tumour g.	Change in weight of rat g	Change in weight of rat per 1 g tumour
♀						Solid growth	10·5	87·5	−3·5	
♀						Tumour very necrotic, ulcerated 21.4.17	22·5	71·5	−10·5	
♀						Tumour small and fibrous	2·0	90·0	−1·0	
♀						Tumour centrally necrotic	24·0	71·0	−4·0	
♂					–	Died 19.4.17	8·5	81·5	+1·5	
						Average	13·5	–	−3·5	−0·26

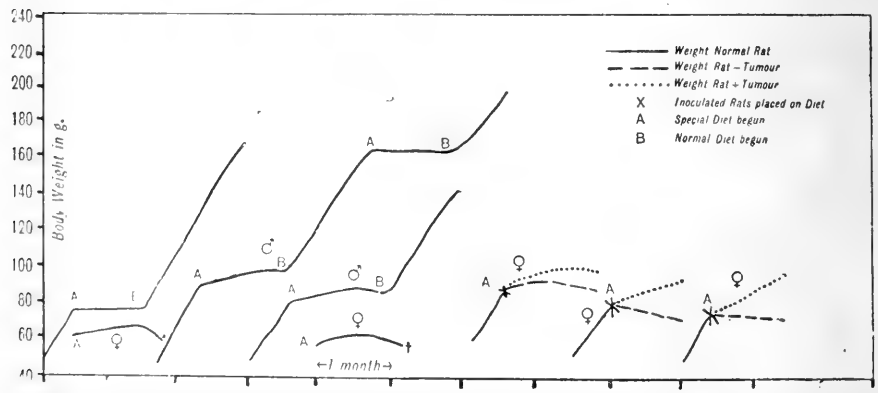


Chart 26. Tumour growth, and growth curves of normal and tumour-bearing rats upon diet deficient in "water-soluble *B*." Tumour *S*/120*d*. Diet similar to that employed for rats in Chart 2, except that "Yeast preparation" was omitted.

From these average figures, as well as from a study of the charts, it may be seen that no retardation of tumour growth followed a deficiency of the fat-soluble *A*, whereas the absence of the water-soluble *B* did cause a certain amount of inhibition. Even in the case of diet *D*, where both factors were

absent, the inhibition of tumour growth is only of the same order as that brought about by the deficiency of the water-soluble factor alone. The explanation of these results may be assisted by a study of the growth curves of the normal animals fed upon these rations. A deficiency in the supply of the fat-soluble factor in the diet of a growing rat is not always followed by

Rat	Days 12	19	26	33	40	Remarks	Weight of tumour g	Weight of rat − tumour g	Change in weight of rat g	Change in weight of rat per 1 g tumour
♂						Tumour firm	7·0	114·0	+21·0	
♀						Large tumour extensively necrotic, small nodule solid	24·5	74·5	+2·5	
♂						Tumour firm	10·0	102·0	+2·0	
♂						Slightly necrotic at centre	38·0	128·2	+27·2	
♂						Hard fibrous growth	0·8	100·0	+12·0	
						Average	16·1	—	+12·9	+0·80

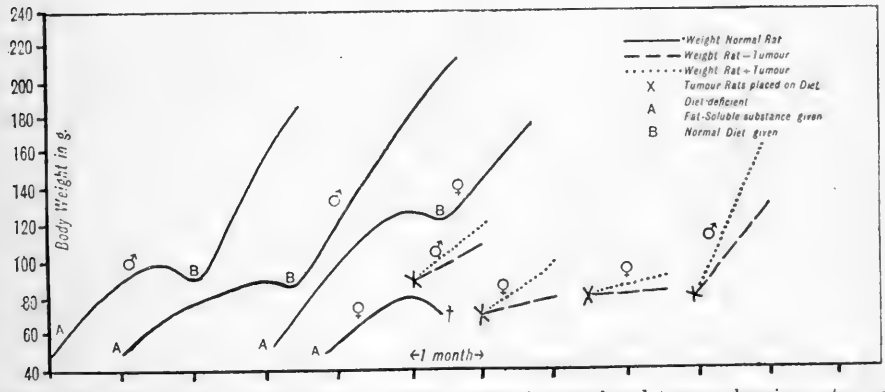


Chart 27. Tumour development, and growth curves of normal and tumour-bearing rats upon diet deficient in "fat-soluble *A*." Tumour *S*/120*d*. Diet similar to that employed in Chart 2, but butter-fat was replaced by lard.

an immediate cessation of growth. More usually, growth continues at a more or less subnormal rate for a period dependent upon the health and age of the animal. An animal which is well nourished at the outset of such an experiment may show such growth for several weeks; sooner or later, however, a cessation of growth will ensue which will be followed by a rapid

decline and death. Only a prompt addition of the missing factor to the diet will prevent this decline and restore health and growth.

These facts have led to the suggestion being advanced that the animal organism can mobilise existing stores of this dietary factor when a deficiency

Rat	Days 12	19	26	33	40	Remarks	Weight of tumour g	Weight of rat – tumour g	Change in weight of rat g.	Change in weight of rat per 1 g tumour
♀						Tumour ulcerated and very necrotic. Rat very thin and anaemic	230	810	−130	
♂						Firm growth	65	845	+05	
♀						Firm growth	85	925	−95	
♀						Somewhat fibrous tumour	30	980	+20	
♂					—	Tumour retrogressed	—	—	—	
						Average	102	—	−50	−0·49

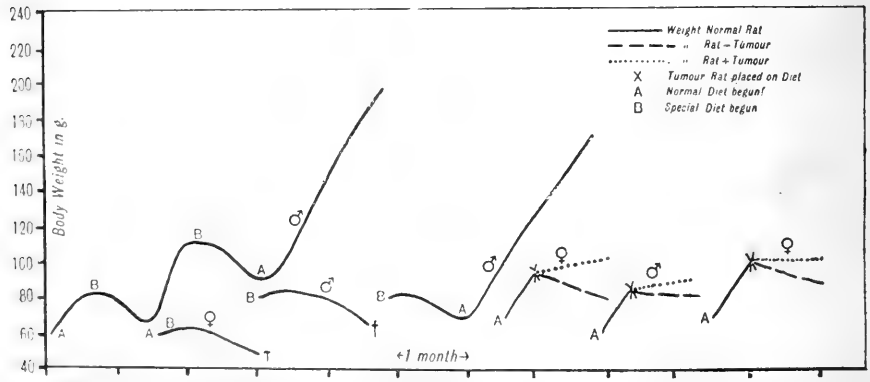


Chart 28. Tumour development, and growth curves of normal and tumour-bearing rats upon diet deficient in "water-soluble *B*," and "fat-soluble *A*." Tumour *S*/120*d*. Diet composed as described under Chart 2, but "Yeast preparation" omitted, and butter-fat replaced by lard.

occurs in its diet [Halliburton and Drummond, 1917]. Since there is evidence which indicates that the animal is able to grow for some weeks upon a diet deficient in the fat-soluble *A*, by making use of its own store of that substance, it is not surprising to find normal tumour growth in hosts placed under the same nutritive condition. Whether a deficiency in this factor

would exert a retarding influence upon tumour development after the available reserves had been exhausted is uncertain.

Animals are much less able to withstand the consequences following the withdrawal of the water-soluble *B* from the diet. As is seen from the curves of the normal rats, the withdrawal of this substance is practically immediate

Rat	Days 12	19	26	33	40	Remarks	Weight of tumour g.	Weight of rat − tumour g.	Change in weight of rat g.	Change in weight of rat per 1 g tumour
♀						Tumour extensively necrotic at centre	320	570	−130	
♂						Slight central necrosis	170	590	−20	
♀						—	70	1030	+30	
♂						Ulcerated. considerable necrosis at centre of tumour	300	820	+60	
♀						ditto	400	570	−190	
						Average	252	—	−50	−020

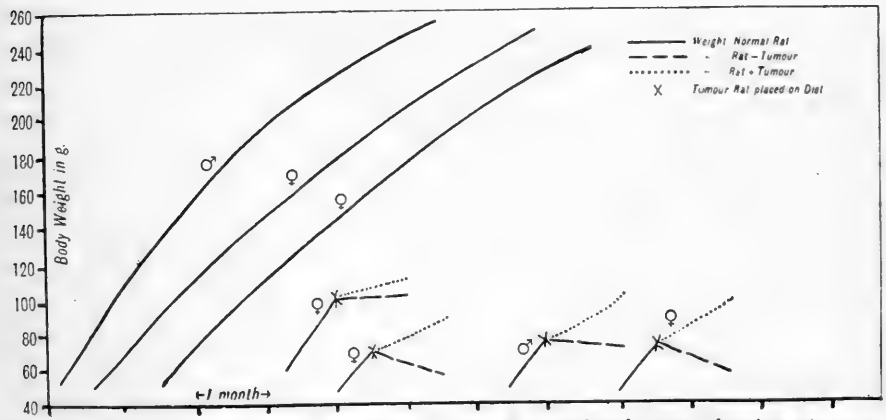


Chart 29. Tumour development, and growth curves of normal and tumour-bearing rats upon a complete artificial dietary. Tumour *S*/117*d*. Diet: Dried milk powder, 50 %, Starch, 40 %, Lard, 10 %.

in causing a cessation of growth. Then follows a period of maintenance or slow decrease in body-weight which eventually ends with a sharp decline and death. What is the state of the animal with regard to this dietary factor during this period is undefined. Drummond [1917] has recently shown that

the properties of the water-soluble growth factor and those of the so-called "beri-beri vitamine" are so closely similar as to render it very probable that the two substances are identical. If this is so certain facts known regarding the latter body may help to throw light on the question. Thus it is well known that the onset of the typical symptoms of the condition known as

Rat	Days 12	19	26	33	40	Remarks	Weight of tumour g.	Weight of rat − tumour g	Change in weight of rat g.	Change in weight of rat per 1 g tumour
♀						Tumour shows slight central necrosis	21	43	−16	
♀						Firm tumour	20	74	−10	
♂						Firm, slow growing	5·5	78·5	−3·5	
♂						Firm tumour	10	70	−1·5	
♀						Centrally necrotic and ulcerated	4·0	39	−2·8	
						Average	19·3	—	−14·5	−0·75

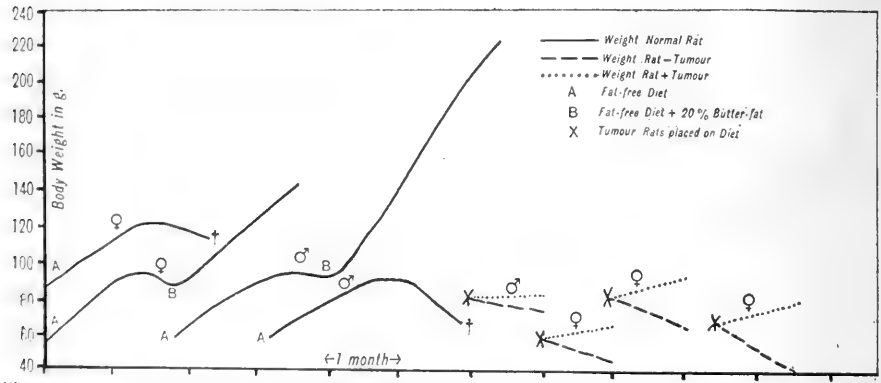


Chart 30. Tumour development, and growth curves of normal and tumour-bearing rats upon diet lacking both fats and the "fat-soluble *A*." Tumour *S*/117*d*. Diet: Ether extracted dried skimmed milk, 50 %, Starch, 50 %.

avian polyneuritis does not occur until the fowl has been fed upon a diet deficient in the so-called "vitamine" for a period of about three weeks. A gradual decrease of body-weight occurs during this period, which apparently corresponds with the period during which many rats show a more or less

successful maintenance of body-weight. The onset of the nerve symptoms in the fowl corresponds roughly with the rapid decline in weight and death of the rat.

Funk [1914, 3] has shown that the period preceding the onset of the nerve disturbances in the pigeon may be shortened by increasing the carbohydrate

Rat	Days 12	19	26	33	40	Remarks	Weight of tumour g	Weight of rat − tumour g	Change in weight of rat g	Change in weight of host per 1 g tumour
♀						Tumour extensively necrotic and ulcerated	27·0	105	+27	
♂						ditto	47·0	105	+30	
♀						ditto	26·0	55·0	−27·0	
♀						Tumour retrogressed	–	–	–	
♀						ditto	–	–	–	
						Average	33·3	–	+1·0	+0·03

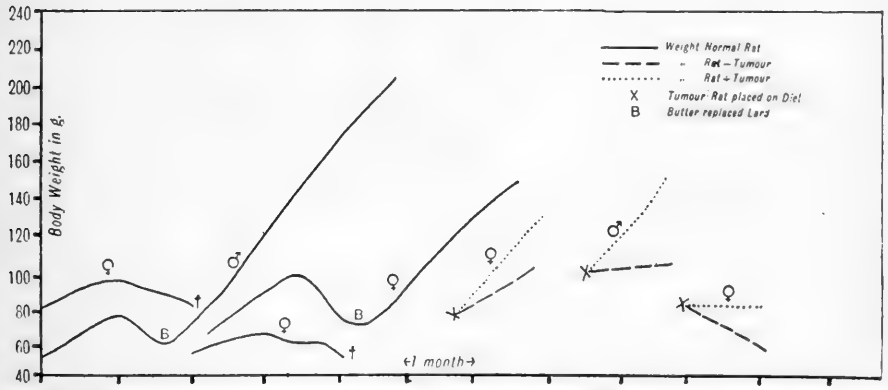


Chart 31. Tumour development, and growth curves of normal and tumour-bearing rats upon diet deficient in "fat-soluble *A*." Tumour *S*/117*d*. Diet: Ether extracted dried skimmed milk, 50 %, Starch, 30 %, Lard, 20 %.

content of the diet, and he has suggested that a relationship may exist between carbohydrate metabolism and the utilisation of "vitamine." He is inclined to believe that the animal organism possesses a store of the latter substance which may be mobilised for use when a deficiency arises in the diet in this respect.

Whether it is the existence of such a reserve that enables rats to live for some time upon diets deficient in this accessory substance or not is hard to say. Certainly if such is the case the substance is utilised for the purpose of helping to maintain the life of the animal rather than in effecting growth. Tumour growth suffered a slight retardation under these dietary conditions, and was also accompanied by a loss of weight upon the part of the host (Chart 26). Post-mortem examination of these tumour-bearing rats revealed a considerable wastage of skeletal muscle, but the other organs were not visibly affected.

Benedict and Rahe [1917] whose work has already received attention, concluded that the tumour cells are as dependent upon exogenous supplies of this dietary factor, as are the normal cells of the growing animal.

Discussion.

Certain of the points which have arisen during this work have been discussed briefly in the preceding pages, so that the present consideration must be devoted to a more general survey of the results taken as a whole. The outstanding feature which is apparent from a glance at the charts presented with this paper is the comparative failure which has attended the attempts to inhibit the rate of tumour growth by dietary restrictions. In other words it is demonstrated that a considerable degree of tumour growth may occur in hosts fed upon diets which will not permit any normal tissue growth.

The established tumours which formed the subject of this investigation invariably appeared to possess the power of commandeering what part of the food supply of the host they required for their own use. Failing this source of nutrition it was usual to find the tissues of the host sacrificed to no inconsiderable extent in order to provide the requirements of the parasitic cells.

Not altogether unconnected with this subject is the question of tumour growth in pregnant females, a condition where two parasitic cell complexes, each possessing a high growth energy, may be said to be competing for possession of the available food supply.

Clinical observation both on human cases and on experimental animals, has repeatedly demonstrated a retardation of tumour growth during pregnancy, whilst in other instances an increased rate of development upon the part of the neoplasm has been noted under what were apparently identical conditions.

Haaland [1907], Cuénot and Mercier [1909], Uhlenhuth and Weidanz

[1909] are amongst those who have reported a slower rate of tumour growth during pregnancy.

It has been suggested by Jannovics [1912] that the pregnant female presents an unfavourable soil for tumour growth; but Rous [1911, 2] has pointed out that pregnancy under normal conditions is a stimulus to growth and nutrition. The inconsistences in the observations upon the relationship between the degree of tumour growth and pregnancy have been explained by Fichera [cited by Apolant, 1911] as being due to the influence of the number of embryos. Whether this explanation is the correct one, or whether it lies in the relative blood supplies of the tumour and embryos is uncertain, but there is much to support the latter view. It must be remembered that cancer cells perform no function, and that they are therefore free to expend the whole of their energies upon cell division and growth.

The development of the embryo may be for some reasons considered to possess certain parallel features to tumour proliferation, so that it is of interest to note the influence of inadequate dietaries, such as have been studied in this work, upon the former process.

It has been repeatedly observed in this laboratory that animals fed upon inadequate diets will not breed. If females are placed upon the diets soon after they are pregnant the results vary according to the nature of the dietary deficiency.

McCollum, Simmonds and Pitz [1916], found that when the accessory factors *A* and *B* are absent from the diet of the nursing mother, she is unable to rear her young, being only able to supply these substances so long as they are present in her own food supply.

Some results, recently obtained in this laboratory, indicate that in the absence of the water-soluble substance from the diet, growth of the young may continue for a short period after the imposition of the restriction upon the mother; but that sooner or later a rapid decline in the body-weight and health of the young will occur.

Nevertheless, there is unmistakable evidence of growth upon the part of the young for some time after the supply of this dietary factor is cut off from the mother. This result presents similar features to the tumour growth observed under similar conditions.

Returning to a consideration of the process by which tumour cells obtain supplies of food units such as certain amino-acids, when the latter are absent from the diet of the host, it is found that considerable light is thrown upon the subject by a study of certain other conditions.

It is well known that during inanition a transfer of protein "Bausteine"

may occur in the animal organism whereby vital organs, such as the heart, are enabled to exist and functionate as long as possible at the expense of the less important tissues, such as the skeletal muscle. Of particular interest are the classical investigations of Meischer, who described the development of the genital organs of the Rhine salmon at the expense of the musculature, during the period of starvation prior to spawning. In this instance he found that the muscle tissue of the fish might lose 55 % of its weight, without the apparent death of a single muscle cell.

Abderhalden, Bergell and Dörpinghaus [1904] showed that the protein of tissues in inanition possess the same amino-acid distribution as in their normal condition.

This result is confirmed by a few analyses made by the author who could trace no appreciable difference in the amino-acid partition of the tissue proteins of normal chickens, and others whose growth and development had been stunted by dietary inadequacies [Drummond, 1916, 3].

Other examples of a transfer of protein from comparatively unimportant tissues to organs where it is urgently needed are those furnished by the growth of the pregnant uterus and the maintenance of the protein content of the milk of a lactating animal. Both of these occur, as far as possible, at the cost of other tissues, when dietary restrictions necessitate the sacrifice.

The process by which this balance is attained is unknown. It appears however that it is tissues exhibiting at the time a high growth or functionating energy that are protected from the immediate effects of malnutrition by this means. Such for example are the cases which have been given, the uterus during pregnancy, the mammary gland during lactation and the genital organs of the salmon during the spawning period. At the same time it appears probable that all-important organs such as the heart are able to obtain the supplies necessary for their repair and function with equal ease.

Returning to the consideration of the growth of tumours under these circumstances, there seems no reason why the above explanation should not be equally applicable. Tumour cells, particularly those of actively growing sarcomas, possess a high growth potential, as compared with the tissues of the host. At the same time there is no waste of energy in the cancer cell upon functionating processes; cell-division being as far as is known the chief activity exhibited by these units. There is therefore no apparent reason why tumours in possession of an adequate blood supply should not be equally capable of satisfying their requirements by enforcing sacrifice of the tissue of the host, as is the developing embryo.

Any consideration of the results of this investigation must take into account

their bearing upon the treatment of cancer, but little if any hope of alleviating the course of the disease in man can be gathered from the preceding pages. All the results of this experimental research indicate that only the most drastic dietary restrictions, involving a very serious loss of weight upon the part of the host, would have any retarding influence upon tumour growth.

Summary.

1. By imposing certain restrictions upon the diet of a young animal, its growth may be completely inhibited. The present investigation has aimed at a determination of the influence which these dietary inadequacies exert upon the growth of tumours in tumour-bearing subjects.

2. The influence of the following dietary inadequacies upon normal and tumour growth has been studied:

(*a*) Low protein content of the diet.

(*b*) Nitrogen of the diet supplied in the form of a protein possessing a relatively low nutritive value.

(*c*) Absence of certain indispensable amino-acids from the diet.

(*d*) Absence of the equally indispensable accessory growth promoting factors, the "fat-soluble *A*" and the "water-soluble *B*."

3. In the event of a deficiency arising in the diet of the host, the tumour, provided it possesses a satisfactory blood supply, will continue to grow, although the host may be quite unable to do so. There is evidence that this proliferation will proceed at the expense of the tissues of the host, until these are no longer able to supply the missing units.

4. When the host is unable to make good the deficiency, by drawing upon its own reserves, the rate of tumour proliferation will decrease. This occurs at a comparatively early stage when the diet is deficient either in tryptophan or in the water-soluble accessory factor, *B*.

5. There is no evidence that the cells of tumours possess powers of synthetical action which the normal cell of similar type does not possess.

6. It does not appear possible to bring about an inhibition of tumour growth by an employment of dietary restrictions, such as have been used in this research, without the nutritive condition of the host being very seriously impaired.

7. There is, therefore, little hope of bringing about an alleviation of the disease in man by the imposition of dietary restrictions, such as are described in this communication.

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INDEX

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