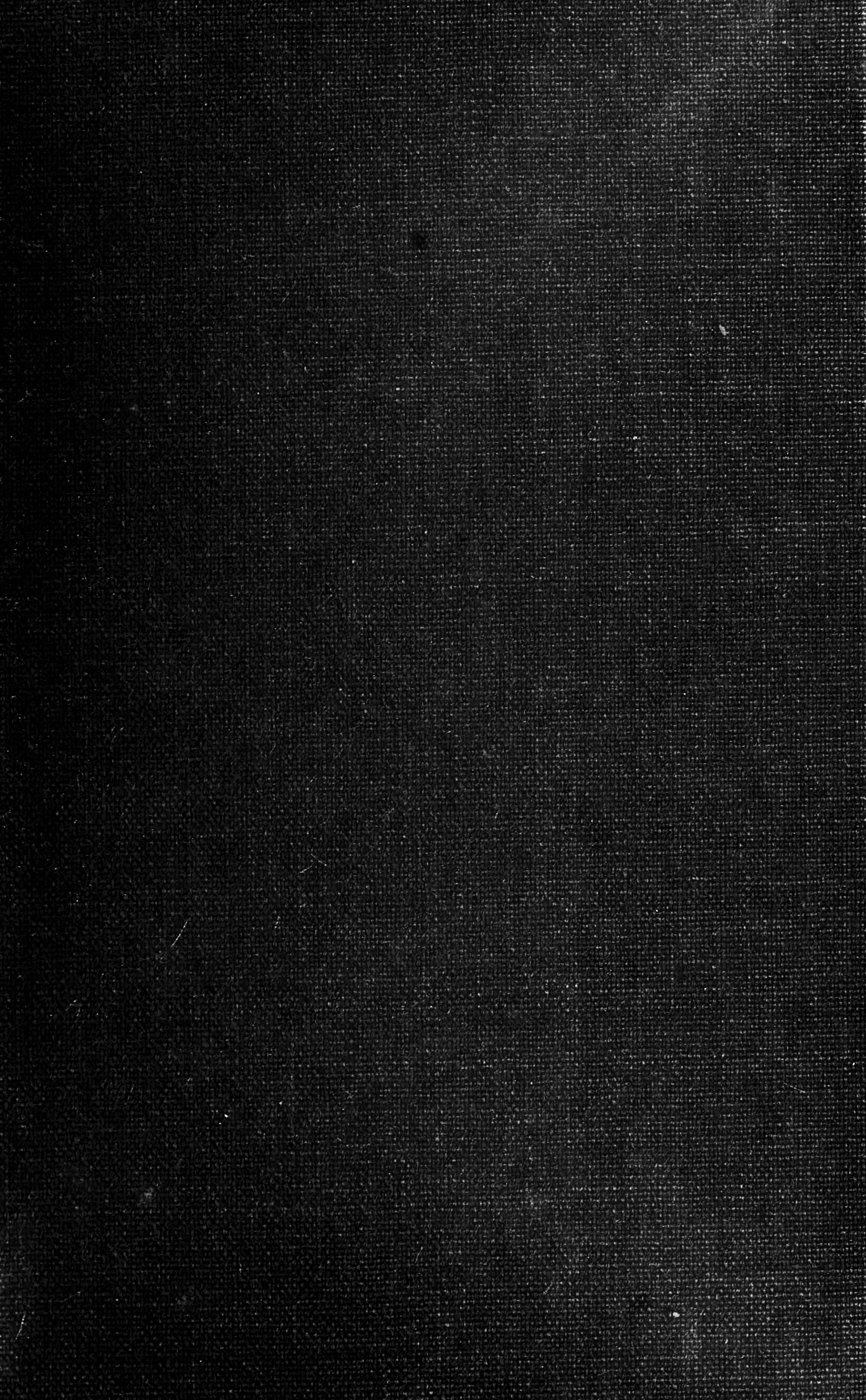

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THE DETECTION OF VOLATILE ALKYLAMINES IN THE PRESENCE OF AMMONIA AND OF VOLATILE TERTIARY ALKYLAMINES IN THE PRESENCE OF VOLATILE PRIMARY AND SECONDARY ALKYLAMINES.*

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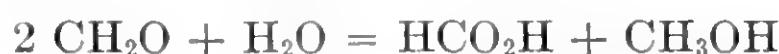
(Received for publication, January 10, 1921.)

The presence of volatile alkylamines in foodstuffs may be regarded in most instances as an index of decomposition. In the incipient stages of decomposition the quantities of volatile amines present may be very small. The detection of such minute quantities of these amines is made difficult by the presence of ammonia which almost always is found at the same time. There are many records in the literature of efforts to develop means of detecting volatile amines in the presence of ammonia. Most of the methods which have been suggested can be used only with amounts of amines greater than 10 mg., although the test for trimethylamine by odor and the test for primary amines by the isonitrile reaction are more delicate. Subsequent to the completion and reporting of the investigation presented herein Weber and Wilson (1) proposed a satisfactory quantitative method depending upon the freeing of the amines from ammonia by means of yellow oxide of mercury and the determination of the amines by the use of nitrous acid. In the present paper methods are suggested for the rapid qualitative detection of volatile alkylamines in the presence of ammonia, and of tertiary volatile alkylamines in the presence of primary and secondary amines.

The method suggested for the detection of volatile alkylamines

* Presented under the title "A new reagent for volatile tertiary amines," September, 1916, at the New York meeting of the American Chemical Society.

in the presence of ammonia depends on the difference in behavior between ammonia and the amines with formaldehyde. Ammonia reacts with formaldehyde to form such compounds as hexamethylenetetramine, while the amines in dilute solution react upon formaldehyde to give formic acid and methyl alcohol, perhaps in accordance with the following scheme:



$$2\ CH_2O + H_2O = HCO_2H + CH_3OH$$

The formic acid is present as the salt of the amine, and the reaction proceeds until enough formic acid has been formed to neutralize the amine. The reaction by which formic acid is produced is not quantitative except within certain limits for the concentration of the amine and the formaldehyde. This observation is in agreement with that of Loew (2), who studied the action of fixed alkalies on formaldehyde and found that the concentration and strength of the alkali influence the formation of formic acid.

A method of separating the alkylamines from each other by the use of formaldehyde was suggested by Delépine (3). In this method the amines are treated in rather concentrated solutions with formaldehyde and the mixture is dehydrated with potassium hydroxide. Under these conditions the primary and secondary amines give condensation products of different boiling points, while tertiary amines do not react. The condensation products are easily hydrolyzed. They are probably not formed in dilute solution.

If, therefore, formaldehyde is allowed to act upon a solution containing both ammonia and amines, the ammonia will be converted into such substances as hexamethylenetetramine and the amine will cause a certain amount of formic acid to be produced from the formaldehyde. Under these conditions presence of formic acid indicates that amines occur. To detect formic acid the reagent that was found most suitable is mercuric bromide, which is reduced by the formic acid to form a white insoluble precipitate of mercurous bromide. Mercuric chloride may also be used as a reagent for formic acid but it was not found to give as delicate a test. Ammonia and mono- and dimethylamine combine with mercuric bromide to give insoluble compounds but interference with the test for formic acid from this source can be

avoided by the addition of an excess of formaldehyde in the presence of which these compounds are soluble. Aromatic amines do not give white precipitates of mercurous bromide in this test. With aniline a brown precipitate is formed; and with dimethylaniline a green precipitate which turns blue.

In practice it is best when making this test for the detection of volatile amines in the presence of ammonia not to use a solution of mercuric bromide in water, in which it is not very soluble, but rather a solution in aqueous potassium bromide. A convenient strength of solution to use is one containing 18 gm. of $HgBr_2$ and 12 gm. of KBr in 1 deciliter. This solution is 0.5 N and each cc. is equivalent to 7 mg. of amine nitrogen.

To detect volatile alkylamines in the presence of ammonia, the following procedure is recommended: The volatile alkali is separated by distillation or aeration and received in a slight excess of acid. This acid solution is evaporated to a small volume and transferred to a small flask, from which it is distilled, after making alkaline with sodium hydroxide, into about 1 cc. of 40 per cent formaldehyde solution in a test-tube. About 1 cc. of reagent, potassium mercuric bromide, is then added and the test-tube is warmed slightly in the steam bath. In the presence of 0.5 mg. of amine nitrogen a fine white precipitate of mercurous bromide appears, and with larger amounts there is a heavy precipitate even before heating. This precipitate will not dissolve when a little more formaldehyde is added.

Within a certain range of concentration of the solutions the reaction is roughly quantitative. The most favorable concentration for the quantitative reaction seems to be about 10 cc. of amine solution which is between 0.01 and 0.02 N with about 1 cc. of formaldehyde. Under such conditions the weight of the precipitate of mercurous bromide is nearly twenty times the weight of the amine nitrogen. For small quantities the use of the nephelometer is of value.

The reagent proposed for volatile tertiary alkylamine is a solution of mercuric iodide and potassium iodide, generally known as Mayer's reagent. This reagent has long been used for the quantitative precipitation of alkaloids (4). It has been known for some time that other compounds including pyridine and quinoline (5), tetramethylammonium, and tetraethylammonium

salts (6) give precipitates with potassium mercuric iodide. There was, however, no reference to the use of Mayer's reagent as a precipitant for trimethylamine, prior to the publication of the work of Jamieson and Wherry (7) undertaken as a sequel to the present investigation. In the present investigation it has been found that in neutral or slightly acid solution, potassium mercuric iodide gives precipitates with salts of trimethylamine and triethylammonium chloride. Whether other volatile tertiary amines behave in the same manner was not investigated. Other compounds which contain a nitrogen atom attached to 3 or 4 carbon atoms, such as dimethyl aniline, choline bromide, betaine bromide, the bromide of betaine aldehyde, and bromoethyl-trimethylammonium bromide, also give precipitates with potassium mercuric iodide.

These substituted ammonium mercuric iodides are readily soluble in the presence of potassium iodide. Therefore, in preparing Mayer's reagent it is of great importance to weigh both the mercuric iodide and the potassium iodide with care in order to avoid an excess of potassium iodide. The Mayer's reagent recommended for the precipitation of trimethylamine is a normal solution containing 45 gm. of HgI_2 and 33 gm. of KI in 1 deciliter.

Mayer's reagent thus prepared does not give a precipitate with salts of ammonia or of monomethyl- or monoethylamine. Salts of dimethylamine and diethylamine, however, are precipitated from solutions of considerable concentration. When 1 cc. of the solution contains more than 20 mg. of diethylamine or more than 4 mg. of dimethylamine, these compounds are precipitated. Trimethylamine on the contrary in solutions as dilute as 0.01 N slowly separates long yellow needles on addition of Mayer's reagent. In solutions as concentrated as 0.1 N a heavy yellow precipitate forms immediately, which quickly turns into flat yellow crystals, while solutions more dilute than 0.01 N give only a slight red precipitate of HgI_2. Triethylammonium chloride behaves in a similar manner, yellow feathery crystals being deposited from concentrations of about 0.01 N, while from concentrations of about 0.1 N a heavy yellow liquid separates.

The limit of the test, as indicated by the above described experiments, is about 3 mg. of trimethylamine in 5 cc. Trimethylamine is, therefore, precipitated from solutions more than twenty times

as dilute as those from which diethylamine can be precipitated, and more than six times as dilute as those from which dimethylamine is precipitated. In studying incipient decomposition of foodstuffs, such high concentrations will not ordinarily have to be dealt with. Hence in such studies, if care is taken to use suitable dilutions, Mayer's reagent may be used to detect trimethyl- and triethylamines in presence of mono- and dimethylamines, of mono- and diethylamines, and of ammonia. Furthermore, the test may be so refined as to make it possible to detect as little as 0.5 mg. of trimethylamine hydrochloride. Only a few drops of Mayer's reagent are added to the amine solution and the mixture is then shaken out in a separatory funnel with a mixture of equal parts of chloroform and ethylacetate. When the chloroform ethyl acetate extract is evaporated to dryness, the yellow color of the trimethylamine mercuric iodide is readily distinguished in the residue from the red mercuric iodide.

To test for trimethylamine in the presence of ammonia and of monomethyl- and monoethylamine and of small quantities of dimethyl- and diethylamine, the following procedure is recommended.

The volatile alkali separated by distillation or aeration is received in a slight excess of dilute acid. Methyl red may be used as an indicator. The acid solution is evaporated to a small volume and transferred to a small flask. It is filtered if necessary. The reagent is then added from a graduated pipette or burette until there is no more precipitate. Each cc. of the reagent precipitates 59 mg. of trimethylamine.

The trimethylamine precipitate crystallizes from alcohol in long yellow needles. When a hot saturated solution is cooled suddenly the crystals are in plate form. The melting point of the needles, after two crystallizations from methyl or ethyl alcohol, is 136°C. Analysis indicates that its formula is $(CH_3)_3N.HI.HgI_2$. Trimethylamine may be recovered by dissolving the crystals in a solution of sodium hydroxide and sodium sulfide and distilling. In this manner it is possible to free trimethylamine from traces of mono- and dimethylamine.

The triethylamine precipitate, as above stated, is an oil which does not readily crystallize. At −20°C. it crystallizes from alcohol in fine yellow needles which have a melting point of 77°C. Analysis indicates that its formula is $(C_2H_5)_3N.HI.HgI_2$.

Both compounds are easily decomposed by water which contains no potassium mercuric iodide with the formation of red mercuric iodide. Both are soluble in several organic solvents, but only in those which also dissolve mercuric iodide or potassium mercuric iodide. Hence small amounts cannot be separated from excess of the precipitating reagent, either by washing with water or with organic solvents. They can, therefore, serve to separate the amine from many other substances; but for the exact estimation of the amine, it must first be regenerated from the compound.

A more detailed study of these and related compounds was entrusted to Jamieson and Wherry (7) who have reported their findings.

In analyzing the trimethylamine compound, nitrogen was determined by distilling from a solution of sodium hydroxide and sodium sulfide and titrating the volatile alkali.

A determination of mercury was made by dissolving in a solution of sodium hydroxide and sodium sulfide, and acidifying with sulfuric acid to precipitate mercuric sulfide. This was filtered in a Gooch filter, washed with hydrogen sulfide water, alcohol, and carbon disulfide, then dried at 100°C., and weighed.

Iodine was determined in the filtrate from the mercury sulfide, by heating with ammonia and hydrogen dioxide to destroy hydrogen sulfide, and then precipitating with silver nitrate after acidifying with nitric acid. A blank was made with the reagents used.

A determination of iodine was made by distillation with sulfuric acid and ferric sulfate. This treatment did not decompose HgI_2, and gave only the iodine from HI.

The following tables give the analytical data:

1 gm. substance gave 15.40 cc. 0.1 N volatile alkali, equivalent to 0.0216 gm. N or 0.0911 gm. $(CH_3)_3N$.

0.500 gm. substance gave 0.1816 gm. HgS, equivalent to 0.1566 gm. Hg.

0.500 gm. substance gave 0.5470 gm. AgI, equivalent to 0.2956 gm. I.

0.500 gm. substance gave a distillate titrating 6.60 cc. of 0.1190 N $Na_2S_2O_3$, equivalent to 0.09967 gm. I.

	Found. *per cent*	Calculated for $(CH_3)_3N.HI.HgI_2$. *per cent*
N as $(CH_3)_3N$	9.11	9.20
Hg	31.31	31.28
I from AgI	59.12	59.36
I (distillation)	19.93	19.79

	Found. per cent	Calculated for $(C_2H_5)_3N.HI.HgI_2$. per cent
N as $(C_2H_5)_3N$	14.24	14.79
Iodine from AgI	55.71	55.72

In analyzing the triethylamine compound, the same procedure was followed but only nitrogen and iodine were determined.

SUMMARY.

A test is described which detects small amounts of volatile alkylamines in the presence of ammonia.

It has been found that potassium mercuric iodide gives a precipitate with small amounts of trimethyl- and triethylamine and that under certain conditions this reagent can be used to detect these amines in the presence of the corresponding mono- and di-amines. The compound formed with trimethylamine has the composition $(CH_3)_3N.HI.HgI_2$, that formed with triethylamine the composition $(C_2H_5)_3N.HI.HgI_2$.

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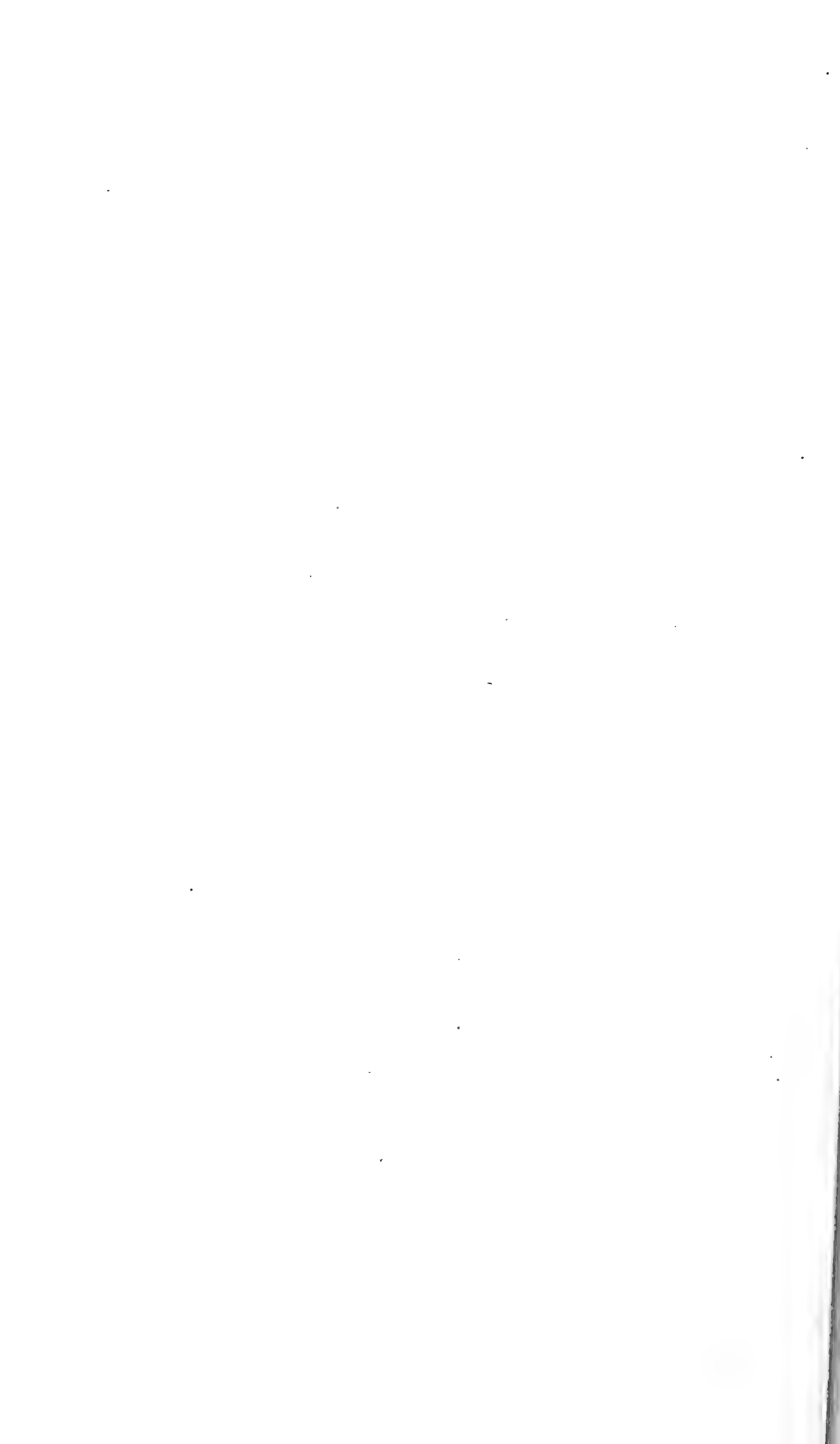

STUDIES ON THE DIGESTIBILITY OF PROTEINS IN VITRO.

I. THE EFFECT OF COOKING ON THE DIGESTIBILITY OF PHASEOLIN.*

By HENRY C. WATERMAN and CARL O. JOHNS.

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(Received for publication, December 17, 1920.)

In a recent publication from this laboratory (1) it has been shown that the proteins of the navy bean, *Phaseolus vulgaris*, are not capable of producing normal growth in white rats when fed as the sole source of protein in an otherwise adequate diet. A determination of the basic amino-acids (2) in the isolated globulin suggested that cystine might be a limiting amino-acid. This was found to be in fact the case. Phaseolin supplemented with 2 per cent of its weight of cystine enabled the animals to gain weight slowly, but growth was still far from normal. If, however, the protein or the bean meal were first boiled for a short time with distilled water, dried, and cystine added as before, normal growth was obtained. Even without the addition of cystine the cooked protein or meal produced markedly better results than did the raw material. To explain this observation Johns and Finks offered alternative hypotheses: (*a*) The cooking may have rendered the protein more readily digestible; (*b*) some other, more radical, change in the composition of the complex and doubtless labile protein molecule may have taken place under the combined influence of heat and water, possibly involving the loss of a protein toxicity or the destruction of an asso-

* Prepared by dilution of the navy bean extract with distilled water. The authors have shown that this precipitate is *not* a single protein, but a mixture containing a small quantity of a hitherto unknown globulin, *conphaseolin* (sulfur over 1.0 per cent). The work is to be offered for publication in this *Journal* as soon as completed.

ciated toxic substance. It was the purpose of the experiments described in this paper to test the first of these theories.

The experimental conditions were made as nearly like those of natural digestion as is practicable with experiments *in vitro*. Each sample was digested with pepsin in 0.1 N acid at 37°, the acid neutralized, and the digestion continued with trypsin in 0.5 per cent Na_2CO_3 or in 1/120 N NaOH. At the end of the digestion period the activity of the enzymes was destroyed by heating in a steam bath at 80° for 5 minutes. The extent to which proteolysis had proceeded was ascertained in each case by determining amino nitrogen in 10 cc. aliquots from the filtered reaction mixtures, corrections being made for all reagents used. The total nitrogen of each preparation was determined; and the digestibility expressed as percentage of amino nitrogen found after digestion, calculated as follows, on the basis of the total nitrogen of the sample:

$$\text{Per cent digestion nitrogen} = \frac{N_d - N_b}{N_t} \times 100^1 \text{ where}$$

N_d = mg. amino N found in the reaction mixture after digestion, corrected for blank due to the reagents used in the Van Slyke amino N apparatus.

N_b = mg. amino N produced by self digestion from the enzymes in a blank digestion; corrected as above.

N_t = mg. total N in the sample.

For instance, Cooked Sample 10, containing 13.46 per cent of nitrogen, weighed 501.6 mg.

N_t = 67.52 mg.

This sample gave 14.70 cc. of nitrogen at 27°C. and 753 mm. from 10 cc. out of 60 cc. of reaction mixture. The blank for the Van Slyke reagents was 0.50 cc. 1 cc. nitrogen evolved = 0.54375 mg. amino nitrogen at the given temperature and pressure.

$(14.70 - 0.50) \times 0.54375 = 7.72$

$N_d = 6 \times 7.72 = 46.32$ mg.

The blank digestion gave 6.60 cc. of gas from 10 cc. out of 60 cc. at 26°C. and 753 mm. 1 cc. gas = 0.54675 mg. amino nitrogen at this temperature and pressure. The reagent blank was 0.50 cc.

---

[1] To obtain figures truly representative of the actual proportion of the protein digested it would of course be necessary to calculate on the basis of the total amino nitrogen yielded by the protein after complete hydrolysis, subtracting the *free* amino nitrogen of the protein both from this total amino nitrogen and from the amino nitrogen found after digestion. See "Discussion."

$$(6.60 - 0.50) \times 0.54675 = 3.34$$
$$N_b = 6 \times 3.34 = 20.04 \text{ mg.}$$
$$N_d - N_b = 26.28 \text{ mg.}$$

$$100 \times \frac{N_d - N_b}{N_t} = 100 \times \frac{26.28}{67.52} = 38.92 \text{ per cent digestion nitrogen}$$

The results indicated a distinct and fairly constant superiority in digestibility of the cooked phaseolin over the untreated protein. When the phaseolin was boiled for about ¾ hour with distilled water, it yielded approximately 30 per cent more amino nitrogen after digestion than did the uncooked material; and when the boiling was continued for 4 hours, the gain was increased in one set of experiments to approximately 60 per cent. The latter result, however, could not be duplicated in subsequent determinations made after the accuracy of the method had been considerably improved. The phaseolin cooked 4 hours, then, showed about the same digestibility as did the material cooked ¾ hour. Even when the protein was cooked but 5 minutes some increase in digestibility could be detected. It would seem probable, therefore, that the effect of replacing raw with cooked phaseolin in the feeding experiments as noted by Johns and Finks is to be explained as due, in large measure at least, to an increase in the digestibility of the protein.

EXPERIMENTAL PART.

Preparation of the Phaseolin.—The raw phaseolin was taken from the lot prepared for the feeding experiments (1). Only that which passed a 100 mesh sieve was used in the digestion. The cooked material was prepared from the 100 mesh powder by boiling with about 50 parts of distilled water and evaporating to apparent dryness on a steam bath. It was then ground again to a 100 mesh powder. Three such preparations were made, the first being cooked 5 minutes, the second ¾ hour, and the third 4 hours.

Digestion with Pepsin.—Samples of approximately 0.500 gm. were suspended each in 25 cc. of 0.1 N sulfuric acid; a few drops of the acid were first added and the sample was rubbed to a smooth, thick paste; then the rest of the acid was added. If all

the acid be added at once to the dry sample, the powder floats upon it and thorough mixture cannot be obtained. To these suspensions were added 25 cc. each of a 0.2 per cent solution of pepsin.[2] The mixtures were then digested in an incubator at 37° for 1½ hours.

Digestion with Trypsin.—In the first experiments digestion with trypsin was carried out in alkaline solution produced by adding to the pepsin reaction mixture 10 cc. of a solution of sodium carbonate of such a concentration that the 50 cc. of 0.1 N acid present were neutralized and the resulting 60 cc. contained 0.5 per cent excess of sodium carbonate. Each 10 cc. of this sodium carbonate solution contained also 0.3 gm. of trypsin.[3] The enzyme was dissolved in the sodium carbonate solution immediately before use. Neun (3) states that 0.5 per cent sodium carbonate is the optimum concentration of this salt for tryptic digestions. The tryptic digestion was continued for 2½ hours at 37°. After digestion the activity of the enzymes was destroyed by heating for 5 minutes at 80°, the solutions were filtered, and duplicate determinations of amino nitrogen made upon 10 cc. samples from the filtrates. The results are given in Table I.

In the first five experiments the considerable discrepancies (which, however, are in no case great enough to contradict or weaken the conclusion that the cooked phaseolin in both groups was distinctly more readily digested than was the raw) were found to be in large measure due to two things: (1) The blank obtained in the first set was used to correct the results obtained in the second set; it seems to be necessary to run a blank digestion with every set of experiments, that is whenever new enzyme solutions are prepared; (2) the percentages of digestion nitrogen for Cooked Preparation A show a regular decrease. By determining nitrogen on this preparation again after the digestion experiment it was found that the preparation had absorbed moisture from the air to such an extent that if the data for the third

[2] For the pepsin used in this investigation we are indebted to Mr. V. K. Chestnut of the Phytochemical Laboratory, Bureau of Chemistry. It is a powdered preparation of approximately 1:10,000 strength, made by the Ray Chemical Company, Detroit, Michigan.

[3] Fairchild Brothers and Forster's powdered trypsin.

sample were calculated on the basis of the new nitrogen determination the result checked within a per cent with the first digestion nitrogen figure for this preparation. Accordingly, all preparations were exposed to a filtered current of air until they came to equilibrium with atmospheric moisture before further experi-

TABLE I.

Peptic-Tryptic Digestions in Which the Trypsin Acted in 0.5 Per Cent Sodium Carbonate Solution.

Sample No.		Amount of sample.	Total N in sample (N_t).	Amino N after digestion (N_d).	Amino N of blank digestion (N_b).	Digestion N calculated on total N.
		mg.	*mg.*	*mg.*	*mg.*	*per cent*
Raw.*	1	500.8	71.47	40.68	22.32	25.7
	2	499.0	71.72	40.56	22.32	25.6
	3	502.5	71.71	41.28	22.32	26.4
Average						25.9
Cooked A.†	1	499.0	73.75	46.44	22.32	35.9
	2	500.5	73.97	47.88	22.32	34.5
	3	501.2	74.08	47.04	22.32	33.7
Average						34.7
Raw.	4	502.5	71.72	38.04	22.32	22.8
	5	502.3	71.69	36.36	22.32	19.6
Average						21.2
Cooked B.‡	4	499.4	67.22	47.82	22.32	37.9
	5	497.4	66.95	45.42	22.32	34.5
Average						36.2

* Nitrogen 14.27 per cent.

† Cooked ¾ hr. Nitrogen 14.78 per cent.

‡ " 4 hrs. " 13.46 " "

ments were made with them, and total nitrogen was again determined in each case. Also in all further experiments a blank digestion was made with the fresh enzyme solutions used in each set of experiments.

Again, considerable difficulty was encountered in the amino nitrogen determination due to the carbon dioxide given off when the samples were acidified in the deaminizing bulb of the Van

Slyke apparatus. The carbon dioxide was, of course, readily absorbed by the alkaline permanganate solution when the gas was transferred to the Hempel pipette, but on returning the gas to the gas burette the permanganate solution cannot be prevented from coming in contact to some extent with the 1 per cent sul-

TABLE II.

Peptic-Tryptic Digestions in Which the Trypsin Acted in 1/120 N Sodium Hydroxide Solution.

Sample No.		Amount of sample.	Total N in sample (N_t).	Amino N after digestion (N_d).	Amino N of blank digestion (N_b).	Digestion N calculated on total N.
		mg.	*mg.*	*mg.*	*mg.*	*per cent*
Raw.*	6	501.0	71.50	38.88	17.64	29.7
	7	501.7	71.60	37.68	17.64	28.0
	8	501.2	71.52	38.22	17.64	28.8
Average						28.8
Cooked A.†	6	500.6	72.99	47.34	17.64	40.6
	7	500.6	72.99	47.40	17.64	40.8
	8	501.3	73.09	47.34	17.64	40.6
Average						40.7
Raw.	9	500.7	71.45	40.74	20.04	29.0
	10	499.6	71.30	40.92	20.04	29.3
	11	485.7	69.31	40.26	20.04	29.2
Average						29.2
Cooked B.‡	9	499.5	67.23	46.02	20.04	38.6
	10	501.6	67.52	46.32	20.04	38.9
	11	501.5	67.50	46.32	20.04	38.9
Average						38.8

* Nitrogen 14.27 per cent.

† Cooked ¾ hr. Nitrogen, 14.78 per cent.

‡ " 4 hrs. " 13.46 " "

furic acid in this part of the apparatus and there was therefore considerable danger of measuring some carbon dioxide with the purified nitrogen.

In order to avoid this difficulty, the sodium carbonate was replaced with sodium hydroxide in the later experiments. Ham-

marsten (4) states that 1/70 to 1/200 N is the range of optimum concentration of hydroxyl ions for tryptic proteolysis. The 50 cc. of 0.1 N acid in the pepsin digests were neutralized with 5 cc. of N sodium hydroxide and 5 cc. of a 6 per cent solution of trypsin in 0.1 N sodium hydroxide were added. The resulting 60 cc. contained, then, 0.5 per cent of trypsin and the hydroxyl ion concentration was approximately 1/120 N. After a digestion of $2\frac{1}{2}$ hours with the trypsin, the activity of the enzymes was destroyed as before by heating at 80° for 5 minutes, and amino nitrogen determined. The results of these experiments are given in Table II.

The increase in digestibility thus demonstrated seems amply sufficient to explain the findings of Johns and Finks that animals fed raw phaseolin rapidly lost weight and soon died, while those given phaseolin which had been cooked $\frac{3}{4}$ to 4 hours maintained their weight for long periods or even made some gain.

Osborne and Mendel (5), however, found that phaseolin cooked but 5 minutes gave them better results in feeding experiments than were obtained with the raw protein. Accordingly, a preparation was made by boiling phaseolin with distilled water for 5 minutes, drying, grinding, and sieving as before (Preparation C). No gain was shown when the digestion period was the same as that used in the preceding experiments. But on increasing the time of digestion to $2\frac{1}{2}$ hours with pepsin and 5 hours with trypsin, Preparation C showed an amount of proteolysis distinctly higher than that of the raw phaseolin (Table III).

The longer digestion gave, of course, considerably higher figures for the *raw* protein than those found for this material in the first two series of experiments. The results given in Table III are therefore not comparable with those given in Tables I and II.

DISCUSSION.

While the experiments described in this paper did not yield very closely concordant results in all cases, it seems possible that some of the principal sources of error may be eliminated and a method developed which will yield valuable comparative results on the digestibility of proteins. Inasmuch as determinations of digestibility using experimental animals require some months

for completion and, on the other hand, experiments of the sort here described may be carried out in a few days, such a method might be of considerable use to the biological investigator; and work will be continued with this end in view.

Obviously, when comparing proteins from different sources with the purpose of obtaining approximately quantitative results it would be necessary to take into consideration certain factors

TABLE III.

*Digestions of Cooked Phaseolin C, Cooked 5 Min. Digestion Period Increased to 2½ Hrs. with Pepsin, 5 Hrs. with Trypsin.**

Sample No.		Amount of sample.	Total N in sample (N_t).	Amino N after digestion (N_d).	Amino N of blank digestion (N_b).	Digestion N calculated on total N.
		mg.	*mg.*	*mg.*	*mg.*	*per cent*
Raw.	12	500.8	71.48	45.90	20.28	35.8
	13	500.3	71.40	46.32	20.28	36.5
	14	501.0	71.50	46.56	20.28	36.8
Average						36.4
Cooked C.†	12	500.1	68.02	46.98	20.28	40.1
	13	500.9	68.13	47.58	20.28	39.3
	14	500.0	68.14	47.16	20.28	39.5
Average						39.6

* Preparation C did not show a definite difference from the raw phaseolin when the samples were digested 1½ hrs. with pepsin and 2½ hrs. with trypsin as in the previous experiments. The time of digestion was therefore increased, as above stated, and a small gain in digestibility as a result of the 5 min. cooking was demonstrated. The digestion nitrogen figures for the *raw* protein were, of course, considerably greater under these conditions than were those obtained with the shorter digestion period.

† The nitrogen content of Preparation C was 13.60 per cent.

which could be, and were, ignored in the present case where the results obtained were to have qualitative significance only, and the protein preparations compared were of the same source and differed only in treatment. In the first place, proteins of different origin would be likely to have a somewhat different ratio of total amino nitrogen to total nitrogen content. In such a case, to obtain comparable results it would be necessary to base the calculation upon the total amino nitrogen of the protein found

after complete hydrolysis instead of upon the total nitrogen of the protein. Again, all proteins even without hydrolysis show a small percentage of free amino nitrogen. This would have to be determined in each case and subtracted both from the total amino nitrogen and from the amino nitrogen yielded after digestion. With the introduction of these two corrections into the calculation of the results, it is clear that even in its present state the method furnishes a means whereby gross differences in the digestibility of proteins may readily be detected.

SUMMARY.

Preliminary work on a method for the comparison of protein digestibilities is described; the possibility of developing an approximately quantitative method is discussed; and further work outlined.

The experiments indicate that phaseolin is rendered more readily digestible by boiling with distilled water. Cooking for 5 minutes gave a detectable increase in digestibility, while cooking for ¾ hour was apparently sufficient to produce the maximum effect.

The finding of Johns and Finks that phaseolin gave better results in feeding experiments after cooking than did the raw protein is apparently to be explained as due to an increase in digestibility.

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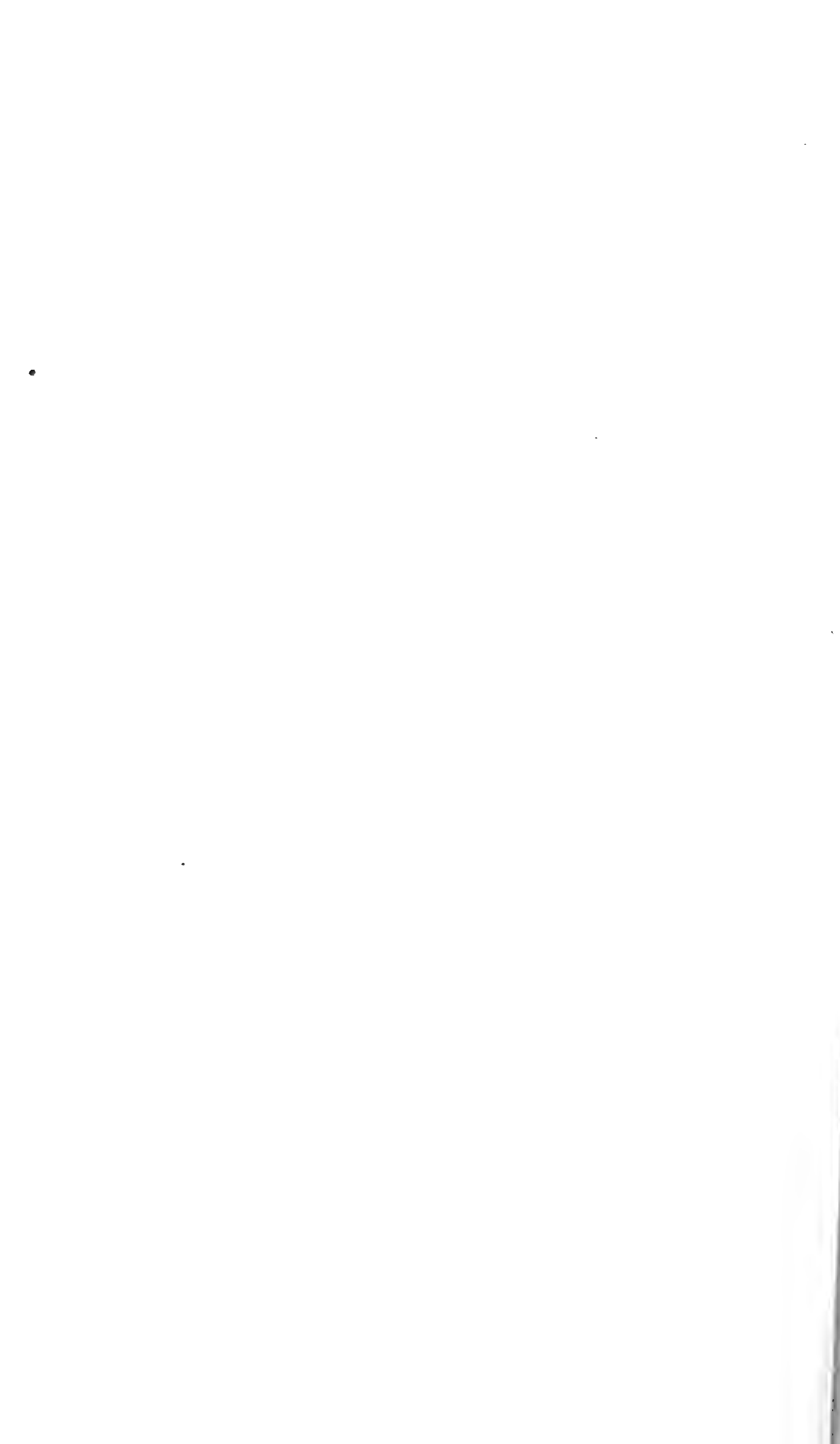

d-RIBOHEXOSAMINIC ACIDS.

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In previous publications[1-4] three pairs of epimeric hexosaminic acids were described. In that series the direction and numerical value of the optical rotation of the α-carbon atom were determined. On the basis of the direction of the rotation of the α-carbon atom all α-hexosaminic acids can be divided into two groups: the dextro and the levo acids.

On addition of prussic acid to a pentose two epimeric hexonic acids are obtained, one form as a rule predominating. On addition of prussic acid to an aminopentoside two epimeric α-hexosaminic acids are obtained, one form predominating. If a given pentose forms predominatingly a levo acid the corresponding aminopentoside also forms the levo-hexosaminic acid in excess over the epimer, and *vice versa*.

This seemed to suggest that in the levo-hexonic and in the levo-α-hexosaminic acids the configuration of the α-carbon atom is analogous, the position of the hydroxyl in one being the same as that of the amino group in the other. Furthermore, it was found that on deamination of the amino-acids the resulting oxy-acids were of the opposite sign. Fischer, on the basis of his extensive studies on Walden inversion, reached the conclusion that the presence of an adjoining carboxyl group is required for the occurrence of the inversion. If this rule holds good also for the α-amino sugar acids, then the observation just referred to offers additional evidence in favor of the assumption that in hexonic acids and in aminohexonic acids of the same sign the configuration of

[1] Levene, P. A., *J. Biol. Chem.*, 1918, xxxvi, 73.
[2] Levene, P. A., and Meyer, G. M., *J. Biol. Chem.*, 1917, xxxi, 623.
[3] Levene, P. A., and La Forge, F. B., *J. Biol. Chem.*, 1915, xx, 433.
[4] Levene, P. A., *J. Biol. Chem.*, 1918, xxxvi, 89.

the α-carbon atom is identical, the position of the hydroxyl in one being the same as that of the amino group in the other.

If the amino sugar or its lactone is deaminized and subsequently converted into the sugar acid, this, as a rule, is of the same sign as the α-aminohexonic acid corresponding to the sugar or the lactone. This observation also is in harmony with Fischer's observations on α-amino-acids. Also, as a rule, there was no inversion in the esters. Exceptions to this rule were observed in the esters of the α-amino-acids as well as in the lactones of the hexosaminic acids.

In the present communication the synthesis of two epimeric acids derived from ribose are reported. Thus the synthesis of the entire series of *d*-hexosaminic acids is completed. The observations made on this new pair agree in every way with the observations made on the other acids, namely:

1. The predominating acid is the levo acid, the same as on the addition of prussic acid to ribose.

2. On deamination of the acids, the change of sign occurs when nitrous acid acts on the acid and does not occur when it acts on the lactone.

The following is a table of the direction of the rotation of the α-carbon atoms in the hexosaminic acid and in their products of deamination.

Acids.	Rotation of the α-carbon.	Deamination product, α-carbon atom.
Chitosaminic	Levo.	Dextro.
Epichitosaminic	Dextro.	Levo.
Chondrosaminic	Levo.	Dextro.
Epichondrosaminic	Dextro.	Levo.
Dextro-xylohexosaminic	"	"
Levo-xylohexosaminic	Levo.	Dextro.
Dextro-ribohexosaminic	Dextro.	Levo.
Levo-ribohexosaminic	Levo.	Dextro.

If one then assumes that the hexosaminic acids and hexonic acids of the same sign have the α-carbon atom of an analogous configuration, then the configuration of the hexosaminic acids and their corresponding sugars are as follows:

A	Chitosaminic (Glucosaminic acid)	acid	..............Manonic	acid.
	Epichitosaminic	"	..............Gluconic	"
B	Dextro-xylohexosaminic	"	..............Gulonic	"
	Levo-xylohexosaminic	"	..............Idonic	"
C	Chondrosaminic	"	..............Talonic	"
	Epichondrosaminic	"	..............Galactonic	"
D	Dextro-ribohexosaminic	"	..............Allonic	"
	Levo-ribohexosaminic	"	..............Altronic	"

Future work may prove that in hexonic and hexosaminic acids of the same sign the configuration of the α-carbon atom is not identical. The observations presented here will nevertheless retain their value, inasmuch as the configuration of all acids of this class will become clear as soon as the configuration of only one of them is definitely established.

There remains still to be mentioned the remarkable identity in the numerical value of the rotation of the α-carbon atom in the hexonic and hexosaminic acids.

The following is a table representing, on one hand, the numerical value of the α-carbon atom of the phenylhydrazides[2] of the hexonic acids and, on the other, of the corresponding hexosaminic acids.

Gluconic Manonic	14.25	Epichitosaminic Chitosaminic	12.5
Gulonic Idonic	14.25	Dextro-xylohexosaminic Levo-xylohexosaminic	12.5
Galactonic Talonic	8.25	Epichondrosaminic Chondrosaminic	12.5
Allonic Altronic	20.8	Dextro-ribohexosaminic Levo-ribohexosaminic	19.12

Only in the pair of galactonic and talonic acids is there noted a great difference in the numerical value of the rotation in the two series. This is probably due to the fact that talonic acid has not been obtained in pure form. This finding also shows that the superposition theory of optical rotation is only acceptable with certain reservations.

The present communication contains also improved directions for the preparation of ribose from yeast nucleic acid.

EXPERIMENTAL.

Preparation of d-Ribose.

The ribose used in these experiments was prepared from the purine nucleosides, guanosin and adenosin, obtained from yeast nucleic acid by ammonia hydrolysis. To prepare these nucleosides 400 gm. of yeast nucleic acid, suspended as a smooth paste in a hot solution of 320 cc. of concentrated ammonia and 1,680 cc. of water, were heated at 145° for 2 hours in an autoclave. After cooling, the solution was removed from the autoclave and placed in the refrigerator over night when a portion of the guanosin separated out. It was filtered off and treated as follows.

The crude guanosin was dissolved in a considerable quantity of boiling water, about 4 per cent solution, and made strongly acid to litmus with acetic acid, after which normal lead acetate was added until no more precipitate was formed. The precipitate was filtered from the boiling solution and the filtrate was then treated alternately with ammonia and basic lead acetate until a drop of either reagent gave no further precipitate. This precipitate was filtered off on a Buchner funnel, ground up in a mortar with water, and then filtered again.

After removing as much water as possible, it was ground up to a smooth paste with hot water, diluted to a considerable volume, made slightly acid with acetic acid, heated to the boiling point, and treated with hydrogen sulfide. When the lead was completely precipitated the solution with the lead sulfide was brought again to a boil and filtered. Upon cooling, guanosin crystallized out. This was filtered off and the mother liquors were evaporated to a small volume when a second crop was obtained.

The crude guanosin thus obtained was purified by repeating the above process with neutral and basic lead acetate, etc., and recrystallizing the resulting product from boiling water with a little charcoal.

To obtain the remaining guanosin and the adenosin, the filtrate from the original hydrolysis was made strongly acid with acetic acid and treated first with normal lead acetate, then with basic lead acetate and ammonia exactly as given above for guanosin.

The filtrate from the treatment with hydrogen sulfide was concentrated to a rather heavy solution. During this operation

more guanosin separates out from time to time. This is filtered off and purified as outlined above. The filtrate was diluted to about 1 liter and made acid to Congo red with sulfuric acid and then treated with a hot concentrated solution of picric acid until no further precipitate was formed. The precipitate was crude adenosin picrate. This was purified by recrystallizing from boiling water.

After the nucleosides were obtained in a pure condition they were hydrolyzed in an acid medium to obtain the sugar. For guanosin the following method was used. 100 gm. of guanosin were dissolved in 10 liters of boiling 0.1 N sulfuric acid and hydrolyzed by gentle boiling for 1 hour. Then to the hot solution an excess of silver sulfate suspended in a little water was added. The solution was allowed to stand over night, after which the guanine silver sulfate compound was filtered off and the clear solution freed from the excess of silver with hydrogen sulfide. The hydrogen sulfide was removed with a current of air and the sulfuric acid with chemically pure barium hydroxide. Great care had to be taken to get the solution exactly neutral to litmus and at no time was it allowed to become alkaline. It was best to add the barium hydroxide slowly in a rather dilute solution testing from time to time with litmus paper.

The barium sulfate was allowed to settle for a short time when most of the solution was drawn off from the precipitate. The remainder was filtered through a Buchner funnel and the combined solutions were then concentrated under reduced pressure at a low temperature to about 800 cc. when it was again made exactly neutral to litmus with dilute barium hydroxide water and filtered. The filtered solution was then concentrated under diminished pressure to a rather thick syrup and taken up in about 500 cc. of absolute alcohol. 300 cc. of ether were then slowly added with constant stirring. The precipitate formed was allowed to settle over night and then the solution was filtered. The filtrate was evaporated to a thick syrup as before and taken up with about 300 cc. of absolute alcohol and again evaporated. This evaporation with alcohol was done three times to remove all water. The last time it was concentrated to about 60 cc., then warmed on a water bath, and removed as completely as possible from the flask by draining about 5 minutes. The residual syrup

was then washed from the flask with 40 cc. of absolute alcohol in small portions and added to the main syrup, making the total volume 100 cc. Dry ether was then added until the syrup became turbid. It was then seeded and allowed to crystallize in a small desiccator. After 2 or 3 days the crystalline mass was broken up and filtered. The sugar was washed with a little absolute alcohol and ether mixture (2 parts alcohol to 1 part ether) then with ether and dried in a desiccator over sulfuric acid. By evaporating the mother liquor and adding ether as outlined above another crop was obtained. The yield is generally 82 to 83 per cent of the theory.

The ribose from the adenosin picrate was obtained as follows. 150 gm. of the recrystallized adenosin picrate were dissolved in 12 liters of boiling distilled water. When solution was complete 70 gm. of 95 per cent sulfuric acid, diluted in about 200 cc. of water, were cautiously added. The hydrolysis was effected by gentle boiling for 1 hour. The solution was then allowed to stand in the refrigerator over night when the adenine picrate crystallized out. This was filtered off and enough dilute barium hydroxide solution to neutralize 60 gm. of the sulfuric acid was slowly added with constant stirring.

The barium sulfate was allowed to settle and as much of the supernatant liquid drawn off as possible, the remainder was filtered from the barium sulfate, and the combined solutions were evaporated under reduced pressure to about 1 liter. This solution was then filtered and extracted with ether to remove the last traces of picric acid. When the extraction was completed the aqueous solution was diluted to about 4 liters and cautiously neutralized with barium hydroxide, then it was evaporated to about 800 cc. and again made exactly neutral to litmus with barium hydroxide. The further treatment was the same as in the preparation from guanosin.

The ribose remaining in the mother liquors from the second crop of sugar in all experiments was recovered by forming the *p*-bromophenylhydrazone. To this end an aliquot portion of the liquors was titrated and the calculated amount of *p*-bromophenylhydrazine added. After a short time the hydrozone began to crystallize out. It was then filtered off and recrystallized from a small amount of absolute alcohol and decomposed in

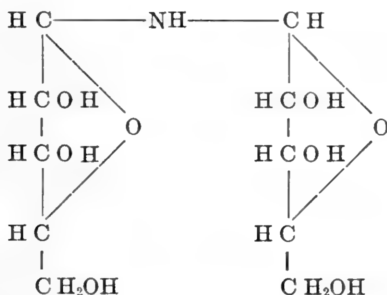
the ordinary way with benzaldehyde. This procedure, however, would hardly be expedient unless the mother liquors from a comparatively large amount of material were used, as relatively little sugar remains after the second crop.

The ribose was recrystallized by dissolving in distilled water, making a 10 per cent solution, and decolorizing with a little charcoal. It was then evaporated to a thick syrup, taken up several times in absolute alcohol, and evaporated so as to remove all the water, as outlined above, and then crystallized.

About 1.5 kilos of ribose have been made by this method with good and uniform results.

Preparation of Amino-d-Riboside.

This substance has been prepared and analyzed previously by Levene and La Forge.[3] It was found in the course of the present work that in order to obtain the maximum yield of the pure substance care must be exercised as to the dryness of the methyl alcohol and of the pentose. When either one of the substances is imperfectly dried the resulting product contains a certain proportion of the dipentose derivative, of the structure:



Under favorable conditions the yield of the amino derivative is between 90 and 95 per cent of the theory.

Preparation of the Dextro- and Levo-d-Ribohexosaminic Acids.

As in the preparation of other α-hexosaminic acids, so also in the preparation of these two acids the yield depends on the temperature of the reaction, on the proportion of prussic acid,

and on the duration of the reaction between prussic acid and the aminopentoside. Optimal yields of 60 to 65 per cent of the employed ribose were obtained only at the very end of the work.

The optimal conditions are as follows. 20.0 gm. of the aminopentoside dried under diminished pressure at room temperature were dissolved in 40.0 cc. of water and 30 cc. of crude freshly prepared prussic acid (of 80 per cent strength) and 5 cc. of ammonia water were added. The solution was rapidly warmed to 30°C. and kept at that temperature for 15 minutes. The temperature should be watched very carefully. The further treatment is similar to that in the preparation of other *d*-hexosaminic acids.

When only 20 cc. of prussic acid were added instead of 30.0 cc., the yield of acids did not exceed 40 per cent and often did not reach that value.

The crystallization of the mixed acid is brought about in the following way. The product of reaction prepared for crystallization is made up with water to 25 cc. and to this hot methyl alcohol is added to marked opalescence. The solution is then placed on a water bath and stirred with a glass rod until the appearance of a crystalline deposit.

The optical rotation of the mixed acids was as follows:

$$[\alpha]_D^{20} = \frac{-0.16 \times 100}{1 \times 2} = -8.0^\circ$$

Separation of the Two Acids.

A. Levo-d-Ribohexosaminic Acid.

Levo-*d*-ribohexosaminic acid is the more insoluble form and is prepared without much difficulty. Two experiments are here reported.

Experiment 1.—57.0 gm. of the mixed acids were dissolved in 70.0 cc. of boiling water and allowed to crystallize over night. The yield of the crystalline deposit was 22.0 gm. This substance had the following optical rotation (No. $638\frac{1}{20}$):

$$[\alpha]_D^{20} = \frac{-0.52 \times 100}{1 \times 2} = -26.0^\circ$$

This rotation indicates that the substance was the pure levo form. On recrystallization out of water two fractions were

obtained (No. 640, 641$\frac{19}{20}$) each having the same rotation, and as will be shown later the substance prepared from the pure crystalline lactone possesses the same optical rotation.

Experiment 2.—142.0 gm. of the mixed acids were dissolved in 300 cc. of boiling water. 35.0 gm. of the levo form crystallized over night. The rotation of the substance was as follows (No. 88$\frac{20}{21}$):

$$[\alpha]_D^{20} = \frac{-0.52 \times 100}{1 \times 2} = -26.0°$$

On further recrystallization the rotation of the substance did not change.

Properties of the Levo-d-Ribohexosaminic Acid.—The substance crystallizes in thin plates resembling those of cholesterol. It is soluble in water and insoluble in the usual organic solvents. The melting point of the substance is M. P. = 212°C. (uncorrected) with decomposition.

Lactone Hydrochloride of the Levo-d-Ribohexosaminic Acid.—12.0 gm. of the acid carefully dried and pulverized were suspended in 600 cc. of alcohol (99.5 per cent) and dry hydrochloric acid gas was passed for 15 minutes. The acid dissolved almost immediately. The solution was concentrated under diminished pressure at ordinary temperature until crystallization took place in the distillation flask. The contents were then transferred to an evaporating dish and allowed to stand over night in a desiccator over sulfuric acid. The yield of the lactone was 12.0 gm. The melting point of the substance was M. P. = 188°C. (uncorrected). It had the following composition (No. 24$\frac{20}{21}$):

0.1076 gm. of the substance gave 0.1304 gm. of CO_2 and 0.0592 gm. of H_2O.

0.2000 gm. of the substance employed for Kjeldahl nitrogen estimation required for neutralization 8.74 cc. of 0.1 N acid.

0.2000 gm. of the substance required 9 cc. of 0.1 N $AgNO_3$ to titrate its hydrochloric acid.

	Calculated for $C_6H_{12}NO_5Cl$. per cent	Found. per cent
C	33.71	33.05
H	5.67	6.17
N	6.55	6.12
Cl	16.25	15.96

The substance still contained 0.46 per cent of mineral impurity. The peculiarity of this lactone was that by the Van Slyke method only 4.71 per cent of N was obtained, even when the reaction was allowed to proceed for 30 minutes.

The rotation of the substance was the following:

$$[\alpha]_D^{20} = \frac{-0.22 \times 100}{1 \times 2} = -11.0^\circ$$

Conversion of the Lactone into Free Acid.—5.0 gm. of the lactone hydrochloride were dissolved in 50 cc. of water, the solution was treated with an excess of barium hydroxide, and allowed to stand over night. The barium and hydrochloric acid were removed from the solution and the free acid was crystallized on concentration of the aqueous solution. For analysis it was recrystallized out of water on addition of a little alcohol. After three recrystallizations the substance was analyzed. The melting point was M. P. = 212°C. (uncorrected). It analyzed as follows:

0.1010 gm. of the substance gave 0.1364 gm. of CO_2 and 0.1010 gm. of H_2O.

0.1990 gm. of the substance employed for Kjeldahl nitrogen estimation required for neutralization 10.10 cc. of 0.1 N acid.

	Calculated for $C_6H_{13}NO_5$. *per cent*	Found. *per cent*
C	36.92	36.83
H	6.66	7.04
N	7.18	7.10

The rotation of the substance was the following:

$$[\alpha]_D^{20} = \frac{-0.52 \times 100}{1 \times 2} = -26.0^\circ$$

B. Dextro-d-Ribohexosaminic Acid.

The separation of the dextro form was found more difficult than that of its epimer, and was accompanied with considerable loss of material. The procedure finally adopted was as follows. All fractions with the optical rotation above 0.0° were combined, dissolved in about 5 to 8 volumes of hot water, and methyl alcohol was added to initial opalescence. The solution was then placed on a boiling water bath and there allowed to crystallize.

The crystalline deposit was filtered off while the mother liquor was still hot. The operation was repeated until a constant rotation was obtained. This was found to be as follows:

$$[\alpha]^{20}_{D} = \frac{+0.25 \times 100}{1 \times 2} = +12.5°$$

After three recrystallizations the rotation remained unchanged. As will be seen later the substance obtained from the lactone possessed the same optical activity. The substance had the melting point M.P. = 186°C. (uncorrected) and analyzed as follows:

0.1057 gm. of the substance gave 0.1422 gm. of CO_2 and 0.0626 gm. of H_2O.

0.2000 gm. of the substance employed for Kjeldahl nitrogen estimation required for neutralization 10.20 cc. of 0.1 N acid.

	Calculated for $C_6H_{13}NO_6$. *per cent*	Found. *per cent*
C	36.92	36.70
H	6.66	6.63
N	7.18	7.14

Lactone Hydrochloride of Dextro-d-Ribohexosaminic Acid.—5.0 gm. of the acid carefully dried under diminished pressure at 50°C. and pulverized were suspended in 300 cc. of absolute alcohol (99.5 per cent) and dry hydrochloric acid gas was passed through the alcohol. Solution was accomplished almost immediately. The gas was passed 7 minutes. The solution was then concentrated under diminished pressure until a considerable sediment began to form in the distilling flask. The material was then transferred to an Erlenmeyer flask. The sediment on standing increased in volume, but was found to be amorphous. On heating, however, the sediment redissolved and on prolonged standing on the water bath with stirring a sediment of heavy crystals of the lactone settled out.

The substance had the melting point M.P. = 150°C. (uncorrected) and analyzed as follows:

0.0977 gm. of the substance gave 0.1212 gm. of CO_2 and 0.0546 gm. of H_2O.

0.1872 gm. of the substance employed for Kjeldahl nitrogen estimation required for neutralization 8.85 cc. of 0.1 N acid.

	Calculated for $C_6H_{12}NO_5Cl$. *per cent*	Found. *per cent*
C	33.71	33.83
H	5.67	6.25
N	6.55	6.62

The rotation of the substance was as follows:

$$[\alpha]_D^{20} = \frac{+0.43 \times 100}{1 \times 2} = +21.5^\circ$$

Conversion of the Lactone into the Dextro-d-Ribohexosaminic Acid.—Three grains of the lactone hydrochloride were dissolved in 25 cc. of water. The solution was rendered alkaline by means of barium oxide and allowed to stand over night. The barium and the hydrochloric acid were then removed and the remaining aqueous solution was concentrated to a small volume. To the concentrated solution alcohol was added to opalescence, and the solution was allowed to digest on a boiling water bath until a heavy crystalline deposit formed. The yield of this substance was 2.0 gm.

The melting point of the substance was M.P. = 186°C. (uncorrected). The rotation was as follows:

$$[\alpha]_D^{20} = \frac{+0.25 \times 100}{1 \times 2} = +12.5^\circ$$

Thus the melting point and the optical rotation of the substance purified through conversion into its lactone and reconversion of this into the acid remained identical with those of the original material.

Dibenzal-Dextro-d-Ribohexosaminic Ethyl Ester Hydrochloride.

The dextro form differed from its epimer in that it formed the above compound under the same condition in which the levo form gave rise to its lactone hydrochloride. 2.0 gm. of the carefully dried and pulverized acid were suspended in 20.0 cc. of absolute alcohol (99.5 per cent) to which 2.0 cc. of redistilled benzaldehyde were added, and dry hydrochloric acid gas was passed through the solution. The acid dissolved rapidly, but the treatment with acid was continued for 7 minutes. The slightly

turbid solution was allowed to stand over night. A crystalline deposit consisting microscopically of long needles was formed. It was filtered off, washed with alcohol and ether, dried, and analyzed. The substance (No. 232) had a melting point M.P. = 221°C. (uncorrected) and the following composition:

0.1069 gm. of the substance gave 0.3270 gm. of CO_2 and 0.0602 gm. of H_2O.

0.1978 gm. of the substance employed for Kjeldahl nitrogen estimation required for neutralization 4.60 cc. of 0.1 N acid.

	Calculated for $C_{22}H_{26}NO_6Cl$. *per cent*	Found. *per cent*
C	60.65	60.47
H	6.34	6.31
N	3.20	3.25

The rotation of the substance dissolved in methyl alcohol was the following:

$$[\alpha]_D^{20} = \frac{-0.26 \times 100}{1 \times 1} = -26.0°$$

From the mother liquor of the dibenzal derivative on standing a second crop of crystals formed, which once recrystallized had the composition and the physical properties of the lactone hydrochloride of the dextro-*d*-ribohexosaminic acid. This observation is important inasmuch as it offers additional evidence of the purity of the dextro-*d*-ribohexosaminic acid.

The substance analyzed as follows:

0.0942 gm. of the substance gave 0.1174 gm. of CO_2 and 0.0512 gm. of H_2O.

0.0918 gm. of the substance employed for Kjeldahl nitrogen estimation required for neutralization 4.5 cc. of 0.1 N acid.

	Calculated for $C_6H_{12}NO_5Cl$. *per cent*	Found. *per cent*
C	33.71	33.99
H	5.67	6.08
N	6.55	6.86

The optical rotation of the substance in 2.5 per cent hydrochloric acid was

$$[\alpha]_D^{20} = \frac{+0.43 \times 100}{1 \times 2} = +21.5°$$

Oxidation of the d-Ribohexosaminic Acids with Nitric Acid.

Levo-d-ribohexosaminic acid on oxidation with nitric acid gave rise to α,α-anhydroallomucic acid. Two 5.0 gm. portions of the acid were dissolved each in 35 cc. of water; to the solution 15 cc. of 10 per cent hydrochloric acid and 5.0 gm. of silver nitrite were added. The mixture was allowed to react over night. Twice during the reaction 2.0 gm. portions of the nitrite and 2 cc. of hydrochloric acid were added. The reaction product was freed from excess of silver and the filtrate reduced to a volume of 75 cc. by distillation under diminished pressure at 40–50°C. of the water bath. To this solution 50 cc. of nitric acid were added and the resulting solution was heated over flame for 20 minutes and then rapidly concentrated to a thick mass on a water bath. The thick residue was dissolved in 10 cc. of a solution consisting of equal parts of water and concentrated nitric acid, then evaporated once with water to remove nitric acid. The final residue was then converted into the calcium salt of α,α-anhydroallomucic acid. For final analysis the salt was suspended in water and its calcium removed by boiling in hot water containing a slight excess over the required amount of oxalic acid.

The calcium salt of α,α-anhydroallomucic differs from that of anhydrotalomucic, first by being optically inactive and second by the difference in behavior on heating. Both salts crystallize with 3 molecules of crystal water. Heated under diminished pressure at the temperature of xylene vapors anhydroallomucic salt loses all its 3 molecules of crystal water, whereas the corresponding anhydrotalomucic salt loses only 2 retaining the third one.

The Ca salt of anhydroallomucic acid analyzed as follows:

0.1228 gm. of the salt on drying lost 0.0244 gm. in weight.

	Calculated for $C_6H_6O_7Ca + 3\ H_2O$. *per cent*	Found. *per cent*
H_2O	19.02	19.87

0.0984 gm. of the substance gave on combustion 0.11 gm. of CO_2, 0.0270 gm. of H_2O, and 0.0984 gm. of CaO.

	Calculated for $C_6H_6O_7Ca$. *per cent*	Found. *per cent*
C	31.30	30.99
H	2.61	3.07
CaO	24.35	25.00

Dextro-d-ribohexosaminic acid on oxidation gave rise to α, α-anhydrotalomucic acid. 5.0 gm. of the substance were deaminized in the same manner as the levo form. For oxidation the solution was brought to 60 cc., to which an equal volume of nitric acid was added. The resulting solution was heated over free flame for 13 minutes. The product of reaction was transferred to clock glasses and rapidly concentrated nearly to dryness. The residue was reoxidized once more with a solution of equal parts of water and nitric acid, and finally once evaporated with water to remove nitric acid. The residue was then converted into Ca salt. The yield was 2.8 gm. For purification the salt was reconverted into free acid by means of oxalic acid and reconverted into the Ca salt. The pure salt crystallized partly in plates and partly in prismatic needles. Heated under diminished pressure at temperature of xylene vapors it lost 2 molecules of water. The salt was levorotary.

It analyzed as follows:

0.1216 gm. of the substance on drying lost 0.0160 gm. in weight.

	Calculated for $C_6H_8O_8Ca + 2\ H_2O$.	Found.
	per cent	*per cent*
H_2O	12.68	13.16

0.1056 gm. of the substance gave 0.1120 gm. of CO_2, 0.0302 gm. of H_2O, and 0.0242 gm. of CaO.

	Calculated for $C_6H_6O_7Ca + H_2O$.	Found.
	per cent	*per cent*
C	29.03	28.98
H	3.22	3.20
CaO	22.58	22.92

The optical rotation of the substance was the following:

$$[\alpha]_D^{20} = \frac{-0.18 \times 100}{1 \times 2} = -9.0^\circ$$

CONTRIBUTIONS TO THE BIOCHEMISTRY OF IODINE.*

IV. THE EFFECT OF THYROXIN ON GROWTH IN WHITE RATS AND IN RABBITS.

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INTRODUCTION.

In Paper III of this series (Cameron and Carmichael, 1920–21) we gave the results of experiments which proved that the administration of thyroid to growing rats and rabbits caused a decrease in the rate of growth; we also confirmed the observations of Hoskins and of Herring that such treatment caused hypertrophy of certain organs, especially the heart, liver, kidneys, and adrenals, and that of Herring, that the thyroid gland itself is relatively decreased. Our experiments showed also that the administration of sodium iodide in much larger relative doses produced no such effect. Since E. R. Hoskins had previously shown that feeding with thymus, pineal, or pituitary does not produce these effects, while R. G. Hoskins noted after adrenal administration only hypertrophy of testes (and ovaries), we suggested that the three factors mentioned, decrease of total growth rate, hypertrophy of heart, liver, kidneys, and adrenals, and decrease of thyroid growth rate, could be used as a rigid test for preparations alleged to be the essential thyroid secretion.

We have now subjected to this test thyroxin, a definite crystalline compound isolated from thyroid tissue in 1915 by Kendall, and subsequently shown by him to be a tryptophane derivative with the formula

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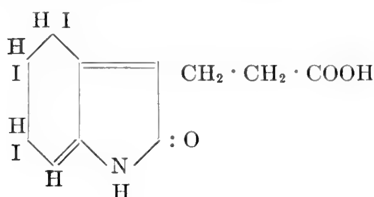

and containing 65 per cent of iodine.

Kendall and others have already advanced considerable clinical evidence that thyroxin is the essential compound of the thyroid secretion. This will be outlined later, along with the discussion of our own results; these show that thyroxin produces both decrease of growth rate and hypertrophy of the body organs, with a less pronounced decrease in the thyroid growth rate.

EXPERIMENTAL RESULTS.

The thyroxin used was purchased from E. R. Squibb and Sons. Two analyses, on 2.25 and 2.43 mg., gave respectively 62 and 63 per cent of iodine. The solution gave the color test with nitrite and ammonia described by Kendall. The thyroxin under the microscope appeared to resemble the enol form. The preparation was considered to be practically pure thyroxin.

In a preliminary experiment an endeavor was made to use a stock solution, kept in the dark. The experiment led to no definite results indicating the rapid decomposition of dilute solutions to which Kendall has drawn attention. Subsequently from 2 to 5 mg. of thyroxin crystals were weighed out accurately every day on a balance weighing to 0.05 mg. (except on a few occasions when one solution was used on 2 consecutive days), suspended in water, brought into solution by the addition of 1 drop of 10 per cent sodium hydroxide, and made up to a definite volume, usually 10 cc.

The doses were based, as in our previous experiments, on the actual daily body weight of each animal. For rats, at first each dose, measured from a graduated pipette, was made up into a paste with flour and fed on a watch-glass. This did not prove sufficiently appetizing for prolonged treatment (contrary to the iodide experiments, when the paste was always immediately

eaten), so that subsequently the solution was dropped on a piece of bread, and the moistened bread given on a watch-glass. This we found to be eaten immediately and completely in all cases. The error of feeding by this method was certainly less than 10 per cent.

The dose selected in the first experiment was chosen for an iodine content comparable to that in the minimal thyroid dose capable of producing distinct effects. As the effect produced was not marked, in the succeeding experiments the dose was gradually increased.

The remaining experimental details were precisely similar to those used previously by us when feeding thyroid and iodide, and described in Paper III. The diet consisted always of unlimited bread and milk.

In order to facilitate reference the experiments, tables, and figure are numbered consecutively with those in Paper III. The details follow.

Experiment 14.—Litter of twelve rats, six male and six female, born Sept. 23rd, 1920. Two of each sex were used as controls, two were fed thyroxin at the rate of $1:4 \times 10^6$ of body weight, and two at the rate of $1:10^6$ of body weight. Treatment was commenced on the 53rd day. The thyroxin solution was fed in flour paste. From the 62nd day Rats 5 and 11, which had commenced to take the paste badly, were given the solution soaked up in bread and ate this satisfactorily; similarly, Rat 9 was fed in this manner from the 68th day. The rats were killed on the 19th day of treatment, and the organs dissected and transferred immediately to stoppered glass vessels and weighed. The figures for body and organ weights are given in Tables XX and XXI.

Experiment 15.—Litter of six rats, four male and two female, born Oct. 5th, 1920. One of each sex was used as control, one male was fed thyroxin at the rate of $1:10^6$ of body weight, and the remaining animals at the rate of $1:0.4 \times 10^6$ of body weight. Treatment was commenced on the 55th day. Up to the 62nd day thyroxin was fed in flour paste. As Rats 2 and 3 then commenced to take it badly in this form, it was subsequently given to all four treated rats with bread. The thyroids of Rats 1 and 2 were transferred to corrosive sublimate, and after hardening were sectioned and stained with hematoxylin-eosin for histological examination. The figures for body and organ weights are given in Table XXII.

Experiment 16.—Litter of eight rats, four male and four female, born Oct. 19th, 1920. Two of each sex were kept as controls and two fed thyroxin with bread at the rate of $1:0.2 \times 10^6$ of body weight. Treatment was commenced on the 53rd day. The figures for body and organ weights are given in Tables XXIII and XXIV.

TABLE XX.

Male Rats.

Age.	Rat 1. Control.	Rat 2. Control.	Average control.	Rat 3. Thyroxin 1:4 × 10⁶.	Rat 4. Thyroxin 1:4 × 10⁶.	Average. Thyroxin 1:4 × 10⁶.	Rat 5. Thyroxin 1:10⁶.	Rat 6. Thyroxin 1:10⁶.	Average. Thyroxin 1:10⁶.
days	*gm.*	*gm.*	*per cent*	*gm.*	*gm.*	*per cent*	*gm.*	*gm.*	*per cent*
47	59	56		55	45		55	52	
50	67	55		56	46		61	57	
53	85	70		74	58		73	75	
56	95.5	81		82	69		81	83	
59	100	92		89	78		84	90	
62	108	101		96	83		97	97	
65	114	111		104	86		103	100	
68	128	120		117	96		116	110	
71	140	133		124	107		122	115	
72	141	133		125	112		122	114	
Weight increase.									
In 19 days	56	63		51	54		49	39	
Percentage	(66)	(90)	(78)	(69)	(93)	(81)	(67)	(52)	(59)
Weight of organs.									
Liver	9.1	8.7		7.0	6.9		8.7	9.0	
Kidneys	1.43	1.29		1.37	1.13		1.48	1.61	
Heart	0.54	0.56		0.70	0.65		0.77	0.72	
Testes	2.02	1.62		1.76	1.11		1.45	1.28	
Spleen	0.516	0.390		0.636	0.510		0.702	0.546	
Adrenals	0.041	0.034		0.045	0.032		0.040	0.041	
Thyroid, fresh	0.0141	0.0130		0.0115	0.0126		0.0095	0.0146	
" dry	0.0034	0.0037		0.0036	0.0035		0.0027	0.0042	

	per cent	per cent		per cent	per cent		per cent	per cent	
Liver	6.5	6.5	6.5	5.6	6.2	5.9	7.1	7.9	7.5
Kidneys	1.01	0.97	0.99	1.09	1.01	1.05	1.22	1.41	1.31
Heart	0.38	0.42	0.40	0.56	0.58	0.57	0.63	0.63	0.63
Testes	1.43	1.22	1.32	1.41	0.99	1.20	1.19	1.12	1.15
Spleen	0.37	0.29	0.33	0.51	0.45	0.48	0.57	0.48	0.52
Adrenals	0.029	0.025	0.027	0.036	0.029	0.032	0.033	0.036	0.034
Thyroid, fresh	0.0100	0.0098	0.0099	0.0092	0.0112	0.0102	0.0078	0.0128	0.0103
" dry	0.0024	0.0028	0.0026	0.2009	0.0031	0.0030	0.0022	0.0037	0.0029

TABLE XXI.

Female Rats.

Age.	Rat 7. Control.	Rat 8. Control.	Average control.	Rat 9. Thyroxin 1:4 × 10⁶.	Rat 10. Thyroxin 1:4 × 10⁶.	Average. Thyroxin 1:4 × 10⁶.	Rat 11. Thyroxin 1:10⁶.	Rat 12. Thyroxin 1:10⁶.	Average. Thyroxin 1:10⁶.
days	*gm.*	*gm.*	*per cent*	*gm.*	*gm.*	*per cent*	*gm.*	*gm.*	*per cent*
47	54	44.5		52.5	53		49	55	
50	61.5	47		55.5	53		50.5	60	
53	77	60		74	64		65	73	
56	84	68.5		81	74		75	83	
59	93	75		87	82		80	88	
62	101	87		96	91		87	93	
65	108	94.5		104	99		92.5	95	
68	116	103		115	110		100	105	
71	122	109		121	115		106	109	
72	123	109		119.5	117		110	109	
Weight increase.									
In 19 days	46	49		45.5	53		45	36	
Percentage	(60)	(82)	(71)	(61)	(83)	(72)	(69)	(50)	(59)
Weight of organs.									
Liver	5.6	6.9		9.3	8.1		8.4	8.1	
Kidneys	1.14	1.05		1.54	1.29		1.25	1.40	
Heart	0.56	0.53		0.62	0.66		0.71	0.70	
Spleen	0.411	0.408		0.533	0.543		0.578	0.556	
Adrenals	0.046	0.043		0.054	0.035		0.037	0.035	
Thyroid, fresh	0.0111	0.0132		0.0112	0.0092		0.0122	0.0115	
" dry	0.0031	0.0025		0.0028	0.0028		0.0033	0.0031	

	per cent	per cent		per cent	per cent		per cent	per cent	
Liver	4.6	6.3	5.4	7.7	6.9	7 3	7.7	7.5	7.6
Kidneys	0.92	0.96	0.94	1.29	1.10	1.19	1.14	1.29	1.21
Heart	0.46	0.48	0.47	0.52	0.56	0.54	0.64	0.64	0.64
Spleen	0.33	0.37	0.35	0.45	0.46	0.45	0.52	0.51	0.51
Adrenals	0.037	0.039	0.038	0.045	0.030	0.037	0.034	0.032	0.033
Thyroid, fresh	0.0089	0.0121	0.0105	0.0094	0.0078	0.0086	0.0111	0.0105	0.0108
" dry	0.0023	0.0023	0.0023	0.0023	0.0024	0.0023	0.0030	0.0028	0.0029

TABLE XXII.

Age.	Male.					Female.	
	Rat 1. Control.	Rat 2. Thyroxin 1:0.4 × 10⁶.	Rat 3. Thyroxin 1:0.4 × 10⁶.	Average.	Rat 4. Thyroxin 1:10⁶.	Rat 5. Control.	Rat 6. Thyroxin 1:0.4 × 10⁶.
days	*gm.*	*gm.*	*gm.*	*per cent*	*gm.*	*gm.*	*gm.*
52	44	53	50		42	45	46
55	61	74	67		56	65	63
58	74	82	80		67	74	75
61	81	89	85		69	80	77
64	96	98	91		76	92	83
67	108	108.5	104		87	102	96
70	116	116	116		94	105	106
73	127	123	127		114	113	110
Weight increase.							
In 18 days	66	49	60		58	48	47
Percentage	(108)	(66)	(90)		(104)	(74)	(75)
Weight of organs.							
Liver	9.0	10.1	9.7		9.5	7.8	11.5
Kidneys	1.29	1.86	1.75		1.61	1.26	1.70
Heart	0.62	0.99	0.98		0.87	0.61	0.88
Testes	1.80	1.88	1.75		1.67		
Spleen	0.449	0.724	0.701		0.751	0.410	0.737
Adrenals	0.022	0.030	0.031		0.029	0.036	0.042
Thyroid, fresh	0.0125	0.0098	0.0125		0.0089	0.0131	0.0119
	per cent	*per cent*	*per cent*		*per cent*	*per cent*	*per cent*
Liver	7.1	8.2	7.7	7.9	8.3	6.9	10.4
Kidneys	1.02	1.51	1.38	1.44	1.41	1.11	1.54
Heart	0.49	0.80	0.77	0.78	0.76	0.54	0.80
Testes	1.42	1.53	1.38	1.45	1.46		
Spleen	0.35	0.59	0.55	0.57	0.66	0.36	0.67
Adrenals	0.017	0.024	0.024	0.024	0.025	0.032	0.038
Thyroid, fresh	0.0098	0.0080	0.0098	0.0089	0.0078	0.0116	0.0102

TABLE XXIII.

Male Rats.

Age.	Rat 1. Control.	Rat 2. Control.	Average control.	Rat 3. Thyroxin 1:0.2×10⁶.	Rat 4. Thyroxin 1:0.2×10⁶.	Average. Thyroxin 1:0.2×10⁶.
days	*gm.*	*gm.*	*per cent*	*gm.*	*gm.*	*per cent*
53	57	63		59	63	
56	64	69		57.5	66	
59	72.5	79		58	69.5	
62	70	80		55	67	
65	75	86		60	70	
68	84	94		62	77	
71	93	105		63	79.5	
Weight increase.						
In 18 days........	36	42		4	16.5	
Percentage.......	(63)	(67)	(65)	(7)	(26)	(16)
Weight of organs.						
Liver..............	5.5	6.2		6.1	7.7	
Kidneys............	0.93	1.06		1.15	1.30	
Heart..............	0.46	0.54		0.55	0.61	
Testes.............	0.91	1.19		0.84	0.78	
Spleen.............	0.270	0.275		0.179	0.271	
Adrenals...........	0.019	0.20		0.023	0.022	
Thyroid, fresh......	0.0098	0.0086		0.0055	0.0075	
" dry.......	0.0027	0.0025		0.0018	0.0024	
Right gastrocnemius muscle...........	0.175	0.203		0.108	0.130	
	per cent	*per cent*		*per cent*	*per cent*	
Liver..............	5.9	5.9	5.9	9.7	9.7	9.7
Kidneys............	1.00	1.01	1.00	1.83	1.64	1.74
Heart..............	0.49	0.51	0.50	0.87	0.77	0.82
Testes.............	0.98	1.13	1.05	1.33	0.98	1.15
Spleen.............	0.29	0.26	0.27	0.28	0.34	0.31
Adrenals...........	0.020	0.019	0.019	0.037	0.028	0.032
Thyroid, fresh......	0.0105	0.0082	0.0093	0.0087	0.0094	0.0090
" dry.......	0.0029	0.0024	0.0026	0.0028	0.0030	0.0029
Right gastrocnemius muscle...........	0.188	0.193	0.190	0.171	0.164	0.167

Experiment 17.—Three males of a litter of four rabbits born Sept. 30th, 1920. Two were fed thyroxin at the rate of 1:10⁶ of body weight and the third was used as a control. Feeding was commenced on the 53rd day. At first the thyroxin solution was given with bread. No. 1 took this satis-

factorily, but No. 2 took it badly in this form, and to this animal the solution was given by allowing a small amount of green food to absorb it. This green food was eaten completely but the error of feeding was probably

TABLE XXIV.

Female Rats.

Age.	Rat 5. Control.	Rat 6. Control.	Average control.	Rat 7. Thyroxin $1:0.2 \times 10^6$.	Rat 8. Thyroxin $1:0.2 \times 10^5$.	Average. Thyroxin $1:0.2 \times 10^6$.
days	*gm.*	*gm.*	*per cent*	*gm.*	*gm.*	*per cent*
53	57	66		58	69	
56	66	76		61	70	
59	73	85		62	73	
62	72.5	88.5		60	74	
65	81	94		64	76	
68	82	99		70	81	
71	90	108		81	87.5	
Weight increase.						
In 18 days	33	42		13	18.5	
Percentage	(58)	(64)	(61)	(22)	(27)	(25)
Weight of organs.						
Liver	5.8	6.3		6.4	8.5	
Kidneys	0.86	1.03		1.11	1.31	
Heart	0.43	0.53		0.56	0.66	
Spleen	0.234	0.359		0.255	0.363	
Adrenals	0.022	0.027		0.019	0.026	
Thyroid, fresh	0.0068	0.0123		0.0050	0.0081	
" dry	0.0021	0.0033		0.0018	0.0027	
Right gastrocnemius muscle	0.184	0.219		0.103	0.136	
	per cent	*per cent*		*per cent*	*per cent*	
Liver	6.5	5.8	6.1	9.0	9.7	9.3
Kidneys	0.96	0.95	0.95	1.56	1.50	1.53
Heart	0.48	0.49	0.48	0.79	0.75	0.77
Spleen	0.26	0.33	0.29	0.36	0.41	0.38
Adrenals	0.024	0.025	0.024	0.027	0.030	0.028
Thyroid, fresh	0.0076	0.0114	0.0095	0.0070	0.0093	0.0081
" dry	0.0023	0.0031	0.0027	0.0025	0.0031	0.0028
Right gastrocnemius muscle	0.204	0.203	0.203	0.145	0.155	0.150

distinctly greater than in the case of the rats. The diet was the same as that in Experiment 13. Rabbit 1 was found dead on the 66th day. No cause could be definitely assigned except the thyroxin feeding. This ani-

mal was dissected and the organs were weighed at once, though partial drying had taken place. The others were killed and examined on the 72nd day. The figures for body and organ weights are given in Table XXV.

TABLE XXV.

Age.	Rabbit 1. Thyroxin 1: 10^6.	Rabbit 2. Thyroxin 1: 10^6.	Rabbit 3. Control.
days	*gm.*	*gm.*	*gm.*
53	465	628	485
54	493	639	529
57	462	657	563
60	420	650	608
63	366	661	687
66	(341)	648	700
69		672	673
72		681	738
Weight increase. In 19 days		53	253
Weight of organs.			
Liver	16.0	40.5	50.3
Kidneys	5.42	8.22	7.35
Heart	1.90	3.31	1.95
Testes	0.066	0.259	0.262
Spleen	0.228	0.290	0.567
Adrenals	0.129	0.143	0.172
Thyroid		0.057	0.057
	per cent	*per cent*	*per cent*
Liver	4.7	5.9	6.8
Kidneys	1.59	1.21	1.00
Heart	0.56	0.49	0.26
Testes	0.019	0.038	0.035
Spleen	0.067	0.043	0.077
Adrenals	0.038	0.021	0.023
Thyroid		0.0084	0.0077

General Observations.

Thyroxin-fed rats showed distinctly less fat than the control animals, but the loss of fat was certainly never so great as that in rats fed thyroid in the ration of 1:5,000 of body weight.

The thyroids of treated rats were always paler in color than those of controls; this we have remarked previously on thyroid-fed animals. The thyroid of Rat 2, Experiment 15, presented

the same histological appearance as those from rats fed thyroid or iodide. The vesicles were swollen with colloid, indicating a resting condition of the gland.

There was marked increase of lymphatic tissue. This was observed in each experiment in the superficial glands of the neck. More complete observations were made in Experiment 16; the lymphatic tissue in the neighborhood of the axillæ was also found to be greatly hypertrophied. Professor William Boyd, of the Department of Pathology of this University, kindly examined the neck glands and also the spleens of Rats 1 to 4 of this series; there was no difference between the tissue from controls and from treated animals; all were normal.

Not only does the heart appear larger in rats treated with thyroxin, but the ventricular walls are actually thickened, so that the exsected heart shows greater resiliency to pressure. The same is true of thyroid-fed rats (*cf.* for example Herring).

All the rats fed thyroxin appeared normal throughout the experiments;[1] there was no change in the appearance of the hairy coat, and no condition resembling tetany occurred in any of these rats.

DISCUSSION.

Our results show conclusively that thyroxin produces a distinct decrease in growth rate, and hypertrophy of liver, kidneys, heart, spleen, and adrenals. The effect on the testes was less certain.

The results for rabbits are in general agreement with those for rats, though the number of animals fed was too few for stress to be laid on them.

In Table XXVI we have endeavored to compare the relative effects of thyroxin and desiccated thyroid when fed to rats. Unfortunately, for direct comparison we can only use our own figures, since not only is comparison with control animals of the same litter required, but also a constant ratio of dosage to body weight. The figures for thyroid-fed animals, from Paper III of this series, are obtained from comparisons of single animals;

[1] One exception may be noted. Paraphimosis was observed in Rats 3 and 4, Experiment 15. It was not remarked in Experiment 16. In Paper III, p. 83, line 2 from bottom and p. 85, lines 3 and 17, *stricture* should read *paraphimosis*.

those in Experiments 14 and 16 with thyroxin are obtained from the mean figures for two animals.

In spite of the few figures available, and the fact that comparison has to be made on animals from different litters, the figures for percentage of normal increase of body weight show a steady decrease for increased thyroid dosage, and a decrease, not so regular, for increased thyroxin dosage for the same period of

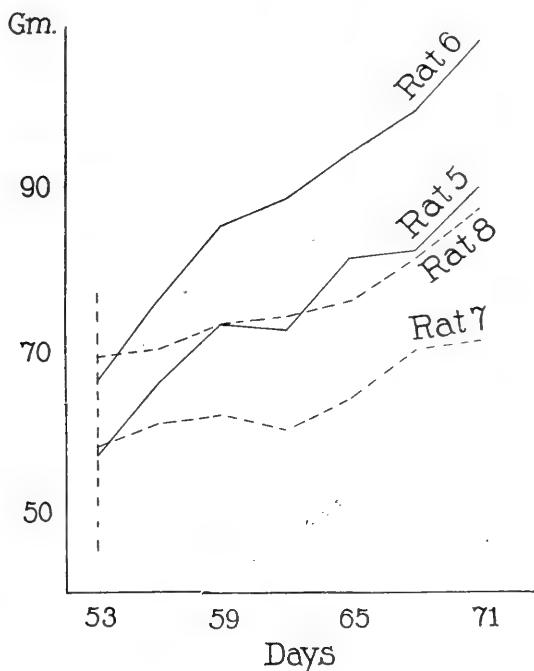

Fig. 3. Experiment 16. The continuous lines represent weights of female rats dosed with thyroxin in the ratio of 1:200,000 of body weight; the dotted lines those for control rats.

Thyroxin feeding was commenced where indicated by the vertical dotted line.

feeding. If comparison is made between the two series, using the iodine content as basis, it will be seen that thyroxin produces relatively less effect, a dose of thyroid containing the same amount of iodine producing from two to four times the effect.

Fig. 3, for female rats of Experiment 16, fed thyroxin in iodine ratio of $1:0.3 \times 10^6$ of body weight, shows a very similar curve

TABLE XXVI.

Comparative Action of Desiccated Thyroid and of Thyroxin.

Experiment No.	Duration.	Treatment (ratio to body weight).	Iodine.	Iodine dose (ratio to body weight).	Percentage of normal increase of body weight.	Percentage hypertrophy of				
						Liver.	Kidneys.	Heart.	Spleen.	Adrenals.
Male rats.										
	days		*per cent*							
2	18	Thyroid, 1:20,000	0.39	$1:5 \times 10^6$	79					
4	18	" 1:20,000	0.39	$1:5 \times 10^6$	72					
4	36	" 1:20,000	0.39	$1:5 \times 10^6$	55	75	44	32	19	
6	18	" 1:5,000	0.34	$1:1.5 \times 10^6$	53					
5	17	" 1:5,000	0.34	$1:1.5 \times 10^6$	38	66	29	14	19	56
6	26	" 1:5,000	0.34	$1:1.5 \times 10^6$	45	39	59	71	72	93
4	18	" 1:5,000	0.39	$1:1.2 \times 10^6$	37					
5	17	" 1:5,000	0.39	$1:1.2 \times 10^6$	33	96	55	33	31	62
6	18	" 1:5,000	0.39	$1:1.2 \times 10^6$	32					
6	26	" 1:5,000	0.39	$1:1.2 \times 10^6$	18	74	190	80	24	357
14	19	Thyroxin, $1:4 \times 10^6$	65	$1:6 \times 10^6$	104	−9	6	42	46	19
14	19	" $1:10^6$	65	$1:1.5 \times 10^6$	76	15	32	57	61	26
15	18	" $1:10^6$	65	$1:1.5 \times 10^6$	96	17	38	55	88	47
15	18	" $1:0.4 \times 10^6$	65	$1:0.6 \times 10^6$	72	11	41	59	63	41
16	18	" $1:0.2 \times 10^6$	65	$1:0.3 \times 10^6$	25	65	73	64	15	68

Female rats.

2	18	Thyroid, 1:20,000	0.39	1:5 × 10⁶	83					
4	18	" 1:20,000	0.39	1:5 × 10⁶	83					
4	36	" 1:20,000	0.39	1:5 × 10⁶	78	34	22	18	19	
7	18	" 1:5,000	0.34	1:1.5 × 10⁶	68	50	51	23	38	29
4	18	" 1:5,000	0.39	1:1.2 × 10⁶	6					
7	18	" 1:5,000	0.39	1:1.2 × 10⁶	67	34	47	25	38	25
14	19	Thyroxin, 1:4 × 10⁶	65	1:6 × 10⁶	101	35	26	15	29	−3
14	19	" 1:10⁶	65	1:1.5 × 10⁶	83	39	29	36	49	−18
15	18	" 1:0.4 × 10⁶	65	1:0.6 × 10⁶	101	51	39	48	86	19
16	18	" 1:0.2 × 10⁶	65	1:0.3 × 10⁶	41	61	60	60	31	17

slope to Fig. 2 (Paper III) for rats fed thyroid in the iodine ratio of $1:1.2 \times 10^6$ of body weight, and very similar curves are also given by the figures for males of this experiment.

The percentage figures for hypertrophy of the various organs show less regularity and are more difficult of analysis. Those for liver are especially irregular (this is partly due to the fact that in the experiments described in Paper III the livers were weighed after blood had drained from them, while in those described in this paper they were transferred immediately to closed vessels and weighed with the greater part of their normal blood content). Nevertheless the same general conclusion can be drawn.

The figures for spleen (thyroxin-fed rats) seem to suggest that there is a certain optimum dose for hypertrophy of this organ; this point is being further investigated.

The figures for thyroid feeding of duration longer than 18 days support the conclusion put forward in Paper III that the degree of hypertrophy increases with increased time of treatment.

While the thyroids of the thyroxin-fed rats tend to be slightly smaller, the effect is distinctly less marked than for thyroid-fed rats; the same is true, as we have stated above, for loss of body fat.

Our results show, therefore, that qualitatively thyroxin and thyroid produce the same effects, as far as our three factors of comparison are concerned, but quantitatively, on the basis of iodine content, the effect of thyroxin is not so marked.

This can perhaps be attributed to bacterial action. Since tryptophane is so easily acted on by intestinal bacteria, it is not unlikely that thyroxin is similarly decomposed, while thyroid protein, undergoing normal digestion, may act in a protective capacity, the thyroxin liberated being absorbed as fast as it is set free.

If comparison is made on the basis of Kendall's hypothesis (1919,*a*) that only one-fourth of the thyroid iodine is in combination in the thyroxin radical, the quantitative difference is accentuated, and leads to the conclusion that either bacterial action on thyroxin fed as such is pronounced, or that the other iodine-containing radicals present in thyroid can produce similar effects on growth, etc.

In any case our results seem to suggest that thyroxin can be controlled best in clinical use by injection.

The marked hypertrophy of certain organs, accompanied by a relative decrease of total weight greater than fat loss can account for, suggests lessened development of such tissue as muscle. Measurements of the right gastrocnemius muscle in the rats of Experiment 16 show that there was a decrease of from 20 to 25 per cent.

The Essential Compound Secreted by the Thyroid.

In order to show that any preparation is the essential secretion of the thyroid gland, we can only compare its clinical, metabolic, and physiological actions with those of thyroid tissue itself. The tests available include the specific effects on myxedema and cretinism, the toxic effects of large doses (tachycardia, etc.), the increase of nitrogen excretion, and the test that we have established in this and the third paper of this series.

Kendall has shown that thyroxin has the same action as thyroid on myxedema and cretinism. Plummer (see Kendall, 1919,*c*) has shown that it, like thyroid, markedly increases the basal metabolism, and Kendall has obtained all the toxic effects produced by large doses of thyroid with large doses of thyroxin. Janney has confirmed Kendall's results as to the toxic effects of large doses, and the beneficial effects on cretinism, and has shown that the nitrogenous excretion is markedly increased. He has further shown that similar effects are produced whether the thyroxin is administered by mouth or by injection.

To this convincing testimony we are now able to add a further rigorous parallelism in the comparative action of large doses on growth and in producing hypertrophy of certain organs.

Hence thyroxin answers satisfactorily to all the tests to which it can be subjected, and is undoubtedly the essential chemical compound (autacoid, hormone) secreted by the thyroid gland.

Comparative Conditions in Hyperthyroidism and after Thyroxin Administration.

Numerous observers have pointed out the similarity in the conditions produced by feeding large doses of thyroid, *i.e.* of increasing the circulating thyroxin in the body, and those occurring in exophthalmic goiter. We have not found any references

drawing attention to a parallelism as regards hypertrophy of body organs with the exception of the heart. In exophthalmic goiter, according to MacCallum, the heart is frequently hypertrophied but there are no obvious changes in the other organs. MacCallum also states that "the lymph-glands, especially in the neck, are often markedly enlarged. In some cases this increase in the bulk of the lymphoid tissue occurs throughout the body, even the solitary nodules in the intestine projecting as gray prominences."

The hypertrophy of the heart (and other organs) following administration of thyroid or thyroxin, and that of the lymphoid tissue to which we have especially drawn attention in this paper, while affording a striking parallelism, have been observed with young growing rats, so that an absolute comparison cannot be made. We are at present commencing experiments with adult rats to see if the parallelism holds true for these.

SUMMARY.

Using decrease of growth rate in growing rats, hypertrophy of heart, liver, kidneys, and adrenals, and decrease of growth rate of thyroid as tests, thyroxin, when administered by mouth, produces the same qualitative effects as does desiccated thyroid.

Quantitatively, when compared on a basis of iodine content, the effects of thyroxin are distinctly less. This is probably due to bacterial decomposition; thyroid acts as a shield.

The hypertrophy of heart and lymphatic tissue resembles that observed in cases of hyperthyroidism.

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COMPARATIVE RESULTS WITH SCALES' METHOD AND DEVARDA'S ALLOY FOR REDUCING NITRIC NITROGEN.

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(Received for publication, January 8, 1921.)

The determination for nitric nitrogen has always been the subject of much investigation. The literature is flooded with various methods and their modifications, none of which is perfectly satisfactory, and most of which are troublesome.

Perhaps of all methods, Devarda's[1] has most rapidly gained favor with many chemists, especially since W. S. Allen[2] presented data showing the accuracy of the method. Nitric nitrogen is reduced in alkaline solution by the action of Devarda's alloy, the composition of which is about 50 per cent of aluminum, 45 per cent of copper, and 5 per cent of zinc.

E. R. Allen[3] made an exhaustive study of several Devarda modifications, as well as of the aluminum reduction method, proposing finally the use of the Valmari-Mitscherlich-Devarda modification, developed by himself. The method, recommended for soil extracts, was described for use with the Mitscherlich apparatus,[4] somewhat modified, a direct air-cooled distillation.

Snyder and Potter[5] found that a modified form of the Devarda procedure, after Allen,[3] was satisfactory for the determination of nitrate nitrogen in soil extracts. They used the usual water-cooled block tin distillation apparatus, taking 2 gm. of alloy and 2 cc. of saturated sodium hydroxide.

[1] Devarda, A., *Z. anal. Chem.*, 1894, xxxiii, 113.

[2] Allen, W. S., *Orig. Com. 8th Internat. Cong. Appl. Chem.*, 1912, i, 19.

[3] Allen, E. R., *J. Ind. and Eng. Chem.*, 1915, vii, 521.

[4] Mitscherlich, E. A., and Herz, P., *Landw. Jahresb.*, 1909, xxxviii, 279.

[5] Snyder, R. S., and Potter, R. S., *Soil Science*, 1918, vi, 441.

But even the method of Devarda has its disadvantages, and any method that tends toward making a nitric nitrogen determination simpler deserves consideration.

Scales[6] published a method employing a zinc-copper couple as the reducing agent, easily and quickly prepared from ordinary scrap zinc and a solution of copper sulfate acidified with sulfuric acid. Common salt is used as the electrolyte and magnesium oxide as the alkali, 1 gm. of oxide being sufficient for reduction, at the same time not strong enough to break down the organic matter of soil solutions, or chemicals easily broken down by strong alkali. Scales showed the accuracy of his method, but did not compare it with any of those in ordinary use. It is to be noted that a reduction method taking place in as weak an alkali as magnesium oxide and producing accurate results, has many commendations.

The author of this paper has used a slightly modified form[7] of the Scales procedure successfully for some time, and believes it desirable to bring out the advantages of the method, especially as it is equally as accurate as the recognized Devarda determination. Scales originally described a special apparatus for use with his couple, enabling the distillation to be carried on satisfactorily without a condenser. However, the regular Kjeldahl rack and condenser may be used. In fact, if the distillate is collected in boric acid,[8] some form of condenser *must* be used, as it is necessary to keep the distillate at room temperature.

As the work of Snyder and Potter[5] was somewhat similar to some of that carried on in this laboratory, it was desirable to compare the Devarda method with the Scales procedure as used here.

A solution containing roughly 0.1 mg. of nitrogen per cc. was prepared and 200 cc. portions were accurately measured into Kjeldahl flasks. To one set, 3 gm. of Devarda's alloy were added, then 2 cc. of saturated sodium hydroxide and the flasks connected

[6] Scales, F. M., *J. Biol. Chem.*, 1916, xxvii, 327.

[7] The difference being in the sodium chloride-magnesium oxide mixture (5 to 1.5 to compensate for an acid extract), and the use of the ordinary Kjeldahl block tin condenser instead of the special apparatus.

[8] Scales, F. M., and Harrison, A. P., *J. Ind. and Eng. Chem.*, 1920, xii, 350.

up to the apparatus and distilled. Another set was prepared by pouring the solution into flasks containing about 80 gm. of the freshly prepared zinc-copper couple, then adding 6 gm. of a mixture of sodium chloride and magnesium oxide, 5 to 1—which may be simply measured into the flasks—and then proceeding with the distillation as before.

The distillate, 150 cc., was collected in flasks containing 50 cc. of 4 per cent boric acid,[8] 200 cc. in all, taking 45 minutes to distill over. 1 drop of brom-phenol blue[9] was added to each distillate, and the titration carried on by artificial light, using N/14 sulfuric acid. A set of ten flasks was run for both methods, and controls were run for the reagents employed. The data are given in Table I.

Both methods are accurate to practically the same degree.

The controls may seem a little high but the correction for the indicator and boric acid throughout the table has not been made. This correction amounts to about 0.35 cc. of N/14 sulfuric acid, making the actual nitric nitrogen content of the reagents equivalent to 0.57 and 0.37 cc. respectively.

The chief advantages of the zinc-copper couple method over the Devarda, are convenience and (because there is no necessary weighing of reagents) a saving of time. After the couple has once been used it may be rinsed, and left in the flask for the next determination. For use, 150 cc. of the copper sulfate solution[6] are left over the metal for about 10 minutes, rinsed once with cold water, the sample is poured in, then a measured 6 gm. quantity of the salt-magnesia mixture added, and the distillation carried on.

The zinc may be used without renewal for several months, for as the zinc wears away approximately the same amount of surface is always exposed and there is thus no loss in the effect of the couple. The author has used the same zinc from March to August before a renewal was necessary, but it was not in constant use.

The salt and oxide mixture may be measured into the flasks roughly.

[9] The brom-phenol blue was prepared as described,[8] diluting only one-third as high.

In the Devarda procedure, the alloy must be weighed each time, and the sodium hydroxide measured carefully as more alloy must be used for strong alkali.[3] The excess alloy is thrown away when the determination is over.

TABLE I.

N/14 Sulfuric Acid Required to Recover the Nitric Nitrogen in 200 Cc. Solution of Sodium Nitrate.

	Devarda's alloy.	Zinc-copper couple.
	cc.	*cc.*
Control on reagents.		
1	0.93	0.83
2	0.86	0.64
3	0.92	0.66
4	0.98	0.74
Total	3.69	2.87
Average	0.92	0.72
200 cc. $NaNO_3$.		
1	19.70	19.68
2	19.81	19.68
3	19.80	19.52
4	19.72	19.50
5	19.86	19.67
6	19.74	19.73
7	19.88	19.59
8	19.78	19.72
9	19.93	19.69
10	19.74	19.49
Total	197.96	196.27
Average	19.80	19.63
Average control	0.92	0.72
Difference	18.88	18.91

CONCLUSION.

The reduction of nitrates by a zinc-copper couple as suggested by Scales is as reliable as by Devarda's alloy, is more convenient, and, as no weighing of reagents is necessary, the determination may be completed sooner.

THE ALKALI RESERVE OF THE BLOOD OF CERTAIN OF THE LOWER VERTEBRATES.

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(Received for publication, December 28, 1920.)

INTRODUCTION.

The alkali reserve of mammalian blood has been a subject of much investigation. The carbon dioxide content and combining power of the plasma and body fluids of certain marine fish and invertebrates have recently been determined (1). There are reported in this communication the results of determinations of the carbon dioxide content and combining power of whole blood and blood plasma of certain representative forms of the lower vertebrates. The carbon dioxide content of the various specimens was determined after they had been equilibrated with room air, while the carbon dioxide-combining power was determined after equilibration with alveolar air of the normal human subject. The apparatus of Van Slyke (2) was used and the carbon dioxide determined by absorption with 10 per cent sodium hydroxide. The blood was collected by bleeding from the carotid artery except in the case of the frog, where the sample was obtained from the femoral artery after the leg had been completely severed.

Results.

The results are expressed in Table I. The figures represent cc. of CO_2, measured at 0°, 760 mm., per 100 cc. of blood or plasma.

DISCUSSION.

As the results may be taken as an index of the alkali reserve of the blood of the forms investigated, it is evident that there is a considerable degree of variation in this factor in different verte-

TABLE I.

Source.	Specimen.	CO_2 Equilibrated with atmospheric air.	CO_2 Equilibrated with alveolar air.
		vol. per cent	*vol. per cent*
Pigeon	Whole blood.	26.8	35.6
"	" "	25.3	36.5
"	" "	41.7	58.3
Duck, domestic	Plasma.	48.2	65.3
Goose, "	"	38.9	51.9
Turkey, "	"	52.5	69.7
Hen, "	Whole blood.	31.5	46.3
" "	Plasma.	36.0	49.5
Prairie chicken	"	38.9	74.8
Duck, mallard	"	40.5	
Hawk	"	28.2	
Guinea fowl	"	46.3	58.4
Turtle	Whole blood.	70.5	89.3
"	" "	65.1	81.8
Frog (*Rana pipiens*), 1 specimen (4 months in tank)	" "		44.5
Frog, 3 specimens (4 months in tank)	" "	24.9	39.2
" 3 " out of water some days	Plasma.	48.6	60.3
Frog, 3 specimens, in tank 2 weeks.	"	37.8	49.5
Frog, 1 specimen (Submerged 9 days)	"	14.4	
" 1 " (Submerged 9 days)	"	14.4	
" 1 " (Submerged 9 days)	"	22.5	

brate types as well as fairly wide range of variation in the blood of different members of the same species. It would also follow from the application of the Henderson equation

$$\frac{\text{Free } CO_2}{\text{Combined } CO_2} = K$$

that the carbon dioxide tension in the alveolar air of the lower vertebrates must vary greatly in different forms. The relatively high alkali reserve of the blood of the turtle is noteworthy, as is also the relatively low value found for certain birds. The great decrease in the alkali reserve of the blood of the frog following submersion is also of interest. The acid-base transfer between red cells and plasma emphasized by Van Slyke and Cullen (3)

was noted also for lower vertebrate blood. Plasma separated from whole blood equilibrated with atmospheric air, and saturated with alveolar air of the normal human subject, contained less carbon dioxide than plasma separated from whole blood previously equilibrated with alveolar air. Also plasma separated from whole blood, which had been equilibrated with atmospheric air, contained less carbon dioxide than plasma, similarly equilibrated with atmospheric air after separation from whole blood equilibrated with alveolar air. An example of this phenomenon is in Table II.

TABLE II.

Specimen.	CO_2
	vol. per cent
Hen's whole blood, Sample 1, equilibrated with alveolar air then centrifuged.	
Plasma from above equilibrated with alveolar air......	59.4
" " " " " atmospheric air...	45.0
Hen's whole blood, Sample 2, equilibrated with atmospheric air then centrifuged.	
Plasma from above equilibrated with alveolar air......	49.5
" " " " " atmospheric air...	36.0

SUMMARY.

1. The carbon dioxide content and combining power of whole blood and blood plasma of certain of the lower vertebrates have been determined.

2. There is considerable variation in the alkali reserve, both of the different species and of different members of the same species.

3. An acid-base transfer between the red cells and the plasma with varying carbon dioxide tension is manifested in lower vertebrate blood.

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THE ACID-BASE EXCHANGE BETWEEN THE PLASMA AND THE RED BLOOD CELLS.

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INTRODUCTION.

Gürber (1) noted that the titratable alkali of the blood plasma could be increased by saturating the whole blood with CO_2. He was of the opinion that the increase in the alkalinity of the plasma under these circumstances was due to the passage of hydrochloric acid from the plasma into the cells, the amount of this latter substance thus entering the cells being equivalent to the gain in the titratable alkali in the plasma. Hamburger (2) has shown that there is some transfer of sodium and potassium between the cells and plasma. Van Slyke and Cullen (3) were able to show a decrease in the chlorine content of the plasma with a concomitant increase in the bicarbonate content, by treating whole blood with CO_2. They also demonstrated an increase in plasma chloride, and a decrease in plasma bicarbonate when whole blood was equilibrated with air. As the change in the concentration of bicarbonate in the plasma was out of proportion to the concomitant change in the concentration of chloride except when the CO_2 tension of the blood was altered within physiological limits, Van Slyke and Cullen (3) concluded that acid not only passes back and forth between the plasma and cells with fluctuating CO_2 tension, but that base is also transferred. However, they are of the opinion that a transfer of hydrochloric acid between red cells and plasma may be sufficient to account for the difference noted in the CO_2-combining power of plasma separated from venous blood on the one hand and arterial on the other. De Boer (4) has shown that treating whole blood with CO_2 causes a

decrease in the sulfate of the plasma. Van Slyke and Cullen (3) also demonstrated that the CO_2 capacity of plasma is greater than that of the whole blood from which it is derived and therefore greater than that of the corpuscles. Joffe and Poulton (5) found that plasma or serum always had a higher CO_2 capacity than the respective corpuscles in the case of both oxygenated and reduced blood. In the latter instance, however, both serum or plasma and corpuscles had a greater CO_2-combining power than in the former. The effect of the reduction of the blood was, they showed, to increase the CO_2-combining power of the whole blood but this increase was most marked in the case of the corpuscles. They attributed this to the fact that oxyhemoglobin has more marked acid properties than reduced hemoglobin and thus competes to a greater degree with the CO_2 for the sodium than the reduced hemoglobin. They are, therefore, in agreement with the work of Christiansen, Douglas, and Haldane (6), Parsons (7), and L. J. Henderson (8). Collip (9) has extended the observation of Van Slyke and Cullen (3) that mammalian plasma has a higher CO_2 content than the corresponding cells, to the blood of lower vertebrates. Van Slyke and Cullen (3), Parsons (7), Campbell and Poulton (10), and others are of the opinion that the combined CO_2 of normal blood is held entirely as bicarbonate, the proteins acting as weak acids and competing with the CO_2 for the sodium. Mellanby and Thomas (11) have recently obtained results which have led them to conclude that the bicarbonate hypothesis is inadmissible. They support the view of Bohr (12) and Buckmaster (13) that a considerable portion of the CO_2 is in combination with hemoglobin and other blood proteins. They also conclude that the transport of CO_2 in the body is effected by protein and not by $NaHCO_3$. These authors do not believe that an exchange of anions such as Cl or of the cations such as Na or K between the red cells and serum can be demonstrated with ease and certainty, and they have explained the decrease in the CO_2-combining power of serum separated from whole blood from which CO_2 has been removed as being due to the production of lactic acid by the red cells. They were able to demonstrate a marked increase in the lactic acid content of the blood when it was freed of CO_2. The production of lactic acid by the corpuscles would therefore, they concluded, be the chief

factor causing the expulsion of the CO_2 from the blood when it is exposed to a vacuum.

As it is possible for one to demonstrate such marked changes in the CO_2 capacity of the plasma and corpuscles by exposing whole blood to varying CO_2 tensions, it would appear that the recently advanced hypothesis of Mellanby and Thomas (11) is insufficient to explain for example a 139.3 per cent increase in the CO_2-combining power of plasma as a result of exposing whole blood to a high CO_2 tension (see Experiment II, Table I), and many other examples of greatly altered CO_2-combining power of blood plasma such as are set down in this paper.

Methods.

Whole blood, oxalated or defibrinated, was equilibrated with CO_2 at different tensions. The plasma or serum was then separated from the cells by centrifuging and pipetted at once into a dry container. The separated plasma or serum was then exposed to CO_2 at various tensions and its CO_2-combining power determined by use of the Van Slyke apparatus (14). The evolved CO_2 was absorbed by the use of 10 per cent NaOH. Correction was made for the CO_2 physically dissolved and the results recorded therefore represent the CO_2 held as bicarbonate under the different experimental conditions.

As Mellanby and Thomas (11) in a recent paper, which came to our notice while this work was in progress, make use of the method of ashing serum separated from whole blood at definite CO_2 tension in order to determine the total available alkali, the method of ashing a definite amount of serum, blood, or cells, and determining the CO_2-combining power of the solution of the ash was also employed. In the experiments in which this latter method was followed the material to be studied was first air oven-dehydrated in a platinum crucible and then cautiously ashed at a dull red heat. The ash was extracted with a volume of distilled water equal to that of the fluid originally used. The extract of the ash was shaken for 2 minutes in a large separating funnel with a mixture of air and CO_2. The fluid was then equilibrated with atmospheric air which was introduced into the separating funnel by the use of the filter pump, and the bicarbonate content determined by the use of the Van Slyke apparatus.

TABLE I.

Experiment No. and source of blood.	Specimen analyzed.	CO_2 as bicarbonate at 0°C. and 760 mm.	Remarks.
		vol. per cent	
I. Turtle.	1. Plasma.	80.0	Equilibrated with alveolar air. Separated from whole blood equilibrated with alveolar air.
	2. "	71.1	Equilibrated with atmospheric air. Separated from whole blood equilibrated with alveolar air.
	3. "	67.4	Equilibrated with alveolar air. Separated from whole blood equilibrated with atmospheric air.
	4. "	57.6	Equilibrated with atmospheric air. Separated from whole blood equilibrated with atmospheric air.
II. Ox.	1. Whole blood.	38.0	Equilibrated with atmospheric air.
	2. Serum.	45.0	" " " " Separated from above blood.
	3. Cells.	31.4	" " "
	4. Serum.	61.2	Equilibrated with alveolar air. Separated from above blood.
	5. Cells.	67.4	" " "
	6. Whole blood.	68.5	Equilibrated with alveolar air.
	7. Serum.	81.8	" " " " Separated from above blood.
	8. "	67.5	Equilibrated with atmospheric air. Separated from above blood.
	9. Cells.	56.9	Equilibrated with alveolar air. Separated from above blood.
	10. Serum.	107.7	Equilibrated with atmospheric air. Separated from whole blood saturated with CO_2 after 24 hrs. on ice.

III. Rabbit.	1. Whole blood.	34.2	Equilibrated with atmospheric air.
	2. Plasma.	39.6	Equilibrated with atmospheric air. Separated from No. 1.
	3. Cells.	27.0	Equilibrated with atmospheric air. Separated from No. 1.
	4. Plasma.	53.9	Equilibrated with alveolar air. Separated from No. 1.
	5. Whole blood.	68.3	Equilibrated with alveolar air.
IV. Rabbit.	1. Whole blood.	30.6	Equilibrated with atmospheric air.
	2. Plasma.	30.6	Equilibrated with atmospheric air. Separated from No. 1.
	3. Plasma.	47.6	Equilibrated with alveolar air. Separated from No. 1.
	4. Whole blood.	63.8	Equilibrated with alveolar air.
	5. Plasma.	70.1	Equilibrated with alveolar air. Separated from No. 4.
	6. Plasma.	57.4	Equilibrated with atmospheric air. Separated from No. 4.
V. Dog.	1. Whole blood.	17.1	Equilibrated with atmospheric air. Animal under ether.
	2. Plasma.	17.1	Equilibrated with atmospheric air.
	3. Plasma.	26.9	Equilibrated with alveolar air. Separated from No. 1.
	4. Whole blood.	41.3	Equilibrated with alveolar air.
	5. Plasma.	44.9	Equilibrated with alveolar air. Separated from No. 4.
VI. Hen.	1. Serum.	54.8	Equilibrated with alveolar air. Separated from whole blood equilibrated with alveolar air.
	2. Serum.	45.0	Equilibrated with atmospheric air. Separated from above blood.
	3. Serum.	44.9	Equilibrated with alveolar air. Separated from whole blood equilibrated with atmospheric air.
	4. Serum.	36.0	Equilibrated with atmospheric air. Separated from above blood.
VII. Ox.	1. Serum.	30.1	Equilibrated with atmospheric air. Separated from whole blood equilibrated with atmospheric air.
	2. Serum.	61.9	Equilibrated with atmospheric air. Separated from whole blood partially saturated with CO_2.

TABLE I—*Continued.*

Experiment No. and source of blood.	Specimen analyzed.	CO_2 as bicarbonate at 0°C. and 760 mm.	Remarks.
		vol. per cent	
VII. Ox (*Concluded*).	3. Whole blood.	37.4	Equilibrated with alveolar air.
	4. " "	37.4	" " " " Saturated with CO_2, then with atmospheric air, finally with alveolar air.
	5. " "	25.5	Equilibrated with atmospheric air.
VIII. Dog.	1. " "	22.4	" " " " Dog under morphine bled from femoral vein.
	2. " "	21.6	Equilibrated with atmospheric air. No. 1 on ice 24 hrs.
	3. Serum.	23.4	" " " " Separated from No. 2.
	4. Cells.	19.7	" " " " Calculated from Nos. 2 and 3. Cells 49 per cent; serum 51 per cent.
	5. Serum.	34.1	Equilibrated with alveolar air. Separated from No. 2.
	6. Whole blood.	43.1	" " " "
	7. Serum.	49.4	" " " " Separated from No. 6.
	8. Cells.	36.6	Calculated from Nos. 6 and 7.
	9. Serum.	81.8	Equilibrated with alveolar air. Separated from whole blood saturated with CO_2.
	10. Cells.	31.4	Equilibrated with alveolar air. Separated from whole blood saturated with CO_2. Cells 51 per cent; serum 49 per cent.
	11. Whole blood.	42.1	Equilibrated with alveolar air. 2 days on ice.
IX. Sheep.	1. " "	52.4	Equilibrated with alveolar air.
	2. Serum.	59.2	" " " " Separated from No. 1.
	3. Whole blood.	29.7	" " atmospheric air.
	4. Serum.	85.5	" " alveolar air. Separated from whole blood staturated with CO_2.

X. Ox.	1. Serum.	42.5	Equilibrated with atmospheric air. Separated from whole blood equilibrated with atmospheric air.
	2. "	97.8	Equilibrated with atmospheric air. Separated from whole blood saturated with CO_2.
XI. Ox.	1. "	46.1	Equilibrated with atmospheric air. Separated from whole blood after 24 hrs. on ice.
	2. "	87.9	Equilibrated with atmospheric air. Separated from whole blood previously saturated with CO_2.
	3. Solution of ash of No. 1.	72.7	CO_2-combining power of ash serum; volume of solution equal to volume of serum ashed.
	4. " " " " " 2.	107.0	" " "
	5. " " " " " 2.	61.2	CO_2-combining power of solution of ash of cells separated from whole blood previously saturated with CO_2 (see No. 2 above).
	6. " " " " " 2.	79.1	CO_2-combining power of solution of ash of whole blood.
XII. Sheep.	1. Fresh defibrinated whole blood.	43.7	Blood aspirated from jugular vein into flask defibrinated as collected.
	2. Whole blood.	64.7	Equilibrated with alveolar air.
	3. " "	30.2	CO_2-free air pumped through for 1 hr. after collecting.
	4. " "	58.8	No. 3 equilibrated with alveolar air.
	5. Serum.	77.3	Equilibrated with atmospheric air. Separated from whole blood saturated with CO_2.
	6. Solution of ash.	116.6	Solution of ash of No. 5 in distilled water.
	7. " " "	49.5	" " " " cells from which No. 5 was separated.
	8. " " "	75.6	" " " " whole blood.
	9. Serum.	44.7	Separated from whole blood 6 hrs. after drawing.

TABLE I—*Concluded.*

Experiment No. and source of blood.	Specimen analyzed.	CO_2 as bicarbonate at 0°C. and 760 mm.	Remarks.
		vol. per cent	
XII. Sheep (*Concluded*).	10. Solution of ash.	66.8	Solution of ash of No. 9.
	11. " " "	82.3	" " " " cells from which Serum 9 was separated.
	12. Serum.	78.2	Equilibrated with atmospheric air. Separated from whole blood partially saturated with CO_2.
	13. Solution of ash.	99.2	Solution of ash of No. 12.
	14. " " "	62.6	" " " " cells from which Serum 12 was separated.
XIII. Ox.	1. Serum.	40.8	Equilibrated with atmospheric air. Separated from whole blood 48 hrs. in ice chest in open beaker.
	2. "	97.7	Equilibrated with atmospheric air. Separated from whole blood after 3 hrs. exposure to CO_2.
	3. Solution of ash.	73.7	Solution of ash of No. 1.
	4. " " "	127.7	" " " " " 2.
	5. " " "	84.9	" " " " cells from which Serum 1 was separated.
	6. " " "	42.5	" " " " " " " " 2 " "
	7. " " "	78.0	" " " " whole blood.
XIV. Turkey.	1. Serum.	38.3	Equilibrated with atmospheric air. Separated from whole blood equilibrated with atmospheric air.
	2. "	47.9	Equilibrated with alveolar air. Same sample as No. 1.
	3. "	78.3	" " atmospheric air. Separated from whole blood saturated with CO_2.
	4. "	95.7	Equilibrated with alveolar air. Same sample as No. 3.
	5. Solution of ash.	60.1	Solution of ash of No. 1.
	6. " " "	102.7	" " " " " 3.

Different types of blood were used. As a general rule no anesthetic was administered prior to bleeding. The blood samples were oxalated in some instances, and defibrinated in others. Only defibrinated blood was used in the experiments in which ashing was carried out.

The electrical conductivity of serum was determined in two instances by the Kohlrausch method.

Results.

The results of a number of experiments designed to demonstrate the acid-base transfer between cells and plasma are shown in Table I. We take it that they are indicative of an actual acid-base transfer between cells and serum brought about by altering the tension of CO_2 to which whole blood is exposed, and that they are confirmatory of the views expressed by Van Slyke and Cullen (3), Joffe and Poulton (5), Campbell and Poulton (10), and others.

DISCUSSION.

The results herein reported would seem to indicate that the transfer of acid and base between corpuscles and plasma or serum is a well defined phenomenon as has been stated by several writers. If one were to explain the decrease in the CO_2-combining power of plasma when the tension of CO_2 is reduced in the blood by the production of lactic acid within the corpuscle and the subsequent passage outward of this into the plasma, there uniting with sodium previously held as bicarbonate, as Mellanby and Thomas (11) suggest, it would be impossible to explain the concomitant increase in the CO_2-combining power of the cells under these circumstances (Experiment II). While it is quite possible, as the work of Mellanby and Thomas (11) demonstrates, that lactic acid may play a part in this phenomenon, yet it also appears that the cells have gained in available alkali, which they could only do by giving up acid such as HCl, as suggested by Van Slyke and Cullen (3), or by taking up base. The fact that lactic acid is produced by the red cells when the CO_2 tension is reduced is of interest in another way not mentioned by Mellanby and Thomas (11). MacLeod and Knapp (15) have demonstrated that lactic acid is produced in experimental alkalosis, and Haldane, Kellas,

and Kennaway (16), and Haggard and Henderson (17) have independently shown that exposure to low oxygen tension produces an alkalosis characterized by the concomitant appearance of lactic acid in the blood and urine. The production of lactic acid in blood *in vitro* from which the CO_2 is being removed, as demonstrated by Mellanby and Thomas (11), may therefore be a reaction confined to an isolated tissue, similar to that manifested by the intact animal during an alkalosis. In other words the lactic acid production in shed blood may be in a sense a protective reaction brought about by the loss of the volatile acid CO_2.

The great alteration in the CO_2-combining power of plasma or serum and red cells separated from whole blood at widely divergent CO_2 tensions shown in the results of experiments set down in Table I is indicative of a very material transfer of acid and basic radicals or ions between the plasma or serum and the red cells under the conditions here imposed. Theoretically the decrease in the CO_2-combining power of the plasma, and the increase of that of the red cells, on exposing the whole blood to a low tension of CO_2, may be explained by one of the following processes: (1) The migration of HCl from cells to serum; (2) the migration of alkali from serum to cells; (3) a combination of (1) and (2). The results of Experiments XI, XII, XIII, and XIV, Table I, in which the respective sera and cells were ashed and the available alkali of the ash of each was determined, taken in conjunction with the observations of Van Slyke and Cullen (3) on the migration of chlorine, would indicate that an actual transfer of base does take place. It is of course quite possible that, as the CO_2 tensions to which the bloods were exposed were far removed from the normal physiological limits, the results are not directly applicable to the blood of the intact animal.

The fact that the CO_2-combining power of whole blood, serum, or cells is less than the CO_2-combining power of the ash of the same would lend further support to the bicarbonate hypothesis of CO_2 transport in the body. Evidently in all the experiments of this nature herein reported the whole blood or serum always held a certain amount of sodium in organic combination over and above that combined as bicarbonate. On ashing, this alkali is set free, and hence the CO_2-combining power of the solution of

the ash is greater than that of the original blood or serum. The fact that the CO_2-combining power of the ash exceeds that of the blood supports the theory that the proteins of the blood act as weak acids competing with the carbonic acid for the available base.

The fact that an acid-base transfer can be demonstrated between red cells and plasma of the blood of lower vertebrates is also of interest.

As there is practically no change produced in the electrical conductivity of the serum (Table II) by treating whole blood with CO_2 at a high tension, it is evident that, although the total available alkali of serum is increased by exposing whole blood to an increased CO_2 tension, the ionic concentration of the serum is relatively unaffected.

TABLE II.

Experiment No.	Specimen analyzed.	Conductivity $\times 10^4$ at 18°C.
I	Sheep serum separated from whole blood equilibrated with atmospheric air	100.0
	Sheep serum equilibrated with atmospheric air after separation from whole blood saturated with CO_2	100.7
II	Ox serum separated from whole blood equilibrated with atmospheric air	117.9
	Ox serum equilibrated with atmospheric air after separation from whole blood saturated with CO_2	118.2

SUMMARY.

Confirmatory evidence is given of the acid-base transfer between the red cells and the plasma of the serum of vertebrate blood with fluctuating CO_2 tension.

The solution of the ash of serum or of whole blood has a greater CO_2-combining power than the original serum or whole blood. This fact indicates that part of the alkali of the blood is bound by an organic substance (probably with protein for the most part) which functions as a weak acid.

The hypothesis that $NaHCO_3$ rather than CO_2 combinations with proteins is the transporting vehicle for CO_2 in the body is supported.

Serum, separated from whole blood saturated with CO_2 and equilibrated with atmospheric air, does not differ materially in its electrical conductivity from serum separated from whole blood equilibrated with air.

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STUDIES ON THE SYNTHESIS OF HIPPURIC ACID IN THE ANIMAL ORGANISM.

IV. A NOTE ON THE SYNTHESIS OF HIPPURIC ACID IN THE RABBIT AFTER EXCLUSION OF BILE FROM THE INTESTINE.

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The source of the glycocoll made available for detoxication after the ingestion of benzoic acid or its salts is not known. The possibility that hippuric acid is formed from the glycocoll secreted as glycocholic acid in the bile either in the intestine or in the circulatory system has been suggested. Such a theory would ascribe to the bile an important function. Little experimental evidence in support of this theory has been presented and the theory must be considered untenable in light of more recent work.

Kühne and Hallwachs (1) fed 1 to 4 gm. of benzoic acid to a dog with a biliary fistula and observed the elimination of hippuric acid in the urine. They were unable to detect hippuric acid in the duodenal contents of a normal animal after benzoate ingestion. From their experiments they reached the conclusion that the glycocoll of the bile acids was not the only source of glycocoll available for conjugation. Rosenberg (2) repeated this work on a dog with a gall bladder fistula and found appreciable quantities of hippuric acid eliminated, and no evidence of leakage of the bile from the fistula. Zimmermann (3), on the other hand, has reported experiments on a woman in whom a temporary gall bladder fistula with drainage of the bladder and of the opened duct had been made for the relief of a suppurative cholecystitis. 7 days after the operation 5 gm. of sidonal (piperazine salt of quinic acid), which is normally converted to hippuric acid in man almost quantitatively, were administered *per os* and the urine was examined for hippuric and benzoic acids. No traces of hippuric acid were detected, but large amounts of benzoic acid were present in the urine. 3 days later after discontinuance of the drainage of the hepatic duct, the administration of sodium benzoate resulted in a marked elimination of hippuric acid in the urine. He concluded that in man the liver as the site of bile synthesis was the sole source of glycocoll.

Inasmuch as there are marked differences in the synthetic processes in man and the dog, as far as concerns the detoxication of benzoic acid, it was considered of interest to note the effect of exclusion of the bile from the intestine in the rabbit, an animal in which the detoxication of small amounts of benzoic acid is accomplished more completely by conjugation with glycocoll than in the dog.

A rabbit was operated upon[1] under ether anesthesia and a cannula inserted in the common bile duct. This was connected with a small rubber sack which was held in place in the abdominal cavity and the wound closed. 16 hours later 17 cc. of bile were removed from the sack and the animal was fed 1.5 gm. of sodium benzoate through a stomach sound. During the ensuing 24 hour period 90 cc. of urine and 32 cc. of bile were secreted. The animal died about 48 hours later. Autopsy showed peritonitis but no evidence of leakage of bile around the duct. The urine was analyzed for hippuric acid (total benzoic acid) by the method of Folin and Flanders (4) and also by the extraction method of Dakin which involves isolation of the hippuric acid. Hippuric acid equivalent to about 30 per cent of the theoretical amount was present. The hippuric acid isolated showed on recrystallization the characteristic crystalline form and after drying melted at 187°. No benzoic acid crystals were observed. The bile was also examined for hippuric and benzoic acids with negative results in each case.

The experiment demonstrates that hippuric acid synthesis in the rabbit occurs after the exclusion of bile from the intestine and that glycocholic acid is not the only source of glycocoll in the rabbit. It should be noted, however, that the percentage of hippuric acid eliminated was smaller than normal. Whether this was due to the poor physical condition of the animal or to the effect of bile exclusion could not be determined. Experiments with other rabbits gave unsatisfactory results due to the death of the animals before the completion of the experiments. It is hoped that the record of the one successful experiment may be of interest and stimulate further work in other laboratories since facilities for further work along this line are no longer available to the author.

[1] The operation was performed by Dr. Max M. Peet of the Division of Experimental Surgery.

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THE NUTRITIONAL REQUIREMENTS OF YEAST.

III. THE SYNTHESIS OF WATER-SOLUBLE B BY YEAST.

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During the past few years it has been shown conclusively that there are other necessary constituents of the diet of the animal besides carbohydrates, proteins, fats, and mineral salts. These food accessory substances are commonly known as vitamines. It has been shown that animals must obtain the vitamines from outside sources, that is from plant life, and cannot synthesize them. No sooner had it been shown that certain vitamines are essential for the growth and well being of animals than certain investigators, Bottomley (1) and others, began to announce that vitamines or similar unknown substances were needed for the growth of plants; that is, that plants cannot synthesize these bodies. If the higher plants cannot synthesize vitamines from whence do the plants derive them? Bottomley (2) claimed that certain plants obtained these food accessory substances, or as he called them "auximones," from the growth of certain organisms in the soil. Recently others have gone further and claimed that vitamines, especially water-soluble B, are necessary for the growth of microorganisms.

Williams (3) maintains that water-soluble B is an essential constituent of the medium for the growth of yeast. He bases his conclusion upon the fact that the addition of alcoholic extracts from various vegetable materials, containing many unknown substances other than water-soluble B, stimulates the growth of yeast when added to a medium of arbitrary composition. He assumes without further evidence that of all the unknown substances present in such an extract only one, water-soluble B, is responsible for the enhanced growth. Williams (4), Bachmann

(5), and Eddy and Stevenson (6) have gone further and perfected quantitative methods for the estimation of water-soluble B by the use of yeast, based upon the assumption that the enhanced growth in the medium of arbitrary composition upon the addition of the alcoholic extracts of the various materials was a measure of the water-soluble B content and of that only. Recently certain investigators, among them being Funk and Dubin (7), Swoboda (8), Miller (9), and Whipple (10), have used this method for the estimation of water-soluble B. Whipple (10), using Williams' method, came to the conclusion that because alkali did not destroy the yeast growth stimulant in extracts from cabbage and onion that water-soluble B is not destroyed by alkali. It is known from the work of McCollum and Simmonds (11), Souza and McCollum (12), and others, that the food accessory substance known as water-soluble B is destroyed by alkali as determined by the only legitimate test; namely, the effect upon the growth of animals. Hence any treatment known to destroy the effect of extracts upon animals which will not destroy the yeast growth-promoting substance shows that the yeast growth-promoting substance is not water-soluble B.

One of the great needs in biological chemistry today is a simple and rapid method for the quantitative determination of vitamines, and since such a great need exists it is all the more necessary to examine critically any method brought forward. In a recent paper it was pointed out by Fulmer, Nelson, and Sherwood (13) that the relative potencies of two substances as yeast growth-promoting substances could not be arrived at by comparing the effects of extracts from equal weights of materials and that any method based upon such procedure would lead to conflicting results; that there are present in alcoholic extracts from wheat embryo and alfalfa substances contributing to the mineral and nitrogenous requirements of yeast and that the yeast growth stimulant in these extracts is not water-soluble B. The same authors pointed out in a second paper (14) that a medium could be developed composed of known constituents in which the growth of yeast is so satisfactory that the addition of vitamine-containing extracts, alcoholic extracts from wheat embryo, and

alfalfa, does not improve it. This shows that none of the unknown substances in the extracts is essential for the growth of yeast. Since it was found that yeast grows satisfactorily upon a medium of known composition the determination whether or not yeast is capable of synthesizing water-soluble B is an obvious procedure.

Souza and McCollum (12) have recently made a study of Williams' method for the quantitative determination of water-soluble B. They come to the conclusion " . . . . that the use of yeast as a test organism for determining the presence or absence of the antineuritic dietary factor is complicated by so many disturbing factors as to make it of little if any value."

EXPERIMENTAL.

The medium used for the growth of the yeast had the following composition: 100 cc. contained 0.188 gm. of NH_4Cl; 0.100 gm. of $CaCl_2$; 0.100 gm. of K_2HPO_4; 0.040 gm. of $CaCO_3$; 10 gm. of cane-sugar. The yeast was plated from a Fleischmann yeast cake. The yeast had been growing continuously in the above medium for a year. It was grown in 50 cc. portions of the medium, 1 cc. of the culture being transferred every other day to 50 cc. of fresh medium thus geometrically diluting out any of the original constituents of the medium or of the yeast. The maximum concentration possible for the original constituents of the medium or yeast would be 1×50^{-180}. It can be concluded that after such an experience any water-soluble B present in the yeast must be synthesized by the organism. Yeast was grown in sufficient quantities and air-dried for feeding experiments as outlined below.

The albino rat was used as the experimental animal. The animals were a vigorous lot and weighed about 50 gm. each when placed upon the experiment. They received per day as much as they could consume of the following ration: 18 per cent of casein, 5 per cent of salt mixture, 5 per cent of butter fat, 72 per cent of dextrin. The casein was repeatedly washed with distilled water containing a small amount of acetic acid. Repeated trials showed that this casein was free from water-soluble B. The salt mixture had the following composition:

	gm.
NaCl	0.173
$MgSO_4$ (anhydrous)	0.266
$NaH_2PO_4.H_2O$	0.347
K_2HPO_4	0.954
$CaH_4(PO_4)_2.H_2O$	0.540
Iron citrate	0.118
Calcium lactate	1.300
KI	Traces.

After a decline in weight for a period of 2 to 3 weeks on the above diet, 2 per cent of the yeast grown on the synthetic medium was added to the ration. Some of the typical curves of growth are shown in Chart 1. Period 1 shows the growth obtained on

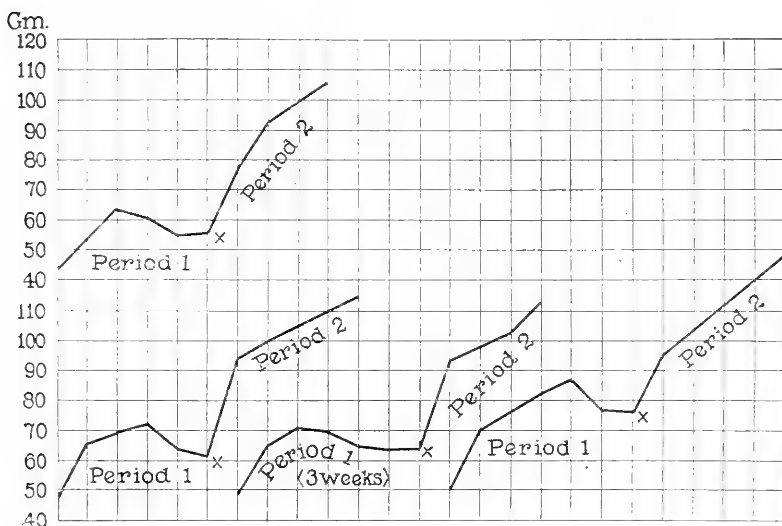

CHART 1.

the synthetic diet. It will readily be seen that after a period of 3 weeks the animals began to decline rapidly. Period 2 shows the rapid gain that was made when the yeast grown on the synthetic medium was added to the ration.

SUMMARY.

Evidence is presented which shows that yeast can synthesize water-soluble B.

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THE AVAILABLE CARBOHYDRATE IN THRICE BOILED VEGETABLES.

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With the introduction of thrice boiled vegetables into the dietary of diabetics it becomes of considerable importance to know more exactly their carbohydrate content which is available to the organism. It has been generally assumed that several of the vegetables with low carbohydrate content are practically free from starches and sugars after being thrice boiled, but very little actual experimental evidence has been produced to confirm this assumption.

Thrice boiled vegetables were first advocated by Allen (1), but little actual data were given. Recently Wardell (2) and Cammidge (3) have published a few analyses of vegetables of low carbohydrate content extracted at 60°C. and at 100°C. Also Cammidge (3) has reported that the vegetables most readily freed from their carbohydrate were celery, spinach, sliced turnips, and sliced carrots. This latter observation was rather contrary to the accepted idea that the tuber vegetables could only with difficulty be freed from carbohydrate. Also the observation that cabbage is a difficult one to free from carbohydrate is important, as this vegetable has been so extensively used.

Method.

The technique adopted was a modification of that used by Olmstead (4). 100 gm. of the material, cut into small pieces, were placed in a one thickness cheese-cloth bag, and boiled for three periods of ½ hour each in an open kettle, using fresh cold water for each period. After draining, 10 gm. of the material were minced to a pulp in a mortar and transferred quantitatively with 150 cc. of water to a 500 cc. Erlenmeyer flask. After boiling for 2½

hours on a reflux condenser, the material was transferred quantitatively to a 250 cc. volumetric flask, allowed to cool, and 0.1 gm. of taka-diastase (Parke-Davis) and a few drops of toluene were added. It was then incubated for 18 to 20 hours at 37°C., cooled, made up to volume, filtered, and 200 cc. of the filtrate were taken. To this were added 10 cc. of a neutral molecular lead acetate solution to precipitate the tannins and gums. When the precipitation was complete 190 cc. of the filtrate (aliquot) were placed in a 250 cc. volumetric flask, 22.5 cc. of 7 N HCl were added, the volume being made up to 220 cc. with water, making it 0.7 N. Hydrolysis was then continued for $2\frac{1}{2}$ hours in a water bath and the solution was cooled, neutralized with 40 per cent NaOH, made up to volume, and filtered. The filtrate was titrated with Benedict's solution. In most instances it was found convenient to add 50 cc. of the filtrate and to complete the titration with a 1 per cent glucose solution.

Table I shows the results obtained when using 1 and 2 liters of water for each boiling. It will be seen that in most instances the larger volume of fluid caused the extraction of carbohydrate to be much more complete. In each instance 10 gm. of the boiled vegetable were used for analysis. The vegetables are arranged in the order of their carbohydrate content as given by Atwater and Bryant (5) for raw values. The values given in the "Carbohydrate" columns are those determined by analysis upon the weight of vegetable given, which weight was that remaining of the original 100 gm. after the three boilings. In the corrected percentage columns are given the carbohydrate values calculating that the change in weight was due to fluid loss or gain during the process of boiling.

Table I shows that in fourteen instances out of seventeen the extraction of carbohydrate was more complete when using 2 liters of water. In the other three there was practically no change when allowing for the usual variation in carbohydrate content of the same vegetable. In no instance was it possible to free the vegetable completely from carbohydrate when using 1 liter of water, but in the case of vegetable marrow, celery, and lettuce this was accomplished with 2 liters.

A small amount of sodium bicarbonate added to the water in which vegetables are boiled has been known to cause the vegetable to become softer in consistency and to retain its natural color more completely. This observation was thought to be worth while investigating, as vegetables of softer consistency would probably liberate their carbohydrate more readily. Ac-

cordingly, analyses were carried out on vegetables boiled in water containing 0.05 and 0.1 per cent sodium bicarbonate. Those prepared in the former dilution were uniformly softer in consistency than when boiled in tap water. Likewise they approached more closely their natural color. In no instance were they objectionable. Those boiled in the 0.1 per cent soda solution were frequently objectionable from their brown color and too soft consistency. This was most noticeable in the case of onions and cauliflower. Table II shows the results obtained.

TABLE I.

Vegetable.	Atwater and Bryant's CHO value.	With 1 liter.			With 2 liters.		
		CHO	Weight.	Corrected.	CHO	Weight.	Corrected.
	per cent	*per cent*	*gm.*	*per cent*	*per cent*	*gm.*	*per cent*
Vegetable marrow	2.6	0.22	62	0.14	0.0	42	0.0
Canned spinach	2.6	0.31	88	0.27	0.20	73	0.15
" asparagus	2.8	0.24	50	0.12	0.20	74	0.15
Lettuce	2.9	0.03	24	0.01	0.0	80	0.0
Celery	3.3	0.07	83	0.06	0.0	68	0.0
Brussels sprouts	3.4	0.72	100	0.72	1.31	100	1.31
Canned string beans	3.8	0.49	78	0.38	0.41	83	0.34
Cauliflower	4.7	0.79	92	0.73	0.51	67	0.34
Pumpkin	5.2	0.29	76	0.22	0.41	57	0.23
Cabbage	5.6	0.45	88	0.39	0.51	86	0.44
Turnip	8.1	0.72	90	0.65	0.20	75	0.15
Squash	9.0	6.90	54	3.73	4.74	72	3.41
Carrots	9.3	0.60	90	0.54	0.55	92	0.51
Beets	9.7	1.17	84	0.98	0.20	93	0.19
Canned green peas	9.8	7.70	60	4.63	3.10	70	0.22
Onions	9.9	0.52	41	0.21	0.14	36	0.05
Parsnips	13.5	6.53	103	6.75	2.49	95	2.36

Table II shows that in twelve out of seventeen instances the presence of sodium bicarbonate in the water caused the extraction of carbohydrate to be more complete. In the case of certain vegetables, especially cauliflower, there was a marked difference. Certain irregularities, as in the case of celery with 0.1 per cent soda, cannot be explained.

Further analyses were carried out upon cauliflower, cabbage, and turnips using 2 liters of water for each boiling with the two concentrations of soda. These are reported in Table III.

TABLE II.

Vegetable.	Atwater and Bryant's CHO value.	Sodium bicarbonate.	With 1 liter.		
			CHO	Weight.	Corrected
	per cent	*per cent*	*per cent*	*gm.*	*per cent*
Vegetable marrow	2.6	0.0	0.22	62	0.14
		0.05	0.0	53	0.0
		0.10	0.0	51	0.0
Canned spinach	2.6	0.0	0.31	88	0.27
		0.05	0.27	84	0.22
		0.10	0.17	100	0.17
Canned asparagus	2.8	0.0	0.24	50	0.12
		0.05	0.0	50	0.0
		0.10	0.0	52	0.0
Lettuce	2.9	0.0	0.03	24	0.01
		0.05	0.0	30	0.0
		0.10	0.0	32	0.0
Celery	3.3	0.0	0.07	83	0.06
		0.05	0.03	72	0.02
		0.10	0.21	67	0.14
Brussels sprouts	3.4	0.0	0.72	100	0.72
		0.05	0.89	100	0.89
		0.10	0.72	100	0.72
Canned string beans	3.8	0.0	0.49	78	0.38
		0.05	0.59	82	0.48
		0.10	0.39	82	0.32
Cauliflower	4.7	0.0	0.79	92	0.73
		0.05	0.35	85	0.30
		0.10	0.07	88	0.06
Pumpkin	5.2	0.0	0.29	76	0.22
		0.05	0.17	78	0.13
		0.10	0.24	71	0.17
Cabbage	5.6	0.0	0.45	88	0.39
		0.05	0.38	92	0.35
		0.10	0.38	93	0.35

TABLE II—*Concluded.*

Vegetable.	Atwater and Bryant's CHO value.	Sodium bicarbonate.	With 1 liter.		
			CHO	Weight.	Corrected.
	per cent	*per cent*	*per cent*	*gm.*	*per cent*
Turnips	8.1	0.0	0.72	90	0.65
		0.05	1.31	80	1.05
		0.10	1.07	84	0.90
Squash	9.0	0.0	6.90	54	3.73
		0.05	5.14	44	2.26
		0.10	4.60	60	2.76
Carrots	9.3	0.0	0.60	90	0.54
		0.05	0.55	88	0.48
		0.10	0.45	88	0.40
Beets	9.7	0.0	1.17	84	0.98
		0.05	1.38	80	1.01
		0.10	1.59	80	1.27
Canned green peas	9.8	0.0	7.70	60	4.63
		0.05	2.00	60	1.20
		0.10	0.34	60	0.20
Onions	9.9	0.0	0.52	41	0.21
		0.05	0.52	44	0.23
		0.10	0.38	30	0.11
Parsnips	13.5	0.0	6.53	103	6.75
		0.05	4.30	106	4.55
		0.10	3.80	102	3.89

Table III shows that in the case of turnips the use of a larger quantity of water improves the degree of extraction to a marked extent.

The remaining point investigated was whether the use of a closed or open kettle would affect the extraction, as with an open kettle frequently the fluid loss during the ½ hour boiling is 50 per cent. Cauliflower was used for this work with results as given in Table IV.

It can be seen that in the case of this one vegetable the use of an open or closed kettle makes little difference.

TABLE III.

Vegetable.	Atwater and Bryant's CHO value.	Sodium bicarbonate.	With 2 liters.		
			CHO	Weight.	Corrected.
	per cent	*per cent*	*per cent*	*gm.*	*per cent*
Cauliflower..................	4.7	0.0	0.51	67	0.34
		0.05	0.03	73	0.22
		0.10	0.07	58	0.04
Cabbage....................	5.6	0.0	0.51	86	0.44
		0.05	0.58	86	0.50
		0.10	0.35	87	0.30
Turnips....................	8.1	0.0	0.20	75	0.15
		0.05	0.10	65	0.06
		0.10	0.10	65	0.06

TABLE IV.

Vegetable.	Kettle.	Sodium bicarbonate.	With 1 liter.		
			CHO	Weight.	Corrected.
		per cent	*per cent*	*gm.*	*per cent*
Cauliflower...............	Open.	0.0	0.89	82	0.73
	Closed.	0.0	0.83	84	0.70
	Open.	0.05	0.55	94	0.52
	Closed.	0.05	0.55	96	0.53

SUMMARY.

Only in the case of a few thrice boiled vegetables is it possible to free them completely from available carbohydrate as determined by taka-diastase. The use of 20 parts of water, as compared with 10, for each boiling makes the carbohydrate extraction more complete. By this latter method vegetable marrow, lettuce, and celery can be completely freed from available carbohydrate, and canned spinach, canned asparagus, turnips, beets, and onions can be rendered approximately carbohydrate-free. Canned string beans, cauliflower, pumpkin, cabbage, and carrots still retain about 0.5 per cent of available carbohydrate.

Sodium bicarbonate added in 0.05 and 0.1 per cent concentration markedly favors the completeness of the carbohydrate extraction in most vegetables. This is very marked in the case of cauliflower.

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FACTORS GOVERNING THE EXCRETION RATE OF UREA.*

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In using the rate of urea excretion as criterion of the functional capacity of the kidney, there are two known factors, besides the excretory ability of the kidneys, which influence the urea excretion rate to such an extent that they must be either reduced to constancy, or measured and allowed for by calculation, before the excretion rate itself can be taken as even an approximate measure of the third factor; *viz.*, the functional capacity. Of these two factors, one has been recognized as the concentration of urea in the blood since the publication of the papers of Ambard and his collaborators (Ambard (1910, 1920), Ambard and Papin (1909), Ambard and Weill (1912)), whose work has inspired many investigations, including our own. The other factor was conceived by these authors to be the concentration of urea in the urine, but we believe that we shall demonstrate in this paper that it is rather the rate of volume output of urine.

Effect of Blood Urea Concentration on Excretion Rate.—Ambard held that, other factors being equal, the excretion rate of urea increases as the square of the blood urea concentration; *i.e.*, that doubling the blood urea quadruples the excretion rate. Marshall and Davis (1914), on the other hand, found that when plenty of water is given "the rate of excretion of urea in normal animals is directly proportional to the concentration of urea in the blood." Further data indicating an approximate proportionality between blood urea concentration and rate of urea excretion were pub-

* A preliminary report of the results published in this paper was presented to the Society for Experimental Biology and Medicine on December 17, 1919 (Austin, J. H., Stillman, E., and Van Slyke, D. D., *Proc. Soc. Exp. Biol. and Med.*, 1919–20, xvii, 59).

lished by Pepper and Austin (1915), by Addis and Watanabe (1916), and by Addis, Barnett, and Shevky (1918).

The conclusions reached in this paper are based chiefly upon data from human subjects. We have, however, performed a sufficient number of experiments on dogs to confirm the latter authors. In some of these experiments we induced marked variation in the blood urea concentration by administering urea by stomach tube, in others by feeding an amount of meat equivalent to 5 per cent of the animal's body weight. The blood samples were drawn from the jugular vein by puncture through the skin. The results of four experiments are shown in Table I.

Simple inspection of the ratios in the seventh column shows that despite wide fluctuations in both D and B, the ratio $\frac{D}{B}$ is fairly constant. In Dog 1 only one value out of eleven for the ratio in the two experiments lies outside the range 57 ± 11, and in the experiments with Dog 2 only one value out of the fifteen lies outside the range 115 ± 26. Fivefold increase in blood urea is accompanied by almost exactly fivefold increase in excretion rate.

The percentage variations in the Ambard constant $\frac{D\sqrt{C}}{B^2}$ are much greater than in the simple $\frac{D}{B}$ ratio.

The ratio $\frac{D}{B}$ appears to be independent of changes in volume output. This independence is only apparent, however. The animals drank as much water as they wished, and this with the urea formed or fed induced such a diuresis that practically all the observations were made when the volume output exceeded the "augmentation limit" which will be discussed later.

The Effect of Urine Volume and Concentration on the Excretion Rate of Urea.—When volume influences are reduced to a minimum by keeping the volume output high, our experiments, and those of Marshall and Davis (1914), indicate that the relationship of the urea excretion is more nearly expressed by the simple relationship $\frac{D}{B} = K$ than by any more complex function of D and B. Addis found the same to be true with rabbits, especially when other factors were reduced to a relative minimum by making B very large (by feeding large amounts of urea).

TABLE I.

Effect of Varying Blood Urea Concentration in Dogs.

Experimental details.	Time of period.	B, blood urea N.	C, urine urea N.	D, rate of urea N excretion.	V, rate of urine volume excretion.	$\frac{D}{B}$	$\frac{D\sqrt{C}}{B^2}$, Ambard constant.
		gm. per liter	*gm. per liter*	*gm. per 24 hrs.*	*liters per 24 hrs.*		
Experiment I. Dog 1, 14.5 kilos. 14.5 gm. urea by mouth at 11.49 a.m.	9.47–10.49 a.m.	0.109	12.3	5.8	0.47	53	1,650
	10.49–11.47 "	0.107	6.13	5.7	0.93	53	1,220
	11.47 a.m.–1.49 p.m.	0.504	20.4	25.9	1.27	51	457
	1.49–2.52 p.m.	0.437	22.5	29.5	1.31	67	725
	9.47–11.53 a.m.	0.120	6.75	7.3	1.08	61	1,320
Experiment II. Dog 1, 14.5 kilos. 14.5 gm. urea by mouth at 10.23 a.m.	9.25–10.21 a.m.	0.127	8.54	6.4	0.75	50	1,040
	10.21–11.26 "	0.611	20.8	28.3	1.36	46	342
	11.26 a.m.–12.27 p.m.	0.594	24.6	36.5	1.48	64	517
	12.27–1.30 p.m.	0.497	7.11	33.6	4.73	68	367
	1.30–2.27 "	0.381	2.80	25.5	9.10	67	294
	9.44–11.01 a.m.	0.235	9.44	18.5	1.96	79	1,000
Experiment III. Dog 2, 23.0 kilos. 23 gm. urea by mouth at 10.26 a.m.	10.21–10.56 a.m.	0.256	10.6	26.1	2.46	102	1,200
	10.56–11.26 "	0.686	14.8	63.1	4.27	92	510
	11.26–11.56 "	0.616	19.7	63.3	3.20	103	740
	11.56 a.m.–12.30 p.m.	0.532	20.0	54.9	2.74	103	860
	12.30–1.26 p.m.	0.429	18.5	45.7	2.47	107	1,070
	1.26–2.25 "	0.347	22.6	32.8	1.45	94	1,300
	2.25–3.25 "	0.278	27.6	24.6	0.89	89	1,680
	3.25–4.25 "	0.215	23.2	22.8	0.98	106	2,380
Experiment IV. Dog 2, 23.0 kilos. 1.15 kilos meat at 10.57–11.00 a.m.	10.09–10.56 a.m.	0.092	3.90	11.5	2.94	125	2,690
	12.02–1.05 p.m.	0.134	10.15	14.4	1.42	107	2,550
	1.05–2.05 "	0.142	7.38	28.4	3.85	200	3,800
	2.05–3.05 "	0.227	10.9	30.6	2.81	135	1,960
	3.05–4.05 "	0.266	7.36	30.4	4.13	114	1,160
	4.05–5.00 "	0.268	10.8	37.9	3.51	141	1,720
	9.12–9.57 a.m.	0.132	5.56	12.8	2.30	97	1,740

Since the $\frac{D}{B}$ ratio tends to be constant when variations of other factors lack relative importance, it seemed that a logical way to measure the influence of the other factors on the excretion rate would be to determine their effect on the $\frac{D}{B}$ ratio when they attain such importance. For this purpose data from experiments on normal men are available in the papers of McLean (1915) and of Addis and Watanabe (1916), and to these we have added a number by experiments of our own.

In the experiments of Addis and of McLean, and in our own, the urine was collected over short periods, usually 60 or 72 minutes (ours varied in all from ½ to 3 hours), and blood was taken at the middle of each period. The rate of volume output of urine was lowered by avoidance of water and food, or raised by drinking water, or, for large volumes, dilute salt solution (about 0.4 per cent). No attempt was made to regulate activity or general diet. Urea was determined by Marshall's urease method with the technique of Van Slyke and Cullen (1914).

DISCUSSION OF DATA.

In Figs. 1*a*, 2*a*, 3*a*, and 4*a*, the data on the four individuals from Table II are plotted, values of $\frac{D}{B}$ being used as ordinates, values of $\frac{1}{C}$ as abscissæ. In Figs. 1*b*, 2*b*, 3*b*, and 4*b*, we have for comparison on scales as nearly like as possible plotted the values of $\frac{D}{B}$ against the values of V. Concerning the nature of the relationships of urea excretion to urine volume and concentration, the results expressed in Table II and in the figures indicate the following:

1. Increase in rate of urine volume excretion up to a certain point, varying between 2.5 and 6 liters per 24 hour time unit in the different individuals, results in a regular increase in the $\frac{D}{B}$ ratio. Quantitatively, this increase is approximately proportional to the square root of the rate of urine volume excretion. This fact is shown by the manner in which the points represent-

ing the experimental data follow the curves, which represent the equation $\frac{D}{B} = K\sqrt{V}$. It does not seem that they would follow so closely curves of any other form. We have tried curves repre-

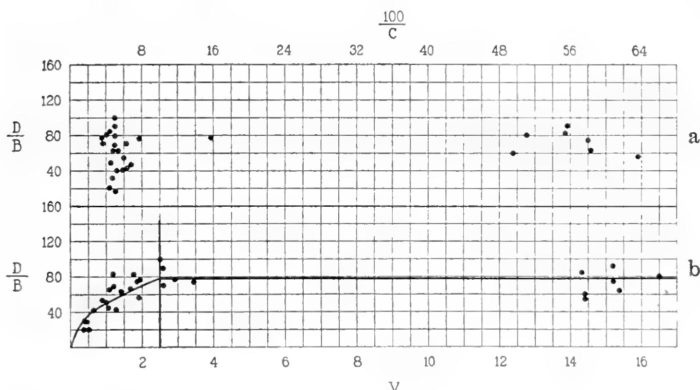

FIG. 1. Data from Addis on Ad.

(*a*) Upper half: Ordinates = $\frac{\text{urea excretion rate}}{\text{blood urea concentration}}$

Abscissæ = urea concentration in urine

(*b*) Lower half: Ordinates = $\frac{\text{urea excretion rate}}{\text{blood urea concentration}}$

Abscissæ = rate of urine volume excretion

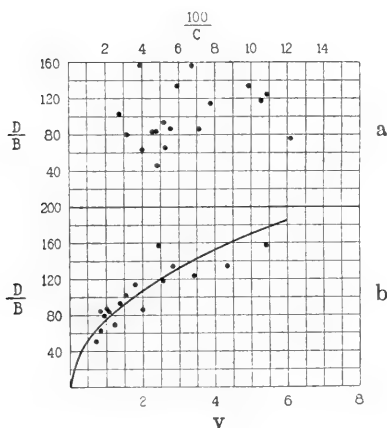

FIG. 2. Data from McLean on himself. Ordinates and abscissæ as in Fig. 1.

senting other functions of V, such as $V^{\frac{2}{3}}$, $V^{\frac{1}{3}}$, and log V without finding one that so nearly approximates the experimental results.

2. It is evident from Figs. 1*b*, 3*b*, and 4*b*, that a limit is reached as the rate of urine volume excretion increases, beyond which

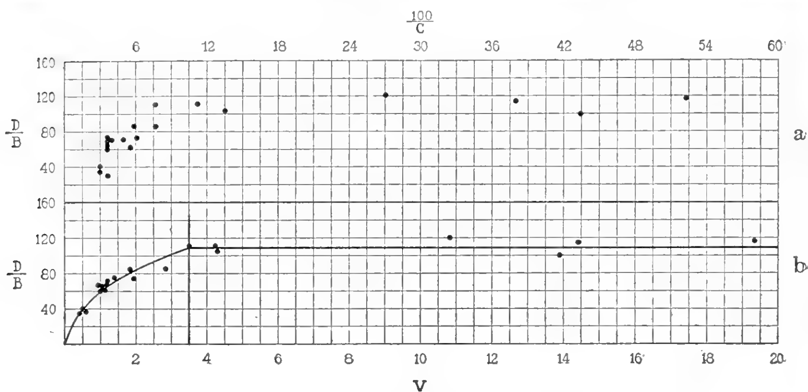

FIG. 3. Data on Austin. Ordinates and abscissæ as in Fig. 1.

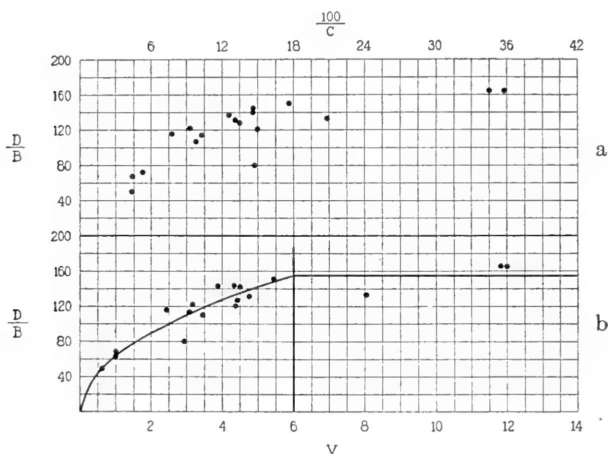

FIG. 4. Data on Van Slyke. Ordinates and abscissæ as in Fig. 1.

further increase in the rate of urine volume excretion has no further effect in augmenting the $\frac{D}{B}$ ratio. This limit we will call the augmentation limit. Of the existence of this limit there

TABLE II.

Effect of Varying Blood Urea and Volume Output of Urine on the Excretion Rate of Urea in Man.

Subject No.		B, blood urea.	D, rate of urea excretion.	V, rate of urine volume excretion.	C, concentration of urea in urine.	$\frac{D\sqrt{C}}{B^2}$, Ambard constant.	$\frac{D}{B}$	$\frac{D}{B\sqrt{V}}$, present constant.	$\frac{D}{B\sqrt{A}}$
		gm. per liter	*gm. per 24 hrs.*	*liters per 24 hrs.*	*gm. per liter*				
Ad.	1	0.450	9.5	0.41	23.3	226	21.1	33.0	
	2	0.300	8.9	0.43	20.6	448	29.7	45.3	
	3	0.495	9.4	0.48	19.5	170	19.0	27.4	
	4	0.276	11.5	0.67	17.1	365	41.7	51.0	
	5	0.442	23.1	0.94	24.6	588	52.3	53.9	
	6	0.427	21.8	0.98	22.3	564	51.1	51.5	
	7	0.322	15.1	1.05	14.4	553	46.9	45.7	
	8	0.322	20.6	1.08	19.1	870	64.0	61.6	
	9	0.367	25.0	1.22	20.5	843	68.1	61.7	
	10	0.346	28.7	1.22	23.5	1,160	82.9	75.1	
	11	0.472	20.1	1.27	15.8	358	42.6	37.9	
	12	0.465	28.9	1.44	20.1	448	62.2	51.9	
	13	0.607	45.4	1.66	27.5	504	74.8	56.8	
	14	0.495	40.7	1.71	23.8	810	82.2	62.9	
	15	0.682	52.0	1.87	27.8	592	76.2	55.8	
	16	0.540	30.4	1.89	16.3	420	56.3	41.0	
	17	0.322	24.7	1.92	12.9	854	76.7	55.3	
	18	0.495	49.8	2.50	19.9	904	100.6	63.7	
	19	0.585	40.9	2.57	15.9	484	69.9	43.6	44.2*
	20	0.577	51.6	2.62	19.7	688	89.4	55.3	56.6
	21	0.765	59.7	2.90	20.6	462	78.0	45.8	49.3
	22	0.300	22.1	3.46	6.40	672	73.7	39.6	46.6
	23	0.312	25.9	14.3	1.81	358	83.0	22.0	52.5
	24	0.402	22.6	14.4	1.57	175	56.2	14.8	35.5
	25	0.489	29.1	14.4	2.02	173	59.5	15.7	37.6
	26	0.300	27.4	15.2	1.80	408	91.3	23.4	57.8
	27	0.354	26.2	15.2	1.72	274	74.0	19.0	46.8
	28	0.414	26.2	15.3	1.71	200	63.3	15.3	40.1
	29	0.408	32.4	16.5	1.96	273	79.4	19.5	50.3
McL.	1	0.299	14.7	0.72	20.7	750	49.2	58.0	
	2	0.321	20.1	0.82	24.8	980	62.6	69.0	
	3	0.214	18.0	0.86	21.0	1,810	84.1	90.0	
	4	0.363	29.3	0.96	31.2	1,250	80.7	84.0	
	5	0.221	19.4	1.07	18.1	1,690	87.8	85.0	
	6	0.298	25.0	1.12	22.3	1,330	83.9	75.0	
	7	0.341	23.2	1.24	18.7	860	68.0	61.0	
	8	0.286	26.5	1.38	19.1	1,420	92.7	79.0	
	9	0.513	52.6	1.54	35.1	1,150	102.5	82.0	
	10	0.206	23.0	1.76	13.1	1,960	111.7	84.0	
	11	0.321	27.8	2.00	13.9	1,010	86.6	61.0	

* The value employed for augmentation limit (A) is for Ad. 2.5; J. H. A. 3.5; V. S. 6.0.

TABLE II—*Concluded.*

Subject No.		B, blood urea.	D, rate of urea excretion.	V, rate of urine volume excretion.	C, concentration of urea in urine.	$\frac{D\sqrt{C}}{B^2}$, Ambard constant.	$\frac{D}{B}$	$\frac{D}{B\sqrt{V}}$, present constant.	$\frac{D}{B\sqrt{A}}$
		gm. per liter	*gm. per 24 hrs.*	*liters per 24 hrs.*	*gm. per liter*				
	12	0.406	63.3	2.42	25.2	1,930	155.9	100.0	
	13	0.206	24.3	2.56	9.5	1,760	118.0	74.0	
	14	0.211	28.2	2.80	10.1	2,020	133.6	80.0	
	15	0.254	31.2	3.40	9.2	1,470	122.8	73.0	
	16	0.542	72.5	4.32	16.7	1,010	133.8	64.0	
	17	0.539	84.7	5.40	15.7	1,150	157.1	68.0	
J. H. A.	1	0.424	15.6	0.47	33.0	500	36.8	54.0	
	2	0.443	17.7	0.51	32.7	520	40.0	56.0	
	3	0.360	14.3	0.53	28.0	631	39.7	54.5	
	4	0.445	29.4	0.97	30.4	818	66.1	67.1	
	5	0.450	27.6	1.00	27.6	716	61.3	61.4	
	6	0.442	28.7	1.04	27.7	780	64.9	64.0	
	7	0.313	19.6	1.09	18.0	848	62.6	59.9	
	8	0.448	30.8	1.15	26.8	794	68.8	64.1	
	9	0.452	32.2	1.19	27.6	820	71.2	65.0	
	10	0.371	27.0	1.32	20.2	882	72.8	63.4	
	11	0.360	31.1	1.81	17.2	995	86.4	64.2	
	12	0.425	31.7	1.94	16.4	710	74.6	53.0	
	13	0.422	36.2	2.77	13.1	740	85.8	52.0	
	14	0.423	46.4	3.53	13.1	940	109.7	58.5	58.8*
	15	0.340	37.7	4.24	8.9	972	110.9	53.8	59.3
	16	0.313	32.7	4.30	7.6	921	104.5	50.4	55.8
	17	0.350	42.3	10.80	3.9	680	120.9	36.8	64.6
	18	0.320	31.6	13.90	2.3	470	98.8	26.6	52.9
	19	0.332	38.0	14.45	2.63	559	114.5	30.2	61.1
	20	0.323	37.9	19.30	1.91	502	117.3	26.7	62.7
V. S.	1	0.301	15.0	0.65	22.9	800	59.8	62.0	
	2	0.355	24.2	1.03	23.3	930	68.2	61.0	
	3	0.300	21.5	1.13	19.0	1,040	71.7	67.5	
	4	0.269	31.2	2.46	12.8	1,540	116.0	74.0	
	5	0.243	19.7	2.90	6.8	870	81.1	47.6	
	6	0.268	30.7	3.12	9.8	1,590	114.6	63.0	
	7	0.283	34.5	3.19	10.8	1,415	121.9	68.3	
	8	0.326	35.6	3.46	10.3	1,075	109.2	58.7	
	9	0.209	28.8	3.64	7.9	1,853	137.8	72.3	
	10	0.213	30.2	4.31	7.0	1,760	141.8	68.3	
	11	0.243	29.5	4.40	6.7	1,292	121.3	57.9	
	12	0.255	32.8	4.42	7.4	1,372	128.6	61.2	
	13	0.219	31.4	4.48	7.0	1,730	143.4	67.7	
	14	0.276	36.5	4.74	7.7	1,328	132.2	60.7	
	15	0.206	31.2	5.47	5.7	1,755	151.5	64.7	
	16	0.285	38.6	8.05	4.8	1,040	135.4	47.7	55.1*
	17	0.204	34.2	11.80	2.9	1,398	167.6	48.8	68.4
	18	0.202	33.6	12.00	2.8	1,376	166.3	48.0	67.9

seems no room for doubt after inspection of the figures named, especially Fig. 1. The variation of individual observations from the mean curve is sufficient, however, to make the exact location of the limit in a given individual somewhat difficult. The method we have employed is to calculate the constant, $\frac{D}{B\sqrt{V}} = K$, for all observations on a given individual, arranging them in the order of increasing values of V as in Table II. Inspection of the series of values for K shows them to lie within a certain range until for the higher values of V they decrease progressively. Omitting these progressively decreasing values, the mean of the remainder is determined and the curve shown in the figures is that obtained by the use of this mean as K in the equation $\frac{D}{B} = K\sqrt{V}$. A horizontal line is then drawn through the mean position of the observations at the higher values of V. The value of V at the intersection of this horizontal line with the curve is considered to be the augmentation limit for the individual. The value of V at this point is designated as A and is used in place of V in calculating the constant for the individual in observations in which the urine volume excretion exceeds the augmentation limit. The values of the constant thus calculated are shown in the last column at the right of Table II.

In this manner we have estimated the approximate augmentation limits of the four individuals studied as follows:

Ad.	Rate of 2.5 liters per 24 hrs.
Austin	" " 3.5 " " 24 "
McLean	" " over 5 liters per 24 hrs.
Van Slyke	" " 6.0 liters per 24 hrs.

It is probably because many of Addis' observations were made at rates of urine volume excretion exceeding the augmentation limit that he concluded that the rate of urine volume excretion is without effect on the rate of urea excretion. In man, in the limited number of observations that we have considered, the augmentation limit lies between the rates 2.5 and 6 liters of urine volume excretion per 24 hours, hence is in excess of the rate of urine volume excretion usually observed except after the drinking of large amounts of fluid.

3. The concentration of urea in the urine was believed by Ambard to be a factor of definite influence upon the urea excretion rate. We have tested this probability by attempting to plot in Figs. 1*a*, 2*a*, 3*a*, and 4*a*, curves showing the relationship of C, the concentration of urea in the urine, to the $\frac{D}{B}$ ratio. When these figures are inspected no such effect is to be seen in two of the four subjects (Ad. and McLean). In the other two (Austin and Van Slyke) the $\frac{1}{C}$ points follow curves similar to those followed by the V points, but follow them less closely (calculation

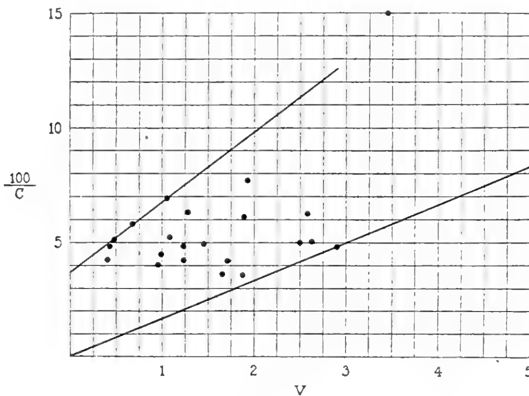

FIG. 5. Data from Addis on Ad.

Ordinates = reciprocal of concentration of urea in urine
Abscissæ = rate of urine volume excretion

shows the average deviation to be twice as great).. The apparent relationship between excretion ratio and urinary concentration in these two subjects may be due to the fact that in all the experiments performed on them the concentration of urea in the urine varies quite consistently in inverse proportion to the volume, so that the urinary concentration happens to be an indirect measure of urinary volume. The presumable cause of this consistency is that in these experiments the changes in rate of excretion were induced by varying the fluid intake. In some of the experiments of Addis and McLean, great. variations in the blood urea were also induced, resulting in urines that were high both in volume

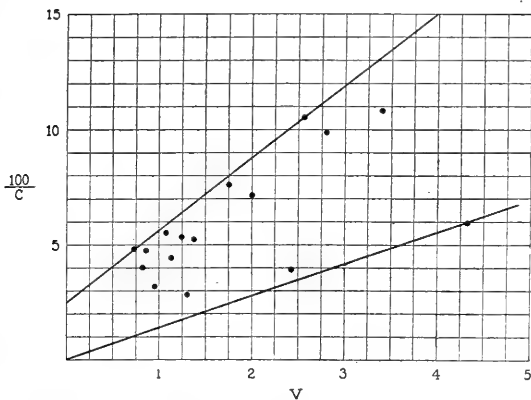

FIG. 6. Data from McLean on himself. Ordinates and abscissæ same as Fig. 5.

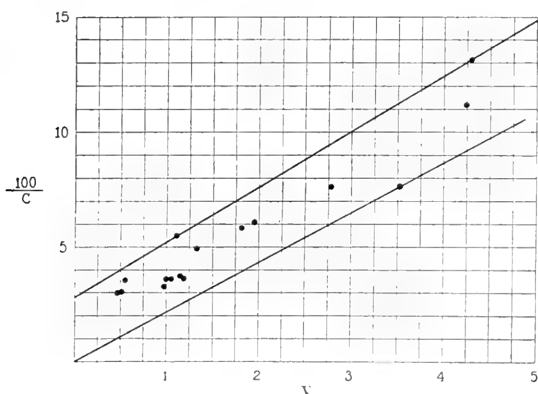

FIG. 7. Data on Austin. Ordinates and abscissæ same as Fig. 5.

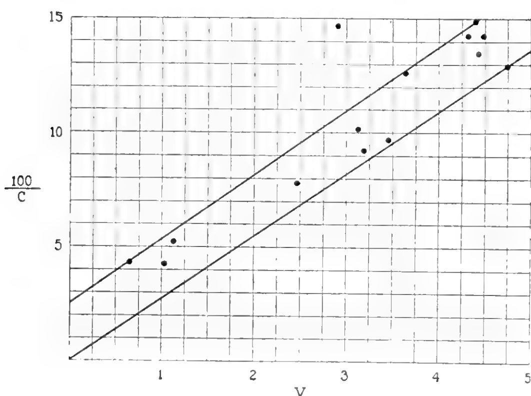

FIG. 8. Data on Van Slyke. Ordinates and abscissæ same as Fig. 5.

and concentration (see Figs. 5, 6, 7, and 8). In these cases, where concentration fails to be an indirect measure of volume, it also fails to show any relationship whatever to rate of urea excretion. The conclusion seems with a considerable degree of probability to be true that C is related to $\frac{D}{B}$ only when it happens to approximate inverse proportionality to V.

Equations Expressing Observed Relationships.

The above relationships of urea excretion rate to (1) blood urea and (2) urine volume, are expressed in the following equations:

$$(1) \quad D = K_1 B$$

$$(2) \quad D = K_2 \sqrt{V}$$

Expressing both relationships in one equation, we have

$$(3) \quad D = KB\sqrt{V}$$

For individuals of varying body weight, an allowance must be made for this factor. For this purpose it appeared most logical, and was found to give the most consistent results, to express the excretion rates of both urea and urine volume excretion on a per kilo basis, as follows:

$$(4) \quad \frac{D}{W} = KB\sqrt{\frac{V}{W}} \quad \text{or}$$

$$(5) \quad D = KB\sqrt{VW} \quad \text{or}$$

$$(6) \quad K = \frac{D}{B\sqrt{VW}}$$

$K = 7.5 \pm 3$ for normal individuals, as will be shown.

When V exceeds the augmentation limit, normally 2.5 to 6 liters per 24 hours, which we will designate as A, the equation becomes

$$(7) \quad K = \frac{D}{B\sqrt{AW}}$$

In Fig. 9 are given all the data on all individuals taken from the published figures of McLean and of Addis and Watanabe,

and from our own observations that have values for V below a mean augmentation limit of 3.5 liters per 24 hours, and plotted in accordance with this formula, values of $\frac{D}{B\,W}$ being plotted as ordinates, those of $\frac{V}{W}$ as abscissæ. It is evident that the data arrange themselves along the curve representing the equation $\frac{D}{B\,W} = K\sqrt{\frac{V}{W}}$, which is identical with $K = \frac{D}{B\sqrt{VW}}$, K having a value of 7.5 ± 3.

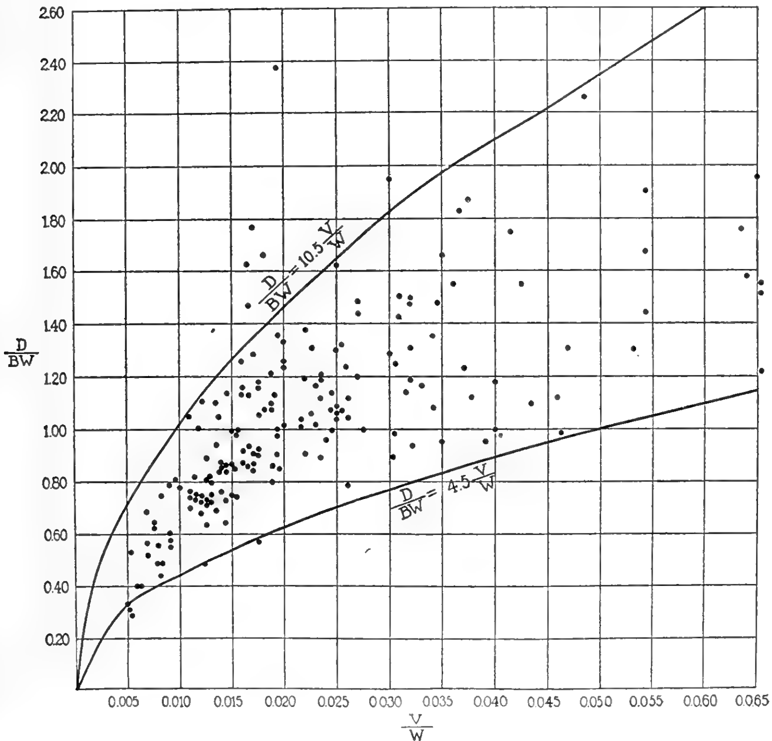

FIG. 9. Showing all observations on normal individuals with urine excretion rates below augmentation limit, reported by Addis, by McLean, and by ourselves.

$$\text{Ordinates} = \frac{\text{rate of urea excretion}}{\text{blood urea concentration} \times \text{body weight}}$$

$$\text{Abscissæ} = \frac{\text{rate of urine volume excretion}}{\text{body weight}}$$

The lower curve represents values for $K = 4.5$, the upper for $K = 10.5$. About 96 per cent of the determinations fall within these limits. Whether the 4 per cent that fall outside are due to unnoted analytical errors, or to occasional failure to empty the bladder completely at one end of the period, or whether they represent the actual frequency with which unrecognized factors deflect the excretion rate outside the usual range, it is at present impossible to say.

The greater part of the determinations yield results in the range midway between the two curves, covered by values of K between 6 and 9.

Variability of Results Obtained with Normal Individuals.

The fact that the "urea secretory constant" calculated even by the present formula, $K = \frac{D}{B\sqrt{VW}}$, varies in normal individuals between limits as wide as 7.5 ± 3 indicates that the two factors, rate of urine volume excretion and blood urea concentration, are not the only ones aside from kidney excretory ability which govern the rate of urea output. The variation in the constant indicates the presence of other factors, as yet unrecognized, perhaps of nervous or chemical nature, which influence to a minor, but at times marked, degree the rate of urea excretion. If these factors, or any one of them, can be both recognized and measured, we shall have advanced another step in the accuracy of our knowledge of renal function.

Relationships of Present Formula to That of Ambard.

1. The First Law of Ambard.—According to the first law of Ambard, with constant concentration of urea in the urine, the urea output varies as the square of the blood urea concentration.

$$\frac{D}{B^2} = K$$

The relation between the first law of Ambard and the present formula becomes clear, if the conditions of the experiments employed by Ambard in arriving at his first law are considered. These experiments were conducted by placing dogs on a diet of

meat and withholding all water other than that present in the meat. Under such conditions Ambard observed that the dog secretes a urine of constant and maximal urea concentration and more or less of both urine and urea per time unit according as the blood urea concentration is increased or decreased by the amount of meat fed.

If the present formula be applied to the special case in which the urea concentration in the urine, C, remains constant, the following is derived:

$$C = k_1 = \frac{D}{V}$$

hence

$$\frac{D}{k_1} = V \text{ or } k_2 D = V$$

substituting in

$$\frac{D}{B\sqrt{V}} = K$$

we have

$$\frac{D}{\sqrt{k_2 D}} = KB$$

combining constants

$$\frac{D}{\sqrt{D}} = k_3 B$$

or

$$\sqrt{D} = k_3 B \text{ or } \frac{D}{B^2} = k_4$$

$\frac{D}{B^2} = K$ is the mathematical expression of Ambard's first law. Therefore, Ambard's first law is a special case of our general formula limited to the condition which obtains when the concentration of urea in the urine is maintained constant.

2. The Second Law of Ambard.—According to the second law of Ambard, when the concentration of urea in the blood is constant, the rate of excretion of urea is inversely proportional to the square root of the urea concentration in the urine.

$$D\sqrt{C} = K$$

or

$$D = \frac{K}{\sqrt{C}}$$

If our general formula $\frac{D}{B\sqrt{V}} = K$ is true, however, the following relations may be derived:

$$(1) \quad \frac{D}{B\sqrt{V}} = k$$

$$(2) \quad \frac{D\sqrt{D}}{B\sqrt{V}\sqrt{D}} = k$$

since

$$(3) \quad \frac{\sqrt{D}}{\sqrt{V}} = \sqrt{C}, \qquad \frac{D\sqrt{C}}{B\sqrt{D}} = k$$

or

$$(4) \quad D\sqrt{C} = kB\sqrt{D}$$

Whence $D\sqrt{C} = K$ not when B is constant, as Ambard's second law states, but when $B\sqrt{D}$ is constant or when $B = \frac{k}{\sqrt{D}}$.

Hence Ambard's second law is inconsistent with our formula.

The Combined Laws of Ambard.

The quantitative differences between the present equation and that conceived by Ambard become clearer when the urine concentration, C, of the Ambard equation is transformed into terms of volume by substituting $\frac{D}{V}$ for C. The equations may be compared by arranging them both to express values of D. The complete Ambard equation in this form becomes

$$D = \frac{KB^2W}{\sqrt{\frac{D}{V}}}$$

whence

$$D^{\frac{3}{2}} = KB^2V^{\frac{1}{2}}W$$

$$D = K'B^{\frac{4}{3}}V^{\frac{1}{3}}W^{\frac{2}{3}} \quad \text{(Ambard equation)}$$

According to the equation developed in this paper

$$D = KBV^{\frac{1}{2}}W^{\frac{1}{2}} \quad \text{(present equation)}$$

The greater accuracy with which the present equation, as compared with that of Ambard, appears to express the relationships observed in man is indicated by Table III, which is a summary of the data presented in Table II.

In order to permit accurate comparison of the constants, we have expressed both Ambard's and ours in such form that D appears in the first power in the numerator of each. Thus Ambard's constant $K = \dfrac{B}{\sqrt{\dfrac{D}{W}\sqrt{C}}}$ we have inverted and squared giving it the form $\dfrac{D\sqrt{C}}{B^2W} = K$ or for comparison of repeated observations on the same individual simply $\dfrac{D\sqrt{C}}{B^2} = K$. Table III shows that in three of the four individuals studied the mean percentage deviation from the average urea secretory constant for the individual, calculated by the equation derived in this paper, *viz.* $K = \dfrac{D}{B\sqrt{V}}$, is approximately half as great as the percentage deviation in the Ambard quotient for the same individual. In the fourth individual it is only one-third as great.

Also, when the weight factor is introduced for each individual by taking the average of all the constants determined on the individual and correcting it by his body weight, the average constants thus determined by the present equation $K = \dfrac{D}{B\sqrt{VW}}$ vary, in the four individuals, between the limits 6.1 and 8.5. The average constants calculated by Ambard's equation $K = \dfrac{D\sqrt{C}}{B^2W}$ vary from 8.1 to 18 for the four subjects. While the number of subjects is too small to base conclusions on this difference, the latter is so marked that it may be significant.

We believe that by directing our attention to urinary volume as well as concentration, and by utilizing data obtained with methods for urea determination perhaps more accurate than those

originally available to Ambard, we have been able to express the influence of the most important blood factor (urea concentration) and the most important urinary factor (rate of urine volume excretion), on the rate of urea excretion in an equation of definitely greater accuracy than that originally conceived by Ambard. If this belief is confirmed, the present work is to be regarded not as a disproof of Ambard's, but rather as an advance which has proceeded along the path opened by his researches, and which has resulted in a somewhat closer approximation to his ideal of accurate functional measurement.

TABLE III.

Summary of Data of Table II (below Augmentation Limit).

Subject.	Weight.	No. of determinations.	$\frac{D\sqrt{C}}{B^2}$ Minimum.	$\frac{D\sqrt{C}}{B^2}$ Maximum.	$\frac{D\sqrt{C}}{B^2}$ Average.	$\frac{D\sqrt{C}}{B^2}$ Mean deviation from average. Numerical value.	$\frac{D\sqrt{C}}{B^2}$ Mean deviation from average. Per cent of average.	$\frac{D\sqrt{C}}{B^2W}$ Average.	$\frac{D}{B\sqrt{V}}$ Minimum.	$\frac{D}{B\sqrt{V}}$ Maximum.	$\frac{D}{B\sqrt{V}}$ Average.	$\frac{D}{B\sqrt{V}}$ Mean deviation from average. Numerical value.	$\frac{D}{B\sqrt{V}}$ Mean deviation from average. Per cent of average.	$\frac{D}{B\sqrt{VW}}$ Average.
	kilos													
Ad.	73	18	170	1,160	594	209	35	8.1	27	75	52	9.0	17.3	6.1
McL.	77	17	750	2,020	1,390	350	25	18.0	58	100	76	9.2	12.1	8.5
J.H.A.	66	13	500	995	750	105	14	11.3	52	67	60	4.6	7.7	7.4
V.S.	72	15	800	1,853	1,356	288	21	17.9	48	74	64	5.0	7.8	7.5

Use of the Creatinine Ratio in Calculating Excretion Rates.

It has been our experience that in determining the urea excretion rate, the most frequent source of error is failure to secure complete emptying of the bladder at the beginning or end of the period of collection of urine. This difficulty is more likely to be encountered in patients than in normal subjects. When the time elapsing between the two emptyings of the bladder is employed for the calculation of D and of V, as has been the custom in the use of Ambard's formula, incomplete emptying of the bladder introduces an error which may be very large.

It has been established by Van Hoogenhuyze and Verploegh (1905) and by Shaffer (1908–09) that the hourly elimination of creatinine throughout the day in a given individual is approximately constant.

Taking advantage of this constancy of creatinine excretion, we have found that often more consistent results were obtained when the calculations of D and V were based on the ratio of the creatinine in the sample of urine collected to the 24 hour excretion of creatinine, than when they were based on the ratio of the time interval of the collection period to 24 hours. The calculation based on the creatinine ratio is made as follows:

TABLE IV.

Excretion Constants in Normal Subjects Calculated by Time and Creatinine Ratios Respectively.

Subject.	Length of period.	Volume urine.	Day's volume calculated by		Urine urea N.	Day's urea N calculated by		Blood urea N.	Urea index calculated by	
			Time.	Creatinine.		Time.	Creatinine.		Time.	Creatinine.
	min.	*cc.*	*liters*	*liters*	*per cent*	*gm.*	*gm.*	*per cent*	$\frac{10\,D}{B\sqrt{VW}}$	$\frac{10\,D}{B\sqrt{VW}}$
J.H.A.										
1	60	20	0.48	0.47	15.4	7.4	7.3	0.198	66	66
2	63	22	0.50	0.51	16.3	8.2	8.2	0.207	69	69
6	59	41	1.00	1.04	12.9	12.9	13.4	0.206	80	78
9	63	49	1.12	1.19	12.6	14.1	15.0	0.211	78	80
10	41	39	1.37	1.32	9.5	13.1	12.6	0.173	80	78
12	195	302	2.23	1.94	7.6	17.0	14.8	0.198	71	66
13	53	130	3.53	2.77	6.1	21.6	16.9	0.197	72	63
V.S.										
1	42	31	0.85	0.65	10.7	9.1	7.0	0.140	83	73
2	60	42	1.01	1.13	8.9	9.0	10.0	0.140	75	79
3	42	31	1.06	1.03	10.9	10.8	11.3	0.166	75	79
4	60	102	2.45	2.46	6.0	14.6	14.7	0.126	87	88
6	39	102	3.77	3.12	4.6	17.3	14.3	0.125	84	76
9	30	80	3.85	3.64	3.7	14.2	13.5	0.098	87	85
11	60	184	4.40	4.40	3.1	13.7	13.7	0.113	68	68

$$V = \frac{\text{gm. creatinine excreted per 24 hrs.}}{\text{gm. creatinine per liter in sample of urine secured}}$$

$$D = V \times \text{gm. urea per liter of urine}$$

In entirely normal individuals, nearly identical results are obtained by both methods of calculation (Table IV).

Clinical Application of the Index of Urea Excretion.

The work presented in this paper has been undertaken with the frank intention of obtaining knowledge of normal urea secretion in order that pathological deviations therefrom may be recognized and studied with additional accuracy. Before application of the results to routine diagnosis can appear justified, however, it is necessary to compare the relationships of urea excretion to its governing factors as established in normal individuals with the relationships found in a sufficient number of patients under the most complete and prolonged clinical observation. The results of such comparison may be expected to indicate the possible variety of interpretations, diagnostic and prognostic, that may attach to observed abnormalities in the excretion. Until this has been accomplished, the functional index must be regarded as an object of investigation, rather than as an aid in the clinic.

CONCLUSIONS.

1. The rate of urea excretion per unit of body weight in a normal dog or man increases approximately (*a*) in simple direct proportion to the blood urea concentration, and (*b*) in proportion to the square root of the rate of volume output of urine per unit of body weight, as long as the volume rate remains within ordinary limits.

2. The increase in rate of urea excretion with volume output of urine holds, however, only up to a certain limit of volume output. This "augmentation limit" varied in different normal individuals between 2.5 and 6 liters per 24 hour time unit. Beyond this limit, rise of urine volume to any height does not further accelerate urea excretion.

3. That an augmentation limit exists not only in volume output of urine, but also in blood urea concentration is indicated by the results of Addis, who found that after the blood urea in rabbits reached 2.5 gm. per liter further increase no longer accelerated excretion. Unlike the augmentation limit of urine volume excretion, however, that of blood urea concentration lies at a

height so greatly above that ever approximated in normal individuals that it may be neglected in formulating naturally occurring relationships.

4. The observed relationships are expressed in the equation

$$\frac{D}{W} = KB\sqrt{\frac{V}{W}}$$

or

$$K = \frac{D}{B\sqrt{VW}} = 7.5 \pm 3 \text{ (for normal man)}$$

D being the urea output (gm. per 24 hour time unit), B the blood urea (gm. per liter), V the volume output (liters per 24 hour time unit), W the body weight (kilos), and K the excretory constant. For values of V above the augmentation limit, the value of the augmentation limit A replaces V in the formula.

Values for K below the minimum normal limit of 4.5 indicate that for the blood urea concentration, urine volume output, and body weight of the individual, urea excretion is abnormally slow.

5. Ambard's first law, $\frac{D}{B^2} = K$ when C is constant, is shown to be a special case of the above equation. His second law, $D\sqrt{C} = K$ when B is constant, is shown to be inconsistent with our formula.

The greater constancy of results calculated by the equation developed in this paper indicates the probability that it expresses the influence of the chief factors governing excretion with a closer degree of accuracy than does Ambard's equation.

6. The use of a creatinine ratio rather than a time ratio for calculating urinary excretion rates is described, and is advocated when, because of retention in the bladder or of other factors, the accuracy with which the time intervals of the collection period can be measured is doubtful.

7. The physiological studies presented in this paper afford a basis for detecting abnormalities in urea excretion, but not for interpreting the significance of such abnormalities.

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VITAMINES AND YEAST GROWTH.

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In an earlier paper (1) evidence was given that yeast needs for its growth an unknown substance which was concluded to be identical with the antineuritic vitamine. Later (2) the test was developed as a quantitative method for determining this vitamine.

It was thought best to check further the original conclusion by comparison of the vitamine content of different foodstuffs, as determined by the yeast method, with their vitamine content as determined recently by Osborne and Mendel (3) by the use of growing rats. Accordingly determinations were made on the materials which had been chosen by Osborne and Mendel as far as they were available.

Difficulties were encountered in extracting with alcohol in that alcohol ordinarily extracts the vitamine only partially compared with a like extraction with water. Therefore, water was used under such conditions as would give a fairly uniform and complete extraction provided the vitamine was not combined in some way so as not to be extracted. 1 gm. of the dry material was ground with carborundum flour to a fine powder, suspended in 100 cc. of water, and kept at 60°C. over night. After filtration the clear extract was tested; an appropriate amount to give a growth of 5 to 20 mg. of yeast was used. The experiments were carried out according to previous directions (2) using two dilutions, one twice as concentrated as the first. The value of the material in question is expressed in mg. of yeast which would be produced under given conditions from the extract of 1 gm. of the material. A proportionality between growth produced and vitamine added is obtained only when a very small seeding of yeast is used and the initial growth is measured. When a larger amount of yeast is used for seeding and growth is allowed to go nearly to

completion, the writer has found increasing vitamine content in some cases to have a distinct retarding effect. This would probably explain the contrary results of Fulmer, Nelson, and Sherwood (4).

Table I is a record of the results obtained and a comparison with the results of Osborne and Mendel obtained from rat feeding experiments.

TABLE I.

	Osborne and Mendel's rat feeding experiments.	Yeast method duplicates.
		mg.
Brewers' yeast...	Richest.	380
		360
Alfalfa..........	About one-fourth value of brewers' yeast.	144
		162
Spinach.........	About one-half value of alfalfa.	116
Tomato (canned).	" " " " "	52
	" " " " "	68
Carrot...........	" " " " "	76
Turnip...........	" " " " "	56
	" " " " "	62
Cabbage.........	" " " " "	104
Potato...........	Less than one-half value of alfalfa.	104
Beet.............	Lower than any other roots tested.	34
		38
Milk.............	Less than one-sixteenth value of alfalfa.	12

In the case of the rat feeding experiments the materials were fed directly while in the case of the yeast experiments, extracts of the materials were tested. Individual samples of a given material are known to vary in vitamine content. For instance, younger plants are found to contain more vitamine than mature plants. Considering these facts and the limitations of each

method, it seems that the agreement so far between the two methods is satisfactory.

In addition to the substances tested by Osborne and Mendel and listed above, ordinary bakers' yeast was tested for its vitamine content using the yeast method. As the extraction was carried out bakers' yeast showed a value over three times that of brewers' yeast. As the vitamine value of bakers' yeast had not been determined by animal feeding experiments, it was desirable to check this result by rat feeding experiments. Seven groups of rats (three in each group, weighing 60 to 90 gm. each) which had been bought from an animal dealer were fed on purified diets to which different additions of bakers' and brewers' yeast were made for comparison. The basal ration was composed of

	per cent
Casein	18
Salt mixture*	4
Starch	69
Butter fat	9

* The same as used by Osborne and Mendel.

To this diet were added 1.0, 0.5, and 0.25 per cent respectively of dried brewers' yeast[1] and for comparison the same percentages of dried bakers' yeast. One group was kept on the basal ration alone, as a control. Each rat was kept in a separate cage and the food consumption of each was recorded.

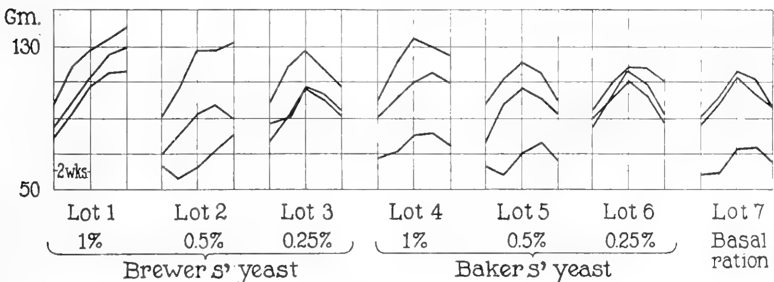

Fig. 1.

The growth curves in Fig. 1 show that the bakers' yeast is not three times as rich as brewers' yeast, as a source of vitamine for rats, but instead is not more than half as rich. The parallel use

[1] Obtained from the Scientific Station for Pure Products, New York.

of the yeast method and the rat growth method *in this case* gives absolutely contrary results. The difference in case of extraction might have been responsible for this discrepancy. This was found, however, by experiments with extracts on rats not to be the case.

This disagreement between the two methods does not necessarily mean that the substance which promotes the growth of rats fails to promote the growth of yeast. There is evidently some substance which is able to promote the growth of yeast which fails to induce rat growth. This substance may be a somewhat specific growth stimulant evidently capable of being produced by the yeast itself, which is more potent for its own growth than any related substance from any other source. The probability of such interpretation is indicated by the following experiment. Water extracts of bakers' yeast and of brewers' yeast were tested in the usual manner in duplicate by the growth of bakers' yeast. In addition, following the same procedure these extracts were tested for their effect on the growth of a pure culture of *brewers'* yeast.[1]

	Bakers' yeast.	Brewers' yeast.
	mg.	*mg.*
0.1 cc. bakers' yeast extract	8.8	1.9
0.2 " " " "	17.5	3.6
0.1 " brewers' " "	6.9	3.2
0.2 " " " "	12.0	5.0

Whereas bakers' yeast extract is better for promoting the growth of bakers' yeast, brewers' yeast extract is more potent in stimulating the growth of brewers' yeast. There is evidently some specificity of growth stimulants in different organisms, but this does not mean necessarily that absolutely different substances are necessary for the stimulation of growth in bakers' and in brewers' yeast. The B vitamine which promotes rat growth may be able to stimulate the growth of either bakers' or brewers' yeast. Substances capable of promoting the growth of bakers' yeast are known to occur in milk, in blood (5), as well as in animal organs and tissues (6). Here they would naturally be expected to have an intimate connection with growth as in the case of the yeast organism.

It was stated in a previous paper (2) that yeast was able to produce vitamine. This was ascertained by determining the relative amount of vitamine in a commercial wort, in the corresponding amount of yeast, and in an equivalent amount of the separated beer. The yeast in some cases contained several times as much vitamine as could have been taken in from the wort. The yeast method was used for these determinations and hence the test was for the substance promoting the growth of yeast. Whether yeast is able at the same time during growth to produce substances also potent for rats is an open question.

TABLE II.

Extract tested.	Heat treatment.	Yeast per gm. of material.	
		Lower concentration.	Higher concentration.
		mg.	*mg.*
1. Cabbage	Over night at 60°C. and 20 min. at 100°C.	104	192
2. New potato	Over night at 60°C. and 20 min. at 100°C.	104	256
3. Spinach	Over night at 60°C. and 20 min. at 100°C.	116	156
4. Carrot	Over night at 60°C. and 20 min. at 100°C.	76	106
5. Malted barley (acrospires overgrown)	Berkefeld filter; no heat.	20	36
6. Barley (beginning to sprout)	" " " "	12	18
7. Barley (unsprouted)	" " " "	11.7	10.7
8. " " different from No. 7	100°C. for 30 min.	20	16.7
9. Barley (sprouted) different from No. 5	100 " " 30 "	22	26.2

In Table I some of the values were omitted as evidently another factor came into play in some cases where the higher concentration was used. If toxic materials are present the higher concentration gives too low results to show a direct proportion. Some of the results in Table II, however, show a disproportionality in the opposite direction, whereas extracts of yeast (and other materials, especially if sterilized thoroughly with heat) ordinarily give a good proportionality.

It was noticed after these results were obtained that the materials which showed a marked disproportionality in this direction are known to be unusually good sources of Vitamine C, or the antiscorbutic vitamine, and that the factor producing this disproportionality appears quite labile to heat as is Vitamine C. This factor seems to be produced during the sprouting of barley which is also true of Vitamine C. As it is not feasible at this time to repeat and extend these experiments the results are presented for their value as they stand.

SUMMARY.

1. The quantitative yeast method for determining vitamine on the materials selected by Osborne and Mendel gives results fairly concordant with the results obtained by Osborne and Mendel in rat feeding experiments.

2. Bakers' yeast, however, by the yeast method shows a much higher vitamine content than brewers' yeast, whereas by animal feeding experiments here reported brewers' is found much richer. A bakers' yeast extract is found to be richer as regards the growth of bakers' yeast than a brewers' yeast extract. A brewers' yeast extract, however, is richer in affecting the growth of brewers' yeast than a bakers' yeast extract. This is interpreted to mean that there is some specificity in growth stimulants, but not necessarily two totally different substances stimulating the growth of the two varieties of yeast.

3. Some results are recorded which present the possibility that Vitamine C, as a secondary factor, may stimulate yeast growth.

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THE USE OF EDESTIN IN DETERMINING THE PROTEOLYTIC ACTIVITY OF PEPSIN.

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Although there are numerous methods for testing peptic strength, there is only one, the method of the United States Pharmacopœia commonly known as the "U. S. P. method," which is official in the United States for the assay of pepsin.

There has been much criticism of the above named method by many who have occasion to use it, chiefly on the ground that the basis of the test is unsound. From eggs which have been boiled 15 minutes, the white is separated from shell, yolk, and membrane, passed through a 40 mesh sieve, and mixed with a standard hydrochloric acid to provide protein for the test. After digestion with pepsin during $2\frac{1}{2}$ hours at 52°, the volume of unliquefied residue is measured. It is often difficult for the analyst to check himself with the method even when running several tests side by side. Not only do the whites of eggs vary in composition, but also errors are readily, though unwittingly, introduced in the preparation of the material.

In working with pepsin and other proteolytic enzymes it occurred to the writer that some of the crystallizable proteins might be used in making the pepsin assay, and might also be employed in general enzyme work, at least where the first stage of digestion or liquefaction is being studied.

Edestin, the globulin of hempseed, has been used by Fuld and Levison (1), and Farrington and Lewis (2) in clinical methods devised by them for testing the peptic activity of stomach contents.

The method of Fuld is admirable in principle and can be applied successfully to the assay of commercial pepsins in a great many cases after certain modifications in concentration of pepsin and

edestin solutions have been made. Some commercial pepsins, however, when in solution give an immediate cloud or slight precipitate when added to Fuld's edestin solution so that difficulty in reading end-points arises. Fuld and Levison have also failed to set down any specifications for the preparation of their edestin and have stated no standard of purity.

Farrington and Lewis employ edestin in 10 per cent sodium chloride solution and give details of the preparation of the edestin, which they regard as sufficiently pure for their purposes.

The Jacoby-Solms (3) method must be mentioned here, since there is much of similarity in their method, that of Farrington and Lewis, and the one described here. Jacoby and Solms make use of so called ricin from the castor bean and recommend that the material be purchased from manufacturers of laboratory supplies. The product supplied cannot be regarded as a pure protein and in fact often contains coarse matter that appears to be hulls. It has also been found that the precipitated ricin sometimes fails to digest even upon standing 15 hours.

An attempt is made in the present paper to show that edestin prepared by crystallization from sodium chloride solution may be adopted as a basis for pepsin assay, the edestin present in a given preparation in turn being measured by a careful nitrogen determination. It is obvious that both the procedure for obtaining edestin and for performing the pepsin test should be standardized. The method of preparing edestin as described below varies little from the usual one and the writer wishes here to acknowledge his indebtedness to Dr. C. O. Johns of the Protein Investigation Laboratory of the Bureau of Chemistry for suggestions regarding it.

Osborne (4) has shown that pure edestin is soluble in neutral salt solutions but insoluble in water alone. If hydrochloric acid is added in definite quantity to a solution of edestin in salt solution there is formed first what is regarded as a monohydrochloride insoluble in pure water but soluble in weak salt solution. If twice this amount of hydrochloric acid is added, there is formed a dihydrochloride soluble in water and in stronger salt solution (10 per cent NaCl). Conversely, if salt solution is added to an aqueous solution of the dihydrochloride the protein is precipitated. Fuld takes advantage of this fact for determining the end-point in his method.

The writer has verified Osborne's statement that the dihydrochloride is entirely precipitated by weaker salt solutions (2.5 to 5 gm. of NaCl per 100 cc.). This was readily done by determining nitrogen in 20 cc. of the acid solution of the protein, then precipitating the protein in an aliquot by adding salt solution, filtering, washing with acid-salt solution of the same concentration as the precipitating medium, and determining nitrogen in the precipitate. The results are given in Table I.

TABLE I.

Completeness of Precipitation of Edestin by Salt Solution.

Sample No.	N present.	N precipitated.
	gm.	*gm.*
1	0.01415	0.01373
	0.01415	0.01429
2	0.03376	0.03334
	0.03418	0.03362

Evidence of the uniformity of edestin content calculated from the determination of nitrogen in the samples is provided. Three preparations of the protein made at different times and from different lots of hempseed were submitted to trial in the method of assay described below against the same pepsin.

The results appear in Table II.

Preparation of Edestin.

The preparation of edestin is performed by extracting most of the fat from the ground hempseed and then extracting the air-dried meal with 5 per cent sodium chloride solution and recrystallizing the edestin which separates. Since the method is a general one it is considered unnecessary to describe details here, as these are to be found in the published work of Osborne.[1] The only differences to be noted are the use of 5 per cent sodium chloride instead of 10 or 20 per cent, and the manner of washing

[1] At the request of several investigators who were interested in the assay of pepsin the author wrote in detail his exact procedure. Mimeographed copies were made and these may be obtained by writing to the Bureau of Chemistry, Washington, D. C.

and drying the edestin. After the recrystallized edestin is filtered it is washed twice with each of the following: 50 per cent alcohol, 95 per cent alcohol, absolute alcohol, and finally three times with ether. The edestin is air-dried at room temperature.

Method of Pepsin Assay.

Apparatus.—A constant temperature water bath with stirring mechanism capable of regulation to within ±0.05° is used. Electrically heated and regulated baths are best. A suitably partitioned oblong wire rack such as is used by bacteriologists is fitted in the bath to receive the test-tubes.

Test-tubes of Pyrex glass 15 mm. wide and about 115 mm. long or bacteriological culture tubes of the same dimensions with heavy walls may be used. Heavy tubes may be immersed sufficiently deep without being disturbed by agitation of the bath.

Four 1 cc. pipettes graduated in 0.01 cc., certified by the Bureau of Standards, are required.

Reagents.—1 Per Cent Edestin Solution.—From the nitrogen determination as stated under "Preparation of edestin" calculate the weight of preparation to furnish 1 gm. of pure edestin. Weigh out this amount, transfer it to a 100 cc. beaker, and add a few drops of water from a burette. Thoroughly mix to a paste before adding more water, making certain that all particles of edestin are broken up and brought into contact with water as the ease of solution depends on first thoroughly wetting the edestin. Some preparations will dissolve readily in water alone. This is due to the fact, as explained by Osborne, that a soluble salt of edestin has been formed in the course of preparation. Obviously this is no disadvantage. When the edestin and water have been thoroughly mixed, more water in small portions is added from the burette to make the total volume used 25 cc. The mixture is stirred to disintegrate the paste and 0.2 N hydrochloric acid in 0.5 cc. portions is run into the mixture from a burette and stirred until 25 cc. have been added. The edestin should be completely dissolved and the solution clear without opalescence. The solution is transferred to a 100 cc. volumetric flask, the beaker thoroughly rinsed with 0.1 N hydrochloric acid, and the rinsings are poured into the flask. The volume of the solution is now

made up to the mark with 0.1 N hydrochloric acid. Edestin solutions in hydrochloric acid which have stood longer than 48 hours should not be employed for the test.

1 Per Cent Pepsin Solution.—The sample of pepsin is pulverized to pass a 60 mesh sieve and dried *in vacuo* over sulfuric acid. The dried sample is placed in a small tube and kept tightly corked. Weigh out quickly 1 gm. of the pepsin, transfer to a 100 cc. beaker, add 5 cc. of 0.05 N hydrochloric acid, and stir to a paste with a glass rod. Add more 0.05 N hydrochloric acid, dissolving the pepsin and transfer the solutions and washings to a 100 cc. volumetric flask, making up to the mark with the same acid. Some pepsin solutions are cloudy and it is an advantage to pass these repeatedly through a dry filter, although it is not always possible to remove all the cloudiness. Repeated filtration does, however, remove filter fibers which may be mistaken in the test for undigested protein.

10 Per Cent Sodium Chloride Solution.—Dissolve 100 gm. of sodium chloride in water and make up to 1 liter. The solution is passed through a dry filter and the filtration repeated through the same filter to clear the solution of all suspended matter.

0.1 N Hydrochloric Acid.—

The Assay.—A series of six thoroughly clean, dry test-tubes is set up in a convenient rack and numbered progressively from 1 to 6. Into these the reagents are introduced successively from the graduated pipettes. All the tubes receive first their quota of 1 per cent edestin, then the 0.1 N hydrochloric acid, then the 10 per cent sodium chloride, and finally the pepsin. The edestin is added in increments of 0.25 cc. in Tubes 1 to 4, beginning with 0.25 cc. in Tube 1. Tubes 5 and 6 each receive 1 cc. the same as Tube 4. It is recommended that Nos. 4, 5, and 6 be made in triplicate to serve as a check on each other for the reason that in the higher concentration of edestin the protein is sometimes precipitated in the form of a curd which becomes very compact and resistant to the action of pepsin. The 0.1 N hydrochloric acid is now added in decrements of 0.25 cc. beginning with 0.75 cc. in Tube 1. Tubes 4, 5, and 6 receive none.

1 cc. of 10 per cent sodium chloride is now added to each of the tubes. This precipitates the edestin. The tubes are not to be shaken at this time. The time of beginning the digestion is now

set down in table form along with the records of the tubes. An example of the entire procedure is to be found in Table II. It is the writer's practice to space the time intervals 1 minute apart as this allows ample time for filling the pipette and delivering its contents. 1 cc. of the 1 per cent pepsin solution in 0.05 N hydrochloric acid is now added. The addition of the pepsin is to begin exactly on the instant the second hand of the watch reaches the 60 second graduation. This, of course, should correspond with the minute set and recorded for its specific tube. As soon as all the pepsin is added, the test-tube held by the top is gently shaken by describing one or two small horizontal circles with the bottom to mix the contents thoroughly. Care is to be taken that particles of edestin are not forced up on the side of the tube. If the mixture is too violently shaken, the edestin tends to form a stiff curd. It is desirable to retain the precipitate in a flocculent condition. The tube and contents are immediately placed in the constant temperature bath which is to be held at 37.5°. During the digestion the tubes containing the mixtures should be gently shaken at 5 minute intervals as above.

The time of the end of reaction may be taken at the instant when the precipitated protein is completely liquefied and the contents of the tube become perfectly clear. This point is usually very sharp, particularly in the lower concentations of edestin. The error in the higher concentrations is not greater than ± 15 seconds. Tube 1 must be closely watched since it is the first to clear and its time interval may be used as a guide in approximating the intervals of the other tubes. It may also be used in determining end-points of the others by comparison.

RESULTS.

The various results obtained with the new procedure are shown in Table II. Pepsin 1 was assayed by the U. S. P. test, and, in terms of the U. S. P. formula was found to be 1 : 2,830 or to possess 94.33 per cent full U. S. P. activity. The other samples of pepsin were not submitted to the U. S. P. assay.

In Fig. 1 curves are plotted from the data of Experiments 2, 5, and 6 (Pepsins 1, 2, and 3 respectively), with time of digestion in minutes as abscissæ and concentration of substrate as

ordinates. As one gains acquaintance with the method the ideal curve, a straight line, is frequently obtainable.

If also one represents volume of substrate by s and time of digestion by t, then $\frac{t}{s}$ = constant.

Thus, for Pepsin 1, $\frac{t}{s} = 40$, for Pepsin 2, $\frac{t}{s} = 38$, and for Pepsin 3, $\frac{t}{s} = 21.5$ and the relative values are found directly. These values may be readily translated into the U. S. P. terms. Assuming Pepsin 2 to possess U. S. P. activity or to be 1:3,000, then No. 1 is 95 per cent of 3,000 or 1:2,850 and No. 3 is 183.1 per cent or 1:5,494.

TABLE II.

Digestion mixture.	Tube No. 1	Tube No. 2	Tube No. 3	Tube No. 4, 5, and 6.	Edestin Preparation No.
1 per cent edestin, *cc.*	0.25	0.50	0.75	1.00	
0.1 N HCl, "	0.75	0.50	0.25	0.00	
10 per cent NaCl, "	1.00	1.00	1.00	1.00	
1 per cent pepsin, "	1.00	1.00	1.00	1.00	
Experiment No.	**Digestion time.**				
	min.	*min.*	*min.*	*min.*	
1. Pepsin 1	9	19	25	40	2
2. " 1	10	20	30	40	2
3. " 1	11	22	30	40	15
4. " 1	12	20	30	40	16
5. " 2	9.5	19	28.5	Undissolved.	2
6. " 3	5	11	15	21	15

If it is deemed desirable to obtain more data on which to base calculations this may be done by increasing the number of tubes in the experiment and changing the spacing of volumes of edestin solutions. The spacing may be arranged in increments of 0.1 cc., beginning preferably at 0.2, thus giving 9 tubes and 9 sets of data. The decrements of 0.1 N hydrochloric acid must be arranged accordingly.

The rest of the procedure is to be carried out precisely as described in the method of assay.

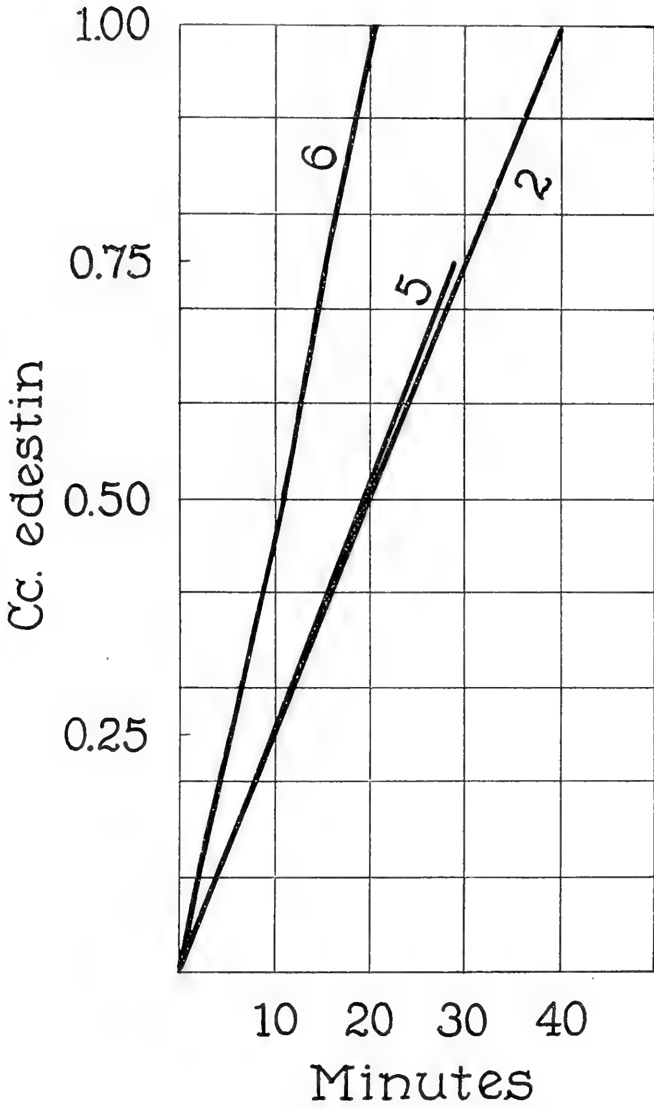

FIG. 1.

SUMMARY.

A method for the assay of pepsin based upon the use of edestin is given.

A method for the preparation of standard edestin for the pepsin test by recrystallization of the protein is given.

The edestin content of a given preparation made by the above method may be calculated from the nitrogen content of the preparation.

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THE ACIDITY OF GOAT'S MILK IN TERMS OF HYDROGEN ION CONCENTRATION, WITH COMPARISONS TO THAT OF COW'S AND HUMAN MILK.

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Goat's milk is occasionally recommended in preference to cow's milk for infant feeding. For that reason the authors considered it of interest to determine the hydrogen ion concentration of goat's milk and thereby ascertain the relation that the acidity of goat's milk bears to that of cow's and human milk. Easy access to two herds of milch goats encouraged these studies. A series of determinations was made on freshly drawn milk, and also a series on milk allowed to sour at room temperature in the laboratory. The latter readings were made to determine the maximum acidity of soured goat's milk, in order that comparisons could also be made with the maximum acidity attained by cow's milk.

Method.

The colorimetric method was employed. The principles and procedures of this method of determining the hydrogen ion concentration of fluids are fully described in an excellent treatise on the subject by Clark.[1] The Sörensen mixtures of primary and secondary phosphates were used as standard buffer solutions for the pH range from 7.2 to 5.2; the Clark and Lubs standard mixtures of potassium hydrogen phthalate and sodium hydroxide from 5.2 to 4.0; and their mixture of potassium hydrogen phthalate and hydrochloric acid from 4.0 to 3.4. Brom-thymol blue was used as an indicator for the pH range from 7.2 to 6.0, methyl

[1] Clark, W. M., The determination of hydrogen ions, Baltimore, 1920 (contains complete bibliography).

red from 6.0 to 4.6, and brom-phenol blue from 4.6 to 3.4. Pyrex glassware was used throughout. The accuracy of the standard solutions was controlled.

The samples of milk were taken directly from the udder into a sterile flask and immediately taken to the laboratory. The readings were made within 1 hour, usually within 30 minutes. About 1 cc. of milk was dialyzed through a collodion sac against 2 cc. of neutral distilled water. (Distilled water gave us the same results as physiological salt solution, and the proportion of milk to water used the same as equal volumes.) $7\frac{1}{2}$ minutes were allowed for dialysis to take place. The proper amount of indicator was then added to the dialysate and the reading made. The maximum acidity of soured milk was determined by making daily readings on each sample until constant readings were obtained. In no case did a sample of sour milk show a decrease in acidity.

Results.

The results obtained from 160 determinations on fresh goat's milk varied from pH 6.7 to 6.4. The average pH for 86 morning samples was 6.52; for 74 evening samples 6.54; and 6.53 for the total number.

The end-points of 73 samples of soured goat's milk varied from pH 4.4 to 3.7, averaging 3.92.

Hydrogen Ion Concentration of Cow's and Human Milk.

Cow's Milk.

Van Slyke and Baker[2] state that the pH in 300 samples of cow's milk ranged from 6.5 to 7.2, 83 per cent of which were between 6.5 and 6.76. Taylor[3] gives a pH of 6.8; Clark[4] of 6.6; Van Dam[5] of 6.74; Alleman[6] of 6.61; Davidsohn[7] of 6.52; and Mil-

[2] Van Slyke, L. L., and Baker, J. C., *J. Biol. Chem.*, 1919, xl, 345.

[3] Taylor, H. B., *J. Proc. Roy. Soc. New South Wales*, 1913, lxvii, pt. 2, 174.

[4] Clark, W. M., *J. Med. Research*, 1914–15, xxxi, 431.

[5] Van Dam, W., *Rev. gén. Lait*, 1908, vii, 121.

[6] Alleman, O., *Biochem. Z.*, 1912, lxv, 346.

[7] Davidsohn, H., *Z. Kinderheilk.*, 1913, ix, 14.

roy[8] of 6.8 to 6.64. These were all determinations on fresh cow's milk. Taylor[3] found the maximum acidity of soured cow's milk to be 4.65.

Human Milk.

Davidsohn[9] found that human breast milk gave a pH reading of 6.97, while Clark's[4] determinations average 7.22. However, the latter author states it is safe to say that the average hydrogen ion concentration of human breast milk lies between 6.86 and 7.46. Terry[10] states that cow's milk has about six times the potential acidity of human milk.

CONCLUSIONS.

The average hydrogen ion concentration of fresh goat's milk is 6.53; that of completely soured milk 3.92. Fresh goat's milk is therefore slightly more acid then fresh cow's milk, and appreciably more acid than human breast milk. Soured goat's milk is considerably more acid than soured cow's milk.

[8] Milroy, T. H., *Pharmacol. J.*, 1914, xciii, 350.
[9] Davidsohn,[7] p. 11.
[10] Terry, R. W., *J. Am. Pharm. Assn.*, 1919, viii, 538.

THE SIZE OF FAT GLOBULES IN GOAT'S MILK.

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The following determinations were made while the authors were conducting studies on the hydrogen ion concentration of goat's milk. They were prompted by the fact that goat's milk rarely forms a cream layer, though the percentage of fat is usually not appreciably below that of cow's milk. That the fat globules are comparatively small is generally recognized, but no definite idea of their size is given in the literature.

The specimens were studied in the fresh state with the aid of a dark ground illuminator and a micrometer attached to an ordinary microscope. The specimens were mounted in the shape of capillary films between a slide and cover-slip, and rimmed with vaseline. Several hundred globules were grouped as to sizes in each specimen. Whole milk, freshly drawn, was used. It came from fifteen milch goats, for the most part of mixed breeding, and during various periods of lactation.

The results obtained are given in Table I.

TABLE I.

Average Percentages of Various Sized Fat Globules in 45 Samples of Goat's Milk.

Below 2 μ.	2 to 4 μ.	4 to 6 μ.	6 to 8 μ.	8 to 10 μ.
57.0	34.0	7.0	2.0	0

DISCUSSION.

It will be noted that 91 per cent of the fat globules are under 4 microns; and that over half of these are under 2 microns. It is interesting to compare these figures with those obtained by

Bitting[1] on whole cow's milk. The results which he obtained may be tabulated as follows:

Size in microns.......	4	6	8	12	16	20	24
Per cent.............	7	19	31.5	18	12	7.5	2

These observations show that in cow's milk 90 per cent of the fat globules are over 4 microns. Of course, the size depends to some extent upon the breed, they being largest in Jersey and smallest in Holstein milk. The fat globules in human milk are said to be even larger than those in cow's milk. Heineman[2] states that they attain a size of 32 microns. According to him the cream layer forms within a few hours and separates more completely than the fat in cow's milk, which is confirmatory evidence of the larger size of the globules.

Bosworth and Van Slyke[3] give the following comparison of the fat content of cow's, goat's, and human milk: cow's milk 3.90, goat's milk 3.80, and human milk 3.30 per cent. With the quantity of fat practically the same, it becomes obvious that the fat in goat's milk, being more highly dispersed, possesses an enormously greater surface area than does that of either cow's or human milk. It should, therefore, be considerably more accessible to the lipase of the digestive juices, and consequently more rapidly and completely digested.

[1] Bitting, A. W., *U. S. Dept. Agric., Bureau of Animal Industry, 19th Ann. Rep.*, 1902.

[2] Heineman, P. G., Milk, Philadelphia, 1919.

[3] Bosworth, A. W., and Van Slyke, L. L., *J. Biol. Chem.*, 1916, xxiv, 173.

CHEMICAL NATURE OF TISSUE COAGULINS.*

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(Received for publication, August 18, 1920.)

The importance of tissue extracts in hastening the coagulation of the blood has roused interest for a great many years. Carpenter in his "Principles of human physiology," 1869, states that in 1834 it was known that brain tissue, or an emulsion of it, often caused intravascular clotting of the blood when mixed with it by accident or intention (for exact quotation see (1)). Nothing was done, however, to determine the chemical nature of this active substance or the manner of its action on the blood until 1883 when Wooldridge (2) began his work in this field. The author wishes to state here that Wooldridge's views on the coagulation of blood and the action of tissue extracts have been of the greatest value and inspiration during the course of the present work. Very little has really been added to the knowledge of intravascular coagulation since his death.

Working with watery extracts of the testes, thymus, lymph glands, and red blood cells, Wooldridge found that the active material was precipitated by strong acidification with acetic acid, and this precipitate, washed in water, was soluble in dilute sodium carbonate. He stated that 1.5 gm. of this precipitate would kill a dog of ordinary size by intravascular clotting and that 0.5 to 1.0 gm. was sufficient to kill a rabbit. Extraction of the precipitate with alcohol and ether to remove the phospholipins was found to destroy its power to produce intravascular clotting, although it did not render it entirely insoluble in dilute alkali. Digestion with pepsin-HCl produced a precipitate which contained all the phospholipin content of the material together with a little of the protein. Neither the solution nor the precipitate from the digestive mixture would cause clotting in the vessels, so he concluded that the important substance was a protein-phospholipin compound and that the phospholipin fraction was essential for its coagulative action although it alone would not produce intravascular coagulation. It would, however, accelerate clotting *in vitro* of peptone plasma. Wooldridge called this phospholipin lecithin, although he showed that pure lecithin from the yolks of eggs was inactive. He was not familiar with the cephalin class of phospholipins at that time and so could not know

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that it was probably cephalin he was working with. This has been shown to be the case by more recent workers, especially by Howell (3). But later workers have missed an important point in Wooldridge's work and have assigned to cephalin the entire coagulative action of tissue extracts. Yet Wooldridge satisfied himself that the active agent was a protein-lecithin combination and that the lecithin present was essential to the activity of the compound. The present work will be seen to bear out Wooldridge's views in regard to the relative importance of the protein and phospholipin in the compound, since a crude laboratory preparation of cephalin does not possess 1/1,000th the activity toward oxalate plasma that is found in a certain protein-phospholipin compound isolated from tissue extracts.

As for Wooldridge's views on the manner of action of this material in causing intravascular clotting, they will be reviewed in the following paper dealing with the author's work in that line (4).

Following up the work of Wooldridge, Wright (5) brought forth the view that the substance was a nucleoalbumin, although nucleic acid alone possessed no activity in coagulation.

Brieger and Uhlenhuth (6) claimed to have discovered the toxicity of organ extracts, making no mention of Wooldridge's work. They produced death in animals by the subcutaneous injection of the toxin, and so missed the coagulative action of the material. It is likely that they were working with the same sensitive compound, however, since they found that it could be extracted with dilute alkalies, was destroyed by acids, was thermolabile, losing its toxicity at 80°C. (30 minutes), and was precipitated almost quantitatively by $(NH_4)_2SO_4$ and chlorides of the heavy metals. Since it was not affected by dialysis, and from the other facts mentioned above, they classed it with the toxalbumins. Dold (7) studied this same toxic material, noting that its toxicity was decreased by treatment with serum. No coagulative action was noted. Dold and Ogata (8) noticed the intravascular coagulation and the delayed coagulation of the remaining fluid blood. They found hirudin to prevent the toxic action of the extracts, as also did a previous injection of peptone. Extraction with ether and alcohol destroyed the activity so they decided it was of a ferment nature. Dold and Kodama (9) rediscovered the fact that treatment with acid to precipitate the proteins removed the toxin, but that resolution of the acid precipitate in dilute alkali restored the toxicity. They also found the material to be completely precipitated by saturation of the extract with $MgSO_4$. Heating to 60–70°C. destroyed the toxicity of rabbit lung extracts, but extracts of guinea pig lungs were not completely detoxicated by heating to 100°C. for some time. Izar (10) removed the toxin from extracts by shaking with chalk, animal charcoal, or brain emulsions. He also found that the cations of certain salts detoxicated the material. Blood serum did the same. Dold and Ogata (11) found that it was not necessary to break up the cells to obtain a toxic extract. By work on different parts of the eye, they concluded that the amount of toxic substance obtained was proportional to the lymph present rather than the cells. Popielski (12) described vasodilatin, which he obtained from peptone solutions and from tissue extracts.

This gave the same fall in blood pressure as did tissue extracts, but was without their coagulative action, or possessed a negative effect on coagulation. He claimed that it was not histamine although their actions were similar. Aronson (13) also isolated a toxic organic base from tissue extracts, which acted like histamine, giving bladder peristalsis and acute death. Biuret test was negative on this substance. Dale and his coworkers (14) isolated a number of toxic amines from putrid tissues, chief among these amines being histamine. They also found this amine to be present in the walls of the intestine and in extracts of other tissues, but believed it was not present except as formed from histidine by bacteria. Ackermann (15) and Mellanby and Twort (16) showed that such a formation of histamine by decarboxylation of histidine was readily brought about by bacterial action, especially by a certain bacillus of the colon group. Abel and Kubota (17) decided that histamine was present in all tissue extracts, especially extracts of the posterior portion of the pituitary gland, hydrolysis alone, without bacterial action, being sufficient to free the histamine from the protein material. Jackson and Mills (18), however, demonstrated that pituitary extracts need not contain any histamine in order to possess their typical action, although some pituitary preparations do apparently contain very small amounts of the base.

Morse (19) in a recent article refers to work in which he took part a few years ago and in which it was concluded that the toxic agent of tissue extracts was related to the phospholipin content of the tissues, being found bound with nucleoproteins, so that it precipitates with them. Different samples of nucleic acid were found to be without action. The inference is that the toxicity of the extracts is due to their phospholipin content, although Wooldridge showed clearly about 35 years ago that the protein fraction was as essential as the phospholipin.

There seem, then, according to the work in the past, to be two different toxins present in tissue extracts, one causing blood to coagulate, and the other causing a great fall in blood pressure and giving other histamine-like reactions. The latter toxin was found by several investigators to delay the coagulation of blood.

It will be shown in the present communication that Wooldridge was essentially correct in his conclusion that the active agent of tissue extracts was a protein-phospholipin compound, and that the phospholipin alone, although possessing a slight effect on blood coagulation, can account for only a very small fraction of the total activity of the extract. It will also be shown that splitting of the compound destroys its activity, and that reunion of the two fragments restores its action. Neither the protein nor phospholipin alone shows more than a slight activity, not sufficient to cause death when injected in any reasonable amount; but the

combination of the two forms an exceedingly powerful coagulant for blood.

As to the possible presence of histamine in tissue extracts, it will only be shown here that there is none in the purified coagulant. This does not exclude its presence among the other constituents of the extracts.

EXPERIMENTAL.

Since lung tissue was found to contain much more of the active material than any other tissue of the body, it was used principally in the course of this work. The lungs of dogs, rabbits, and cattle were used, all being about equally active. All tests for the active material were made on oxalated beef plasma since it was found possible to obtain fresh plasma in any amount and at any time from the abattoir. This gives in some ways an ideal testing fluid when fresh, for by means of it a noticeable shortening of the coagulation time can be detected with lung extract diluted several thousand times. The coagulation time of the plasma by calcium is found to lengthen gradually on standing in the ice box, due to a settling out of a granular precipitate—the A-fibrinogen of Wooldridge, which is so important in coagulation. This fact does not in the least interfere with its value as a test fluid for the presence of coagulation-accelerating agents. With proper care in measuring the fluids used, in keeping a constant temperature, and in handling the tubes, duplicate tests will usually vary less than 5 seconds in the coagulation time. With very rapid clotting the variation is much less.

Table I will serve to show how the coagulation time varies with the strength of the tissue extract used.

The lung extract used in these tests was a saturated extract made by powdering beef lung, previously dried in an air current at room temperature, and grinding well with 0.9 per cent NaCl solution in the ratio of about 4 cc. of salt solution to 1 gm. of dried lung tissue. Only a few minutes were necessary to insure saturation of the extract with the active substance, so that it must be readily soluble in dilute salt solution. Using a smaller proportion of salt solution does not result in a stronger or more active solution, as comparative tests on the coagulative activity showed. A larger amount of salt solution per gm. of dried lung

may be used and a saturated extract still be obtained, if a longer time is given for extraction.

From such a dilution table, the amount of active material in other extracts may be estimated merely by determining the amount of hastening of coagulation produced by them and then finding the dilution of lung extract that will produce coagulation in the same time.

TABLE I.

Dilution of Lung Extract.

1 cc. oxalate plasma, 0.04 cc. lung extract, 0.2 cc. 1 per cent $CaCl_2$.

Dilution.	Log of dilution.	Coagulation time.	Log of coagulation time.
		seconds	
1/1	0.00000	10	1.00000
1/2	0.30103	12	1.07918
1/4	0.60206	14	1.14613
1/8	0.90309	17	1.23045
1/16	1.20412	20	1.30103
1/32	1.50515	25	1.39794
1/64	1.80618	30	1.47712
1/128	2.10721	40	1.60206
1/256	2.40824	55	1.74036
1/512	2.70927	65	1.81291
1/1,024	3.01030	80	1.90309
1/2,048	3.31133	100	2.00000
1/4,096	3.61236	120	2.07918
1/8,192	3.91339	140	2.14613
1/16,384	4.21442	170	2.23045
1/32,768	4.51545	200	2.30103
1/65,536	4.81648	240	2.38021
1/131,072	5.11751	250	2.39794
None.		250	2.39794

A chart, showing that the logarithm of the dilution of the extract plotted against the logarithm of the coagulation time gives almost a straight line, is to be found in the following paper (4). A formula for the line is also suggested there, together with the method of calculating the strength of any solution in terms of lung extract, and also for calculating the actual concentration of the active material as mg. per cc.

1. Phospholipin Extraction of Dried Tissues.—In order to study the rôle of cephalin in the action of tissue extracts on blood clot-

ting, phospholipin extraction by ether and alcohol was carried out. Dried lung tissue was extracted four or five times successively with boiling 85 per cent alcohol and ordinary anesthesia ether under a reflux condenser. Quantitative figures for this extraction will be given in a later paper. This crude phospholipin material, after evaporation of the ether and alcohol on a water bath, was tested for activity on blood coagulation. About 0.5 gm. of it was rubbed up thoroughly with 10 cc. of 0.9 per cent NaCl solution to a fine milky emulsion which remained emulsified for weeks.

Intravenous injections of this emulsion into rabbits in amounts up to 5 cc. at a single injection were ineffective, whereas 0.1 cc. of the saturated lung extract would produce death in less than a minute. Blood clotting *in vitro* was quickened slightly, but quite definitely, by the phospholipin emulsion, the quickening being about the same as that induced by saturated lung extract diluted 10,000 times with 0.9 per cent NaCl (see Table II).

TABLE II.

1 cc. oxalate plasma, 0.2 cc. 1 per cent $CaCl_2$.

Other addition.	Coagulation time.
	min.
None	$4\frac{1}{2}$
0.2 cc. phospholipin emulsion	$3\frac{1}{2}$
0.04 " lung extract (× 1/10,000)	3

Phospholipin extracts were also made at room temperature by the use of absolute ether, benzene, chloroform, carbon bisulfide, and carbon tetrachloride on separate portions of the dried lungs. The solvent in each case was evaporated at room temperature in an air current. The extracted phospholipin was tested as described above.

The results were similar to those obtained in the preceding experiment. It was noticed that this activity of the phospholipin decreased on standing exposed to the air.

"Laboratory cephalin," obtained from Armour and Company, was also emulsified similarly and tested. The same, or slightly less, activity was found.

The lung tissue freed from phospholipins by boiling alcohol and ether was finely ground with sand and extracted with 0.9 per cent

NaCl solution to see if any active material was left. Such extraction yielded practically nothing, since the proteins had been rendered insoluble by the boiling alcohol. No trace of activity was found in the saline extract. Similar extraction of the lung tissue previously extracted with benzene, chloroform, etc., at room temperature, yielded an extract rich in proteins, but with only about 1/10,000th the activity possessed by a similar extract of untreated beef lungs.

It was possible that the phospholipins were oxidized or otherwise changed in the process of their removal from the lung tissue and evaporation of the solvent. Still the dried lung tissue can be kept exposed to the air and light at room temperature for months with only a slow loss of its action on blood coagulation.

2. Shaking the Watery Lung Extract with Ether.—Since it was thought that the active compound might be extracted from the watery extract, prolonged and vigorous shaking with ether was tried. Following such shaking three layers always separated. The top layer consists of ether with some dissolved fatty material which is without detectable action on blood coagulation. The bottom layer is the watery extract, but with its activity considerably reduced. Repeated extractions with ether will remove very nearly all the active material from the watery extract. The middle layer consists of solid protein material which is very active. It is not coagulated or changed by the ether, apparently, but separates out because of having collected in the surface film surrounding the ether globules and as these coalesce and rise to the surface, the material in the surface films is carried along.

This separation out in a surface film on shaking with another liquid insoluble in water is not peculiar to this protein, but will also occur with the casein of milk and egg albumin in solution.

3. Precipitation of the Active Material by Salting out.—The protein fraction of the extract was now examined.

Half saturation with $(NH_4)_2SO_4$ gave a grayish white precipitate which contained all the active material. The precipitate, redissolved in 1 per cent sodium chloride solution and made back to the original volume, contained all its original activity in hastening coagulation. Complete saturation of the extract after removal of the precipitate separating at half saturation gave about the same amount of protein precipitate, but this was inactive.

Complete saturation with $MgSO_4$ or NaCl gave the same results as half saturation with $(NH_4)_2SO_4$.

As regards "salting out," the active material behaves like a globulin. It does not redissolve well in pure water, but requires the addition of a small amount of NaCl.

4. Precipitation with $HgCl_2$ and Sulfosalicylic Acid.—To a 0.9 per cent NaCl solution extract of finely ground lung tissue was added a 1 per cent $HgCl_2$ solution, drop by drop, until no further precipitation resulted, being careful to avoid any large excess of the $HgCl_2$. The precipitate was collected on a filter, suspended in salt solution again, and the mercury removed by H_2S. The HgS was allowed to settle out and the protein solution decanted. No attempt was made to remove the excess of H_2S, as it seemed to be without effect on the activity of the material. The solution thus obtained possessed the full activity of the original extract. The same volume was maintained so that comparative tests could be made.

The filtrate, after precipitation of the extract with $HgCl_2$, was without a trace of activity on blood coagulation.

Precipitation of another portion of the same extract with 3 per cent sulfosalicylic acid, added carefully drop by drop to avoid excess, removed all the active material in the precipitate, the clear filtrate being left with no trace of activity. No effort was made to recover the active substance from its union with the sulfosalicylic acid. This could probably have been done by making slightly alkaline with ammonia and dialyzing to remove the ammonium salt of the sulfosalicylic acid.

Either of these two methods would be very likely to remove the phospholipins from a solution with the proteins so that such precipitaton has no bearing on the question whether the activity depends on the proteins present, or on the phospholipins.

5. Effect of Heat on the Activity.—By keeping the extract in a water bath and slowly raising the temperature it was found that the greater part of the proteins coagulated at 56–57°C. only a small amount being left to coagulate at 70–75°C. After removing the proteins coagulating at 56–57°C., the remaining solution has only a faint trace of activity on coagulation.

Heating the extract below 57°C. slowly destroys its activity without actually producing a precipitate. Table III shows this.

The heating above 45°C. produces an opalescence in the extract which probably indicates a partial coagulation of the proteins, without an actual precipitation until 57°C. is reached. This slight change in the proteins, however, at once decreases the coagulative activity of the extract.

Heating dried lung tissue also decreases the amount of active material that may afterward be extracted from it. Heating at 100°C. for 2½ to 3 hours leaves only about one-sixteenth of the original amount of active substance to be extracted by 0.9 per cent NaCl solution. Heating for 24 hours at 110°C. completely destroys all activity of the material. All the proteins are coagulated, apparently, for extraction with salt solution yields practically nothing.

TABLE III.

1 cc. oxalate plasma, 4 drops 1 per cent $CaCl_2$.

Lung extract (0.9 per cent NaCl).	Coagulation time.
None	7 min.
1 drop	45 sec.
1 " (kept at 46°C. for 30 min.)	60 "
1 " (" " 50°C. " 12 ")	60 "
1 " (" " 54.5°C. " 8 ")	2 min.
1 " (" " 56°C. " 10 ")	2¾ "
1 " (" " 56–57°C. " 12 ")	4½ "

Autoclaving the fresh lung at 15 pounds pressure for 15 minutes leaves only a trace of active material to be extracted.

6. Berkefeld Filtration.—Filtering the extract through a Berkefeld filter leaves only a faint trace of activity in the filtrate. The substance must be in large molecules or aggregates, although it will pass through ordinary filter paper until the pores of the paper become clogged with the material, after which filtration even under suction is very slow. The activity of the filtrate decreases as the filter becomes clogged with the material.

7. Effect of Hydrolysis or Digestion on the Activity of the Extract.—Roger (20) showed that sterile autolysis of tissue extracts served to destroy their toxicity. Wooldridge also showed that digestion with pepsin-HCl destroyed the activity by digesting off the protein fraction. However, in autolysis cephalin might also be hydrolyzed, and in digestion by pepsin-HCl, the acid might

affect the cephalin fraction so neither of these methods will serve to prove whether cephalin is necessary for the activity of the compound.

The experiments detailed below will show the effect of different digestive or hydrolytic agents on lung extract.

Experiment 1. Autolysis.—To 25 cc. of lung extract (0.9 per cent NaCl solution) were added 5 cc. of toluene and 8 drops of 10 per cent HCl. The mixture was well shaken, placed in an incubator at 38°C., and left for 27 hours. Then 10 cc. of the fluid were removed, and the toluene was removed as completely as possible by evaporation *in vacuo* and by air currents.

The HCl was neutralized by adding 10 per cent Na_2CO_3 drop by drop, and the liquid tested for activity on coagulation. The autolyzed extracts had lost their activity.

TABLE IV.

1 cc. oxalate plasma, 5 drops 1 per cent $CaCl_2$.

Amount of solution used.	Kept at 38°C.	Coagulation time.
drops		
2	1 min.	12 sec.
2	1 hr., 15 "	25 "
2	2 hrs., 15 "	30 "
2	4 "	1 min.
2	6 "	6 "
4	11 "	8 "
10 (neutralized by Na_2CO_3).	28 "	No clot.
None.		15 min.

Injected intravenously into a rabbit, 0.3 cc. of the original extract killed a 2,500 gm. rabbit in 20 seconds by intravascular clotting. Injection of 3 cc. of the neutralized extract after autolysis was entirely without effect on a rabbit.

Experiment 2. Action of HCl.—To 10 cc. of lung extract were added 5 cc. of 0.1 N HCl. The mixture was kept at 38°C. and the test made on the activity as shown in Table IV.

The acid caused a gradual decrease in the activity of the extract until it not only did not accelerate blood coagulation, but seemed actually to retard it. A precipitate formed in the tube, but both it and the liquid above were unable to accelerate blood clotting.

Since acid alone destroyed the activity of the material, it was necessary to find an enzyme that would act in neutral solution. It was desired to find an enzyme that would possess proteolytic

action, but be lipase-free, or else to find a protease-free lipase. This was finally accomplished by using hogs' pancreas.

Experiment 3. Lipase-Free Trypsin Solution.—This trypsin solution was made according to the directions of Cole (21). It was carefully tested and found to be lipase-free but strong in tryptic action.

Pancreatic Lipase Solution.—The lipase was prepared according to the directions of Cole,[1] and tested for lipase and protease activity. Very little protease action was shown, but the lipolytic activity was high. Thus, 2 cc. of the solution added to 5 cc. of neutralized olive oil and kept at 43°C., with occasional shaking, for 1 hour, developed an acidity equivalent to 8.3 cc. of 0.1 N NaOH in excess of that developed by the control tube containing boiled lipase and olive oil.

Watery Pancreatic Extract.—30 gm. of fresh fat-free hogs' pancreas were hashed and finely ground with sand. To this were added 90 cc. of distilled water, and the mixture was allowed to stand 20 hours in the ice box with occasional shaking. It was then strained through eight layers of cheesecloth and tested exactly as were the two previous extracts. The results showed that 8.25 cc. of 0.1 N NaOH should be taken as a measure of the lipolytic activity of this extract. This activity is almost exactly the same as that of the alcoholic lipase tested just previously. This extract was tested on fibrin, now, together with the other two extracts. The results on the fibrin digestion experiments showed that the lipase-free trypsin solution and the watery extract of the pancreas possessed equal proteolytic activity, while the alcoholic lipase solution, very nearly trypsin-free, and the watery extract had equal lipolytic powers. That is, the watery extract possessed the equivalent of the combined digestive powers of the other two solutions.

These three pancreatic extracts were next tested as to their power to destroy the activity of lung extract in blood clotting.

The following tubes were prepared and placed in a water bath at 43°C. The blood clotting tests were also carried out in the same water bath.

A. 10 cc. lung extract + 2 cc. 0.9 per cent NaCl solution.
B. 10 " " " + 2 " lipase-free trypsin solution.
C. 10 " " " + 2 " watery extract of pancreas.
D. 10 " " " + 2 " alcoholic lipase solution.

Table V shows the rate of inactivation of the lung extract in the different tubes.

Here, then, we have complete and very rapid destruction of the activity of lung extract by a trypsin solution which was shown to be absolutely lipase-free, so that protein digestion alone is shown to be sufficient for inactivation of the material. There was no apparent change in the character of the mixture in this

[1] Cole (21), p. 158.

tube, that is no precipitation occurred, so that the phospholipins apparently were left in solution as finely divided as ever. If the

TABLE V.

1 cc. oxalate plasma, 0.2 cc. 1 per cent $CaCl_2$.

Other addition.	Kept at 43°C.	Coagulation time.
		seconds
None		105
0.04 cc. original lung extract		18
0.04 " watery extract of pancreas		100
0.04 " cephalin emulsion		100
0.04 " of A	5 min.	18
0.04 " " "	19 "	18
0.04 " " "	39 "	19
0.04 " " "	50 "	19
0.04 " " "	60 "	20
0.04 " " "	75 "	20
0.04 " " B	1 "	20
0.04 " " "	13 "	30
0.04 " " "	24 "	60
0.04 " " "	30 "	100
0.04 " " "	45 "	100
0.04 " " "	67 "	100
0.04 " " C	10 sec.	18
0.04 " " "	14 min.	60
0.04 " " "	27 "	95
0.04 " " "	45 "	100
0.04 " " "	60 "	100
0.04 " " D	½ "	18
0.04 " " "	13 "	24
0.04 " " "	24 "	30
0.04 " " "	30 "	33
0.04 " " "	45 "	37
0.04 " " "	60 "	50
0.04 " " "	2 hrs.	100
None		105

activity of the extract was dependent on the phospholipin content alone, it should not have been affected here, and yet we see complete inactivation in 30 minutes. Likewise, with the lipase

solution, which was practically trypsin-free, the inactivation of the substance was complete although brought about at a much slower rate. Therefore, it appeared that digesting off the phospholipin fraction, leaving the protein in solution, also destroyed the activity. Both protein and phospholipin fractions appeared essential to the activity, neither alone being effective. It is possible that the activity of cephalin preparations on blood clotting may be due to a slight trace of the protein fraction still present in the material.

8. Precipitation by Acids. Isoelectric Point.—If CO_2 is passed through the solution of the active material for $\frac{1}{2}$ to 1 hour there is almost complete precipitation of the active material, leaving the coloring matter in the solution.

Sulfuric acid produces similar precipitation at N/250 to N/1,000, with most complete and rapid separation at N/500. The precipitated material retains all its activity, and most of the coloring matter remains in the solution. Optimum acidity for precipitation varies with the concentration of material, weaker extracts precipitating best at N/1,000 to N/1,500. The final H^+ concentration of the solution after the precipitate has settled is always 10^{-5} to 10^{-6} N.

9. Effect of Treatment of Lung Extract with Acid or Alkali.—If the activity of the tissue extract depended on the protein content it would probably be affected by changing the neutral proteins to acid or alkali *m*-proteins. For testing out this possibility a series of tubes was prepared, with the calculated acidity varying as follows: 10 N, 4 N, 2 N, N, N/2, N/10, N/50, N/100, N/500, N/1,000, N/5,000, N/10,000. H_2SO_4 was used.

The precipitates found in Tubes 8, 9, 10, and 11, when dissolved in 0.9 per cent salt solution, were found to be very active. The contents of Tube 7, when neutralized with NaOH, were found to be slightly less active than Tube 12 similarly treated, while Tube 6 was found to be almost devoid of activity. All tubes above Tube 6 were entirely lacking in coagulative power on blood. It is seen then, that acid added to give a calculated acidity of N/50 or greater destroys the power of the substance to hasten blood coagulation, even while leaving a perfectly clear solution. Since the proteins precipitate from these solutions on neutralization it is evident that acid *m*-proteins have been formed.

Whether this change alone is responsible for the inactivation cannot be stated.

The precipitation that occurred in Tubes 8 to 11, with the maximum in Tube 9 at a calculated acidity of N/500, probably represents the same sort of precipitation as occurred with CO_2, since all the activity of the precipitated material was retained.

The action of different concentrations of NaOH was also tested in the same manner. Lung extracts are always opalescent, no matter how long they are centrifuged, provided they are very active on blood coagulation. This normal opacity is not affected by alkalies until NaOH has been added sufficient to produce N/100 NaOH, when a slight clearing of the solution is seen. At N/50 NaOH the clearing is more marked and at N/10 the solution is almost transparent, having lost almost all its opalescence. There is little or no loss of activity of the material with the increasing concentration of alkali, until this clearing of the solution starts, and from then on the activity decreases as the clearing increases. At N/10 NaOH only a trace of activity of the material remains. Neutralization of the contents of Tubes 1, 2, and 3 with H_2SO_4 leads to the formation of a protein precipitate, so that here again it may be assumed that *m*-protein formation has taken place, alkali proteins being formed at the same time that a loss of activity of the material results.

This loss of activity with a presumably slight change in only the proteins of the solution would certainly seem to indicate that some protein in the extract is highly important in the production of the activity on the coagulation of blood.

10. Cataphoresis Experiment with Lung Extract.—Cataphoresis experiments with fresh lung extract in 0.9 per cent NaCl showed that the proteins moved toward the anode. Therefore these proteins, including the active substance, are electronegative in neutral saline solution.

11. Final Method of Purification Adopted.—The precipitation of the material at its isoelectric point was chosen as the method of its purification for several reasons. First, such precipitation removed the material completely from the solution without any apparent change in it as far as its activity was concerned. Second, although the substance possesses the characteristics common to all globulins as far as concerns salting out of solution with

neutral salt such as NaCl, $MgSO_4$, and $(NH_4)_2SO_4$, it differs from other globulins in its precipitation at very faint acidity. Thus, if blood plasma is half saturated with $(NH_4)_2SO_4$, a very heavy precipitation of globulins occurs, whereas if to another sample of the same plasma N/2 H_2SO_4 is added to give a calculated acidity of N/500, the only result is the formation of a very small amount of precipitate which, when collected and redissolved in 0.9 per cent NaCl solution, hastens the clotting time of normal oxalate plasma. In blood plasma there are, then, at least three different globulins: fibrinogen, precipitated by half saturation with NaCl; the globulin active in quickening blood coagulation, which precipitates in weakly acid solution; and other globulins forming the greater part of the total globulins of the plasma, which are not affected by such weak acidity, but are salted out by saturation with NaCl or $MgSO_4$, or by half saturation with $(NH_4)_2SO_4$.

Since the substance active in hastening clotting could be so clearly isolated from such a composite protein solution as the blood by this method, it was considered sufficient for the purification of the material from lung extract. In precipitating lung extract in this manner with weak acid, the surprising observation was made that this one active substance was practically the only globulin present in the extract. That is, if the extract was first half saturated with $(NH_4)_2SO_4$, and the resulting globulin precipitate redissolved in 0.9 per cent NaCl and made very slightly acid in the usual manner, the whole of the globulins present precipitated. Also by first removing the active substance from lung extract by acid precipitation, and then half saturating the remaining solution with $(NH_4)_2SO_4$, it was shown that the acid precipitate contained the total globulin content of the extract.

The method adopted in the purification was as follows: Extracts of fresh or dried beef lungs were made by finely grinding the material and adding 0.9 per cent NaCl solution. After settling thoroughly in the ice box, the supernatant extract was siphoned off and tested to find the optimum concentration of acid for precipitation of the active material. The first few extracts of the lung material usually require a concentration of N/500 acid for precipitation. If the extract is very strong, the first precipitation will not remove all the active material so that a second

addition of acid is necessary. H_2SO_4 was used as the acid in all this work. The lung tissue was extracted repeatedly with 0.9 per cent NaCl solution until the active material was practically exhausted, as shown by testing the extract for its action on the clotting of blood.

In this way 566 gm. of beef lungs, dried at room temperature, were finely powdered and extracted with 0.9 per cent NaCl solution, using 8 liters for each extraction. The seventh extract contained only a trace of activity so no further extraction was made. These extracts were acidified as described, and the precipitate collected and washed twice with distilled water made about N/5,000 acid by adding N/2 H_2SO_4. This removes all traces of salt from the precipitate and thus leaves it insoluble in pure water. Washing several times with distilled water now removes most of the acid combined to the precipitate and all else except the materials insoluble in pure water. The precipitate was recovered each time by allowing it to sediment in the ice box and then removing the supernatant liquid through a tube attached to a suction pump. Filtration was not used because of the nature of the precipitated material, the pores of the filter rapidly becoming clogged so that filtration was extremely slow even under suction. The material was finally dried by placing the precipitate, after drawing off all the water possible, in several shallow evaporating dishes and allowing a warm air current to play over it until the water had been completely removed, as far as possible. The material thus dried was collected and weighed. A yield of about 45 gm. was obtained from the 566 gm. of dried lung. This represents a yield of about 8 per cent from the dried lung, or about 1.9 per cent of the weight of the fresh lung. (Fresh beef lungs in drying at room temperature lose about 76.4 per cent of their weight.)

The material finally obtained has a rather dark gray color when powdered. When dissolved in 0.9 per cent NaCl solution it exhibits the usual activity on oxalated plasma and intravascular clotting. The sample was kept in a stoppered bottle in the ice box for analysis and tests.

12. Analysis of the Purified Material.—The material prepared as described above was used for the analysis. It was first tested qualitatively to determine its constituents. The following facts were established.

The biuret, xanthoproteic, and Millon's tests were very strong. Tryptophane tests weak. Molisch test for carbohydrates quite distinct. Reduced sulfur tests fairly strong and phosphorus very strong. Ehrlich's test for imidazole compounds negative. Traces of iron found.

Since tests for phosphorus in the substance were so strong, it was desired to see in what form this phosphorus occurred. Phospholipin extraction with boiling 80 and 95 per cent alcohol, and absolute ether gave yields of 39.9, 41.3, and 41.6 per cent phospholipin, depending on the duration of the extraction. It was very difficult to be certain that the last traces of phospholipin had been removed from the compound.

Quantitative analysis of the whole compound and of the protein and phospholipin fractions gave the following results:

Nitrogen determinations on the whole compound carried out by the Arnold-Gunning modification of the Kjeldahl process showed (1) 10.63 per cent N, and (2) 10.76 per cent N_2, average 10.7 per cent N.

Like determinations carried out on the phospholipin-free fraction gave (1) 16.10 per cent N, and (2) 16.01 per cent N, average 16.06 per cent N.

Phosphorus percentage in the whole compound, determined by the Pemberton-Neumann method, was (1) 1.52 per cent P, and (2) 1.558 per cent P, average 1.539 per cent P.

Phosphorus percentage in the phospholipin-free fraction, determined by the same method, was (1) 1.056 per cent P, and (2) 1.061 per cent P, average 1.058 per cent P.

Phosphorus percentage in the phospholipin fraction was found to be (1) 2.12 per cent P, and 2.13 per cent P, average 2.125 per cent P.

A complete analysis of the active substance will be carried out and reported in full at a later date.

The finding of so much phosphorus remaining in the protein fraction raised the question as to the form in which it was present. Tests for phosphoprotein (boiling with NaOH of different concentrations for different periods) and for nucleoproteins (examination for purine bases after boiling with 14 per cent HNO_3) showed that the phosphorus remaining in the protein fraction is not there as ordinary phosphoprotein or as nucleoprotein. Either it must be present in a very stable form of phosphoprotein, or else as phospholipin which cannot be removed by extraction with boiling alcohol and ether. This latter possibility is made improbable, however, by the fact that the nitrogen percentage of the compound (16.06 per cent) is such as to indicate a rather pure protein,

while if enough phospholipin were present to account for the 1 per cent of phosphorus the nitrogen percentage would be considerably lower than this.

13. Is the Phospholipin Fraction Necessary for the Activity of the Compound?—The finding of as much as 41.6 per cent of phospholipin in the substance at once raised the question as to the importance of it there. The only way to determine the relative importance of the protein and phospholipin fractions would be to separate them without change in either and study their action separately. This is rather difficult, since many methods of removing the phospholipin alter the protein in some way. Extraction with boiling solvents is excluded because of the coagulation of the protein resulting. Alcohol as a fat solvent is excluded for the same reason. Ordinary ether likewise alters the protein, leaving it very much less soluble, probably as a result of the action of the impurities, aldehydes, etc., present. A number of other solvents for the phospholipin were tried, dried beef lung tissue being extracted for several days at room temperature with successive portions of the solvents until the filtered solvents showed only a faint color due to phospholipin. Extraction with 0.9 per cent NaCl solution of the lung tissue so treated gave varying results. Absolute ether, chloroform, carbon disulfide, carbon tetrachloride, and benzene were used as the fat solvents. Each of them left the proteins little altered as far as their solubility in salt solution is concerned, although ether showed the greatest effect in this respect, the saline extract appearing poorer in proteins than normal. Saline extracts of the lung tissue extracted by the other solvents, CS_2, CCl_4, etc., looked just as strong as normal lung extract, but, when tested for their action on blood clotting, were found to possess only a slight action, comparable to the normal lung extract diluted more than 1,000 times. To show that the protein portion of the active substance was present in the inactive extracts they were treated with acid to give N/500 calculated acidity, at which point the precipitation occurred just as in normal extracts, most of the proteins present remaining in solution.

The isoelectric points of the protein alone and of the protein-phospholipin compound are the same. This would lead to the supposition that the protein alone is responsible for the precipita-

tion in slightly acid solution. The protein fraction is present then, unchanged, and possessing only a very slight degree of activity as compared to that of the whole compound.

If both protein and phospholipin have remained unchanged during the extraction they should reunite when mixed together. This was tried by thoroughly rubbing up a small amount of the extracted phospholipin with the inactive protein in a mortar. An immediate and marked increase in activity resulted. Frequent shakings of this mixture by hand caused its activity to continue to increase for several minutes, equilibrium finally being reached with its action on coagulation about 250 times as strong as that of either of the fragments alone. This was still

TABLE VI.

Table Showing Reactivation.

1 cc. oxalate plasma, 0.2 cc. 1 per cent $CaCl_2$.

Other addition.	Kept at 40°C.	Coagulation time.
None		6 min.
0.04 cc. inactive protein solution		3 min., 30 sec.
0.04 " phospholipin emulsion		3 " 30 "
0.04 " protein + phospholipin	1 min.	70 "
0.04 " " + "	7 "	45 "
0.04 " " + "	13 "	40 "
0.04 " " + "	50 "	40 "
0.04 " " + "	15 hrs. in ice box.	40 "
0.04 " normal lung extract		12 "

somewhat below the strength of the normal lung extract, so that apparently the normal amount of phospholipin was not yet attached to the protein.

The tests given in Table VI show this reactivation.

The inactive protein solution was obtained by thoroughly extracting dried powdered beef lung tissue at room temperature with benzene, freeing the extracted tissue from all traces of benzene by an air current, and then grinding the tissue well with 0.9 per cent NaCl solution in quantity small enough to give about a saturated solution of the soluble proteins. The phospholipin emulsion was made by taking the phospholipin obtained by evaporating the benzene extracts at room temperature in an air

current, and grinding it well with 0.9 per cent NaCl solution in the proportion of about 0.1 gm. of phospholipin to 20 cc. of salt solution. This gave an emulsion of milky appearance after the excess phospholipin had risen to the surface and been removed.

In adding the phospholipin to the protein solution, about 0.1 gm. of the phospholipin was thoroughly rubbed up with 5 cc. of the inactive protein solution in a mortar, and then transferred to a test-tube and well shaken by hand. The test on the normal lung extract (saturated) is given to show that the complete activity had not been obtained in the reactivation. By reference to Table I it will be seen that the increase in activity of the combined fractions over either separately was fully a 250-fold increase.

Benzene and carbon tetrachloride were found to be the fat solvents that left the two fractions least altered, since reactivation occurred most readily after their use in the extraction of the phospholipins. It might be added that great care was taken throughout in making the different saline extracts in order to keep as nearly as possible the same concentration of the substances concerned as occurred in the normal extract.

It might be argued here that the protein merely acts by finely emulsifying the cephalin and thus increasing its activity, considering the total effect of the solution on coagulation as being due to the cephalin. Experiments were tried, therefore, in which the phospholipin extracted from lungs as described above, was shaken in a machine for 30 minutes with solutions of soluble starch, soap, and 0.9 per cent NaCl.

A rather thick milky emulsion of the phospholipin in 0.9 per cent NaCl solution was made, and these mixtures made:

(*a*) 1 cc. of phospholipin emulsion + 1 cc. of 5 per cent solution of soluble starch.

(*b*) 1 cc. of phospholipin emulsion + 1 cc. of 1 per cent soap solution.

(*c*) 1 cc. of phospholipin emulsion + 1 cc. of 0.9 per cent NaCl solution. When shaken as described above, a fine and stable emulsion was obtained with the soluble starch, less so with the soap solution, and still less with only the 0.9 per cent NaCl, but the activity on blood clotting remained the same for the three solutions.

14. Increasing the Activity of Lung Extract.—The conclusion that a protein-phospholipin compound is the active principle led naturally to the question whether the compound as it occurs in tissues and tissue extracts is saturated with phospholipin, or whether the addition of more of this fraction would still further enhance the activity of the extract on the clotting of blood. To test this out, saturated lung extract, made as described in the first section of this paper, was thoroughly rubbed up in a mortar with a small amount of phospholipin freshly extracted from dried beef lungs by benzene at room temperature, the benzene being evaporated in an air current at room temperature. Thus to 5 cc. of such saturated lung extract was added about 0.2 gm.

TABLE VII.

Activation of Lung Extract.

1 cc. oxalate plasma, 0.2 cc. 1 per cent $CaCl_2$.

Other addition.	Dilution of extract, 0.9 per cent NaCl.	Coagulation time.
0.4 cc. lung extract		10 sec.
0.4 " " "	2 times.	12 "
0.4 " " "	4 "	14 "
0.4 " same extract + phospholipin		8 "
0.4 " " " + "	4 "	10 "
0.4 " " " + "	8 "	12 "
0.4 " " " + "	16 "	14 "
None		4 min.

of fresh phospholipin. The mixture was well shaken by hand in a test-tube (air being present) for several minutes, and was then tested for its activity as indicated in Table VII.

This same fourfold increase in activity was obtained by adding phospholipin to more dilute lung extracts. Kidney extracts were also rendered more active in the same manner. An analysis to determine the phospholipin percentage in the active substance after such activation as shown in Table VII has not yet been made.

The effort was next made to increase the strength of brain extracts in the same way, but much less of an increase in activity resulted, the activity after phospholipin addition being less than

double the original activity of the extract. This was to be expected since the brain was so very rich in phospholipin already. There is probably an excess of this fraction in brain extracts, so that the addition of more phospholipin has little further effect. Possibly the addition of the inactive protein constituent of the lung would increase the activity of brain extracts, but this has not yet been tried. It would be expected that the active substance as it occurs in brain extract would have the same phospholipin percentage as the lung compound after activation with further phospholipin. It is intended to make such comparative analyses soon.

15. Is the Protein Fraction of a Specific Nature, or May Different Proteins be Used?—Whether the protein in the active compound is of a specific nature is difficult to prove, since methods for isolating and identifying animal proteins of closely similar character are very unsatisfactory. Experiments were carried out in which phospholipin freshly extracted from dried beef lungs with benzene was thoroughly shaken with a 2 per cent solution of dried egg white in 0.9 per cent NaCl solution, and with a 2 per cent casein solution. An emulsion of the phospholipin in 0.9 per cent NaCl was first made and to this milky emulsion the other solutions were added as follows:

(*a*) 2 cc. phospholipin emulsion + 2 cc. 2 per cent egg white solution.
(*b*) 2 " " " + 2 " 2 " " casein solution.
(*c*) 2 " " " + 2 " 0.9 " " NaCl.

After shaking well by hand for about 15 minutes, these different mixtures were tested for their action on blood coagulation as shown in Table VIII.

After standing for a few minutes following the shaking, it was noticed that there had occurred a heavy precipitation in the tube containing the egg white, probably resulting from the union of the colloidal phospholipin with the colloidal protein.

No such precipitation occurred in the casein solution, so it cannot be stated whether union occurred between the casein and phospholipin.

Another interesting experiment pointed to the specificity of the protein fraction. Liver extracts contain a large amount of globulins which possess the same solubility characteristics as the

active compound in the lungs. In fact they could not be differentiated except by the difference in activity, the liver extracts possessing only a slight trace of activity in hastening the clotting of blood. It was thought this lack of activity might be due to a lack of phospholipin, and so this was added just as it was added to lung extract. But only a faint increase in activity resulted, no more than might be accounted for by the free phospholipin added. Kidney extracts, on the other hand, possess considerable activity, and this can be increased markedly by the addition of further phospholipin.

It would seem, therefore, that the protein fraction is of a definite and specific nature, even very similar globulins, such as those from the liver, being ineffective. This gives a clear-cut proof of the importance of the protein fraction of the compound.

TABLE VIII.

1 cc. oxalate plasma, 0.2 cc. 1 per cent CaCl.

Other addition.	Coagulation time.
None.	5 min., 30 sec.
0.2 cc. of (*c*).	3 " 30 "
0.2 " " (*a*).	3 " 30 "
0.2 " " (*b*).	3 " 40 "

16. The Nature of the Phospholipin Concerned.—Howell has showed that of all the phospholipins present in various tissues, only cephalin possesses the power of hastening blood coagulation. This conclusion has been widely accepted and with justification. It is only his statement that cephalin is the active principle of tissue extracts which requires further proof. This is discussed elsewhere in this paper.

In order to see whether it is cephalin that is concerned in the formation of the active material described in these pages, the phospholipin was extracted from the protein fraction, dissolved in ether, and four volumes of absolute alcohol were added. Very nearly the whole of the dissolved material was precipitated and may therefore be considered to be cephalin. It is probably all cephalin, since the last traces of this substance are never entirely precipitated from ether by alcohol addition.

The character of this cephalin and of the total phospholipins of the lungs is very different from brain phospholipins which contain much lecithin. The lung phospholipin is a yellowish brown, sticky, waxy material, very difficult to dry. Some of it was dissolved in ether and absolute alcohol added as above to see what part of the mixture was cephalin. Practically complete precipitation again resulted just as with the phospholipin from the pure compound. It is rather surprising that only cephalin should occur in this tissue to any great extent. It is very possible that all, or nearly all this cephalin, will be found to be present in the new compound described in this paper. The work so far has given that impression. Quantitative work to determine to what the phospholipins are attached in the lung tissue is being carried out at present.

17. Comparative Tests on the Different Coagulant Preparations in Use at Present.—Samples of coagulant preparations were kindly furnished by a number of firms. Tests were made on these different preparations in the usual way, and the results are given in Table IX.

Thromboplastin (Squibb), Hemostatic Serum (Mulford), and Hemogulen (Lilly) are all brain extracts in different degrees of saturation. Hemogulen probably represents a complete saturation of salt solution with the active principle of the brain. These three solutions are similar to lung extract in that boiling destroys their activity and that the active material may be isolated in the same way. Coagulen-Ciba, Coagulose (Parke, Davis Company), and Thromboplastin Hypo. (Squibb) may be boiled without effect on their action so that they contain none of the protein responsible for the action of the tissue extracts. Their activity is comparable to that of cephalin and it is very likely that they are composed of this in large measure. Coagulen and Coagulose are prepared from the blood, supposedly from the platelets, but we might also say from the A-fibrinogen of Wooldridge. However, in the preparation, the protein fraction of the compound is lost and with it goes the greater part of the activity on coagulation. These two preparations, then, would probably be safe for intravenous use, just as is cephalin, unless an abnormal tendency toward clotting was present in the blood. Intravenous injection of any coagulant, however, should be undertaken with great

care, on account of the serious results that often follow even slight thrombosis. What has just been said of Coagulose and Coagulen holds good for Thromboplastin-Hypo. (Squibb), although it is prepared from the brain. It will be shown that the A-fibrinogen

TABLE IX.

Comparative Tests on Coagulant Preparations.

1 cc. oxalate plasma, 0.2 cc. 1 per cent $CaCl_2$.

Other addition.	Coagulation time.
None	6½ min.
1 drop Coagulen-Ciba solution	5 "
5 drops " "	4½ "
10 " " "	5 "
1 drop Coagulose, Parke, Davis Co.	4 " 30 sec.
5 drops " " " "	2 " 45 "
10 " " " " "	2 "
1 drop Hemostatic Serum, Parke, Davis Co.	8 "
5 drops " " " " "	8 "
10 " " " " " "	8 "
1 drop Kephalin solution, Armour Co.	6 "
5 drops " " " "	4 " 30 sec.
10 " " " " "	3 " 30 "
1 drop Thromboplastin-Hypo., Squibb	5 " 40 "
5 drops " "	4 "
10 " " "	3 "
1 drop Thromboplastin, Squibb	20 sec.
5 drops " "	12 "
1 drop Hemostatic Serum, Mulford	40 "
5 drops " " "	20 "
10 " " " "	18 "
1 drop Hemogulen, Lilly	14 "
5 drops " "	10 "
1 drop lung extract	10 "
1 " " " (activated)	8 "
1 " " " (diluted four times with 0.9 per cent NaCl)	10 "

of the blood is identical with the active material of the tissues. This was also stated by Wooldridge.

Solutions as active as the brain extracts tested, or lung extract, can be used only for *local* application to arrest hemorrhage, unless they be greatly diluted, in which case the danger of thrombosis

from intravenous injection is very slight. Application of such materials for intravenous use, however, will be discussed by the author in another paper.

A few points in regard to the local application of this active tissue material in hemorrhages are: First, it is the only substance, apparently, in the tissues of the body and in the body fluids which will markedly accelerate the clotting of blood. It is then the principal factor in the natural mechanism for the arrest of hemorrhages. Second, it is by far the strongest blood coagulant known. Third, it greatly increases the amount of fibrin to be formed from a given plasma, thus assuring a firmer clot to withstand the pressure of the blood from behind. These three points, when properly appreciated, should make the use of tissue extracts, or of the purified material, almost universal in all types of local hemorrhage, whether in surgical or accidental cases. Whether this will apply to hemophilia cases as well has not yet been investigated by the writer.

The thing of importance to remember in their use is that the strongest preparation possible should be applied to the bleeding area, since it is certain to be greatly diluted by the blood issuing from the small vessels. Back in the open ends of the vessels is where the clots must form to be effective and here is where the concentration of the material applied will be least. Pressure to slow the flow of the escaping blood would result in much less dilution of the active material and give quicker clotting. For the purpose of obtaining this maximal concentration in the blood to be clotted, lung extract activated by the addition of further phospholipin would appear to be the ideal preparation to use since it may be diluted fully sixteen times and still be of equal strength with the strongest brain extract undiluted. Reference to Table I will show the slight effect which dilution has on the action of the material. A comparison of the different commercial preparations, as shown in Table IX, with different dilutions of lung extract will show their relative value in directly accelerating blood coagulation.

DISCUSSION.

The experimental facts presented are in direct conflict with very few of the observations made in the past as to the nature of the active coagulant in the tissues. It can readily be shown how

many of the conclusions of others have been erroneous, although their observations were usually correct. Take, for instance, the widely held opinion as to the importance of cephalin in coagulation. On the basis of Howell's very valuable work it has been generally conceded that this phospholipin is the active material of the tissues, with the result that cephalin preparations have appeared on the market as blood coagulants. Cephalin has indeed some action but by no means so powerful as when united with the protein. Even preparations made supposedly from the blood platelets (A-fibrinogen of Wooldridge) were always carefully purified of coagulable proteins so that they could be boiled without injury, with the idea that the all important cephalin was the only constituent that needed to be retained. The reason for all this importance being assigned to cephalin lies in the fact that in its extraction from the tissue the real active protein-phospholipin compound was split into its two fragments each of which possesses slight activity under the best of conditions, but as the extraction was really conducted all the proteins were coagulated by heat and alcohol and that fraction of the active material was lost completely. Of the phospholipins thus extracted cephalin alone showed coagulative activity and so to it was assigned the all important rôle mentioned. It is difficult to see how this conclusion could be arrived at on such grounds, since a simple comparison of the cephalin with tissue extracts would have shown that it alone could not account for a thousandth part of the activity of certain tissue extracts. Apparently no such comparative tests were made in a quantitative way.

Regardless of the work of Wooldridge in showing both the protein and phospholipin to be necessary for a maximum activity, it has been concluded that methods of removing the proteins from active solutions, such as by boiling, by precipitation with alcohol, or by salting out, removed the activity only because the cephalin was adsorbed onto the protein aggregates and thus removed from an active state. Why this idea should have persisted is difficult to see, since any method of removing the proteins from solution or of splitting them by hydrolysis will invariably destroy the activity of the material. It is true that the cephalin is removed along with the proteins but why assign to cephalin all the importance, rather than to the proteins? Evi-

dently it was because cephalin had been shown to possess some slight action while no pure protein had ever been found to possess such, although this point had really hardly been touched, except by Wooldridge. The proof presented in this paper as to the equal importance of the phospholipin and protein in producing the activity should be conclusive. The slightest change in the nature of the protein as by the production of acid or alkali proteins destroys the activity of the material, even though the state of solution of the substance is markedly improved. This loss of activity is impossible to explain if cephalin alone is considered responsible for the action of the compound. Also the hydrolysis of the protein by a trypsin solution almost or entirely lipase-free, very rapidly destroys the activity of the compound and furnishes further proof of the importance of the proteins. But the final and conclusive proof comes when it is shown that extraction of the phospholipins, without injury to the proteins, destroys most of the activity, while reunion of the separated fragments restores the original action.

The importance of the amount of phospholipin thus combined with the protein is clearly shown also by the fact that addition of further amounts of it to the active compound in lung extract results in a fourfold increase in the specific activity. It must be remembered, however, that it is not the cephalin alone that is responsible for this increase, but the union of the phospholipin with the specific protein molecules. Emulsification of the phospholipin with other agents, or union with other proteins does not thus increase its action. Even emulsification of it with brain extract gives no results, probably because the specific protein in such extracts is already saturated with such material.

From a scientific point of view, the proof of the nature of this material should be of interest. It has been thought that the phospholipins of the tissue cells were very probably united with the proteins in some sort of a union forming a very complex molecule, but the actual proof for this was lacking. If such a state of actual union could be proved, it might supply a basis for explaining many pathological changes in functions of the cell, especially for the effects of the anesthetics, at least those which are fat-soluble. The facts here presented should furnish this needed proof, since for the specific action of this substance on coagula-

tion, the close union of the phospholipin and protein has been shown to be absolutely essential. That the compound occurs in the tissues in this form is proved from the fact that the tissue material itself, upon disorganization of the cell structure with a minimum of chance for chemical change, as by merely grinding in isotonic salt solution, exhibits this same marked action on coagulation. There are probably other such compounds between phospholipins and proteins present in the cells, but this is difficult to prove on account of there being no specific test for them such as we have for this material. It is hoped soon to see just how much of the total phospholipin of the tissues is present in this one compound.

It is likely that there may be a practical application of some value of the discovery described here by which a coagulant is produced at least sixteen times as strong as any at present on the market, and 1,000 times as strong as several that have been widely used in the past. When it is remembered that the greatest possible concentration of the active material in the escaping blood is needed to insure quick coagulation, the possible value of thus increasing the power of these coagulants is clear.

The synthesis of such a substance as this active material has proved to be, cannot occur until specific proteins and phospholipins can be built up. At present the science is far from this perfection.

SUMMARY.

The results of this work may be summarized briefly as follows:

1. The phospholipins to be extracted from tissues possess only a very small part of the total activity of the tissue material on the clotting of blood.

2. The active principle of tissue extracts is in part protein in nature, so that any treatment that removes the proteins from solution or alters them in any way destroys or greatly reduces the activity of the material.

3. The protein nature of the compound must be considered in its purification, if its activity is to be retained.

4. The isoelectric point of the compound lies between $N \times 10^{-5}$ and $N \times 10^{-6}$ acidity. At this point the material precipitates from its solutions without loss of its activity on coagulation.

This fact is made use of in the isolation and purification of the active principle.

5. The purified substance possesses the solubility characteristics of the globulin class of proteins and is heat-coagulable.

6. It consists of about 41.6 per cent of phospholipin and 58.4 per cent of protein, the protein fraction containing about 1.06 per cent of phosphorus. This phosphorus is apparently present as very stable phosphoprotein, since no purine bases were found after thorough hydrolysis of the protein by acid. That the phosphorus is very firmly combined in the phosphoprotein is evident from the fact that several hours boiling with 5 per cent alkali fails to split off more than a small part of it.

7. The activity on blood coagulation depends on the union of the phospholipin and protein fractions. Separation of the two parts as completely as can be by extraction at room temperature with fat solvents, leaves each with only a very small part of the original activity. Reunion of these two fractions restores the greater part of this activity.

8. Addition of further amounts of phospholipins to the active material as it exists in the lungs increases its activity about fourfold. It is therefore evident that the percentage of phospholipin united to the protein fraction is a very important factor in the production of the activity.

9. This further activation of tissue extracts should be of considerable practical importance, since it is possible thereby to get a solution fully sixteen times as strong in activity as the best of those at present on the market, and about 1,000 times as strong as many that have been widely used.

10. The presence in the tissues of an actual union between the protein and phospholipin elements is here clearly shown. It is also shown that any alteration in this relation causes a great change in the specific activity of the compound. This fact may afford a future basis for working out the reasons for many cell reactions, especially reactions to any fat solvent materials.

The work reported in this and an accompanying paper on the action of tissue coagulins, as well as an earlier paper on the distribution of the active material in the various tissues, has been carried out under the direction of Dr. A. P. Mathews, for whose

valuable aid and numerous suggestions the author here desires to express his deep appreciation. Much credit is also due Dr. Shiro Tashiro for many valuable suggestions during the course of the work.

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THE ACTION OF TISSUE EXTRACTS IN THE COAGULATION OF BLOOD.*

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It has often been demonstrated that blood drawn from a vessel without contact with any injured tissue surface and kept in such a manner as to prevent destruction of the blood corpuscles, exhibits a considerable delay in its coagulation. Bird blood especially will remain fluid almost indefinitely when drawn and kept under such conditions. If, however, the escaping blood flows over an injured tissue surface, or the corpuscles are broken up as by whipping, it is found that the blood exhibits a much more prompt coagulation. The addition of saline extracts of tissues, especially such tissues as the lungs and brain, accelerates the clotting still further, it being possible thus to shorten the coagulation time to a small fraction of a minute.

There is evidently then in tissues and cells in general a substance which possesses the property of quickening the clotting of the blood. This substance has been variously named. Wooldridge (1), who was the first to study the nature and action of it, held that it was a protein-phospholipin compound and that in the process of coagulation it reacted with the fibrinogen to form fibrin, an actual union occurring between the two substances with a loss of phospholipin. Since the tissue material entered into the formation of the fibrin, he termed it tissue fibrinogen. He also showed that there was a substance in the blood itself which was practically identical with the tissue fibrinogen, and this he termed A-fibrinogen. The reaction of this with the ordinary fibrinogen of the blood which he termed B-fibrinogen, resulted in the forma-

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tion of fibrin. Precipitation of this A-fibrinogen by letting the plasma stand in an ice box for some time destroyed the spontaneous coagulability of the plasma. Addition of a new supply of the substance or of the tissue fibrinogen restored the power of clotting to such plasma. Wooldridge was also the first to show that the intravenous injection of the tissue fibrinogen in sufficient amounts would upset the equilibrium existing in the blood between the A-fibrinogen and B-fibrinogen and result in the formation of fibrin in the vessels, causing death from thrombosis. The fluid blood remaining after such intravascular coagulation showed a delayed coagulation time, or absolute non-coagulability. The increased coagulability of the blood he termed the positive phase of coagulation, while the tendency toward non-coagulability was called the negative phase. The negative phase was also produced by sublethal injections of tissue fibrinogen without the formation of solid clots to any great extent, and he considered this to be a protective reaction on the part of the blood itself or of the vascular endothelium. Wright (2), following up the work of Wooldridge, considered the negative phase to be due to the partial digestion or hydrolysis of the tissue fibrinogen in the blood with the liberation of albumoses and peptones, these bodies being more directly responsible for the negative phase reaction on the part of the vascular system. His basis for this conclusion was that the urine always gave a reaction for albumoses after injection of tissue extracts and also that in pneumonia there occurred a delayed coagulation of the blood at the same time that albumoses and peptones were being excreted in the urine, the origin of these bodies being from the resorption of the imperfectly digested exudate in the lungs. Since the direct injection of such albumoses or peptones into the blood was known to produce varying degrees of non-coagulability in the blood of dogs, Wright considered the process here to be of the same order.

It would not be practical to review in detail all the more recent work on the action of tissue extracts, so only a few of the more important articles will be mentioned here. Morawitz (3) and Fuld and Spiro (4) held that the tissue substance acted in the rôle of a kinase to initiate and hasten the formation of thrombin from the calcium and prothrombin of the plasma. Fuld and Spiro termed the substance cytozyme while Morawitz called it thrombokinase. Many workers have since accepted this theory of the action of the tissue material and based upon it their explanations of ex-

perimental observations. Nolf (5) and Howell (6) hold views somewhat different from the above. Nolf believes that fibrinogen is held in solution by being combined with a substance, hepatothrombin, produced by the liver, and that the breaking of this union by removal of the hepatothrombin permits the fibrinogen to precipitate as insoluble fibrin. The hepatothrombin may be removed by a substance derived from the leucocytes and hence called leucothrombin. Howell considers that tissue extracts act by neutralizing the antithrombin present, the antithrombin acting to prevent the interaction of prothrombin and calcium in the formation of thrombin. On neutralizing the antithrombin, then, the thrombin formation proceeds and clotting results. He called the substance thromboplastin or thromboplastic substance. Loeb (7) terms the active tissue substance coagulin because he considers it to act in the same manner as thrombin. Thus he finds that it will clot a solution of fibrinogen in the presence of calcium, presumably without the presence of any prothrombin. Mellanby (8), however, has brought forth proof that the prothrombin is very intimately associated with the fibrinogen and probably is not removed in the purification of the fibrinogen.

According to the theories of Morawitz, Fuld and Spiro, Nolf, and Howell, the tissue substance is not concerned in the formation of fibrin except indirectly in the formation of thrombin. That is, it is not a fibrinogen in the sense that it enters directly into the formation of the fibrin. Wooldridge, on the other hand, considered it to be a true tissue fibrinogen, furnishing a part of the material that entered into the fibrin molecule. The present work will be found to support Wooldridge's work in this, as in many other respects, although differing from it in certain important points. Most attention in this work, however, was paid to the effects of tissue extracts on the blood *in vivo*, especially to determining the nature of the negative phase.

EXPERIMENTAL.

1. Relation of Concentration of Active Material to Time of Clotting.

In a previously published article (9) the author showed that different tissues of the body vary in their content of material active in hastening the clotting of blood. Lung extract was found to be considerably stronger in this respect than extracts of any other tissues made in a similar manner, so it was chosen for use in most of this work. The method found most efficient for testing the activity of these extracts, and that which most nearly approximates the normal conditions of blood coagulation was the use of fresh oxalated blood. Upon recalcification with the optimum amount of $CaCl_2$, clotting occurred in fresh oxalate plasma in approximately the same time as in normal blood drawn from a vein and let stand. Standing in the ice box causes a gradual

lengthening of the coagulation time of the oxalate plasma, the time ranging from 3 to 15 minutes during 2 to 4 weeks standing, the reason for this apparently being a gradual precipitation of the A-fibrinogen of Wooldridge which is essential for spontaneous coagulation. Addition of very small amounts of this substance or of tissue fibrinogen shortens the coagulation time, the shortening bearing a definite relation to the amount of tissue substance added.

The plasma used throughout this work as normal plasma was obtained from the abattoir by letting the blood flow directly from the slaughtered animals into a vessel containing sufficient potassium oxalate to make the whole amount collected about 0.5 per cent. This made the plasma alone, after centrifuging, about 0.8 per cent oxalate, and this gave too heavy a precipitate of calcium oxalate on recalcification to make a reliable test fluid for accurate coagulation time determinations. To such cell-free plasma $CaCl_2$ was added in amount just below that necessary for clotting, and the precipitate of calcium oxalate removed by sedimentation in the ice box and then by centrifuging out the last traces. This resulted in a fairly clear plasma which required only a small amount of $CaCl_2$ to induce clotting in a normal manner. It was found necessary to put so much oxalate in at the time of the drawing of the blood because it was mostly done by inexperienced hands, and with smaller amounts of oxalate there frequently occurred clotting due to insufficient mixing of the oxalate with the blood. The plasma finally used for the test required usually about 0.2 cc. of 1 per cent $CaCl_2$ per cc. of plasma for optimum recalcification. Such a plasma forms a very delicate medium for detecting the presence of any amount of active coagulant material.

Table I in the preceding paper (10) giving dilution of lung extract shows clearly that the shortening of the coagulation time bears a definite relation to the concentration of the tissue extract.

The lung extract used in these dilution tests was a saturated solution made by extracting dried ground beef lung tissue with 0.9 per cent NaCl solution (4 cc. of salt solution per gm. of dried lung tissue). Fresh beef lungs were thoroughly washed in tap water, hashed in a meat chopper, spread on a glass plate in a thin layer, and dried in a warm air current for 24 hours. This

drying caused a loss in weight of about 76 per cent, but did not affect the blood-coagulating properties of the active material as was determined by comparative tests. The dried material was now powdered and extracted with 0.9 per cent NaCl solution in the proportion stated above. The use of less salt solution per gm. of tissue did not increase the activity of the solution so saturation was probably complete. Only a few minutes standing was necessary to obtain this saturation, after which all solid tissue particles were removed by centrifuging the solution for 20 minutes at about 3,000 revolutions per minute.

The relation of coagulation time to the dilution of the extract may be represented as shown in Chart 1. The plot of the logarithm of the dilution against the logarithm of the clotting time is nearly a straight line.

Considering the line in Chart 1 to be a straight line, the following formula was very kindly suggested by Dr. Mathews:

$$\text{Log } x = 3.4 \log y - 3.4$$

where x represents the dilution of the extract, and y the coagulation time in seconds. By using this formula it would be possible to express the amount of active material in any extract in terms of lung extract, merely by determining its degree of acceleration of the normal coagulation time, and, letting the observed coagulation time equal y, calculate x. However, it is readily seen that this would only hold good for tests in which the normal clotting time of the plasma used was 250 seconds. If it differed from this figure, which it would do in all likelihood, an approximate value could be arrived at by simple proportion.

Thus, if an extract of unknown strength is tested on oxalate plasma having a normal clotting time of 5 minutes, *i.e.* 300 seconds, and it is found that 0.04 cc. of the extract (or about 1 drop) accelerates the clotting time to 80 seconds, the strength of the extract as compared to saturated lung extract could be found as follows:

$$300 : 250 :: 80 : y$$
$$y = 67 \text{ seconds}$$

Substitute this value of y in the formula

$$\text{Log } x = 3.4 \log y - 3.4$$
$$\text{Log } x = (3.4 \times 1.82607) - 3.4$$
$$\text{Log } x = 2.80864$$
$$x = 644$$

That is, the extract tested is equivalent in strength to saturated lung extract diluted with 643 volumes of 0.9 per cent NaCl solution.

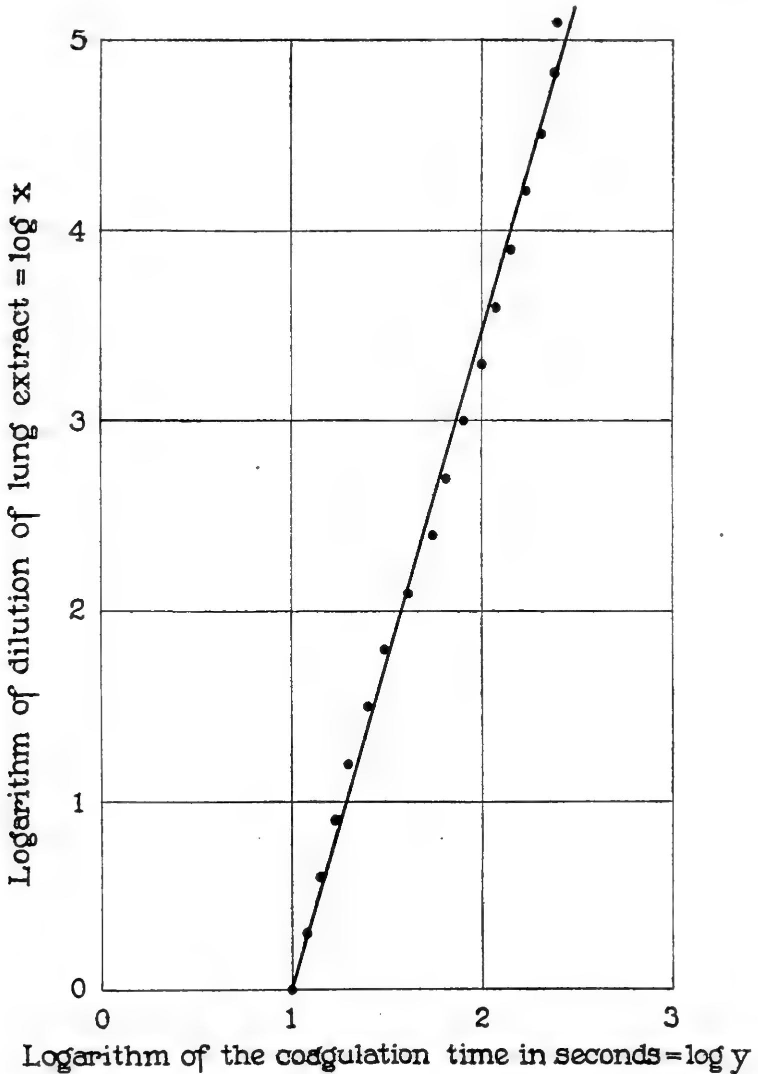

CHART 1.

A method of arriving at a quantitative expression of the amount of active substance in any solution would be to define arbitrarily a unit of the substance as that amount present in a certain amount of a given dilution of lung extract, as, for instance, in 0.04 cc. of lung extract diluted 100,000 times, and then express the activity of other solutions in terms of this unit. However, it is hoped that a definite statement can be made very soon as to exactly how much active substance by weight is present in any strength of extract, and thus save establishing any arbitrary unit of measuring the activity.[1]

[1] Since the preparation of this manuscript, experiments have been carried out to determine the concentration of active tissue substance in solutions of known effect on hastening coagulation of blood. For this determination, about 300 gm. of fresh beef lungs were hashed, ground well with sand, and extracted twice with 0.9 per cent NaCl solution. These two extracts, after removal of all solid particles by sedimentation in the ice box over night, were acidified with 0.5 N H_2SO_4 to make a calculated acidity of 0.002 N. The resulting precipitate of the active substance was washed free of other materials, as described in a later section of this paper. The active material, thus purified, was not dried, but was redissolved in 0.9 per cent NaCl solution, 0.5 N NaOH being added to give a calculated concentration of 0.002 N to aid the resolution of the material. This solution of the active principle diluted to the same volume as the combined original extracts possessed the same amount of activity in hastening coagulation as did the combined extracts before precipitation of the active substance.

Duplicate determinations of the N content of the solution were now made in order to compute the concentration of the active material present. In the preceding paper it is shown that the active substance contains 10.7 per cent N. Using this figure as a basis, the average of the two determinations gave 4.16 mg. of active substance per cc. of solution.

This solution was now tested for its accelerating influence on clotting, and the coagulation time was found to increase almost exactly the same, with dilution, as it did in diluting lung extract, as given in Table I of the preceding paper. Apparently it was about one-fourth as strong, undiluted, as was the saturated lung extract used in the tests recorded in Table I (10).

The saturated lung extract used contained about $4 \times 4.16 = 16.64$ mg. of active substance per cc. of solution. Diluted 65,536 times, at which point the last trace of acceleration of clotting was in evidence, there was then $16.64 \div 65{,}536 = 0.000256$ mg. of active substance per cc. of the diluted solution. 0.04 cc. of this was added to 1 cc. of oxalate plasma in the test, so that this final concentration in the plasma was $0.000256 \div 25 = 0.00001024$ mg. per cc., or approximately 1 part of active substance to 100,000,000 parts of plasma.

2. Is the Active Tissue Substance a True Tissue Fibrinogen?

In all the tests with active tissue extracts the clots formed were very much firmer and more resistant to breaking up on shaking than were clots formed from the same plasma without tissue extract addition. This suggested that this active material not only hastened the clotting of the plasma, but also increased the fibrin yield. Schmidt (11), in 1872, described what he termed a "fibrinoplastic substance" which he obtained from blood plasma by dialysis or treatment with weak acid, and which probably corresponded, in part at least, to the active material from the tissues. He claimed to obtain as high as 1 per cent yield from beef plasma, which many times exceeds the amount the writer has been able to demonstrate in such plasma. However, Schmidt demonstrated increases in fibrin yields from given plasmas of from 10 to 30 per cent, under the influence of the addition of different amounts of his "fibrinoplastic substance." Rettger (12), in 1909, showed that from a given fibrinogen solution varying amounts of fibrin could be produced by adding varying amounts of thrombin. However, he says that probably not all the fibrinogen had been changed over to fibrin with the addition of the smaller amounts of thrombin, thus showing that the thrombin acts in a quantitative manner, rather than that it actually increases the amount of fibrin to be obtained from a given amount of blood fibrinogen.

A number of experiments have been carried out to determine the effect of tissue extract addition on increasing the fibrin yield from a given plasma, and results showing increases up to 156 per cent in the fibrin yield have been produced by varying the amount of active tissue substance added. A number of other substances, such as starch paste, boiled dilute egg albumin, and milk, have also been added to plasma in order to see if such inactive solutions or suspensions would increase the weight of the fibrin to be obtained. Thus, a 0.4 per cent starch suspension (boiled) mixed with citrated horse plasma in equal volumes causes only 3.66 per cent increase in the fibrin yield. Out of 1.6 gm. of starch so added, only 0.0868 gm. was retained in the fibrin after thorough washing with 0.9 per cent NaCl solution and distilled water. With the use of lung extract, containing 1.8

per cent of proteins, in the same way, an increase of 152 per cent in the fibrin yield was obtained, 1.641 gm. of the lung material out of 5.448 gm. being bound in the fibrin. Fresh egg white, which is without any marked effect on the clotting time of horse plasma, was diluted with five volumes of distilled water, filtered, and boiled. A milky opalescent suspension resulted, acting like lung extract as far as passing through a filter is concerned; that is, a few cc. pass through readily and then the pores of the filter become blocked and filtration practically ceases. Such a suspension of boiled egg white, containing 2.13 per cent of solids, was added to horse plasma and the fibrin yield observed. An increase of 39.5 per cent in the fibrin occurred, 0.466 gm. of the egg albumin being bound out of 6.366 gm. added.

These experiments, together with many more, will be reported more in detail in the near future. It is greatly to be desired that definite proof be found whether the active coagulant of tissue extracts produces the increase in fibrin by chemically uniting with the latter, or is merely held mechanically due to its coarse state of dispersion in the extracts. Further experiments on this subject are at present being conducted with the aid of Mr. G. M. Guest.

The few results cited above seem to indicate strongly that the active coagulant of lung extract is present in the fibrin in some other form than as mechanically held material. That is, the substance appears to be a true tissue fibrinogen, the name given to it many years ago by Wooldridge. If it does enter chemically into the fibrin formation, such a fact would be difficult to explain under the theories of coagulation that are commonly accepted at present.

3. Tissue Fibrinogen and Blood Fibrinogen Unite only in the Presence of Calcium.

The addition of tissue fibrinogen to oxalate plasma does not result in the formation of fibrin until the plasma has been recalcified, nor does the active material appear to be bound in any way, since its activity persists for days and weeks and it may be precipitated out in active form by making the plasma very weakly acid (10).

Experiments were also tried on the blood of rabbits *in vivo* to see if the blood could be rendered non-coagulable by decalcification and death from lung extract injection thus be avoided.

Attempts were therefore made to decalcify the blood of rabbits by intravenous injections of potassium oxalate and sodium citrate. The former proved too toxic to permit of even close approach to complete decalcification. After the injection of as much oxalate as the rabbit could withstand, thrombosis resulted when lung extract was injected. The blood of a rabbit completely prostrated by oxalate, clots in the normal time after withdrawal from the vessels, so that this salt is not at all suitable for intravenous use as desired here. Sodium citrate, on the other hand, is much less toxic, as high as 9 cc. of a 5 per cent solution having been injected into an 1,800 gm. rabbit intravenously over a period of 15 minutes, with only spasmodic tremors and twitchings resulting. The following protocol of an experiment will serve to show the effects of lung extract injection after such citrate treatment.

Rabbit, male, 1,800 gm. 9 cc. of 5 per cent sodium citrate injected intravenously over a period of 15 minutes, keeping the rate of injection just below that necessary to produce muscular twitching or evolutions.

This was followed at once by an injection of 1 cc. of lung extract (saturated). Lethal dose of this extract in a normal rabbit was less than 0.1 cc.

Spasms occurred 20 seconds after the extract injection, and the rabbit was dead in 1½ minutes.

Immediate examination for clots showed only slight fibrin strings suspended from the chordæ tendineæ of the right heart. Remainder of blood was fluid, and a sample of it kept in the ice box showed only a slight clot in 24 hours. To another sample was added 1 drop of serum. Clotting occurred in 3 hours. To a third sample were added 2 drops of 1 per cent $CaCl_2$ which resulted in coagulation in 15 minutes.

40 cc. of the blood were oxalated to 0.5 per cent and centrifuged to obtain a clear plasma. The following tests were performed on this plasma:

1 cc. plasma + 2 drops 1 per cent $CaCl_2 \rightarrow$ clot in 65 sec.

1 cc. normal beef oxalated plasma + 2 drops of 1 per cent $CaCl_2 \rightarrow$ clot in 6 min.

1 cc. normal beef oxalated plasma + 5 drops of rabbit plasma + 2 drops of 1 per cent $CaCl_2 \rightarrow$ clot in 65 sec.

The rabbit plasma contained plenty of fibrinogen to form a normal clot, but was lacking in calcium. Therefore the decalcification by the citrate injection was almost complete. The rabbit plasma contained sufficient tissue fibrinogen to accelerate

markedly the clotting of normal beef plasma. Whether the slight clots which were found were sufficient to cause death cannot be stated. There is also the possibility that something else in the tissue extract, other than the tissue fibrinogen, might have caused the death of the animal.

4. Results of Intravenous Injections of Tissue Extracts.

Since the work of Wooldridge and of Wright on intravascular coagulation following injections of tissue fibrinogen little has really been added in this field, except repeated demonstrations that thrombin injections will not cause coagulation. Gutmann (13) found that rabbits killed by single injections of rabbit lung extract exhibited the negative phase of coagulation in the portion of the blood remaining fluid, and that this non-coagulability was due to a decrease in fibrinogen, the amount being decreased nine to eleven times. He supposed that this loss was to be accounted for by absorption of the fibrinogen by the clots formed in the vessels. Mellanby (14) showed that certain snake venoms were strongly coagulative for blood and that slow repeated injections of very small amounts rendered the blood of animals non-coagulable (negative phase) by gradual removal of the fibrinogen as fibrin, but without solid clot formation. It will be shown here that tissue extracts possess the same power of defibrinating the blood.

All the lung extracts used in the following injections were of about equal strength, ten parts by weight of 0.9 per cent NaCl solution being used for each gm. of lung tissue taken. This gives a much weaker extract than that used in the preceding section to study the effect of dilution. These extracts possess about one-thirty-second of the activity of the saturated extract.

A. Single Injections of Lethal Doses.—Injections of large doses of lung extract, that is 1 cc. or more, into rabbits intravenously cause respiratory symptoms in 20 seconds, followed about 10 to 15 seconds later by violent spasms and convulsive struggles. Death usually occurs within 1 minute of the time of injection. Defecation and urination usually occur during the struggles. On opening the thorax and abdomen immediately, it is noticed that the intestines are in very active peristaltic motion and that

the heart is usually beating weakly. Clots are always found in the portal vein, usually in the inferior vena cava and right heart, and sometimes in the veins above the heart. Only in a very few cases was the blood in the left ventricle found clotted. Wooldridge commented upon the frequency with which the clotting occurred in the portal vein and stated that it occurred here even more readily when the animal was in full digestion. Wright held that the CO_2 content of the blood was the main factor in deciding its tendency to coagulate, thus accounting for the prevalence of clotting in the venous system but its absence from the arterial system.

The blood remaining fluid in the vessels, after death from clotting as described above, showed a partial or complete negative phase of coagulation. Such blood contains no substances which will inhibit the coagulation of normal blood, but instead it still contains some of the active tissue fibrinogen so that its action is to accelerate the clotting of normal blood. It is not deficient in calcium, but contains an amount sufficient to recalcify normal oxalate plasma and cause it to clot. The following tests demonstrate these facts:

1 cc. oxalate plasma + 2 drops 1 per cent $CaCl_2$ solution → clot in 4 min.

1 cc. oxalate plasma + 1 cc. non-coagulable plasma → clot in 2 min.

Here the 1 cc. of the non-coagulable plasma not only furnishes the calcium required for the clotting of the oxalate plasma, but also quickens the clotting time from 4 minutes to 2 minutes. Fibrinogen tests on the plasma, as by half saturation with NaCl, usually show some small amounts to be present, although in some cases there is not the slightest precipitate. Where there is some fibrinogen present, clotting occurs slowly if the blood is not disturbed, the time varying from a few hours to several weeks. These clots are not firm, and are easily broken up by shaking. The negative phase, or non-coagulability, then seems to be due to a lack of fibrinogen, either partial or complete. The fact that the clotting in the vessels occurs while the blood is actively circulating probably accounts for a portion of the blood not being caught in the clot. It is well known that agitation of plasma or blood during clotting will cause the fibrin to precipitate out more in strings than as a fine network, so that most of the fluid is left free.

B. Negative Phase Production in Rabbits.—Intravenous injection of sublethal amounts of tissue extracts, repeated at intervals of 1 to 2 minutes and in increasing amounts, causes the development of a certain immunity to the material after a few injections, so that about ten times the original lethal dose may be given without symptoms. The following protocol will illustrate this.

Rabbit, 2,500 gm. No anesthesia used. Injections were made into the marginal ear vein.

Lung extract injected.	
cc.	
0.2	9.20 a.m.
0.3	9.21½ "
0.5	9.22½ "
0.8	9.23 "
1.2	9.24 "
1.6	9.25 "
2.0	9.26½ "
2.6	9.28 "
2.6	9.29½ "
2.6	9.32 "
2.6	9.37 "

(0.3 cc. of this extract is the lethal dose for a 2,500 gm. rabbit.)

The only symptoms were a slight, but steadily increasing weakness. The animal was killed by a blow on the head 3 minutes after the last injection, and examined for the presence of clots. No trace of clotting was found anywhere. The blood drawn from the inferior vena cava was kept in the ice box for several days without evidence of coagulation occurring. It was very rich in the tissue fibrinogen, as shown by its power to hasten the clotting of normal blood, contained sufficient calcium to clot normal oxalate blood, but was found very poor in fibrinogen. It would not clot with fibrin ferment, CO_2 gas, dilution with water, or the addition of any amount of lung extract.

Thus, although the plasma is itself entirely non-coagulable, it contains sufficient calcium per cc. to recalcify the oxalate plasma and cause it to clot. It must then contain the equivalent of 4 drops of 1 per cent $CaCl_2$ per cc. Not only does it effectively recalcify the oxalate plasma, but it also quickens the coagulation time to half the normal period. This indicates the presence of some of the tissue fibrinogen still in the plasma. Similar experiments with peptone plasma from a dog show a similar recalcification of the oxalate plasma, but also indicate clearly the presence

of some powerful inhibitory substance or condition which retards or prevents the coagulation even with sufficient calcium present.

1 cc. oxalate plasma + 1 drop lung extract + 1 drop 1 per cent $CaCl_2$ → clot in 12 sec.

1 cc. oxalate plasma + 0.25 cc. peptone plasma + 4 drops lung extract → clot in 15 sec.

1 cc. oxalate plasma + 0.5 cc. peptone plasma + 4 drops lung extract → clot in 60 sec.

1 cc. oxalate plasma + 1.0 cc. peptone plasma + 4 drops lung extract → no clot.

The peptone plasma used here had stood several weeks in the ice box without clotting. It is evident from the above tests that 1 cc. of this plasma contains more than enough of the inhibitory material to counterbalance the accelerating effect of the 4 drops of the lung extract. The first test shows that the lung extract is very strong also, 1 drop of it causing 1 cc. of oxalate plasma to clot in 12 seconds when recalcified.

A further experiment was tried, using blood freshly drawn from a dog and mixing with it the peptone plasma and non-coagulable plasma used above.

1 cc. fresh dog blood drawn and let stand → clot in 4 min.

1 cc. fresh dog blood drawn into 1 cc. non-coagulable plasma → clot in 1 min.

1 cc. fresh dog blood drawn into 1 cc. lung extract → clot in 40 sec.

1 cc. fresh dog blood drawn into 1 cc. 0.9 per cent NaCl solution → clot in 4½ min.

1 cc. fresh dog blood drawn into 1 cc. peptone plasma → very slight clot in 18 hrs.

Here it is evident that, while peptone plasma does strongly inhibit the clotting of normal blood, the non-coagulable plasma obtained by tissue extract injection is not only free from such action, but contains some of the tissue material in active form so that its action is to hasten normal clotting markedly. Since the decrease in fibrinogen in this plasma was demonstrated by half saturation with NaCl and comparison with normal plasma similarly treated, the non-coagulability was probably due to such fibrinogen deficiency.

This condition was induced in four rabbits, with complete non-coagulability of the blood resulting. In many other cases

the immunization was not so well accomplished, the animal being killed by too sudden an increase in the dosage. In such a case there were usually found slight clots either in the portal vein or on the chordæ tendineæ of the right heart. The fluid blood presented the characteristics described above, however. In no experiment was there any substance present in the non-coagulable blood which would inhibit the clotting of normal blood.

C. Negative Phase Production in Dogs.—This same immunization was carried out in seven different dogs with the animals under anesthesia. Blood pressure records were made and blood samples drawn at frequent intervals to study the blood changes. The following protocol will serve as a typical example of these experiments.

Dog, male, 9 kilos. Anesthetized with ether. Tracheal cannula. Blood pressure from right carotid artery. Injections from a burette into left femoral vein. Cannula in right femoral artery for blood samples. Table I shows the injections made and the results obtained.

In Table I, by lung extract ($\times \frac{1}{4}$) is meant lung extract diluted with three volumes of 0.9 per cent NaCl solution. Lung extract ($\times$ 1) is the undiluted lung extract. The coagulation time was taken as the time when the tube containing the blood could be carefully inverted without loss of its contents. By "activity" of the blood samples is meant their accelerating effect on the coagulation of normal oxalate beef plasma. All the samples drawn for these tests and for the alkali reserve were oxalated to about 0.5 per cent to prevent clotting, and the corpuscles centrifuged out to obtain clear plasma for the tests. Degree of activity of the plasma samples was tested as follows:

1 cc. oxalate beef plasma + 2 drops 1 per cent $CaCl_2 \rightarrow$ clot in $4\frac{1}{2}$ min.
1 " " " " + 5 " Plasma 1 + 2 drops 1 per cent $CaCl_2 \rightarrow$ clot in 2 min., 40 sec.

It is shown in the table of dilution of lung extract (Table I (10)) that the hastening of the coagulation time bears a very definite relation to the concentration of the active tissue fibrinogen present, so that, by testing the amount of shortening of the coagulation time induced by a given amount of the fluid to be tested, one may rather accurately estimate the amount of the active substance present. Now, by reference to the results listed in Table I of the

TABLE I.

Negative Phase in a Dog.

Time.	Lung extract injected.	Blood samples.			
		No.	Coagulation time.	Activity.	Alkali reserve.
	cc.				*cc.* CO_2
2.34 p.m.		1	4 min.	2 min., 40 sec.	38.6
2.50½ "	0.5 (×¼)				
2.51¼ "	0.5 (×¼)				
2.52 "	1.0 (×¼)				
2.53¾ "	1.0 (×¼)				
2.53½ "	1.2 (×¼)				
2.54 "	1.5 (×¼)				
2.54½ "	2.0 (×¼)				
2.55¼ "	3.0 (×¼)				
2.56 "		2	4 min., 10 sec.	2 min., 20 sec.	38.6
2.56½ "	4.0 (×¼)				
2.57 "	5.0 (×¼)				
2.58 "	6.0 (×¼)				
2.59 "	7.0 (×¼)				
3.00¼ "	8.0 (×¼)				
3.01½ "	9.0 (×¼)				
3.02½ "	10.5 (×¼)				
3.03½ "	12.0 (×¼)				
3.05 "	15.0 (×¼)				
3.09 "	1.0 (×1)				
3.11 "	2.0 (×1)				
3.12½ "	2.5 (×1)				
3.13¼ "		3	34 min.	1 min., 50 sec.	25.3
3.14½ "	3.5 (×1)				
3.16½ "	4.5 (×1)				
3.18½ "	6 (×1)				
3.21½ "	8 (×1)				
3.25 "	10 (×1)				
3.27 "		4	60 min.	1 min., 25 sec.	24.25
3.28½ "	12 (×1)				
3.31 "	15 (×1)				
3.33½ "	25 (×1)				
3.40 "	35 (×1)				
3.42 "	50 (×1)				
3.44½ "		5	Slight clot, 5 min.	45 sec.	23.8
4.06½ "		6	Slight clot, 15 min.	50 "	
4.12½ "	50 (1 per cent $NaHCO_3$)				

TABLE I—*Concluded.*

Time.	Lung extract injected.	Blood samples.			
		No.	Coagulation time.	Activity.	Alkali reserve.
	cc.				*cc.* CO_2
4.14½ p.m.		7	Slight clot, 14 min.	45 sec.	23.32
4.20 "	50 (1 per cent $NaHCO_3$)				
4.22 "		8	Slight clot, 9 min.	47 sec.	25.16
4.35½ "	50 (1 per cent $NaHCO_3$)				
4.38½ "		9	Slight clot, 10 min.	45 sec.	24.68
4.51 "		10	Slight clot, 13 min.	50 "	21.47
5.00 "	Death. Bled from heart.	11	Slight clot, 13 min.	50 "	29.32

present paper under "Activity," it is seen that the blood normally contained a quite definite amount of the material, since the clotting time of oxalate plasma was shortened from 4½ minutes to 2 minutes, 40 seconds, by 5 drops of the dog plasma. During the injections of the lung extract, this accelerating effect of the samples of dog plasma on normal clotting of beef oxalated plasma became more and more marked, so that it must be concluded that the concentration of the active substance in the blood of the dog was steadily increasing. Sample 5, taken 2½ minutes after the last lung extract injection, was able to shorten the clotting time of normal oxalate blood to 45 seconds, while Sample 6, taken 24½ minutes after the last injection, only shortens the clotting time to 50 seconds. Thus during that 22 minute period there occurred a noticeable decrease in the concentration of the tissue fibrinogen in the dog's blood. This decrease is probably to be accounted for by an excretion of the material into the urine, since the urine collected at the end of the experiment contained almost as much of the substance as the blood. 5 drops of the urine were able to

accelerate the clotting time of 1 cc. of normal oxalate plasma to 60 seconds, whereas the same quantity of blood plasma drawn at death gave a clotting time of 50 seconds to normal plasma. This excretion of the material in active form by the kidney is discussed later in this paper.

The coagulation time of the blood of the dog is seen to be unaffected by the injections until injections of 2 to 3 cc. of undiluted lung extract are used. (About 3 cc. of the extract are the fatal dose for a dog this size when only a single injection is given.) More frequent drawing of blood samples before this point always shows a quickening of the clotting time to 1½ to 2 minutes, this then being followed by the negative phase. Samples 5 to 11 formed only slight clots which were readily broken up by the slightest agitation. Several days standing in the ice box failed to show any further clotting. Tests for fibrinogen in the corresponding oxalated samples showed the presence of a small quantity of fibrinogen, but nothing at all to be compared to the normal amount. The negative phase seems, then, to be due to a loss of fibrinogen from the blood. It is not due to any inhibiting substance present, or to lack of calcium, since this plasma will recalcify normal oxalate plasma and cause it to clot in a shorter time than will the addition of $CaCl_2$ alone. Tests similar to those given for the non-coagulable rabbit blood were carried out on this plasma and the results were so similar that they need not be given here. The fate of this lost fibrinogen is not known, since it is not present as visible clots anywhere in the vascular system. Sometimes slight fibrin strings were to be found attached to the chordæ tendineæ of the right ventricle, but no clots elsewhere. Furthermore the dog gave no symptoms of intravascular clotting during the injections. A possible explanation for the fate of the lost fibrinogen will be set forth in the discussion at the end of this paper.

The alkali reserve was determined with the Van Slyke CO_2 apparatus, following the method of Van Slyke and Cullen (15). There is a very sudden fall in the alkali reserve just at the time of the development of the negative phase in the blood. This occurred in each such experiment performed on dogs. It is not due to the anesthesia, for dogs kept under anesthesia, as controls, for 2 hours show a very much smaller fall. The signifi-

cance of such a sudden fall is unknown. It is probably due to negative phase production, since it is so constantly associated with it, but it is not the cause of the loss of coagulability, since bicarbonate injections later in the experiment show no effect on the coagulation time.

D. Effect of Acid and Alkali Injections. Asphyxia.—In order to see whether or not the fall in alkali reserve was a possible causative factor in the negative phase production, weak acid was injected into a dog until the alkali reserve reached an extremely low level. Instead of a lessened coagulability of the blood, there was found a greatly shortened clotting time. The protocol of the experiment follows.

Dog, male, 5 kilos. Anesthetized with ether. Blood pressure from carotid artery. Injections from burette into left femoral vein. Blood samples from right femoral artery.

The procedure followed and the results obtained are shown in Table II.

This experiment is inserted here merely to show that the great fall in alkali reserve during the production of the negative phase by tissue extract injections probably has in itself nothing to do with the non-coagulability. In this experiment it is to be noted that the CO_2-combining power of the plasma falls rapidly as the coagulation time of the blood markedly shortens, and again that it rises as the coagulation time lengthens.

Although this experiment has little bearing on the mode of action of tissue extracts on the blood *in vivo*, it merits attention as regards the action of acids and alkalies on the blood and its coagulability.

In text-books on physiology it is often found stated that death from asphyxia leaves the blood non-coagulable. In such a death the hydrogen ion concentration of the blood probably increases after the CO_2-combining power of the plasma has been exhausted, and so a preliminary experiment on asphyxia was carried out on this dog. Complete occlusion of the air supply for $2\frac{1}{2}$ minutes was all the dog could stand and still be able to recover. The blood became almost black in color, but the coagulation time was not affected. An increase of over 40 per cent in the CO_2-combining power of the plasma was found. Such an increase has been found by others in the past and has been taken to mean that the

corpuscles have contributed material to the plasma for binding the CO_2. It has been shown that the alkali reserve of the whole blood increases much less than does the plasma alone. It is doubtful whether in this experiment, the CO_2-combining power

TABLE II.

Time.	Treatment and injections.	Blood samples.		
		No.	Coagulation time.	Alkali reserve.
				cc. CO_2
3.10 p.m.	15 min. after etherization.	1	5 min., 30 sec.	37
3.16 "	Trachea clamped to cut off air.			
3.18½ "	Air supply restored.	2	5 min., 45 sec.	52.8
3.26 "	10 cc. 0.1 N HCl injected.			
3.27½ "		3	6 min., 30 sec.	31.4
3.30½ "	25 cc. 0.1 N HCl injected. (No more ether given.)			
3.33¼ "		4	5 min., 45 sec.	23.2
3.36¼ "	50 cc. 0.1 N HCl injected.			
3.41½ "		5	1 min., 30 sec.	18.4
3.43½ "			2 "	
3.50½ "	50 cc. 0.1 N HCl injected.			
3.56½ "		6	1 min.,	9.9
3.59½ "			1 "	
4.06½ "			1 "	
4.13 "		7	3 " 45 sec.	9
4.16 "	50 cc. 0.1 N $NaHCO_3$.			
4.20 "		8	12 min.	18.8
4.27 "	50 cc. 0.1 N $NaHCO_3$. (Ether given again.)			
4.31 "		9	23 min.	27.2
4.32½ "	35 cc. 0.1 N $NaHCO_3$.			
4.38 "		10	42 min.	31
4.49 "				
4.50 "	12 cc. 1 per cent $CaCl_2$. (Death during injection, intravascular clotting.)			
5.02 "	Blood taken from left heart.	11	2 min.	24.9

of the blood was exhausted, and, if it was not, the rise in H^+ concentration would have been negligible.

Injection of 0.1 N HCl was without marked effect until after 85 cc. had been given, when the blood in the vessels became so

viscid that it would scarcely issue from a medium sized cannula in the femoral artery. Breathing was very labored and no ether was necessary until during the alkali injections. Blood pressure fell to a very low level although the heart action was apparently well maintained. All these effects probably came as a result of the greatly increased viscosity of the blood. Injection of another 50 cc. of the 0.1 N HCl only increased the above effects. The blood would issue from the cannula so slowly that it would clot before it could be mixed with oxalate. It was with difficulty that enough could be oxalated, unclotted, for the alkali reserve determination. From the very great decrease in the CO_2-combining power of the plasma here it is probable that the H^+ concentration had risen considerably.

The first 50 cc. injection of 0.1 N bicarbonate restored the blood to its normal fluidity, caused a cessation of the labored respiration, and a marked elevation of the blood pressure. Further bicarbonate injections restored the dog to apparently normal condition so that it was necessary to restore the ether administration. The most noticeable peculiarity to be observed in the results is the marked slowing of the coagulation time as the proper bicarbonate concentration is restored to the blood. However, the injection of $CaCl_2$ that followed, with the resulting solid intravascular clotting in the heart and all large veins examined, showed that the delayed coagulation during the bicarbonate injections was probably due to a partial and increasing decalcification of the blood by precipitation of the calcium as $CaCO_3$. The $CaCl_2$ injection seemed to restore the coagulability to the blood through recalcification, and the intravascular clotting probably was caused by the presence throughout the blood of $CaCO_3$ particles which acted as centers for the initiation of the clotting process.

The action of the acid in so markedly increasing the viscosity of the blood suggested that the affair was probably a matter of increasing the hydration capacity of the blood proteins, and, since the coagulation time of the fibrinogen was affected, this protein must have been one of those affected. To see if a similar action could be found *in vitro* some fibrinogen was precipitated from oxalate plasma by half saturation with NaCl, and redissolved in 0.9 per cent NaCl solution. This fibrinogen solution

was placed in a series of tubes and acid added to give a calculated concentration ranging from 0.0002 to 0.5 N. No change in the contents of the tubes occurred until the acid concentration reached 0.01 N when a distinct opalescence was to be seen. At 0.02 N acidity this opalescence was more marked and at 0.1 N there was almost complete precipitation of the fibrinogen. Now the first effect of the acid injection on the dog occurred after 85 cc. of 0.1 N HCl had been injected. This 5 kilo dog had probably 400 cc. of blood, figuring one-tenth to one-thirteenth of the body weight as blood, and of this blood about 60 per cent, or 240 cc., would be fluid plasma. 85 cc. of 0.1 N HCl added to 240 cc. of plasma would make approximately a N/40 HCl solution, if no acid was lost. However, the blood proteins would bind considerable of the acid, and it would be lost rapidly into the tissue lymph and secretions. At any rate, it is seen that the acid concentration in the blood probably fell within the limits of concentration which cause an opalescence to appear in a fibrinogen solution *in vitro*.

E. Effect of Tissue Extracts on Blood Pressure.—One further point of interest in regard to the intravenous injection of tissue extracts is the effect on blood pressure. With the injection of amounts sufficient to cause extensive intravascular clotting, the blood pressure rises abruptly at the time of the clotting. With sublethal doses, however, when no clotting occurs, the blood pressure effects are almost identical with those of histamine (ergamine, β-imidazolylethylamine). There occurs the same abrupt fall in pressure followed by the same more gradual rise back to normal. Like histamine, also, lung extract will cause such an effect time after time, at intervals of 1 to 3 minutes; that is, the sensitivity of the vascular system to its effects is not diminished except very slowly. Besides this similarity to histamine in its vasodilator effect, there is also its stimulating action on other smooth musculature. Urination and defecation nearly always occur following the injection of a moderately large or lethal dose into rabbits while with much smaller doses the increased peristaltic movements of the intestines can be readily observed even without opening the abdomen. Also the gurgling of the contents becomes very marked as a result of such peristalsis.

As has been mentioned before, it is considered by some that tissue extracts contain a mother substance capable of freeing

histamine when injected into the blood, so that it is supposed to be the effects of the histamine that give the above described results. In another place the writer has mentioned that the purified tissue fibrinogen gives a negative test for the presence of imidazole ring compounds when tested by Ehrlich's diazo reaction, although the crude tissue extract gives a distinctly positive test when thus tested. Whether the positive test in the crude extract is due to histidine or to histamine has not been determined.

Since the purified material gave a negative test for histamine, it is interesting to note that intravenous injections of it into rabbits produced death in exactly the same fashion as did the crude lung extracts. It has not yet been tried on dogs, nor has its effect directly on smooth musculature been observed. This would make it seem as though the presence of histamine is not necessary for the characteristic effects of the substance, but rather that the effects probably depend on changes induced in the blood during the transformation of the fibrinogen to fibrin.

5. Excretion of Tissue Fibrinogen in Urine.—Tissue fibrinogen injected intravenously into dogs or rabbits so as to produce a non-coagulability of the blood always results in the excretion of some of the material unchanged in the urine. It so happened that all the urine samples collected from the animals were just about neutral to litmus. What would happen if the urine were acid cannot be stated, although it is likely the material excreted might lose some of its activity. The urine collected at the end of the dog experiment described on page 181, showed the presence of almost as much tissue fibrinogen as did the blood plasma. By reference to the protocol, it will be noted that the dog was not killed until 1 hour and 20 minutes after the last injection of lung extract, and that during this time the concentration of the material in the blood decreased, as shown by the activity tests. This active tissue fibrinogen in the urine retained all the properties of the material injected, the most characteristic of which was its activity on the clotting of blood. It could readily be salted out of the urine by half saturation with $(NH_4)_2SO_4$, just as it could from the lung extract.

It has been shown by the author in the preceding paper (10) that a presumably slight change in the protein-phospholipin compound,

which is here termed tissue fibrinogen, results in a partial or complete loss of its activity on coagulation. Since the size of the molecules of the substance must be very great (as judged by present molecular weight figures, the substance would probably consist of 1 protein molecule to which are united about 12 phospholipin molecules) it is extremely difficult to imagine any process by which it must have passed intact from the blood stream into the urine, unless the two living membranes through which it passed possess pores through which it might pass, just as the white corpuscles pass through the capillary walls.

6. Specificity of Tissue Fibrinogen.—Loeb (16) has shown that a substance derived from the tissues, or blood cells, is necessary for the completion of the coagulation process in the blood of various lower forms of animal life. He termed this substance "tissue coagulin." It is very likely identical with what is here termed tissue fibrinogen. Loeb (17) also states that he found a definite class specificity to exist for these tissue coagulins; that is, the extracts of the tissues of one species of animal will hasten the clotting of the blood of other animals of the same class, but will be without effect on the blood of animals of a different class. Thus the tissue extracts made from any mammalian tissue would hasten the clotting of the blood of any mammal but would be ineffective on bird, reptilian, amphibian, or fish blood.

The writer has not gone deeply into this question, but has found that extracts of the lungs of cattle, dogs, rabbits, rats, and guinea pigs all show marked activity in the coagulation of the blood of any of the animals mentioned, either in *vitro*, or when injected intravenously. The intravenous injections were made mostly into rabbits, although rats and dogs were used to some extent. The only animal outside the mammalian class so far tested was the frog. It was found that extracts of frog lungs would very readily clot frog blood *in vitro*, or cause solid intravascular clotting, but such extracts were entirely without effect on the coagulation of mammalian plasma. Mammalian lung extract, however strong, would not cause intravascular clotting of frog blood, although it would hasten the clotting of such blood *in vitro* to some extent.

SUMMARY.

1. Tissue extracts accelerate the clotting of blood in a very definite manner, the coagulation time of the blood increasing from a minimum of about 10 seconds up to the normal time as the tissue extract added is diluted. The logarithm of the coagulation time in seconds plotted against the logarithm of the dilution of the extract gives almost a straight line. It is possible to get a noticeable quickening of the clotting process with the active tissue substance added to blood plasma in the proportion of 1 part of active substance to 100,000,000 parts of plasma.

2. The active tissue substance will not react with the blood fibrinogen to form fibrin, either *in vitro* or *in vivo*, except in the presence of soluble calcium salts.

3. Injected intravenously, rapidly, and in sufficient amounts, tissue extracts, or the purified active substance, cause intravascular clotting and death in a very definite manner. Injected slowly and in smaller amounts the blood is rendered non-coagulable, partially or completely, the non-coagulability apparently depending on a gradual removal of the greater part of the fibrinogen from the blood stream. A marked decrease in the alkali reserve of the plasma develops along with the development of the negative phase of coagulation, but is apparently not the cause of it.

4. The injection of active tissue extracts into the blood stream so as to produce non-coagulability without clot formation, is followed by an excretion of the active tissue substance apparently unchanged in the urine (dogs and rabbit).

5. As observed by Loeb, there is present a class specificity in regard to the action of the tissue extracts on blood clotting. The specificity, however, is not absolute, since mammalian lung extract will accelerate the clotting of frog blood.

6. There is a latent period in the coagulation process which is remarkably constant under similar conditions. Upon this depended the success of showing accurately the result of dilution of the tissue extracts upon their coagulative action. But it may be demonstrated also for intravascular clotting of the blood. Thus, after the injection of a fatal dose of lung extract into the ear vein of a rabbit, the time that elapsed before the onset of the

convulsive struggles did not vary more than 10 seconds in different rabbits, being 15 to 25 seconds after the injection. This gave time for at least a partial distribution of the active substance through the blood with the resulting great dilution of it. Just as plasma *in vitro* cannot be made to clot much quicker than 10 seconds, regardless of the concentration of the tissue material, so it was also found to be with the blood in the vessels. Always if coagulation occurred in any degree in the vessels following tissue extract injection, it occurred within 30 seconds after the injection. If the amount injected was not sufficient to give results in that time, then no solid clots formed, but a negative phase of coagulation set in. Further injections into the same animal of like or gradually increasing amounts, only served to increase the negative phase, until finally the blood was totally non-coagulable. The diminution, or lack of fibrinogen in such blood appears to be the cause of its non-coagulability.

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LECITHIN.

III. FATTY ACIDS OF LECITHIN OF THE EGG YOLK.

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Many considerations call for a reinvestigation of lecithins in regard to the character of their fatty acids.

The two earliest investigations on the fatty acids of lecithin gave occasion for the question of the existence of several lecithins varying from one another in the character of the fatty acids contained in their structure. Variations were claimed both on the point of the saturated and unsaturated acids. Each one of these points merits special discussion.

The first time an unsaturated acid was separated by Strecker[1] it was assumed by him to be oleic acid. The data on which the assumption was based could not be considered sufficient from a modern point of view. Subsequent workers, including Thudichum,[2] all referred to the unsaturated acid as oleic, on evidence of the same nature as that of Strecker. A very careful investigation on the unsaturated fatty acids of lecithin was published by Cousin,[3] who separated and conclusively identified oleic and linolic acids. More modern investigators following Cousin have added little to the results obtained by him. Stern and Thierfelder,[4] Baskoff,[5] Rollett,[6] and more recently Cruickshank[7] have brought forth evidence confirmatory to that of Cousin.

[1] Strecker, A., *Ann. Chem.*, 1868, cxlviii, 77.

[2] Thudichum, J. L. W., A treatise on the chemical constitution of the brain, London, 1884.

[3] Cousin, H., *Compt. rend. Acad.*, 1903, cxxxvii, 68; *J. pharm. chim.*, 1906, xxiii, series 6, 225.

[4] Stern, M., and Thierfelder, H., *Z. physiol. Chem.*, 1907, liii, 370.

[5] Baskoff, A., *Z. physiol. Chem.*, 1909, lxi, 426.

[6] Rollett, A., *Z. physiol. Chem.*, 1909, lxi, 210.

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As regards the saturated fatty acids, Diacanow[8] was the first to separate one in pure form. It analyzed for stearic acid. All subsequent workers have found that the saturated acid contained also, besides stearic, a lower fatty acid. Some isolated an acid having the composition of palmitic (Strecker). No investigator has identified both of them in pure form, but the mass of evidence was in favor of the assumption of the presence of palmitic and stearic acids in the so called lecithin.

Recently (1912), a very important contribution was made by Delezenne and Fourneau.[9] By the action of cobra venom on commercial lecithin they obtained an intermediate product of lecithin degradation. The substance contained in its molecule all the components of lecithin save the unsaturated acid. The product was crystalline. From this product they isolated an acid which analyzed correctly for palmitic. The melting point of their acid was 59–60°C., and that of pure palmitic, 64–65°C. They did not determine the molecular weight of their acid. It is, however, evident that their acid was, in the main, palmitic and might have contained only traces of impurity. The finding of Delezenne and Fourneau is of great importance as it reopened the question of the existence of more than one lecithin.

Indeed, the observations of all previous writers, granting that they were correct, could be equally well explained by one of the two alternative assumptions. First, that there existed several lecithins. Second, that lecithin contained only oleic and palmitic acid in its molecule, and that the linolic and stearic acids originated in the cephalin present in all crude lecithins. Cephalin, as is now known, contains linolic and stearic acids.

However, a pure lecithin free from cephalin was recently analyzed by Levene and Ingvaldsen.[10] This substance was derived from liver. The saturated acid contained in it was stearic and the unsaturated acid was not oleic but what seemed to be a homologue of linolic. Thus again the question of the existence of more than one lecithin was forced to the front. All these considerations led to the reinvestigation of the problem of the fatty

[8] Diacanow, C., *Centr. med. Wissensch.*, 1868, vi, 2.

[9] Delezenne, G., and Fourneau, E., *Bull. Soc. chim.*, 1914, iv, series 15, 421.

[10] Levene, P. A., and Ingvaldsen, T., *J. Biol. Chem.*, 1920, xliii, 361.

acids of lecithins freed from all traces of cephalin. The present work is a report on the lecithin of the egg yolk. Reports on the work on lecithin of other organs will be communicated later.

Lecithin of the egg yolk free from cephalin was found to contain only one unsaturated acid, namely oleic, and two saturated acids; namely, palmitic and stearic. The bulk of the material employed for this work was the cadmium chloride salt.

The oleic acid was identified by the iodine number and by the analysis of the hydrogenated product. The saturated acids were identified by their elementary composition, by their melting points, and by their molecular weights.

Furthermore, it was found that saturated and unsaturated acids were present in lecithin in equimolecular proportions. All these facts point towards the assumption of the existence of more than one lecithin in egg yolk.

An objection to these conclusions might be found in the assumption that the cadmium chloride product contained impurities which were not cephalin. To meet this objection hydrolyses were made on a smaller sample of dihydrolecithin prepared from the cadmium salt free from cephalin, and also on a smaller sample of free lecithin prepared from the same material. The results obtained from these experiments are confirmatory of those based on the analysis of the cadmium chloride derivative.

EXPERIMENTAL.

Preparation of Lecithin Cadmium Chloride from Acetone Extract of Egg Yolk.

The cadmium chloride salt of lecithin obtained from the acetone-soluble fraction of egg powder was used in this investigation as the source of amino-free lecithin. The following procedure has been found satisfactory. After exhaustively extracting egg powder with acetone, the extract is concentrated to a small bulk and the residual syrup is allowed to stand at 0°C. for at least 24 hours. During this period the higher melting fats separate in a crystalline condition and are easily removed by filtration under suction, if the temperature is kept below 10°C. Such fat contains a relatively constant quantity of lecithin, which is practically

amino-free and forms, therefore, the best source of lecithin, uncontaminated by cephalin.

The cake of fat is melted on the water bath with two or three volumes of alcohol, the liquids are thoroughly mixed, and the fat is again allowed to crystallize at 0°. After filtration the mother liquor is concentrated under diminished pressure to half its original volume and an alcoholic solution of cadmium chloride is added until no further precipitation occurs. The cadmium chloride salt thus obtained is free from unsaturated oils and is therefore almost white in color, with no tendency to darken on exposure to air.

We have found the following method very efficient in reducing the amino content and improving the physical properties of any cadmium chloride salt of lecithin. With salts obtained from the fat cake, one such purification is sufficient to yield amino-free material.

The pulverized salt is suspended in approximately its own volume of toluene. If the material contains any moisture, a clear solution occurs immediately or on slight warming; if a colloidal suspension is formed, the addition of a few drops of water results in its immediate dissolution. An opalescent effect indicates the presence of finely divided cerebrosides. These may be entirely removed by centrifugalization. The clear toluene solution is then poured into four volumes of ether containing 1 per cent of water. Precipitation occurs within a few minutes, if thoroughly cold ether is used. The precipitate is separated by centrifuging, well washed with ether, and finally, in order to remove the toluene as completely as possible, suspended in acetone and filtered.

A sample of material prepared in this manner analyzed as follows:

No. 240. 2 gm. of substance were hydrolyzed with HCl, neutralized, and concentrated to 10 cc.

5 cc. of this solution required for Kjeldahl determination 3.16 cc. of 0.1 N HCl equivalent to 0.00302 gm. of N.

2 cc. of this solution for Van Slyke determination gave 0.03 cc. of N at $T = 21°$, $P = 766$ equivalent to 0.000017 gm. of N.

$$\frac{NH_2\ N}{\text{Total N}} = \frac{1.4}{100}$$

0.1942 gm. of substance used for Kjeldahl determination required 1.96 cc. of 0.1 N HCl.

0.2913 gm. of substance gave 0.0322 gm. of $Mg_2P_2O_7$.

	Calculated for $C_{43}H_{86}O_9NPCdCl_2$. *per cent*	Found No. 240. *per cent*
N	1.43	1.41
P	3.18	3.08

From 25 pounds of egg yolk powder, 80 to 100 gm. of such material may be obtained, depending upon the extent to which the acetone extract is concentrated before filtration. Great variation has been experienced in the yield of the lipoids extracted from different samples of egg yolk powder.

Good material may also be obtained from the acetone filtrate from the fat cake, although with greater difficulty. From 25 pounds of lipoid-rich egg yolk powder an additional 250 gm. of amino-free cadmium chloride salt may be obtained by the following procedure. The filtrate is treated with an alcoholic solution of cadmium chloride until no further precipitation occurs. Several volumes of warm acetone are added to the mixture, and the supernatant liquid is decanted as soon as the precipitate has settled. By allowing the precipitate to stand too long, the oil, which is originally suspended in the acetone and easily decanted, settles out with the cadmium salt, whereby the difficulty of effective separation is greatly enhanced. Four or five repetitions of this process bring the cadmium salt to a condition in which it is easily filtered. The amino content of the material at this state of purity varies from 15 to 25 per cent.

Several steps have been employed to effect its further purification, each one accomplishing a special end. These have been selected with a view to their efficiency; the first of removing fats and oils, the second of reducing the amino content, and the third of removing the final traces of cerebrosides and saturated lipoids.

1. The cadmium salt is suspended in warm ether. To this water is added until the suspended material is dissolved. The solution is then allowed to stand at 0°C. for 24 hours, and the salt separates in flocculent form easily filterable by suction. If it is desired to dry the product, subsequent suspension in acetone and refiltration give a precipitate more easily dried and handled than that separating directly from ether. The amino content of this material is about 7 to 10 per cent.

2. This material is then subjected to the toluene-ether purification as described above; its amino content is thereby reduced approximately 50 per cent. On suspension in acetone a white or very pale, yellowish white waxy precipitate is formed which does not change color on drying and which is easily pulverized, having lost any sticky or gummy character.

3. The dry powder is suspended in ether, dissolved by the addition of water, and precipitated by adding the ethereal solution to several volumes of alcohol. If a Van Slyke determination still indicates the presence of amino nitrogen-containing material, the substance may be again subjected to any of these processes until the cadmium salt is amino-free.

Of such a sample the following is a typical analysis. It contained no amino nitrogen.

No. 18. 0.200 gm. of substance required for a Kjeldahl estimation 2.10 cc. of 0.1 N HCl equivalent to 0.00294 gm. of N.

0.300 gm. of substance gave 0.0330 gm. of $Mg_2P_2O_7$.

	Calculated for $C_{43}H_{86}O_9NPCdCl_2$. *per cent*	Found No. 18. *per cent*
N	1.46	1.47
P	3.14	3.07

There was no difference apparent between the fatty acids isolated from the two fractions.

Preparation of Lecithin Cadmium Chloride from Ethereal Extract of Egg Yolk.

Egg powder which had been exhaustively extracted with acetone was further extracted with ether. This extract contains not only the unsaturated but also the saturated lipoids. These are removed in the following manner. The extract is reduced to a small volume by distilling off the greater part of the ether. The residue is precipitated by acetone. The precipitate thus formed is dissolved in ether and allowed to stand at 0°C. for 24 hours. The cerebrosides and sphingomyelin settle out in the form of a white precipitate. This process is repeated until no further precipitate is deposited from ether on standing. The material which is precipitated by acetone from the clear ethereal solution contains the so called "cephalin," pure cephalin, and lecithin. The lecithin is contained in the fraction soluble in alcohol. This is obtained in the following way. The material is taken up in ether and alcohol is added until a definite precipitation

occurs. After several repetitions no further solution occurs. The decanted alcoholic liquors are concentrated to a smaller bulk under diminished pressure. The lecithin is then precipitated as the cadmium chloride salt. Purification of the salt is continued according to the methods described in the preceding section, until no amino nitrogen is found in the hydrolyzed material by a Van Slyke estimation.

Isolation of the Fatty Acids.

Hydrolysis was effected by boiling the cadmium chloride salt for 10 hours with ten parts of 10 per cent hydrochloric acid. The crude mixture of saturated and unsaturated fatty acids was filtered off, on cooling. The yield of the crude acids was invariably 95 to 98 per cent of that anticipated by the theory.

They consisted apparently of equal parts of saturated and unsaturated acids, as is seen from the following experiment. The mixed acids tranformed directly into lead salts yielded 46 gm. of total lead salts. Of these, 25 gm. were insoluble in ether. In a second experiment, in which the separation of the acids was effected by recrystallization from acetone, as described below, 8 gm. of saturated acids were obtained, as compared with a yield of 7.5 gm. of unsaturated acids.

Furthermore, the crude mixture of fatty acids gave an iodine number of 50.2. Since the unsaturated acid, as will be shown below, is oleic, this value again points towards the presence in the lecithin molecule of equimolecular proportions of saturated and unsaturated acids.

A. Fatty Acids of the Lecithin Prepared from the Acetone Fraction. Acids from Dihydrolecithin Prepared from This Fraction.

A sample of hydrolecithin was prepared from a cadmium salt of lecithin free from amino nitrogen. The reduction was accomplished by Paal's method. The mixed fatty acids obtained from this sample gave the following data:

All samples were dried by fusion on an electric hot plate, and to insure absolute freedom from moisture in the material used for combustion, were remelted under diminished pressure at the temperature of xylene vapor until constant weight was attained.

In all cases the melting points recorded in this paper are corrected and were taken at such a rate that the time interval per degree rise in temperature was 6 seconds. The molecular weights were calculated by the titration of approximately 1 gm. of acid, dissolved in 10 cc. of toluene and 25 cc. of methyl alcohol (neutral to phenolphthalein), with 0.5 N NaOH, using phenolphthalein as an indicator.

They melted at 57–58°, gave a titration value corresponding to a molecular weight of 273, and the following analysis:

0.1004 gm. of substance, dried by fusion, gave on combustion 0.1118 gm. of H_2O and 0.2780 gm. of CO_2.

1.049 gm. of substance, dried by fusion, required for neutralization 7.67 cc. of 0.5 N NaOH, corresponding to a molecular weight of 273.

Sample No.	Analysis.		Molecular weight.	Melting point.
	H	C		
	per cent	*per cent*		*°C.*
132	12.46	75.51	273	57–58

Saturated Acids Isolated from Lecithin Prepared from This Fraction.

The cake of mixed fatty acids was freed from water-soluble impurities by melting on the water bath and collecting the acids with a little benzene. On cooling, the cake was again readily separable. It was then dissolved in a small quantity of acetone, from which the saturated fatty acid crystallized almost quantitatively on cooling to −10°C. After filtration and thorough washing the separation of saturated from unsaturated acid was practically complete.

An additional recrystallization of the saturated acids from acetone completed the first crude separation of the saturated from the unsaturated acids. The saturated acids were then transformed into their lead salts, which were thoroughly extracted with boiling ether in order to remove all traces of unsaturated salts. By decomposition with hydrogen sulfide, the acids were regained. The analysis of several samples follows.

No. 76. 0.1000 gm. of substance, dried by fusion, gave on combustion 0.1137 gm. of H_2O and 0.2760 gm. of CO_2.

1.0082 gm. of substance, dried by fusion, required for neutralization 7.57 cc. of 0.5 N NaOH corresponding to a molecular weight of 266.

No. 452. 0.0992 gm. of substance, dried by fusion, gave on combustion 0.1106 gm. of H_2O and 0.2730 gm. of CO_2.

1 gm. required for neutralization 7.44 cc. of 0.5 N NaOH corresponding to a molecular weight of 268.3.

No. 317. 0.1002 gm. of substance, dried by fusion, gave on combustion 0.1144 gm. of H_2O and 0.2757 gm. of CO_2.

Sample.	Analysis.		Molecular weight.	Melting point.
	H	C		
	per cent	*per cent*		°C.
Calculated for $C_{16}H_{32}O_2$ (palmitic acid)	12.58	74.93	256	64–65
Calculated for $C_{18}H_{36}O_2$ (stearic acid)	12.72	75.98	284	70–71
Found No. 76	12.72	75.26	266	58–59
" " 452	12.48	75.05	268	58–59
" " 317	12.77	75.03	264	61

Fractional crystallization from acetone, ether, or alcohol caused no decided change, nor was repeated preparation of the lead salt with subsequent decomposition effective in fundamentally altering the analytical data.

With acetic anhydride, however, we separated a fraction which corresponded to palmitic acid. The mixed acids were dissolved in ten parts of warm acetic anhydride. A substance crystallized on cooling, which after recrystallization consisted, according to the indications given by its molecular weight and analysis, of a mixture of acid and anhydride. Purification by conversion into the sodium salt and extraction of the latter with acetone with subsequent decomposition gave an acid of the following analysis:

No. 131. 1.0020 gm. of substance, dried by fusion, gave on combustion 0.1177 gm. of H_2O and 0.2755 gm. of CO_2.

1.0887 gm. of substance, dried by fusion, required for neutralization 8.33 cc. of 0.5 N NaOH corresponding to a molecular weight of 257.

Substance.	Analysis.		Molecular weight.	Melting point.
	H	C		
	per cent	*per cent*		°C.
Calculated for $C_{16}H_{32}O_2$ (palmitic acid)	12.58	74.93	256	64–65
Found No. 131	13.14	74.98	257	64–65

The isolation of both constituents of this mixture was effected by the fractional distillation of the methyl esters under diminished pressure. The mixture of methyl esters was prepared by the usual method of esterification with methyl alcohol and sulfuric acid.

The mixed esters, No. 126, the saponification value of which corresponded to a molecular weight of the acid equal to 266, were distilled at a pressure of 1.6 mm. into four fractions. These fractions were then saponified. The molecular weights, melting points, and analyses of their respective acids are recorded below under the corresponding numbers.

Sample.	Boiling point of ester.	Molecular weight of acid.	Melting point of acid.	Analysis of acid.	
				H	C
	°C.		°C.	*per cent*	*per cent*
Mixed esters, No. 126		266	58–59	13.06	75.34
First fraction, No. 133	145–159	255	61–62	12.96	74.70
Second " " 134	155–170	260	61–62	12.83	75.49
Third " " 135	160–178	264	61–62	13.20	75.07
Residue, " 136		278	72–72.5	12.98	75.56

No. 126. 1.004 gm. of substance, dried by fusion, gave on combustion 0.1172 gm. of H_2O and 0.2774 gm. of CO_2.

1.0542 gm. of substance, dried by fusion, required for neutralization 7.98 cc. of 0.5 N NaOH corresponding to a molecular weight of 266.

No. 133. 0.1010 gm. of substance, dried by fusion, gave on combustion 0.1170 gm. of H_2O and 0.2764 gm. of CO_2.

1.0169 gm. of substance, dried by fusion, required for neutralization 7.97 cc. of 0.5 N NaOH corresponding to a molecular weight of 255.

No. 134. 0.1005 gm. of substance, dried by fusion, gave on combustion 0.1152 gm. of H_2O and 0.2782 gm. of CO_2.

1.0213 gm. of substance, dried by fusion, required for neutralization 7.85 cc. of 0.5 N NaOH corresponding to a molecular weight of 260.

No. 135. 0.1012 gm. of substance, dried by fusion, gave on combustion 0.1194 gm. of H_2O and 0.2786 gm. of CO_2.

1.0058 gm. of substance, dried by fusion, required for neutralization 7.60 cc. of 0.5 N NaOH corresponding to a molecular weight of 264.

No. 136. 0.1008 gm. of substance, dried by fusion, gave on combustion 0.1170 gm. of H_2O and 0.2793 gm. of CO_2.

0.6550 gm. of substance, dried by fusion, required for neutralization, 4.70 cc. of 0.5 N NaOH corresponding to a molecular weight of 278.

The combined low boiling fractions from two distillations were esterified and again distilled. This fraction boiled constantly at 155–159° at a pressure of 1.6 mm. After saponification and recrystallization from acetic anhydride, followed by purification through the sodium salt as described above, an acid corresponding in its analytical data to palmitic acid was obtained.

No. 169. 0.0994 gm. of substance, dried by fusion, gave on combustion 0.1134 gm. of H_2O and 0.2726 gm. of CO_2.

0.9539 gm. of substance, dried by fusion, required for neutralization 7.40 cc. of 0.5 N NaOH corresponding to a molecular weight of 258.

Sample.	Analysis.		Molecular weight.	Melting point.
	H	C		
	per cent	*per cent*		*°C.*
Calculated for $C_{16}H_{32}O_2$ (palmitic acid).........................	12.58	74.92	256	64–65
Found No. 169..................	12.77	74.79	258	64–65

By esterification and fractional distillation of the higher boiling fractions, an ester was isolated which distilled at 170–175° under a pressure of 1.6 mm. After saponification this substance gave analytical figures, molecular weight, and melting point corresponding to those of stearic acid.

No. 153. 0.1101 gm. of substance, dried by fusion, gave on combustion 0.1248 gm. of H_2O and 0.3062 gm. of CO_2.

0.7749 gm. of substance, dried by fusion, required for neutralization 5.50 cc. of 0.5 N NaOH corresponding to a molecular weight of 282.

Sample.	Analysis.		Molecular weight.	Melting point.
	H	C		
	per cent	*per cent*		*°C.*
Calculated for $C_{18}H_{36}O_2$ (stearic acid).........................	12.76	75.98	284	70–71
Found No. 153..................	12.69	75.84	282	70.5–71

The Unsaturated Fatty Acid of Lecithin Isolated from This Fraction.

From the acetone mother liquors from which the saturated fatty acids had been removed by filtration, the unsaturated acid was isolated by precipitation as the lead salt. The gummy mass

was dissolved in a small volume of ether, and the ethereal solution kept at a temperature of 0°C. over night. Any contaminating saturated salts crystallized under these conditions and were easily removed by filtration. The unsaturated salt was freed from ether, dissolved in toluene, and decomposed with hydrogen sulfide. The liberated acid was freed from acetone-insoluble impurities by repeated solution in acetone followed by filtration. After concentration of this solution, the residual acid was dark brown in color. A Wijs determination showed the following iodine absorption:

No. 72. 0.2863 gm. of substance absorbed 0.25848 gm. of iodine.

	Calculated for $C_{18}H_{34}O_2$.	Found No. 72.
Iodine number	90	90.4

This acid was then hydrogenated by Paal's method. The saturated acid after two recrystallizations from acetone gave an analysis, molecular weight, and melting point corresponding with those of stearic acid as shown by the following figures:

No. 68. 0.1007 gm. of substance, dried by fusion, gave on combustion 0.1162 gm. of H_2O and 0.2812 gm. of CO_2.

0.9476 gm. of substance, dried by fusion, required for neutralization 6.68 cc. of 0.5 N NaOH corresponding to a molecular weight of 284.

Sample.	Analysis.		Molecular weight.	Melting point.
	H	C		
	per cent	*per cent*		*°C.*
Calculated for $C_{18}H_{36}O_2$ (stearic acid)	12.76	75.98	284	70–71
Found No. 68	12.91	76.15	284	70–71

B. Fatty Acids from Lecithin Prepared from the Ethereal Extract. Fatty Acids of Dihydrolecithin Prepared from This Fraction.

On the acids isolated from a sample of this lecithin, which before hydrolysis had been reduced to the dihydrolecithin, we obtained the following analytical data, corroborating their identity with those isolated in an analogous manner from the lecithin of egg oil.

No. 156. 0.1022 gm. of substance, dried by fusion, gave on combustion 0.1166 gm. of H_2O and 0.2846 gm. of CO_2.

0.7134 gm. of substance, dried by fusion, required for neutralization 5.20 cc. of 0.5 N NaOH corresponding to a molecular weight of 274.

Sample.	Analysis.		Molecular weight.	Melting point.
	H	C		
	per cent	*per cent*		*°C.*
Calculated for $C_{18}H_{36}O_2$ (stearic acid)	12.76	75.98	284	70–71
Found No. 156	12.76	75.88	274	64–65

The Saturated Fatty Acids of Lecithin Cadmium Chloride Prepared from This Fraction.

No difference was apparent between the acids isolated from the cadmium chloride salt of the lecithin of the ethereal extract of egg yolk and those isolated from lecithin of the acetone extract. The mixed esters were fractionated into three fractions and a residue.

Sample.	Boiling point of esters.	Analysis of acid.		Molecular weight of acid.	Melting point of acid.
		H	C		
	°C.	*per cent*	*per cent*		*°C.*
Mixed esters, No. 140		12.20	75.16	267	58–59
First fraction	152–170				
Refractionated, No. 144 (No. 159)	157–160	13.07	74.95	258	64–65
Residue, No. 145		13.21	75.26	262	59–60
Second fraction, No. 146	154–175	13.04	74.96		60–61
Residue, No. 157		13.04	75.93	282	70–70.5

The detailed data on these analyses are as follows:

No. 140. 0.1005 gm. of substance, dried by fusion, gave on combustion 0.1096 gm. of H_2O and 0.2770 gm. of CO_2.

1.0819 gm. of substance, dried by fusion, required for neutralization 8.08 cc. of 0.5 N NaOH corresponding to a molecular weight of 267.

No. 159. 0.0998 gm. of substance, dried by fusion, gave on combustion 0.1166 gm. of H_2O and 0.2743 gm. of CO_2.

0.9948 gm. of substance required for neutralization 7.70 cc. of 0.5 N NaOH corresponding to a molecular weight of 258.

No. 145. 0.1088 gm. of substance, dried by fusion, gave on combustion 0.1190 gm. of H_2O and 0.2782 gm. of CO_2.

1.0080 gm. of substance required for neutralization 7.70 cc. of 0.5 N NaOH corresponding to a molecular weight of 262.

No. 146. 0.1004 gm. of substance, dried by fusion, gave on combustion 0.1170 gm. of H_2O and 0.2760 gm. of CO_2.

No. 157. 0.1007 gm. of substance, dried by fusion, gave on combustion 0.1174 gm. of H_2O and 0.2804 gm. of CO_2.

0.6874 gm. of substance, dried by fusion, required for neutralization 4.87 cc. of 0.5 N NaOH corresponding to a molecular weight of 282.

The Unsaturated Acid of Lecithin Prepared from This Fraction.

These results were similar to those obtained on the unsaturated acid of the previous fraction. The acid was purified by the process described above. Its iodine value was 87.

No. 141. 0.3995 gm. of substance absorbed 0.34308 gm. of iodine when titrated according to the method of Wijs.

	Calculated for $C_{18}H_{34}O_2$.	Found No. 141.
Iodine number	90	87

Saturated Fatty Acids from a Sample of Lecithin Prepared from This Fraction.

A sample of the lecithin cadmium chloride used in the preceding experiment was decomposed with ammonium carbonate. After purification it gave the following analysis:

No. 50. 0.1957 gm. of substance used for a Kjeldahl determination required 2.85 cc. of 0.1 N HCl, equivalent to 0.00399 gm. of N.

0.2936 gm. of substance gave 0.0408 gm. of $Mg_2P_2O_7$.

0.1096 gm. of substance, dried under diminished pressure at temperature of water vapor, gave on combustion 0.1050 gm. of H_2O, 0.2604 gm. of CO_2, and 0.0110 gm. of ash.

	Calculated for $C_{43}H_{86}O_9NP$.	Found No. 50 (calculated ash-free).
	per cent	*per cent*
C	65.27	65.56
H	10.95	10.85
N	1.77	2.04
P	3.92	3.87

This material was hydrolyzed and the mixed fatty acids after purification through the lead salt analyzed as follows:

No. 173. 0.0995 gm. of substance, dried by fusion, gave on combustion 0.1150 gm. of H_2O and 0.2758 gm. of CO_2.

1.1333 gm. of substance required for neutralization 8.60 cc. of 0.5 N NaOH corresponding to a molecular weight of 264.

Sample No.	Analysis.		Molecular weight.	Melting point.
	H	C		
	per cent	*per cent*		*°C.*
173	12.93	75.59	264	57–58

THE CHEMISTRY OF NEOARSPHENAMINE AND ITS RELATION TO TOXICITY.

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Although arsphenamine[1] has within recent years attained the leading place in the treatment of syphilis, its condensation product with sodium formaldehyde sulfoxylate, known as neoarsphenamine, is beginning to find preference with physicians. There are several reasons why it is considered superior. First, it is easier to prepare a solution of neoarsphenamine for intravenous injection, since it is instantly soluble in water and is ready for use without addition of alkali. Arsphenamine on the other hand must be dissolved in water and carefully neutralized with alkali, a procedure requiring time and experience. Second, neoarsphenamine is more readily tolerated by patients. Injections of arsphenamine more often give rise to certain alarming symptoms called "reactions," than its condensation product. Third, the ratio of the therapeutic to the tolerated dose, *i.e.* the chemotherapeutic index, is more favorable in the case of neoarsphenamine (1).

Notwithstanding its wide employment in the therapy of syphilis, very little is mentioned in the literature about the chemical composition of neoarsphenamine. Ehrlich (2) in 1912 was the first to mention this substance, and he described it merely as a condensation product of salvarsan with sodium formaldehyde sulfoxylate. In order to prevent the autooxidation of salvarsan since it contains an "easily oxidizable arseno group and is also a derivative of the oxidizable amido-phenol," Ehrlich endeavored to combine it with different reducing agents (2). The addition of

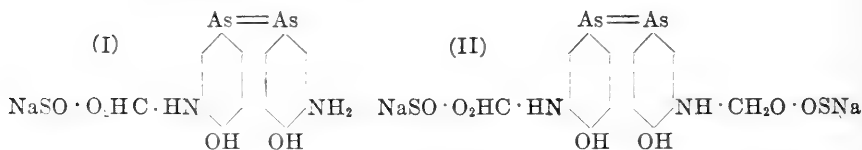
[1] Arsphenamine is the official American name for the dihydrochloride of diaminodioxyarsenobenzene. It is known also as salvarsan, karsivan, diarsenol, arsenobenzol.

sodium formaldehyde sulfoxylate or "hyraldite" not only satisfied the above requirement but also yielded the very desirable neutral reacting sodium salt. No analytical data on neosalvarsan were furnished by Ehrlich or his collaborators, leaving the chemical constitution of the product a matter of surmise.

Bertheim (3) in his monograph on organic arsenic compounds casually speaks of two possible formulas for neosalvarsan:[2] one in which arsphenamine is combined with 1 molecule of sodium formaldehyde sulfoxylate and the other with 2 molecules. Either one, he thinks, can be produced depending upon the conditions of the experiment. He fails, however, to specify them. These chemical formulas are also given in the patents on neosalvarsan without analytical data to support them.

Lehmann (4) analyzed neosalvarsan for arsenic and found only 22.02 per cent. As this is approximately two-thirds of the value required by the formula corresponding to a mono-substituted compound, he concluded that in neosalvarsan the second amino group is not entirely free. He failed to observe that the organic arsenical compound comprises only about two-thirds of the sample of neoarsphenamine, the remaining third consisting of free sodium formaldehyde sulfoxylate, inorganic salts, and other non-arsenical impurities, as will be shown later.

This work has been undertaken mainly with the object of determining whether sodium formaldehyde sulfoxylate is attached to one or both amino groups of the arsphenamine molecule according to the following formulas:



This is an important chemotherapeutic problem, since recently Schamberg, Kolmer, and Raiziss (1) demonstrated that not only is the toxicity of neoarsphenamine lower in man and experimental animals than arsphenamine, but that this diminution in toxicity is proportionally greater than the decrease in arsenic content.

[2] For the sake of convenience we designated these formulas as I and II, frequently referring to these designations in the body of the paper.

Neoarsphenamine, containing an average of 20 per cent of arsenic, is tolerated in doses of from 200 to 300 mg. per kilo of body weight, while the tolerated dose of arsphenamine, containing approximately 30 per cent of arsenic, is only 100 mg. per kilo. Undoubtedly, the decrease in toxicity is due to the change in the amino group. The latter probably is a factor in determining the toxicity, as well as the therapeutic potency of arsphenamine.[3]

In the following are described methods of analysis, which were developed in order to study the problem quantitatively. The results obtained with various samples of neoarsphenamine manufactured by different laboratories are tabulated, each of the letters D, M, and T referring to a different laboratory.

Alcohol or Water of Crystallization.

By drying neoarsphenamine in an inert gas at 60–80°C. it loses in weight. This loss cannot be regarded as due to decomposition since constant weight was obtained in every case. Apparently it contains either alcohol or water of crystallization. The same has been found previously for arsphenamine (2).

About 100 mg. of substance were placed in a small porcelain boat and heated to about 80°C. in a stream of carbon dioxide purified by passing successively through solutions of sodium carbonate, potassium permanganate, and concentrated sulfuric acid. It took usually from 2 to 6 hours before the compound would show constant weight.

As noticed in Table I, the loss in weight varies with different samples; *e.g.*, Sample M_1 showed a loss of 3.63 per cent, while Sample D_1 showed 4.46 per cent. As will be seen later (Table V) the samples of neoarsphenamine analyzed contain from 57.0 to 65.7 per cent of organic arsenicals.

In Table II, calculations are made on the basis of the arsenic content. The method of calculation is very simple. Since Compounds I and II contain 2 atoms of arsenic, 1 molecule of methyl alcohol of crystallization, for example in Sample D_1, can

[3] To prove the correctness of this assumption, the authors in collaboration with Dr. Jay F. Schamberg and Dr. John A. Kolmer carried out further experiments which are being prepared at present for publication ("The significance of the amino group in the chemotherapy of arsenicals").

TABLE I.

Loss upon Drying.

Sample No. of neoarsphenamine.	Substance taken for analysis.	Weight after drying.	Loss.		Average loss.
	mg.	*mg.*	*mg.*	*per cent*	*per cent*
D_1	100	95.4	4.6	4.60	4.46
	55.7	53.3	2.4	4.31	
D_2	100.6	96.1	4.5	4.47	4.28
	101.2	97.0	4.2	4.15	
M_1	101.6	98.0	3.6	3.54	3.63
	107.4	103.4	4.0	3.72	
T_1	103.4	99.1	4.3	4.16	4.15

TABLE II.

Calculations Based upon Arsenic Analyses and Losses by Drying.

Sample No. of neoarsphenamine.	Arsenic found.	Calculated for 1 mol CH_3OH.	Calculated for 1 mol C_2H_5OH.	Calculated for 2 mols H_2O.
	per cent	*per cent*	*per cent*	*per cent*
D_1	19.70	4.20	6.00	4.70
D_2	19.61	4.20	6.00	4.70
M_1	18.04	3.80	5.60	4.30
T_1	19.03	4.10	5.80	4.60

TABLE III.

Arsenic to Nitrogen Ratios.

Sample No. of neoarsphenamine.	Arsenic.	Nitrogen.	$\frac{As}{N}$ found.	Deviation.
	per cent	*per cent*		*per cent*
D_1	19.70	3.42	5.76	+7.50
D_2	19.61	3.56	5.51	+2.80
D_3	19.06	3.41	5.59	+4.29
D_4	19.08	3.57	5.34	−0.40
D_5	19.07	3.57	5.34	−0.40
M_1	18.04	3.30	5.47	+1.80
M_2	17.63			
T_1	19.03	3.38	5.63	+5.00

The theoretical value for the arsenic to nitrogen ratio is 5.36.

be calculated from the following equation: $2As:CH_3OH$ = per cent As found: x, or $150:32 = 19.7:x$. The figures corresponding to 1 molecule of methyl alcohol when compared with the results obtained agree very closely. The results calculated for 2 molecules of water show less agreement, as do those for 1 molecule of ethyl alcohol.

Arsenic and Nitrogen.

Qualitative tests for inorganic arsenic in samples of neoarsphenamine were negative. Arsenic and nitrogen (Table III) are present in the same ratio as in arsphenamine.

Raiziss and Proskouriakoff (5) have shown that with arsphenanime this ratio almost equals the theoretical value of 5.36. Table III shows that Samples D_4 and D_5 have practically the theoretical $\frac{As}{N}$ ratios and other samples deviate but slightly. Sample D_1 is the only one which exhibits a marked discrepancy.

Sulfur.

Most of the sulfur in neoarsphenamine represents the formaldehyde sulfoxylate condensed with the amino group of arsphenamine. Some of it, however, is due to free sodium formaldehyde sulfoxylate. Occasionally sulfur is present as sodium sulfate. In addition a small quantity may possibly be linked either to the nuclear carbon or arsenic. This is firmly bound and can be oxidized only after the complete breakdown of the molecule, whereas the sulfur of the formaldehyde sulfoxylate can be easily oxidized to sulfate and precipitated as barium sulfate.

In order to determine quantitatively the distribution of sulfur among the compounds just mentioned, a plan of procedure has been devised which is based on studies of the chemical properties of neoarsphenamine and sodium formaldehyde sulfoxylate. It was found that at low temperature (below 5°C.) Compounds I and II are quantitatively precipitated by diluted hydrochloric acid without decomposition. The formaldehyde sulfoxylic acid formed from the free sodium formaldehyde sulfoxylate remains in solution, also undecomposed, thereby effecting a complete separation from the formaldehyde sulfoxylate attached to the amino groups.

TABLE IV.

Results of Sulfur Analysis.

Sample No. of neoarsphenamine		D_1	D_2	D_3	D_4	D_5	M_1	M_2	T_1
Substance analyzed, *mg*	Method (*a*)	170.7	204.5	145.8	151.6	160.6	143.5	164.5	196.7
		203.7	202.5	152.6	151.0	151.7	149.9	177.1	196.5
Barium sulfate found, *mg*		115.4	135.9	100.2	100.7	106.8	87.8	102.3	159.0
		140.7	135.3	101.9	97.6	99.5	88.3	105.2	159.7
Total sulfur, *per cent*		9.29	9.13	9.44	9.12	9.14	8.40	8.54	11.10
		9.49	9.18	9.17	8.88	9.01	8.10	8.16	11.16
	(mean)	9.39	9.16	9.31	9.00	9.08	8.25	8.35	11.13
Substance analyzed, *mg*	Method (*b*)	200	200	200	200	200	180	200	200
		200	200	200	200	200	180	164.7	200
Barium sulfate found, *mg*		129.6	129.5	128.8	118.8	117.2	101.5	112.6	155.8
		130.5	129.0	127.7	123.3	120.4	101.3	92.2	155.4
Sulfur, *per cent*		8.90	8.90	8.85	8.16	8.05	7.74	7.73	10.70
		8.96	8.86	8.77	8.47	8.27	7.73	7.69	10.68
	(mean)	8.93	8.88	8.81	8.32	8.16	7.74	7.71	10.69
Sulfur of nuclear carbon, *per cent*		0.46	0.28	0.50	0.68	0.92	0.51	0.64	0.44
Substance analyzed, *mg*	Method (*c*)	300	300	300	300	300	300	300	300
		300	200	300	300	300	300	300	300
Barium sulfate found, *mg*		86.9	72.1	91.5	72.8	89.0	76.0	86.7	124.8
		81.8	43.0	92.5	67.8	85.6	80.0	92.6	127.7
Sulfur, *per cent*		3.98	3.27	4.19	3.30	4.08	3.48	3.97	5.72
		3.80	2.97	4.24	3.12	3.92	3.66	4.24	5.85
	(mean)	3.89	3.12	4.22	3.21	4.00	3.57	4.10	5.79
Sulfur of amino group, *per cent*		5.04	5.76	4.59	5.09	4.16	4.17	3.61	4.90

Substance analyzed, *mg*	Method (*d*)	300 300	300 300				300 300		300 300
Barium sulfate found, *mg*	Method (*d*)	33.0 38.5	31.2 29.9				Trace. "		Trace. "
Sulfur as sodium sulfate, *per cent*	Method (*d*)	1.51 } 1.64 1.76 }	1.43 } 1.40 1.37 }				"		"
Sulfur as free sulfoxylate, *per cent*.		2.25	1.72				3.57		5.79

Our plan, then, for the estimation of various sulfur compounds requires four quantitative analyses:

(*a*) The total sulfur determination by the method of Carius.

(*b*) The determination of total sulfur exclusive of that attached to either nuclear carbon or arsenic by oxidation with iodine solution and precipitation of the sulfate formed as barium sulfate by the following method:

0.2 gm. of neoarsphenamine is dissolved in about 100 cc. of water and a large excess of iodine solution is added and gently boiled in a covered beaker for about 6 hours. After acidulation with hydrochloric acid and addition of barium chloride, the sulfur of both the free and combined formaldehyde sulfoxylate and sulfates is quantitatively precipitated.

The difference between (*a*) and (*b*) represents the sulfur linked to nuclear carbon or arsenic.

(*c*) Total sulfur as uncombined sulfoxylate and sulfate.

0.3 gm. of neoarsphenamine is dissolved in about 25 cc. of water, the solution cooled in ice, and 5 cc. of diluted hydrochloric acid are added. After standing 15 minutes in the cold, the precipitate is filtered off quantitatively by suction through an asbestos and cotton filter (6) and washed many times with small amounts of 1 per cent hydrochloric acid. The filtrate together with the combined washings was boiled with iodine solution, and sulfur determined by the addition of barium chloride, etc. as in (*b*).

The sulfur of the combined sulfoxylate is represented by the difference between (*b*) and (*c*).

(*d*) The sulfur present as sulfates is determined by the same procedure as in (*c*) except that the filtrate was not boiled with iodine, but precipitated by barium chloride in the usual way. The difference between (*c*) and (*d*) represents sulfur in the uncombined formaldehyde sulfoxylate.

An examination of figures presented in Table IV shows that closely checking results have been obtained for the various sulfur analyses, proving the accuracy of the above described methods.

The total sulfur found in various samples of neoarsphenamine obtained from Laboratory D is quite uniform, ranging from 9.00 to 9.39 per cent. Samples from different laboratories exhibit greater variations, the smallest value being 8.25 and the largest 11.13 per cent. The quantity of sulfur attached to either a

nuclear carbon or arsenic atom is small, in all samples varying from 0.28 to 0.92 per cent. Sulfur present as combined sulfoxylate in some samples is greater than that of the uncombined, *e.g.* in Samples D_1, D_2, M_1; while in others it is smaller. In Table V are given the amounts of combined and uncombined sodium formaldehyde sulfoxylate, which are calculated on the basis of arsenic and sulfur values. In Sample D_2 there are more than three times as much combined sulfoxylate as uncombined, in Sample M_1 they are almost equal, while Sample T_1 has more uncombined than combined sulfoxylate.

TABLE V.

Calculations Made on the Basis of Arsenic and Sulfur Analyses.

Calculated for 100 mg. of neoarsphenamine.

Sample No. of neoarsphenamine.	Organic arsenical component.	Combined sulfoxylate.	Uncombined sulfoxylate.	Mono-substituted product.	Di-substituted product.	Mono-substituted product.*	Di-substituted product.*
	mg.	*mg.*	*mg.*	*mg.*	*mg.*	*per cent*	*per cent*
D_1	63.8	18.6	8.3	48.9	14.9	76.6	23.4
D_2	65.7	21.2	6.3	37.9	27.8	57.7	42.3
D_3	60.8	16.9		51.6	9.2	85.0	15.0
D_4	62.4	18.7		44.6	17.8	71.6	28.4
D_5	63.3	15.3		63.3	0	100.0	0
M_1	57.0	15.2	13.3	51.4	5.6	90.2	9.8
M_2	58.5	13.3		58.5	0	100.0	0
T_1	61.7	18.1	21.3	46.8	14.9	75.9	24.1

* These figures represent percentages of the organic arsenical component and not of the original sample of neoarsphenamine.

Still more interesting are the figures indicating the relative amounts of Compounds I and II. Samples D_5 and M_2 contain only mono-substituted arsphenamine; Sample D_2 has 57.7 per cent of the mono-substituted and 42.3 per cent of the di-substituted product; and Sample T_1 has 75.9 per cent of mono-substituted and 24.1 per cent of the di-substituted.

Since there was found in several samples more combined sulfoxylate than can be accounted for by substitution in merely one amino group, the only conclusion which can be drawn is that the second amino group of arsphenamine was also involved, with the production of a di-substituted product. Hence it is logical to assume that neoarsphenamine consists of a mixture of mono- and di-substituted products.

The Oxygen Requirements.

As has been shown by Ehrlich and Bertheim (7), Gaebel (8), Kober (9), and Raiziss and Proskouriakoff (5), arsphenamine is easily oxidized in the cold by iodine solution to the corresponding 3-amino-4-hydroxy-phenylarsenic acid. This oxidation is quantitative, and each molecule requires 4 atoms of oxygen. The same reaction takes place with neoarsphenamine.

Table VI shows that Sample D_1 required 13.25 cc. of 0.1 N iodine solution which is equivalent to 10.6 mg. of oxygen. The arseno group, however, as calculated on the basis of arsenic found requires 8.4 mg. of oxygen. But neoarsphenamine, as was previously pointed out, contains free sodium formaldehyde sulfoxylate. Reinking, Dehnel, and Labhardt (10), and others found that the latter is also oxidized by iodine in the cold. We were able to confirm this for by working with pure recrystallized sodium formaldehyde sulfoxylate we found that it required quantitatively 2 atoms of oxygen. According to Table V, Sample D_1 contains 8.3 mg. of uncombined sodium formaldehyde sulfoxylate, which requires 2.5 mg. of oxygen. Therefore the total amount of oxygen required by the arseno group and sulfoxylate is 10.9 mg. This figure agrees fairly well with the 10.6 mg. actually found.

TABLE VI.

Oxygen Requirements.

Sample No. of neoarsphenamine.	Oxygen calculated for arseno group.	Oxygen calculated for free sulfoxylate.	0.1 N iodine found for 100 mg.	Corresponding oxygen found.	Total calculated oxygen.
	mg.	*mg.*	*cc.*	*mg.*	*mg.*
D_1	8.4	2.5	13.25	10.6	10.9
D_2	8.4	1.7	12.95	10.4	10.1
D_3	8.1		12.80	10.2	
D_4	8.2		11.85	9.5	
D_5	8.2		13.10	10.5	
M_1	7.7	3.6	14.15	11.3	11.3
T_1	8.1	5.8	16.96	13.4	13.9

In Sample M_1 the calculated oxygen is exactly the same as that found by experiment. Values for other samples are also similar to the calculated amounts.

These results are particularly gratifying as they confirm the results obtained for the distribution of sulfur in neoarsphenamine.

Sodium formaldehyde sulfoxylate attached to the amino group is not oxidized by iodine in the cold. This phenomenon is of theoretical importance since it indicates the firmness of the union between the sulfoxylate and arsphenamine.

Chlorides.

Every sample of neoarsphenamine which has been examined by us contained chlorine. Qualitative tests indicated that the chlorine is present in inorganic form. As there are no other metals in neoarsphenamine except sodium, we calculated for sodium chloride (Table VII).

TABLE VII.

Chlorides.

Sample No. of neoarsphenamine.	Substance taken.	AgCl found.	Cl		Calculated for NaCl.
	mg.	*mg.*	*per cent*		*per cent*
D_1	300	35.4	2.92	2.93	4.80
	224	26.5	2.93		
D_2	198.8	39.3	4.89	4.96	8.11
	202.3	41.0	5.02		
D_3	166.4	38.0	5.64	5.47	8.96
	200	42.8	5.30		
M_1	198.5	92.2	11.49	11.38	18.60
	198.7	90.5	11.27		
M_2	200.9	86.0	10.59	10.67	17.40
	204.9	89.0	10.75		
T_1	198.2	16.8	2.10	2.10	3.40
	153.5	13.0	2.10		

Comparatively large amounts of sodium chloride were found in Samples M_1 and M_2, M_1 containing 18.6 per cent. The smallest amount was found in T_1, 3.4 per cent.

Methods of Analysis.

Arsenic was analyzed gravimetrically by digesting with concentrated nitric and sulfuric acids, precipitating as magnesium ammonium arsenate, and weighing as magnesium pyroarsenate

(11). Nitrogen was analyzed by the ordinary Kjeldahl method. Total sulfur was determined by the method of Carius. The oxygen requirement was obtained by dissolving the sample in water at ordinary temperature, adding an excess of 0.1 N iodine, titrating the excess with 0.1 N sodium thiosulfate, and using starch as an indicator.

In all the analytical work only very closely checking results were considered.

SUMMARY.

1. Methods were devised for the quantitative study of the distribution of sulfur in neoarsphenamine.

2. The low arsenic content is due to the presence of non-arsenical compounds such as uncombined sodium formaldehyde sulfoxylate, sodium sulfate, and sodium chloride.

3. Upon drying to constant weight a loss occurs indicating methyl alcohol or water of crystallization.

4. The arsenic to nitrogen ratio, which may be regarded as a comparatively good indicator of the purity of the arsenical component, in some samples equals the theoretical value. Where deviations occur, they are greater than those obtained with arsphenamine.

5. The values obtained for the oxygen requirements (as calculated from the amounts of 0.1 N iodine necessary for complete oxidation) are in excess of that required merely by the arseno group. This excess is attributed to the uncombined sodium formaldehyde sulfoxylate. The latter, when combined to an amino group, evidently is not oxidized by iodine.

6. The amount of combined sulfoxylate found is greater than that necessary for one amino group. It is less than the calculated value for a substitution product with both amino groups. Hence, it is assumed that neoarsphenamine contains a mixture of both the mono- and di-substituted products.

7. It is possible that the variation in the completeness of substitution in the amino groups accounts for the irregularity of the toxicity and therapeutic effect of the drug.

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CERTAIN CHEMICAL CHANGES IN THE BLOOD AFTER PYLORIC OBSTRUCTION IN DOGS.

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The operation of closure of the pylorus was performed on eight dogs in an attempt to reproduce the clinical condition known as gastric tetany. Whether or not this, our original, object was attained we are unable to state definitely; in fact, this matter became of secondary interest as soon as we found and became absorbed in the study of the great disturbances in certain fundamental physicochemical equilibria which took place in the blood after this operation.

Tetany is a condition characterized clinically by contractions in the flexor muscles of the extremities and occasionally by generalized convulsive seizures or muscular spasms in other parts. Increased response to galvanic stimulation when applied over the peripheral nerves has been found in about 80 per cent of the cases (Holmes). When this state of nerve hyperirritability is associated with dilatation of the stomach the condition is termed gastric tetany. The gastric dilatation is often consequent to pyloric obstruction, the latter most frequently the result of cicatricial contraction of an ulcer. A common history of such a case would be that of a man who, with a previous story of gastric indigestion, pain, and vomiting, is one day overcome by a more severe attack and after several inordinate fits of vomiting is taken with tetanic convulsions and dies. Autopsy reveals a much dilated atonic stomach with a stenosed pylorus.

A number of theories were advanced in the past to explain the tetany in such cases. None of these, however, was verified by experiment and they simply mirror the fashions and extent of physiological reasoning possessed by the practitioners of the epoch. For instance, there was the mechanical theory that the

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contractions of the stomach against an obstructed pylorus, or in the act of vomiting, initiated afferent impulses which sensitized the brain and central nervous system to such a degree that any added stimulation, such as cold or the passage of a stomach tube, would inaugurate convulsions and spasms. More recently other ideas such as those of dehydration and intoxication have been presented, supported only by insufficient experimentation and hasty conclusions.

It has been found that gastroenterostomy would entirely cure the condition (Jonnesco and Grossman) and that the administration of calcium would temporarily alleviate the symptoms (Kinnicutt). These are important findings.

As far as we know, few attempts have been made to produce gastric tetany experimentally. In this laboratory Dr. E. H. Pool performed pyloric closure by various methods on many dogs. The animals developed slight muscular tremors and spasms of various kinds, but only in one case, out of about forty, was he able to demonstrate increased electrical irritability with a cathodal opening current of less than 5 milliamperes. In his judgment, therefore, it was not demonstrated that tetany had been produced by the operations. Little significance was attached to the one exception, as it was considered that this was probably due to some technical error. Because of this experience of one so familiar with the subject of tetany, and because we did not in our experiments make the electrical tests, we cannot claim to have reproduced the condition which is clinically known as gastric tetany.

Seven out of our eight dogs showed muscular twitchings, tremors, spasticity, and fibrillary movements in a room where the temperature was constantly maintained above 70°F. Such manifestations were not seen in dogs undergoing various other gastric or abdominal operations. The eighth dog became paralyzed without showing any increased rigidity as far as could be seen. The electrical tests were omitted, not because we were unaware of their importance in the diagnosis of tetany, but because they would have to have been done to the exclusion of the chemical studies in which we were more particularly interested. MacCallum and coworkers, however, have just published the results of electrical tests performed on dogs with pyloric occlusion which

definitely indicate an increased irritability of the nerves to the galvanic current. In one dog, of the present series, pedal spasms in the fore extremities occurred to such a degree that at times it was almost impossible to straighten his paws. Whether or not these signs were manifestations of tetany we cannot say. The subject is pregnant with possibilities and it is with considerable misgivings that we have had to interrupt our work in its bare inception and present the results of such a short series of tests. However, it is believed that the striking regularity of the figures, to be recorded, not only confirm certain results of other investigators but point to further basic disturbances which offer a field for profitable research in the future.

An interesting study of the effects of pyloric occlusion was that of McCann who recently published a short paper giving figures for the combined CO_2 of the blood. In four dogs he showed that, after closing the pylorus, the alkaline reserve of the blood increased rapidly up to the time of death. Since McCann, as well as Wilson and his coworkers, got similar results after parathyroidectomy, and the latter had showed that injections of hydrochloric acid relieved convulsions, the former thought that tetany in both instances (*i.e.*, after pyloric closure and parathyroidectomy) was the result of a condition of alkalosis. We were unable to verify the results of these investigators after removal of the parathyroids, and so we determined to repeat McCann's gastric experiments. The operative procedure consisted merely in tying a stout string very tightly around the pylorus. The CO_2-combining capacity of the plasma was determined by the Van Slyke method in the usual fashion. The results are given in Table I.

In Dog M, our first, it was noted at death that the blood was very dark and viscous. Because of this observation we were prompted in the next experiments to make hematocrit readings on each sample of blood. In most cases there seemed to be a tendency towards an increase in the proportion of solid to fluid elements, probably from dehydration, but in those which showed no such changes the symptoms and chemical changes in the blood were equally well marked. In some cases rectal infusions were given to supply fluid. No blood volume estimations were made.

TABLE I.*

Dog N. Before operation, CO_2 74.8 vol. per cent; 1st day after operation, 84.5; 2nd day, 93.8; 3rd day, 86.2 and 101.2; 4th day, 98.8; 5th day, 91.7; 6th day, 123.7; 7th day, 117.0; 9th day, 117.0; 14th day, 131.1; killed.

	Dog M.	Dog O.	Dog P.	Dog Q.	Dog R.	Dog S.
	vol. per cent	*vol. per cent*	*vol. per cent*	*vol. per cent*	*vol. per cent*	*vol. per cent*
Before operation......	66.3	55.6	40.8	52.5	46.7	47.5
1 day after operation..	158.0	80.2	62.6 74.9	75.8	61.3 63.2	60.3 58.3
2 days " " ..	102.0	77.7 83.6 85.0	76.8	90.0 95.7	70.0 74.8	73.9 96.2
3 " " " ..	Death.	Death.	90.0 Killed.	95.0 Killed.	73.9 Killed.	79.7 Killed.

* Only the figures obtained from samples of blood from the jugular vein have been inserted.

TABLE II.

	Dog M.	Dog N.	Dog R.	Dog S.	Dog O.
	pH	*pH*	*pH*	*pH*	*pH*
Before operation......	7.80	7.82	7.90	7.76	7.74
1 day after operation...	7.87	7.88	7.88	7.83	7.75
2 days " " ...	7.87				7.63 (15 min. before death.)
3 " " " ...	Death.				7.54 (15 min. after death.)

TABLE III.

Calcium per 100 Cc. Serum.

	Before operation.	After operation.
	mg.	*mg.*
Dog M.	10.0	14.0
Dog O..	9.6, 10.3 } 9.95 (Cells 31.3 per cent.)	12.2, 12.5 } 12.35 (Cells 35 per cent.)
Dog P..	12.3, 12.3 } 12.3 (Cells 39 per cent.)	13.7 (Cells 42 per cent.)

There seems to be only one conclusion to be drawn from the above results; namely, that partial occlusion of the pylorus is followed by a marked increase in the alkaline reserve of the blood, a condition which has been called alkalosis.

At the same time as these determinations of CO_2 were being made, the pH of the blood was measured by the gas-chain method. The results are given in Table II. The figures seem to show that there is a very slight rise in pH (decrease in H ion concentration) after the operation, but the figures for Dogs M and O show that after this initial rise there is no further increase. There is also the interesting observation that as death approaches, the blood does not become more alkaline as one might suppose if the mechanisms for maintaining the acid-base equilibrium were suddenly overwhelmed by rapidly increasing basic radicals, but on the contrary, just as in death from other causes, there is a rapid premortem fall in pH.

As calcium salt infusions have been found beneficial in gastric tetany, we tested the concentration of this substance in three dogs before and after operation as shown in Table III.

These tests show that the concentration of calcium is not decreased, but on the other hand, is slightly higher than normal. MacCallum using a crude analytical method purely for comparative purposes found no change or an insignificant drop in calcium in these cases. Variable results were obtained for calcium by the analyses of Dr. Greenwald. The latter, who has been perfecting a method for the estimation of certain inorganic constituents of blood, kindly analyzed samples from three of our dogs with results as given in Table IV.

The results of these salt analyses are significant. It is at once apparent that the most definite change is a marked drop in the concentration of chlorides. This pronounced fall (about 50 per cent) was also found by MacCallum, and in fact, it is just what one would expect after pyloric occlusion. The inverse relationship between the concentration of bicarbonate and chloride ions in the plasma has recently been established by McLean and coworkers. Their experiments were concerned with the well known Zuntz phenomenon. In the present series the rise in bicarbonate and fall in chloride concentrations involve the whole blood. Other changes to be noted are (1) the rise in sulfur and

(2) the rise in phosphorus. The concentration of sodium we rather expected to find above normal on account of the tremendous increase of bicarbonate in the blood; in fact, we had turned

TABLE IV.

Dog N.

	Plasma. Before operation.	Plasma. 7 days after operation.	Cells. Before operation.	Cells. 7 days after operation.	Whole blood. Before operation.	Whole blood. 2 days after operation.	Whole blood. 7 days after operation.
	mg.	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*
Cl	411.0	195.0	189.0	103.0	304.0	188.0	151.0
S	13.7	22.7	13.5	17.3	13.6	14.8	20.1
P	5.07	8.52	41.7	54.5	22.8	40.0	30.3
Ca	12.8	13.3	1.01	8.4	7.10	7.62	11.0
Mg	2.65	Lost.	8.05	Lost.	5.26	7.03	Lost.
K	19.4	12.1	29.0	25.3	24.0	25.1	18.4
Na	338.0	265.0	214.0	271.0	278.0	275.0	268.0

Dog Q.

	Plasma. Before operation.	Plasma. 2 days after operation.	Cells. Before operation.	Cells. 2 days after operation.	Whole blood. Before operation.	Whole blood. 2 days after operation.
	mg.	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*
Cl	383.0		194.0		304.0	148.0
S	10.67		10.6		10.7	17.03
P	6.45		44.6		22.5	43.8
Ca	15.13	Blood	4.27	Blood	10.6	7.21
Mg	2.24	clotted.	6.29	clotted.	3.41	6.80
K	15.6		30.66		21.9	26.1
Na	351.0		251.0		328.0	265.0

Dog T.

	Plasma. Before operation.	Plasma. 2 days after operation.	Cells. Before operation.	Cells. 2 days after operation.	Whole blood. Before operation.	Whole blood. 2 days after operation.
Cl	408.0	245.0	212.0	152.0	306.5	185.8
S	8.61	19.3	9.45	14.2	9.05	15.95
P	2.34	4.53	54.7	58.9	29.6	39.09
Ca	10.5	9.83	6.45	6.58	8.4	7.74
Mg	2.54	3.46	8.47	8.12	5.63	6.41
K	20.47	19.8	29.5	31.1	25.21	27.0
Na	343.0	295.0	273.0	254.0	306.7	268.6

to this expected rise as the most likely explanation for the nervous irritability. MacCallum did not test the sodium but suggested, in his paper, that this be done, intimating, it seems, that the

metal might be found in abnormal amounts. These analyses, however, show that the concentration of sodium is slightly decreased.

In none of our dogs did we see the violent convulsive seizures described by MacCallum. The latter, however, cut across the pylorus, completely closed the lower end of the stomach with sutures, and brought out the proximal end of the duodenum through the abdominal wall. Through this fistula water was regularly injected into the duodenum and periodically the stomach was washed out with a pump. In the present series the pylorus was cut across in only one experiment (Dog T); in the other cases a strong cord was simply tied tightly around the pylorus. At autopsy it was found that the latter procedure had never completely shut off communication with the duodenum, but it was nevertheless sufficient to inaugurate the same chemical disturbances as occurred after complete closure. The animal washed out his own stomach, many times a day, by drinking large quantities of water and then immediately vomiting it up again.

With one exception (Dog O) all our animals had twitches, fibrillations, spasms, and abnormal muscular contractions of various kinds. We feel convinced that these were not the result of such factors as operation, temperature of the room, etc., as there was plenty of opportunity to control such obvious conditions, but rather do we think that they were probably due to the changes in the blood which were invariably found to take place after pyloric occlusion. In Dog O marked paralysis occurred and there were signs of weakness which simulated paresis in two other dogs. This finding is of interest in connection with the work of von Wyss and later that of Grünwald who both believed, as a result of experiment, that paralysis could be produced by decreasing the chloride content of the blood.

A specific function of the chloride ion was also shown to exist when Loeb (1912) discovered that the toxicity of $NaBr$, Na_2SO_4, $NaNO_3$, etc., for the adult *Fundulus* may be abolished by the addition of $NaCl$ or other chlorides but not by other sodium salts.

RESULTS.

The following changes in the blood were found to occur after closure of the pylorus in dogs:

1. A marked increase in the CO_2-combining power of the blood.
2. A striking fall in the concentration of Cl ions.

McCann had already demonstrated the rise in alkaline reserve, and, as these experiments were being completed, MacCallum's article was published showing both the increase in bicarbonate and the fall in chloride concentration. In these particulars the tests set down above may be regarded as confirmatory evidence. We differed from MacCallum in that we found:

3. A slight increase in the concentration of calcium in the serum.

It was further shown that:

4. The pH of the plasma, after operation, showed only an insignificant rise; at death it fell rapidly.
5. The concentrations of sulfur and phosphorus were markedly increased.
6. The concentration of sodium was diminished in two out of three cases.

DISCUSSION.

Practically all the dogs in this series developed evidence of hyperirritability. Regardless of whether this was a condition identical with gastric tetany, whether it was another form of tetany, or whether it was not tetany at all, there must be some cause for the phenomenon and one is tempted to provide an explanation on the basis of the blood findings.

The two previous investigators, MacCallum and McCann, who studied the chemical nature of the changes following pyloric occlusion seemed to favor the view that the nervous manifestations were the result of the alkalosis. Particularly did the latter think so, since he also found an increase in the alkaline reserve in parathyroid tetany. Since we believe it has been shown that there is no increase in the bicarbonate content of the blood after parathyroidectomy and as there are no fundamental studies to our knowledge which indicate that an alkalosis, as such, can produce hyperirritability of nerves, we believe that it cannot be

fairly maintained that this is the cause. However, we have no conclusive proof that this theory is untenable nor have we any other satisfactory explanation to offer. Possibly it is concerned with the equilibrium of certain electrolytes in the body, particularly the disturbance of the ratio between monovalent and divalent anions and cations. The work of Loeb, Mathews, Lillie, and others on the antagonism of ions is very suggestive. Their experiments tend to show that an increase in the relative proportion of monovalent cations and divalent anions will augment the normal irritability of nerve tissue; a condition which may be counteracted by the addition of a salt such as $CaCl_2$ having a divalent cation linked with a monovalent anion. The most marked changes in the present series involve an increase in carbonate and phosphate ions (divalent) and a diminution of chloride ions (monovalent) which according to the theory should bring about hyperirritability. The slight increase in calcium and decrease in sodium in the blood may represent an adaptation on the part of the body to the above mentioned disturbance in the *milieu interieur*.

The explanation for the blood changes observed seems fairly clear. The alkalosis is evidently an exaggeration in duration and extent of the alkaline tide occurring normally after meals. The formation and secretion of HCl by the cells of the gastric mucosa necessitate the removal from the blood stream of H ions and Cl ions. The former exist in the blood mostly as carbonic acid, the latter in the form of sodium chloride. This process of selective secretion liberates bicarbonate ions and sodium ions which unite to form sodium bicarbonate and, in this fashion, bring about an increase in the alkaline reserve.

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OBSERVATIONS ON PARATHYROIDECTOMIZED DOGS.*

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Recent researches have resulted in the formulation of three independent, but not necessarily contradictory, theories concerning the blood changes that follow parathyroidectomy, and the relationship of these changes to the symptoms.

The first of these holds that the symptoms following the removal of the parathyroids are due to a disturbance of salt metabolism, particularly that of calcium. This hypothesis is based essentially upon the fundamental studies of Loeb (1902, *a*) and of J. B. MacCallum relating to the effect of various ions on nerve excitability, upon the suggestive experiments of Sabbatani on the opposing effects of applying calcium chloride solutions and calcium-precipitating solutions to the cerebral cortex, and finally upon

* The present research had its origin in some tests made on four dogs whose parathyroids had been removed as part of the regular course of surgical instruction given to the third year class at the College of Physicians and Surgeons. The results of these early experiments were not entirely in accord with generally accepted opinion, and so we were impelled to repeat them in a more systematic manner with the addition of a few other tests which seemed to be of importance. After eleven thyreoparathyroidectomized dogs had been studied, force of circumstances brought about the termination of the investigation. It has been repeatedly shown, and it seems to be almost universally accepted, that the immediate effects of thyreoparathyroidectomy are similar to those of parathyroidectomy. As parathyroid tissue is not infrequently imbedded within the body of the thyroid gland, by removing the latter the chances of complete parathyroidectomy are enhanced. If the number of experiments in this series is considered meager it is hoped that this deficiency will be somewhat compensated for by the consistency of the results.

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Quest's quantitative analyses of the brain for calcium after tetany. Parhon and Ureche were the first to note the curative effect of calcium salts in tetany and quite independently MacCallum and Voegtlin found in 1908 that the calcium concentration in the blood and tissues was decreased and in the feces and urine was increased after parathyroidectomy. Other chemical investigations soon followed among which may be mentioned the researches of Greenwald (1911) which indicated that the phosphates were increased in the blood and urine during tetany.

The second theory introduced by Wilson, Stearns, and Thurlow placed emphasis on a disturbance of the acid-base equilibrium. These investigators gave evidence to support the view that up to the onset of tetany there was a gradual increase in the alkaline reserve of the blood. During the convulsive seizures enough acid was formed, they thought, to neutralize the excess base and in some cases even to produce a condition of acidosis. The beneficial effects of injections of hydrochloric acid upon the tetany seemed to confirm their theory (Wilson, Stearns, and Janney).

Finally, there may be mentioned a third or toxic theory brought forward in 1915 by the British physiologists, Paton, Findlay, and Burns, which called attention to a highly toxic tetany-producing substance of protein origin found in the blood and urine following parathyroidectomy. This hypothesis seems to have been suggested by the work of Pekelharing and van Hoogenhuyze on the relation of the creatine of muscle to its tonicity, and by the finding of methyl guanidine, a product of protein metabolism, in relatively large amounts in the urine of parathyroidectomized dogs by Koch. Paton and his collaborators showed that injections of guanidine and methyl guanidine would produce a condition of hyperirritability associated with convulsions very much like parathyroid tetany.

Other important experiments, principally along the three lines outlined above, have been performed in various laboratories, but their review would scarcely be relevant to the subject matter of this article. For bibliographies the reader is referred to the theses of Paton and Findlay, Howland and Marriott (1917–18), Ochsner and Thompson, and for a very interesting general discussion of the present status of the subject to a recent article by Voegtlin.

The present research has been limited to an investigation of (1) the blood serum calcium, (2) the alkaline reserve of the blood plasma, (3) the hydrogen ion concentration of the blood plasma, (4) the blood sugar, and (5) the symptomatology.

1. Calcium.

MacCallum and Voegtlin found that the blood of dogs killed during parathyroid tetany contained on an average about 5.4 mg. of calcium per 100 cc. of whole blood; whereas, normally there were found about 13.3 mg. 4 years later, MacCallum and Vogel using a different analytical method found an average of 2.7 mg. of calcium per 100 gm. of whole blood in tetany as against 6.1 mg. in normal dogs.[1] Numerous other experiments were done later by MacCallum and his coworkers, all of which helped to substantiate their original hypothesis that the tetany was the result of calcium deficiency (MacCallum, Lambert, and Vogel, MacCallum and Vogel). Among other things it was found that the injection of calcium salts was the most efficacious temporary remedy for the tetanic convulsions. Some of this work has been brilliantly confirmed in a recent article by Howland and Marriott (1917–18) on infantile tetany, but contested, on less conclusive evidence to be sure, by Cooke, Musser and Goodman, Leopold and von Reuss, Stoeltzner, and others who report contradictory or negative calcium findings. In view, then, of this disagreement, and because no estimations to our knowledge had been made of the calcium content of the blood at various stages after parathyroidectomy, the following tests were undertaken.

The method devised by Halverson and Bergeim for the quantitative estimation of calcium in blood serum was used throughout. Although in our hands it was subject to an error of ± 5 per cent, it was found satisfactory for the purpose. It should be noted that, as there is little calcium in the corpuscles (Cowie and Calhoun), the concentration of this substance in 100 cc. of whole blood is only about two-thirds of the quantity in 100 cc. of serum. The calcium concentrations at various stages after parathyroidectomy are shown by the results given in Table I and Fig. 1.

[1] Normal figures for whole blood obtained recently by more accurate methods would indicate that these latter figures were more nearly correct.

TABLE I.

	Ca per 100 cc. serum.
Dog A.	
	mg.
Before operation	11.5
10 days after operation and 8 hrs. after first and only attack of mild tetany	8.2
18 days after operation, apparent recovery	10.8
Dog B.	
Before operation	11.2
10 days after operation, no tetany. Complete recovery	11.1
(All parathyroid tissue was probably not removed in this dog.)	
Dog C.	
Before operation	10.4
4 days after operation, at onset of third attack of tetany	6.2
10 " " " and $\frac{1}{2}$ hr. before death	4.3
Dog E.	
Before operation	11.7
$21\frac{1}{2}$ hrs. after operation, apparently well	8.0
5 days " " immediately before first attack of mild tetany	4.6
20 days after operation, very weak, continuous fibrillary twitchings	4.9
Dog F.	
Before operation	10.6
26 hrs. after operation, restless	9.0
$33\frac{1}{2}$ " " " early mild tetany	6.7
$47\frac{1}{2}$ " " " at height of first attack of acute tetany	7.0
3 days after operation, 3 hrs. after start of second attack of acute tetany	5.1
Dog H.	
Before operation	12.0
5 hrs. after operation	9.5
23 " " " slight rigidity	8.1
39 " " " at onset of first attack of acute tetany	6.2
3 days " " slight spasticity	5.3
8 " " " spastic, very weak, and cachectic	5.0

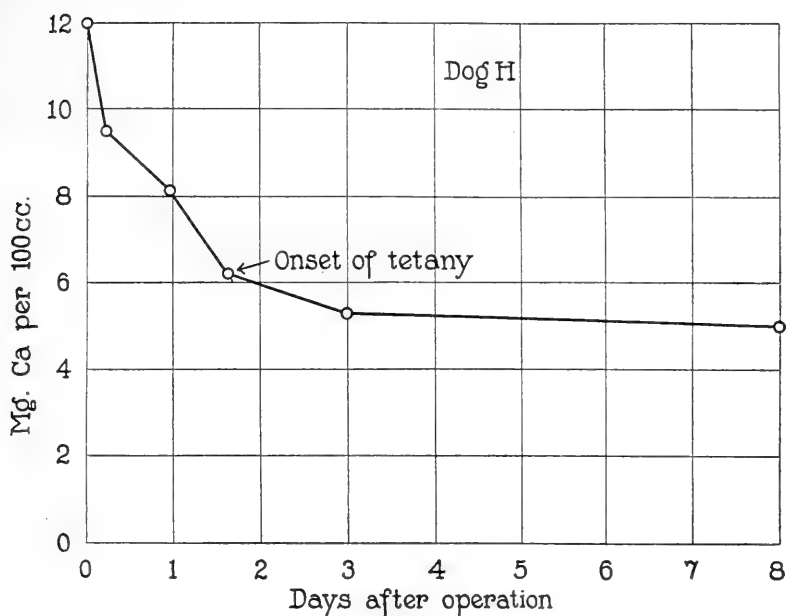

Fig. 1.

The effect of the injection of calcium chloride on the concentration of calcium in the blood is shown by the following figures:

Dog F.

	Ca per 100 cc. serum.
	mg
During acute tetany	5.1
Injection of 40 cc. of 1 per cent $CaCl_2$ solution; followed by complete relaxation and cessation of convulsions 30 min. later	9.0

These tests show that the concentration of calcium in the blood commences to decrease rapidly soon after the removal of the parathyroids, and in a few days reaches a value which is about 40 per cent of the normal. From then on until death there is little, if any, drop. These findings are consistent with the values in infantile tetany and in two parathyroidectomized dogs as found by Howland and Marriott (1916), and in a case of adult tetany recently reported (Barach and Murray). The injection of 40 cc.

of 1 per cent calcium chloride raised the amount in the blood to normal. This temporary curative power of intravenous $CaCl_2$ injections for tetany we were able to demonstrate repeatedly.

TABLE II.

	100 gm. plasma.			100 gm. cells.		
	Before.	After.	Change.	Before.	After.	Change.
Dog E. Before, and 20 days after, thyreoparathyroidectomy.						
	mg.	*mg.*	*per cent*	*mg.*	*mg.*	*per cent*
Chlorine...	284.0 (?)	336.0	Uncertain.	197.0 (?)	184.0	Uncertain.
Sulfur......	6.33	8.85	40+	10.7	12.1	13+
Phosphorus	5.08	7.05	38+	45.7	49.0	8+
Calcium....	9.14	4.91	46−	3.19	1.87	41−
Magnesium.	2.09	Lost.		5.81	Lost.	
Potassium..	20.2	"		29.6	"	
Sodium.....	344.0	"		214.0	"	
Dog J. Before, and 6 days after, thyreoparathyroidectomy.						
Chlorine...	385.0	378.0	None.	245.0	218.0	10−
Sulfur......	9.60	11.1	16+	9.31	12.7	36+
Phosphorus	5:03	5.95	18+	46.9	47.3	None.
Calcium....	13.1	8.41	36−	2.32	6.47	180+
Magnesium.	Lost.	2.07		Lost.	Lost.	
Potassium..	"	14.63		"	30.7	
Sodium.....	"	319.0		"	258.0	
Dog K. Before, and 2 days after, thyreoparathyroidectomy.						
Chlorine...	401.0	412.0	None.	211.0	244.0	15+
Sulfur......	9.52	10.3	8+	9.09	10.7	17+
Phosphorus	2.71	4.19	55+	51.9	50.8	None.
Calcium....	11.6	7.85	41−	6.98	8.2	17+
Magnesium.	1.83	1.84	None.	6.08	5.53	9−
Potassium..	22.3	17.2	23−	32.9	35.8	10+
Sodium.....	322.0	319.0	None.	251.0	289.0	15±

Dr. Greenwald, who was in the process of perfecting a method for the estimation of the principal inorganic constituents of blood, very kindly consented to do some determinations for us. His results are presented in Table II.

Unfortunately these statistics are not complete. The figures corroborate our serum calcium findings and show an increase in

sulfur and phosphorus. Greenwald himself in a former paper (1911) has already drawn attention to the rise in the phosphates of the blood after parathyroidectomy. The high sulfate concentration is in harmony with the observation of Marine that feeding large amounts of sulfur favors the development of tetany. The other figures are too meager to furnish material for deduction.

2. The Alkaline Reserve of the Blood Plasma.

Morel was probably the first investigator to state that a condition of acidosis followed parathyroidectomy, and to affirm that this was the essential cause of the ensuing pathological phenomena. He based his opinion mostly on the high concentration of ammonia and lactic acid in the blood.

Quite an opposite view was presented in 1915 by Wilson, Stearns, and Thurlow, who reported some interesting experiments performed upon parathyroidectomized dogs in the Hunterian Laboratory of the Johns Hopkins University. Determinations of Barcroft's dissociation constant of oxyhemoglobin in venous blood brought into equilibrium with a constant tension of carbon dioxide and measurements of the carbon dioxide tension in the alveolar air seemed to show an increasing alkaline reserve (so called alkalosis) up to the onset of tetany. This was based on the work of Barcroft, who found that the dissociation of oxyhemoglobin varied with the H ion concentration in samples of normal blood, but he did not show, and it does not necessarily follow, that the same relationship will hold for pathological blood, especially in a condition which has been shown to be associated with a disturbance in the equilibrium of electrolytes. The vitality of the alkalosis theory, however, was strengthened in 1918 when McCann published figures for the combined CO_2 after parathyroidectomy. To be sure, this investigator only removed the parathyroids in two dogs, one of which was subjected to gastric lavage—a procedure which may affect the CO_2 tension in the blood—but, as his results showed such a decided rise in alkaline reserve after operation, we were entirely prepared to obtain much the same values.

In the determinations which are about to be recorded, the blood was usually collected from the external jugular vein into a centrifuge tube containing oxalate crystals, and immediately tested

according to the technique of Van Slyke and Cullen. In a number of instances, however, blood was taken from the external saphenous vein, and these samples gave appreciably lower figures than the specimens obtained from the jugular vein. In one series, in which the blood was collected under oil, the results seemed to be a trifle higher than usual; but as no particular advantage seemed to be associated with this extra precaution it was discontinued.

In the following incomplete preliminary tests blood was taken from the saphenous vein:

Dog A.

	Plasma CO_2 capacity. *vol. per cent*
1 day after operation	33.8
2 days " " and 9 days before onset of tetany	40.4

Dog C.

1 day after operation	46.6
2 days " "	40.4
3 " " "	39.5

In the next two experiments (Table III) blood was collected under oil from the jugular veins unless otherwise stated.

In the experiments given in Table IV, the blood was collected into an open tube from the jugular vein.

The determinations given in Tables III and IV and Fig. 2 indicate that after parathyroidectomy the CO_2-combining capacity of the blood is slightly diminished. This drop is only appreciable, however, for a short period after the operation (the usual postoperative acidosis) and to a lesser degree at the onset of acute tetany. With the exception of a single value noted above, the figures do not present the slightest evidence of an alkalosis; in fact, they represent relatively normal values for venous blood, the collection of which was made at different times with respect to meals and attended at times by more or less struggling on the part of the animal.

An unusual observation was made in Dog E. It was found that after running on the treadmill for an hour or more the combining capacity of the plasma for CO_2 and the CO_2 content were greater than before. This is contrary to the findings in Hastings'

55 experiments with normal dogs in which a decrease in CO_2 capacity was invariably found after exercise. Moreover, in normal dogs no significant changes in the H ion concentration have been

TABLE III.

	Plasma CO_2.	
	Content.	Capacity.
Dog E.		
	vol. per cent	*vol. per cent*
Before operation	66.7	73.3
1 day after operation, apparently well	61.5	76.7
2 days " " restless	60.5	60.6
3 " " " no change	52.5	61.1
4 " " " seems quite normal (after which dog was run on treadmill for 1 hr.)	52.2	64.3
5 days after operation, early signs of tetany	54.8	64.3
7 " " " apparently recovered		72.9
14 " " " thin, but apparently well (after which dog was run on treadmill for 2 hrs.)		73.9
19 days after operation, very cachectic and sick	57.7	61.3
31 " " " very much emaciated, limbs spastic.		
12.15 p.m.		65.3
Gasping respirations, 12.45 p.m.		51.7
9.30 p.m.		65.3
32 days after operation, found dead, 7.30 a.m.		
Dog F.		
Before operation		51.0*
1 day after operation, 4 hrs. before onset of mild tetany		51.0*
2 days after operation, at onset of violent convulsions		38.5*
3 days after operation, quiet	50.5	51.5
4 " " " mild tetany	51.6	51.5
6 " " " fibrillary twitchings	56.7	47.8
7 " " " very sick	61.7	79.7†
8 " " " extreme cachexia	62.9	62.4
9 " " " found dead		

* Blood from saphenous vein.

† No explanation for this wide variation is available.

TABLE IV.

		Plasma CO_2 capacity.
Dog H.		
		vol. per cent
2 days before operation		68.1
1 day " "		64.3
5 hrs. after "	good recovery	51.3
23 " " "	slight rigidity	54.8
29 " " "	seems well	58.6
39 " " "	onset of acute tetany	49.0
3 days " "	resting quietly	54.8
8 " " "	very sick and spastic	43.8
Dog J.		
4 days before operation		52.8
1 day " "		57.9
3 hrs. after "	excellent recovery	48.5
21 " " "	nervous and restless	51.9
27 " " "	restless	53.8
2 days " "	twitches and tremors	49.4
3 " " "	4 hrs. before attack of acute tetany	51.9

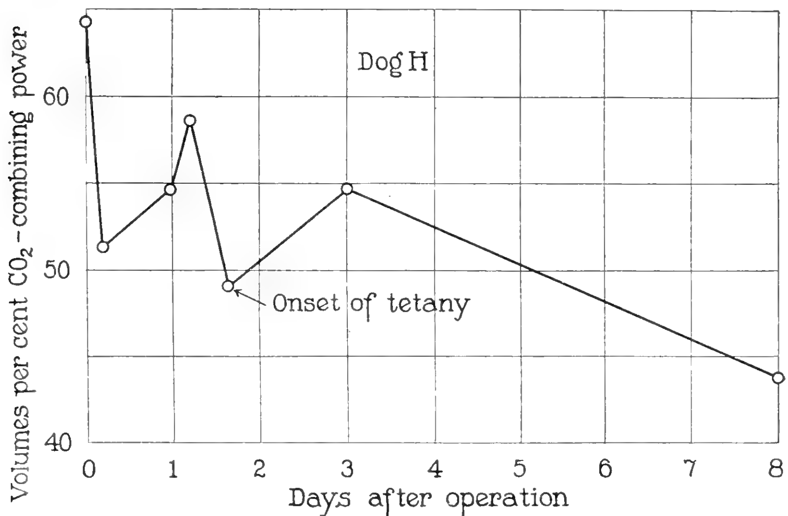

FIG. 2.

detected after exertion, whereas in this animal a marked fall in pH was noted.

Dog E.	CO_2 capacity.	CO_2 content.	pH
	vol. per cent	*vol. per cent*	
Nov. 25, 1919. Before exercise	64.3	52.2	7.61
After "	70.0	74.2	7.52
(Exercise consisted in running on the treadmill for 1 hr.; distance 4.76 miles.)			
Dec. 5. Before exercise	73.9		
After "	76.8		
(Exercise lasted 2 hrs.; distance 10.2 miles.)			

The increase in the content of plasma carbon dioxide and the definite fall in pH after exercise would point, it seems, to some disturbance in the mechanism for eliminating CO_2, but how this might have been brought about in this instance is not clear.

3. The H Ion Concentration of the Plasma.

Wilson estimated the alkalinity of the blood in a few parathyroidectomized dogs by the method of Levy, Rowntree, and Marriott both before and after shaking out the excess of CO_2. No variations in pH were found in blood samples tested immediately after collection, but in those which were shaken, small increases were noticed. This, however, simply confirmed the original high values for the alkaline reserve found by other methods and did not give indications of variations in the pH of the blood in the body. Recent demonstration of the fundamental mechanisms involved in maintaining the acid-base equilibrium had taught us not to look for any appreciable change in the H ion concentration of blood except, possibly, just before death (Henderson). With the idea, however, that there might be slight variations during life and greater changes just before the death of the parathyroidectomized animal, H ion determinations were made by the gas-chain method.

Aside from the following modifications, the technique of these determinations was that employed in most exact H ion measurements made by means of the potentiometer. A Clark hydrogen

TABLE V.

	Reaction.	Temperature.
Dog E.		
	pH	*°C.*
Before operation	7.64	15
1 day after operation, good recovery	7.80	16
2 days " " very lively	7.80	16
3 " " " restless	7.70	16
4 " " " apparently well	7.61	17
(After removing the blood, dog was run for 1 hr. on treadmill.)		
5 days after operation, onset of mild tetany	7.68	20
19 " " " emaciated	7.80	13
31 " " " cachexia, spastic, 12.15 p.m.	7.73	14
Gasping respirations, 12.45 p.m	7.45	14
9.30 p.m	7.56	11
32 days after operation, found dead, 7.30 a.m.		
Dog F.		
Before operation	None taken.	
3 days after operation, 6 hrs. after attack of acute tetany had been cured by $CaCl_2$ injection	7.70	16
4 days after operation, mild tetany	7.76	15
6 " " " fibrillary twitchings	7.59	16
7 " " " apathy	7.57	17
8 " " " extreme cachexia, 12.00 m.	7.65	17
Found dead, 9.00 a.m.		
Dog H.		
Before operation	7.77	12
8 days after operation, spastic but no real convulsions	7.77	13
Dog K.		
Before operation, 9.45 a.m	7.60	20
Operation, little bleeding, 2.30–3.00 p.m		
After operation, good recovery, 4.55 p.m	7.63	20
1 day after operation, twitching, 9.15 a.m	7.61	20
Restless, 2.30 p.m	7.63	20
2 days after operation, spasmodic twitches, 6.00 a.m	7.63	17

TABLE V—*Concluded.*

	Reaction.	Temperature.
Dog L.		
	pH	*°C.*
Before operation, 9.50 a.m	7.70	20
Operation, moderate hemorrhage, 3.10–4.00 p.m.		
After operation, good recovery, 5.00 p.m	7.63	20
1 day after operation, shivering, 9.20 a.m	7.63	20
Twitches, 2.35 p.m	7.64	20
2 days after operation, mild tetany, 6.10 a.m	7.65	17
Onset of acute tetany, 12.45 p.m	7.64	18

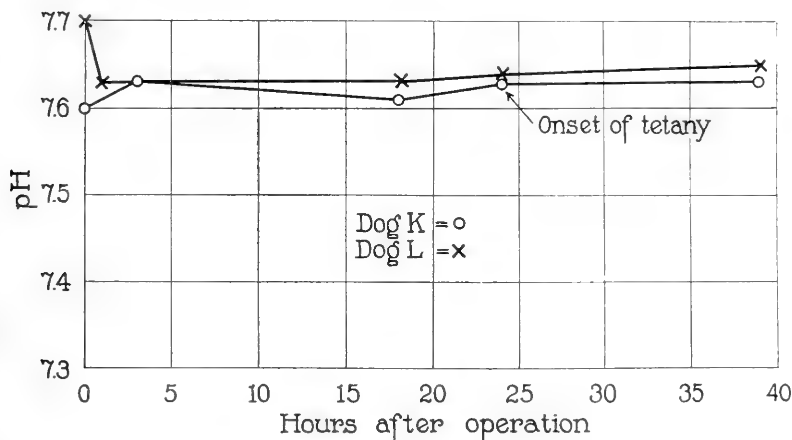

FIG. 3.

electrode was so adapted that the blood plasma, collected, separated, and kept under oil, could be admitted to the gas chamber without exposure to the air. After equilibrium had been reached by shaking, the plasma was replaced by a new sample, and this procedure repeated until no change in the H ion concentration, due to loss of CO_2, occurred. The temperatures at which the H ion determinations were made are given in the last column of Table V. Because of the present disagreement between investigators regarding the magnitude of the change in pH determinations made at 18° and 38°, we have not attempted to report our

values at body temperature. With the provisional acceptance of Michaelis' temperature correction of 0.21, however, it is seen that the average of all our pH determinations is 7.45, a value very close to that regarded as the reaction of normal blood.

It is to be noted that, just as in our estimations of calcium, CO_2, and sugar, the later experiments (*i.e.* in this case Dogs K and L) gave the most uniform results (Fig. 3). We are of the opinion that the increased constancy was mostly due to better technique and, therefore, the latter figures are probably more accurate. If this is true we may infer that the H ion concentration of the blood remains relatively constant after parathyroidectomy.

4. Blood Sugar.

Underhill and Blatherwick have found that blood sugar was low after parathyroidectomy. Their determinations made with the method of Forschbach and Severin showed extraordinary varia-

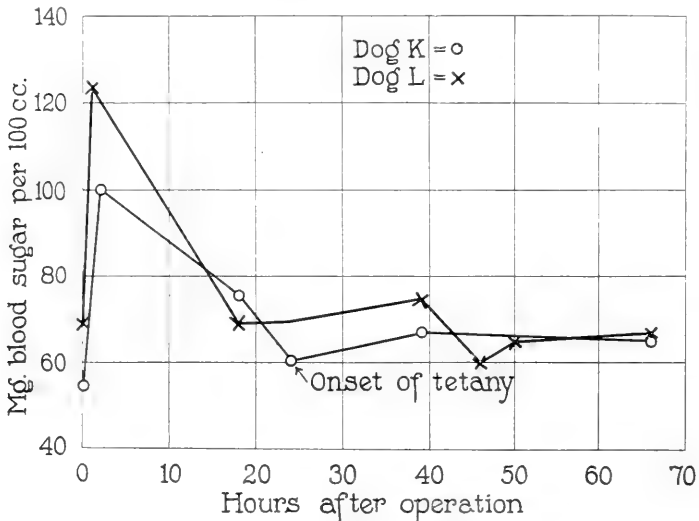

FIG. 4.

tions and were not accompanied by sufficient data to allow for much comment. Watanabe's figures before and after the injection of the tetany-producing methyl guanidine were more uniform,

TABLE VI.

	Glucose per 100 cc. blood.
Dog E.	
	gm.
Before operation, 9.00 a.m.	0.074
1 day after operation, well, 9.00 a.m.	0.095
2 days " " restless, 8.00 a.m.	0.070
3 " " " no symptoms, 8.00 a.m.	0.071
4 " " " " " 9.00 "	0.096
5 " " " onset of mild tetany, 8.00 a.m.	0.079
7 " " " no symptoms	0.087
19 " " " marked emaciation, sick	0.111
31 " " " very sick, last stages, 12.15 p.m.	0.109
Gasping respirations, 12.45 p.m.	0.121
32 days after operation, death.	
Dog F.	
3 days after operation, and 5 hrs. after cessation of acute tetany following $CaCl_2$ administration, 9.00 a.m.	0.135*
4 days after operation, mild chronic tetany, 4.00 a.m.	0.079
6 " " " fibrillary twitchings, 9.00 a.m.	0.083
Dog K.	
Before operation, 9.45 a.m.	0.054
2 hours after operation, 4.55 p.m.	0.100
1 day " " twitches, 9.15 a.m.	0.075
Restless, 2.35 p.m.	0.060
2 days after operation, twitches, 6.00 a.m.	0.067
3 " " " very lively, 9.30 a.m.	0.065
Dog L.	
Before operation, 9.50 a.m.	0.069
1 hr. after operation, 5.00 p.m.	0.123
1 day " " shivering, 9.20 a.m.	0.069
Twitches, 2.35 p. m.	0.069
2 days after operation, mild tetany, 6.10 a.m.	0.075
Onset of acute tetany, 12.45 p.m.	0.060
Acute tetany, 2.15 p.m.	0.065
3 days after operation, mild " 9.40 a.m.	0.067

* This high value may be a sequela of the convulsion, or the result of the calcium injection.

however, and likewise showed a fall. Our few estimations done by the MacLean method,[2] however, do not confirm their results.[3]

The tests recorded in Table VI and Fig. 4 show that, in our series, at least, there was no marked disturbance in sugar metabolism for the first few days after operation. The previously observed postoperative hyperglycemia seems to be the only variation from the normal.

5. Symptomatology.

The phenomena following removal of the parathyroid bodies in dogs have been described many times in the past, and in these experiments there were abundant opportunities to witness and verify the train of events as usually recorded. We could not find in the literature any but the most incomplete and casual mention of certain other less obvious symptoms which may have some bearing on the final solution of the problem and, therefore, will be briefly discussed at this point. In the first series of experiments a number of striking signs were noted which pointed to a widespread stimulation of the parasympathetic nervous system and immediately suggested an explanation for the much discussed but exceedingly obscure function of the parathyroids. These symptoms were as follows: epiphora, followed by purulent conjunctivitis, enophthalmos with narrowed eye-slit, increased watery nasal secretion leading to rhinitis, increased salivation with foaming at the mouth just before tetanic attack, increased labored respirations with expiratory wheeze, respiratory pulse, irregular heart rate characterized (in the last stages) by dropped beats and sinus arythmia, vomiting, diarrhea, frequency of urination and pollakiuria, sexual excitement, and increased coagulation time of the blood. Some of these manifestations have been proved experimentally, whereas others have been supposed on clinical grounds to be of parasympathetic origin. The very characteristic tachypnea, however, did not seem to be the result of a constriction of the bronchial musculature since neither large doses of adrenalin nor atropine had an alleviating effect. The phenomenon might,

[2] The average of about 100 normal determinations of dog blood done by the MacLean technique in this laboratory is 0.069 gm. per 100 cc.

[3] We are indebted to Mr. T. H. Ford for most of the determinations of blood sugar reported in this paper.

however, be the result of increased irritability of the respiratory center. The coagulation tests were made in a small glass tube into which blood was admitted from the bottom through a pet-cock attached to a Luer needle. Blood was collected from the external saphenous vein. The technique has recently been described by King and Murray. The summary of the results is given in Table VII.

Taken as a whole these symptoms represent a rather clear picture of vagotonia, as described by Eppinger and Hess. In the later experiments after more careful observation it became evident that the parasympathetic system was not exclusively affected.

TABLE VII.

	Before operation.		After operation.
	min.		*min.*
Dog A.	11	2 days after operation, 8 days before tetany..	14
Dog B.*	18	No tetany..................................	16
Dog C.	9	2 days after operation, 3 hrs. before tetany.	12
Dog D.	10	1 day " " 1 day " " .	12
Dog E.	10	20 days " " chronic tetany.....	15
Dog H.	11	8 " " " " "	16

* There was no apparent cause for the high figures obtained in this dog.

The evidence of sympathetic excitability was given by the following: dilation of the pupil, the projection of the nictitating membrane across the eyeball, tachycardia, and fever. The following symptoms may be included in this review although their interpretation on the basis of the autonomic system is questionable: sneezing, hiccough, loss of appetite, Cheynes-Stokes respirations, itching of the skin, and weakness. Falta and Kahn, Halsted, and others have recorded some of these signs but little significance has been attributed to them. It is of more than passing interest to note in connection with the conjunctivitis of these dogs with low blood calcium the finding of Chiari and Januschke that calcium chloride injections would prevent the inflammation of the eyes produced by oil of mustard.

We believe that the most likely explanation of these abnormal physiological events is that there exists after parathyroidectomy

an increased autonomic excitability which parallels similar changes in the voluntary nervous system and that this state involves both the parasympathetic and sympathetic divisions, but more particularly the former. This conclusion agrees, in the main, with that of Falta and Kahn, and Hoskins and Wheelon.

RESULTS.

After thyreoparathyroidectomy in a series of dogs, the findings were as follows:

1. The pH of the plasma remained within normal limits.
2. The carbon dioxide-combining capacity of the blood was slightly diminished. This fall was appreciable, however, for only a brief period immediately after operation, and during the hyperpnea at the onset of tetany. No other changes were regularly noted in the alkaline reserve.
3. The calcium content of the serum decreased in a few days from a normal value of about 11 mg. per 100 cc. to about 5 mg., a concentration which was hardly diminished subsequently. When the serum calcium reached a concentration of approximately 7 mg. tetany developed.
4. Two analyses by Greenwald showed that phosphorus and sulfur were increased in the blood.
5. Sugar analyses demonstrated the previously recorded postoperative hyperglycemia, but showed no other significant changes in glucose metabolism during the first few days following operation.
6. Certain of the less obvious symptoms were noted. They seemed to be manifestations of an increased irritability of both the sympathetic and parasympathetic systems.

Since these experiments were finished, there have appeared in the literature two papers confirming the above results for the CO_2-combining power of the blood; a short series of tests included in an article by MacCallum and his coworkers (1920) on gastric tetany, and a more complete series of Van Slyke CO_2 determinations made by Togawa. The latter got slightly lower figures than ours, and believed they were sufficiently abnormal to entitle the condition to consideration as an acidosis. In looking over the more difficultly obtainable literature, we also found that another investigator (Segale) had measured the pH of serum after parathyroidectomy and had likewise found it unaltered. We have discovered no confirmation for our sugar values.

DISCUSSION.

The results of our experiments have not led us to any clear conclusion as to the function of the parathyroid glands or the fundamental disturbances which follow their removal. The decrease in the calcium content of the blood is one very definite and important change which occurs, the significance of which rests upon the fundamental work of Loeb and others who showed that the maintenance of the normal irritability of muscle and nerve is dependent upon an undisturbed equilibrium between various electrolytes in the surrounding medium, and that certain divalent cations were antagonistic to monovalent cations in their influence upon nerve excitability. More specifically, it was found that nerves bathed in a solution of NaCl became, after a certain latent period, chemically stimulated. If, however, a definite concentration of $CaCl_2$ was added to the solution, no such effect was obtained. Loeb (1899–1900) regarded the Na and Ca ions as antagonistic and the solution which contained them both in proper proportions as a physiologically balanced solution. Führer, attracted by Loeb's work, suggested in 1908 the possibility of an increase in the ratio $\frac{\text{monovalent ions}}{\text{divalent ions}}$ as the cause of tetany. Later Loeb and Ewald considered the chemical stimulation of nerves in more detail, stressed the greater importance of the cations as compared with the anions, and discussed the possible relationships between stimulation and rate of diffusion. Loeb (1915) found that to preserve normal irritability, the ratio of $\frac{[Na] + [K]}{[Ca] + [Mg]}$ must be maintained fairly constant and the amount of Ca required to neutralize Na would vary in direct proportion to the concentration of the latter. That the cations are not alone concerned has been shown by Mathews, Lillie, Loeb, and others. Mathews listed a number of sodium salts according to their power to stimulate nerves; as the cation was the same in each case the differences observed were apparently due to the anions. The list was very similar to one made by Loeb a few years before. Finally, Raber has recently reported the results of researches on the effect of anions on the conductivity of living protoplasm. All these experiments seem to show the same thing; namely, that

anions have a definite effect (although to a less degree than the cations) on the chemical stimulation of nerves. The monovalent anions tend to act in the same fashion as the divalent cations, *i.e.* they have an inhibiting effect; whereas the salts with divalent anions are relatively more irritating. If these results may be applied to human problems one would expect to find that the injection of a salt with monovalent cations and divalent anions would provide a maximal irritating effect, whereas a salt with a divalent cation and monovalent anion would be inhibitive.

In 1898 Münzer produced tetany by injecting various sodium salts. According to the theory, any sodium salt, if injected in sufficient quantity, should increase nerve irritability, because cations are more important than anions, and adding sodium would, of course, serve to increase the ratio $\frac{\text{monovalent cations}}{\text{divalent cations}}$. He found that sodium bicarbonate and sodium phosphate brought on convulsions sooner than sodium chloride, which also agrees with the theory since the injection of the former would tend to increase the ratio $\frac{\text{divalent anions}}{\text{monovalent anions}}$. Binger induced convulsions in dogs by injecting sodium phosphate solutions of various H ion concentrations. He found that tetany was more readily obtained with Na_2HPO_4 than with NaH_2PO_4 solutions. He attributed the potency of these mixtures to the phosphate ion, which was doubtless in some measure correct. But the sodium ion is probably the more important of the two and the greater toxicity of the disodium mixture would be explained on this basis. A case of transient tetany recently occurred at the Presbyterian Hospital, New York, after sodium bicarbonate administration, another case was reported by Harrop from the wards of the Johns Hopkins Hospital, and other similar instances have been recorded elsewhere. There is a case on record of tetanoid seizures following a rectal infusion of large amounts of saline (Campbell).

All available data, however, do not agree with the theory. Greenwald, for instance, found that in his hands tetany was produced with more facility by sodium chloride than by sodium phosphate solutions. However, other complicating factors such as osmotic phenomena and the rate of excretion of the various

ions by the kidney might account for some of the apparently discordant results. In the light of our carbon dioxide determinations it is hard to understand the beneficial results obtained by Wilson and coworkers when HCl was injected intravenously. It may be that the explanation lies in the fact that such an injection besides adding monovalent anions greatly alters the ratio between other important ions in the blood, the maintenance of which seems to be important. Much conflicting evidence has been presented in regard to various therapeutic measures in tetany which it is difficult to explain. The attacks of tetany following parathyroidectomy vary both in severity and extent. It is difficult to pass judgment on the many temporary cures for the tetanic seizures which have been reported, as the attacks are usually self-limited, often ending abruptly without treatment. After a few days, the animal lapses into a torpor which is not characterized, as a rule, by typical convulsions, but rather by a continuous spasticity with intermittent twitchings and fibrillary movements of such mildness in comparison to the terrible seizures of the previous period that the unwary may easily ascribe the apparent improvement to medication. The dogs in the present series of experiments were kept in the room where the chemical tests are made and were watched day and night for at least the first 5 days following operation.

The theoretical considerations outlined above seem to be useful in explaining the effect on nerve irritability of various salt solutions when injected intravenously. They also appear to harmonize in a rough way with the findings in tetany and to account for the therapeutic effect of certain solutions. It is not maintained, however, that the disordered salt equilibrium (*i.e.* decrease in calcium, increase in phosphate, etc.) is the underlying disturbance after parathyroidectomy. In fact, many signs seem to point to a fundamental disturbance in protein metabolism, even though evidence in favor of the accumulation of any one toxic protein decomposition product in the blood is as yet incomplete. An observation made in these experiments which suggests a relationship between muscular exercise and tetany may have some bearing on this phase of the question. On two separate occasions attacks of tetany were brought on in a parathyroidectomized dog, which, at the time, gave no evidence of hyperexcitability and, in fact,

seemed on the road to recovery by running 1 to 2 hours on a treadmill.

Further work on this subject should be devoted, we believe, to studying the disordered protein metabolism and the connection between protein metabolites and salt equilibrium. In this field, Watanabe has recently published some interesting experiments, among which are those showing a decrease in the concentration of calcium in the blood after guanidine injections.

CONCLUSION.

The effects of parathyroidectomy upon the calcium, sugar, combined carbon dioxide, and H ion concentrations of the blood have been studied.

The previously observed calcium deficiency in parathyroidectomized dogs is verified, but no support is found for theories based on a disturbed acid-base equilibrium.

General theoretical considerations are outlined which may be of some value in explaining the relationship between tetany and the relative concentration of certain ions in the blood.

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THE VARIATION AND THE STATISTICAL CONSTANTS OF BASAL METABOLISM IN MEN.

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I. INTRODUCTION.

Physiologists now generally accept the so called basal metabolism of the individual as a standard in all comparisons involving the consideration of energy transformation. Furthermore, the technical conditions under which measurements of the basal metabolism shall be made are now generally agreed upon. The opinion has even been widely maintained that heat production per square meter of body surface area is a constant.

A detailed review and discussion of the literature and the results of a biometric analysis of a series of basal metabolism data for 136 men and 103 women have been given elsewhere.[1] The results of this analysis show the following coefficients of variation for the averages[2] of the daily basal metabolism constants for the individual subjects.

	Men.	Women.
Total calories per 24 hrs.	12.54	11.50
Calories per kg. per 24 hrs.	9.36	14.14
Calories per square meter per 24 hrs.	8.05	9.17

[1] Harris, J. A., and Benedict, F. G., *Carnegie Inst. of Washington, Pub. 279*, 1919, 129-200.

[2] These are averages of daily means in all cases in which measurements could be made on more than 1 day. For 35 subjects a single daily mean only was available.

In practise the catabolism of a subject is almost invariably measured in two or more short periods on a given day in a respiration apparatus. Technically these are generally designated as *minimum periods*, since the lowest values are generally found in the absence of muscular activity. The term is somewhat misleading when averages of two or more periods are taken,

These results indicate a rather wide variability in basal metabolism from individual to individual, even when heat production is corrected for body size by expressing it in calories per kilo of body weight or in calories per square meter of body surface area.[3]

The problem now arises whether, under conditions such that the age factor[4] is practically eliminated, the basal metabolism of the individual is essentially constant from day to day or whether it shows sensible variations. In 1915 one of us[5] discussed the question of changes in the metabolism of the individual from day to day, and showed that, contrary to the early assumption that the basal metabolism of an individual remains essentially constant from day to day, it is really variable.[6]

since there can be only one term minimum. The calories produced or the volume of the gaseous exchange for any one of these periods which seems to those acquainted with the technical details of the experiment reasonably free from experimental error, might serve as a measure of the metabolism. Since, however, the basal metabolism of the individual can probably be assumed to be sensibly constant for 1 day, it seems wisest to regard the individual periods which appear the most free from possible experimental error as of the nature of duplicate, triplicate, etc. analyses, and to average these values. This daily mean has served as the unit in work at the Nutrition Laboratory.

[3] The frequency distributions of total calories and calories per square meter are shown in Figs. 1 and 2 of Harris and Benedict (Harris, J. A., and Benedict, F. G., *Scient. Month.*, 1919, viii, 388–389).

[4] The results of a previous study of the relationship between age and metabolism (Harris and Benedict,[1] pp. 107–127) have shown that there is a gradual and practically linear decrease in metabolism with age during the period of adult life. The annual rate of decrease amounts to about 7.15 calories (per 24 hrs.) per year in men and 2.29 calories (per 24 hrs.) per year in women. Correction for body size by expressing the results in calories per kilo of weight gives a rate of decrease of 0.112 calorie in men and 0.124 calorie in women. If the results are expressed in calories per square meter of body surface area as estimated by the Du Bois height-weight chart, the decrease for men is 3.69 calories and for women 2.96 calories per 24 hrs. of life. The change due to age is not, therefore, large and cannot be assumed to be an important factor for subjects observed during a short period of time only.

[5] Benedict, F. G., *J. Biol. Chem.*, 1915, xx, 290–295.

[6] This conclusion was based on a study of the difference between the highest and the lowest daily basal metabolism constant expressed as a percentage of the minimum value as a base. The study showed that the differences varied greatly from subject to subject. In one case the maximum and minimum oxygen consumption varied as widely as 31.3 per cent

The purpose of the present paper is threefold:

1. To obtain some measure of the variability of the basal metabolism of the normal individual.

2. To consider the relationship between the length of time over which the observations extend and the variation in the metabolism of the individual.

3. To consider the most suitable method for determining the population mean from measurements on a series of individuals.

II. PRESENTATION OF RESULTS.

1. Variation of Metabolism in the Individual.

For a certain number of men investigated at the Nutrition Laboratory the number of days on which measurements were made is sufficiently large (20 or more) to justify the calculation of statistical constants for the individual subjects.

These appear in Tables I to III.

The constants indicate that individuals are differentiated among themselves with respect to basal metabolism even when expressed in calories per kilo of body weight or calories per square meter of body surface area as estimated by the Du Bois height-weight chart.[7]

Furthermore it is clear that the metabolism of each individual subject is to a considerable degree variable. This is shown by the rather wide range between the maximum and minimum daily metabolism for each individual as shown in these tables. These ranges are expressed as percentages of the minimum value found in the tables. It is also evident in the absolute variabilities as

of the minimum value. In another case it varied only 3.5 per cent. While it was pointed out that it was hardly correct to obtain an average value for the oxygen consumption for individuals with such wide differences in the time covered by the observations, an average value was determined in the absence of any better available method, and found to be 13.9 per cent.

[7] Since a discussion of the differentiation of individuals with respect to basal metabolism is not a primary purpose of this paper the subject is not pursued farther. The statement above may be verified by taking differences between the various constants and comparing them with their probable errors. It is to be noted that these constants are uncorrected for age, and that the age differences will, in general, tend to increase slightly the differentiation of the subjects.

given in terms of the standard deviations (S. D.). Expressing the total amount of variation as measured in terms of the standard deviation as a percentage of the means we have the relative variabilities expressed as coefficients of variation $\left(\text{C.V.} = \frac{100\,\text{S.D.}}{\text{Mean}}\right)$.

TABLE I.

Statistical Constants for Basal Metabolism in Eleven Men.

No. and individual.	Days.	Total calories per 24 hrs.					
		Minimum.	Maximum.	Percentage range.	Mean.	Standard deviation.	Coefficient of variation.
47. F. P. R....	20	1,446	1,684	16.5	1,540.0±11.2	74.6±8.0	4.8
45. K. H. A....	25	1,505	1,765	17.3	1,648.3± 9.2	68.2±6.5	4.1
96. A. J. O....	25	1,679	1,804	7.4	1,741.5± 4.5	33.5±3.2	1.9
41. C. B. S....	26	1,592	1,785	12.1	1,699.2± 6.5	49.0±4.6	2.9
61. J. K. M....	27	1,458	1,650	13.2	1,546.6± 6.6	51.2±4.7	3.3
54. H. H. A....	28	1,327	1,686	27.1	1,488.3±10.3	80.7±7.3	5.4
66. L. E. E....	31	1,596	1,848	15.8	1,705.6± 7.9	64.9±5.6	3.8
59. H. L. H...	35	1,548	1,890	22.1	1,694.4± 9.2	80.4±6.5	4.7
70. H. F. T....	41	1,205	1,514	25.6	1,350.4± 7.7	73.1±5.4	5.4
9. M. A. M...	53	1,562	1,917	22.7	1,696.0± 6.9	74.7±4.9	4.4
48. J. J. C.....	53	1,511	1,740	15.2	1,583.8± 4.4	47.3±3.1	3.0

TABLE II.

Statistical Constants for Basal Metabolism in Eleven Men.

No. and individual.	Days.	Calories per kg.					
		Minimum.	Maximum.	Percentage range.	Mean.	Standard deviation.	Coefficient of variation.
47. F. P. R....	20	22.1	25.7	16.3	23.65±0.16	1.08±0.12	4.57
45. K. H. A....	25	22.8	27.1	18.4	24.84±0.15	1.10±0.10	4.42
96. A. J. O....	25	23.3	26.6	14.2	25.13±0.11	0.81±0.08	3.24
41. C. B. S....	26	21.9	25.4	16.0	23.92±0.11	0.81±0.08	3.40
61. J. K. M....	27	24.5	27.4	11.8	25.63±0.09	0.70±0.06	2.75
54. H. H. A....	28	22.2	26.9	21.2	23.89±0.14	1.10±0.10	4.60
66. L. E. E....	31	26.8	31.5	17.5	28.47±0.14	1.15±0.10	4.03
59. H. L. H....	35	25.8	31.5	22.1	28.05±0.16	1.39±0.11	4.95
70. H. F. T....	41	21.1	26.0	23.2	23.35±0.12	1.18±0.09	5.06
9. M. A. M...	53	23.8	28.5	19.7	25.72±0.10	1.04±0.07	4.06
48. J. J. C.....	53	22.6	26.7	18.1	24.39±0.07	0.81±0.05	3.32

We note that the coefficients for total calories range from 1.9 to 5.4 per cent with a general average of 3.97. Those for calories per kilo of body weight range from 2.8 to 5.1 per cent with a general average of 4.40, and those for calories per square meter of body surface from 2.3 to 5.3 per cent with a general average of 3.95.

The suggestion will naturally arise that the variation in basal metabolism in these longer periods is due merely to the uniform decline in metabolic activity characteristic of adult life.

TABLE III.

Statistical Constants for Basal Metabolism in Eleven Men.

No. and individual.	Days.	Calories per square meter.					
		Minimum.	Maximum.	Percentage range.	Mean.	Standard deviation.	Coefficient of variation.
47. F. P. R....	20	808	936	15.8	864.0±6.1	40.8±4.3	4.7
45. K. H. A....	25	814	954	17.2	885.6±5.0	36.9±3.5	4.2
96. A. J. O....	25	879	970	10.4	927.2±2.9	21.5±2.0	2.3
41. C. B. S....	26	834	947	13.5	898.2±3.6	27.6±2.6	3.1
61. J. K. M....	27	851	948	11.4	897.4±3.5	26.7±2.5	3.0
54. H. H. A....	28	804	998	24.1	884.3±5.6	43.9±4.0	5.0
66. L. E. E....	31	917	1,074	17.1	980.5±4.7	38.6±3.3	3.9
59. H. L. H....	35	905	1,105	22.1	986.5±5.4	46.9±3.8	4.8
70. H. F. T....	41	700	870	24.3	779.2±4.3	40.9±3.0	5.3
9. M. A. M...	53	863	1,042	20.7	935.2±3.6	39.0±2.6	4.2
48. J. J. C.....	53	840	956	13.8	883.8±2.4	26.3±1.7	3.0

Evidence that this is not the case will be adduced in the following section in which it will be shown that for periods of not more than 10 or 15 days the magnitude of the variation in metabolism is positively correlated with the duration of the period over which the observations extended. We have, furthermore, applied a correction for age[8] to the measurements of three of the individuals with results as given in Table IV. The standard deviations and the coefficients of variation are practically the same after correction for age as for the original observations. The result shows clearly that age is not a primary factor underlying the variations.

[8] Calculations from unpublished data.

TABLE IV.

Comparison of Basal Metabolism Constants Corrected and Uncorrected for Age.

Subject and range.	Constant uncorrected for age.	Constant corrected for age.	Difference in constants.
88. T. M. C., range = 1,624.			
Standard deviation	54.04	53.27	−0.77
Coefficient of variation	4.18	4.12	−0.06
59. H. L. H., range = 905.			
Standard deviation	80.39	82.07	+1.68
Coefficient of variation	4.74	4.84	+0.10
48. J. J. C., range = 819.			
Standard deviation	47.27	47.15	−0.12
Coefficient of variation	2.98	2.98	±0.00

2. The Time Factor in the Variation of the Metabolism of the Individual.

In the preceding section we dealt with the problem of the variability within the individual on the basis of data for a few men upon whom more extensive series of measurements had been made. By the application of other methods it is possible to push the analysis somewhat farther.

While the ultimate purpose of studies of variation in the metabolism constants of the same individual should be to determine something of the proximate causes underlying these variations, it is worth while to obtain some general idea of the amount of variation which may be expected to occur in the individual subject with a lapse of time.[9]

[9] In the first discussion of this subject (Benedict,[5] p. 292) the simple method of range of variation in metabolism led to the following conclusion: "A general inspection of the data will show that, as a rule, the greatest variations were found with the subjects studied over the longest periods. While it is hardly correct to obtain an average value for the oxygen consumption for so many different individuals with such wide differences in the time covered by the experiments, yet such a value has been found and shows that on the basis of these observations there may be an average variation of 13.9 per cent in the basal metabolism, when measured in the post-absorptive condition and with complete muscular repose, during a period of two years or, in the majority of cases, considerably less. With no attempt to analyze the causes of these differences, it is sufficient here simply to call attention to their magnitude."

If the metabolism of the individual changes from time to time irrespective of changes in bodily dimensions it would seem reasonable to presume that these changes would be greater for more widely separated periods. Thus the observations made on a single day can be reasonably regarded as based upon a subject in practically stationary physical and physiological conditions. Those made at widely separated dates more probably represent the individual in somewhat different physiological states. Thus while the active protoplasmic mass is probably essentially identical in the two cases (if the periods are not too widely separated) the unknown stimulus to metabolic activity may differ to a considerable extent from one period to the other.

As a measure of variation of the metabolism of the individual we have adopted the standard deviation[10] of the measurements of each subject. As a measure of time covered by the observation we have taken the actual number of days, including the days upon which the measurements were made, *i.e.* $(T_2 + 1) - T_1$, where T_1 and T_2 are the times of the first and last measurements. Thus if the metabolism of an individual were measured on July 1 and 2 the range would be 2 days. If three observations were made, one on July 1, one on July 10, and one on September 3 of the same year, the range would be 65 days. Correlating between the range in days and the standard deviation of total calories per 24 hours we have for 101 individuals

$$r = +0.276 \pm 0.062, \quad \frac{r}{E_r} = 4.45$$

The coefficient measuring the relationship between range in days and the standard deviation of calories per square meter as estimated by the Du Bois height-weight chart, which will be the only approximation to the body surface used, is

$$r = +0.254 \pm 0.063, \quad \frac{r}{E_r} = 4.03$$

The correction between the range of days over which the experiments extended and the standard deviation of calories per kilo of body weight is

$$r = +0.248 \pm 0.063, \quad \frac{r}{E_r} = 3.94$$

[10] The coefficient of variation might have been used with equal propriety.

These coefficients are not large, but since all are about four times as large as their probable errors they are apparently statistically significant. They indicate that the basal metabolism, whether measured in total calories produced or corrected for the very slight variations in body size by reduction to calories per square meter of body surface area or calories per kilo of body weight, is not constant but variable for the individual subject and that the magnitude of these variations is dependent upon the magnitude of the period of time over which the observation extended.[11]

There are, however, certain statistical difficulties in interpreting these coefficients of correlation. Unfortunately, for the purposes of the present investigation, the duration of the periods over which the observations extended varied enormously from individual to individual, ranging from 2 to 1,624 days. The mean range is 128.0 while the standard deviation of range is 237.5 days. These give a coefficient of variation of 185.5 per cent. In the very long periods there is a possibility that the standard deviation of metabolism constants may be influenced by age changes, but no evidence of material influence of the age factor has been found in these men to whose empirical measurements a reasonable age correction was applied.

Again, many of these subjects were examined on 2 or 3 days only. Standard deviations based on such small numbers give only a very inadequate picture of the variability which might be expected if a larger number of measurements could have been made.

Furthermore it seems unlikely that the standard deviation of basal metabolism will steadily increase with the duration of the period of observation.[12] This *a priori* assumption is based on both statistical and biological grounds. From the physiological side it would seem probable that relatively short periods of time would be sufficient to bring out the minor variations in the stimulus to metabolic activity. If this is true we should expect the cor-

[11] The coefficient for total calories is slightly but not significantly larger than the other values. This is probably due to the slight changes in body weight during the course of the work. In adults such changes will be largely due to fat or water which cannot greatly influence the metabolism constant.

[12] The slight increase due to the change in metabolism with age is disregarded.

relation constants to remain the same or even to be increased by rendering our materials more homogeneous by the elimination of the subjects with extremely long periods of observation.

The difficulty in interpreting the above coefficients has been to some extent obviated in the following manner.

We have split the data up into groups according to the range of days over which the observations extended, and have determined separately the correlation between the range of days and the standard deviation of daily (24 hours) heat production in calories per kilo for groups of increasing range of time. For example, there are nineteen subjects with a range of 2, 3, 4, or 5 days, thirty-two subjects with a range of 2–10 days, etc.

The coefficients appear in Table V, and are represented graphically in Fig. 1. While they differ considerably among themselves they are without exception positive in sign. They are, furthermore, practically without exception more than 2.5 times as large as their probable errors and would, therefore, be generally considered statistically significant.

The reader will note that the first coefficient—that based on the nineteen individuals with a range of 2–5 days—is conspicuously higher than either of the other twenty coefficients. Furthermore, the nineteen coefficients based on periods ranging from 2–15 to 2–1,624 days are all lower than that based on 2–10 days.[13]

The question will naturally arise whether the magnitude of the coefficients for the wider ranges of time is not primarily due to the correlation existing in the groups with shorter range of time.

To answer this question we have determined the correlations for groups of individuals falling in the classes with longer time intervals.

Because of the inherent difficulties the number of individuals that could be studied over the longer periods is small. The constants for seven groups appear in Table VI.

The correlation coefficients in this table are very small indeed. No one is as large as its probable error. Nevertheless, the three coefficients for the wider range of time (121–1,624, 85–1,624, and

[13] We have no explanation to offer for the abnormally low values in the 2–30 day and 2–50 day periods. The question is hardly susceptible of further investigation on series no larger than that available for present purposes.

TABLE V.

Correlation between Duration of Period of Observation and the Standard Deviation of Basal Metabolism in Calories per Kg. per 24 Hrs.

Range.	No. of subjects.	Correlation.		Mean standard deviation for whole range.	Mean standard deviation for individuals added.
		$r \pm E_r$	$\frac{r}{E_r}$		
days					
2–5	19	+0.509±0.115	4.43	0.463	
2–10	32	+0.398±0.100	3.98	0.544	0.662
2–15	37	+0.297±0.101	2.94	0.555	0.626
2–20	42	+0.343±0.092	3.73	0.591	0.856
2–30	49	+0.232±0.091	2.55	0.599	0.646
2–50	59	+0.139±0.086	1.62	0.605	0.638
2–125	73	+0.266±0.073	3.64	0.648	0.829
2–160	81	+0.344±0.066	5.21	0.679	0.963
2–240	86	+0.363±0.063	5.76	0.697	0.977
2–360	90	+0.370±0.061	6.07	0.709	0.979
2–370	91	+0.327±0.063	5.19	0.708	0.585
2–380	92	+0.337±0.062	5.44	0.712	1.059
2–415	93	+0.349±0.061	5.72	0.716	1.099
2–425	94	+0.334±0.062	5.39	0.717	0.813
2–430	95	+0.314±0.062	5.06	0.717	0.704
2–600	96	+0.280±0.063	4.44	0.717	0.700
2–775	97	+0.297±0.063	4.71	0.721	1.147
2–800	98	+0.245±0.064	3.83	0.720	0.572
2–820	99	+0.231±0.064	3.61	0.720	0.809
2–905	100	+0.280±0.062	4.52	0.727	1.390
2–1,624	101	+0.248±0.063	3.94	0.729	0.918

TABLE VI.

Correlation between Duration of Period of Observation and the Standard Deviation of Basal Metabolism in Calories per Kg. per 24 Hrs.

Range.	No. of subjects.	Correlation.	
		$r \pm E_r$.	$\frac{r}{E_r}$
days			
332–1,624	12	+0.116±0.192	0.60
236–1,624	16	+0.018±0.169	0.11
159–1,624	21	−0.067±0.147	0.46
131–1,624	28	−0.014±0.127	0.11
121–1,624	31	+0.007±0.121	0.06
85–1,624	37	+0.020±0.111	0.18
51–1,624	42	+0.062±0.104	0.60

51–1,624 days) are positive in sign, as are two of the other four coefficients. There is, therefore, a suggestion of positive correlation in the longer time groups. It is clear from the magnitude of these correlation coefficients that, as suggested above, the standard deviations do not steadily increase with the lapse of

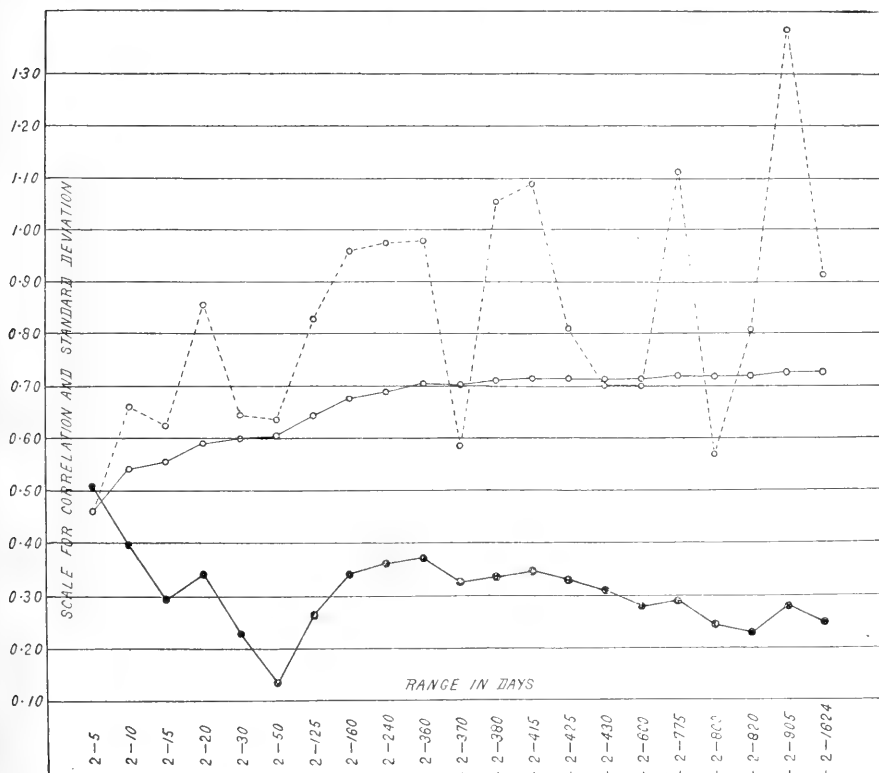

Fig. 1. Abscissæ represent maximum range in days; ordinates show standard deviations of daily means in the individual or correlations between range of time covered by observations and standard deviation of daily metabolism. Solid dots and line represent correlation coefficients; circles and solid line represent the mean standard deviations for the individuals of each group for which the correlations were computed; circles and broken line represent the mean standard deviation of the individuals added to each preceding group to form the group upon which the correlation coefficient is based. Because of the great variation in the length of the periods no attempt has been made to represent abscissæ on a uniform scale.

time. This may be shown to be the case by determining the average values of the standard deviations of the metabolism of the groups of individuals for whom the correlations in Table V were calculated. These means have been calculated for the groups of 19 to 101 subjects with the range indicated in Table V and for the individuals added to each group to give the next wider range group. These mean standard deviations are shown in the last two columns of Table V. The first series is represented in Fig. 1 by the circles connected by continuous lines while the second is shown by the circles connected by broken lines. The wide variability of the latter averages is attributable to the very small number of individuals added in the longer range periods.[14] Both series of means show that the standard deviation at first increases rapidly as the length of the period becomes longer but the rate of increase becomes smaller as the periods over which the observations extend increase in length.

The last paragraph on page 264, written as it stands before the calculation of the constants in this table, is therefore fully substantiated by the available data for individuals who have been studied over longer periods of time.

3. The Statistical Constants for Basal Metabolism in Man.

A primary requisite for continual progress in the investigation of human metabolism under various special conditions, *e.g.* of exercise, rationing, or disease, is the determination of some standard which may serve as a basis of comparison.[15] Such a constant, based upon a considerable series of individuals and designed to serve as a standard of comparison, may be conveniently designated as a *population constant.*

The average value of the basal metabolism constant has generally served this purpose, whether expressed in actual calories per unit of time uncorrected for the influence of body mass, in calories per kilo of body weight, or in calories per square meter

[14] In the twelve groups in which the range is wider than 2–360 days the positions of the circles represent the standard deviations for single individuals only, not means.

[15] The general principles underlying the establishment of control series have been discussed elsewhere (Harris and Benedict,[1] pp. 223–227; also, Harris and Benedict,[3] p. 385).

of body surface area as estimated by some one of the various formulas which have been proposed for this purpose.

When observations for a number of days are available, and when it is desirable to define the basal metabolism of the individual more precisely than can be done as the result of experimentation for 1 day, the question naturally arises whether the *average* of the *daily means* or the *minimum daily mean* shall be used to represent the basal metabolism of the individual. The exact method of calculating the average and the standard deviation therefore requires consideration.[16]

In a preceding publication[1] we used the average of the daily means for the determination of the population constant. Our protocols of data[17] show these values and indicate the number of days and the total number of periods upon which they were based.

One of our helpfully critical correspondents has suggested that it may be quite improper to lump together and treat as of equal value basal metabolism constants for individual subjects some of which rest upon an observation for only a single day while others depend upon many days measurements.

This criticism has probably also occurred to others. It seems desirable, therefore, to consider the problem of the best method of deducing a constant for a series of individuals—a population constant—from the experimental readings.

There are seven possible ways of determining the statistical constants of a series of metabolism measurements some of which rest upon work for but a single day while others depend upon several days observation.

A. The average of the daily averages may serve as the units representing the individuals.

If the measured metabolism is variable it seems illogical, as our correspondent suggests, to treat the constant obtained from an observation for a single day as equal in value to that deduced from a number of days observation. The selected constant may, therefore, be (1) used only once in the calculation of the statistical constant for the population; (2) may be weighted with the num-

[16] While we have elsewhere proposed the use of multiple prediction equations for calculating the basal energy requirements, the consideration of the most suitable method for the determination of the statistical constants to represent the individual is pertinent, since the calculation of these equations involves the determination of the means and standard deviations.

[17] Harris and Benedict,[1] Tables A to D.

ber of days observation; or, (3) weighted with the square root of the number of days observation.

B. The minimum value of the daily averages, *i.e.* the constant for the single day giving the lowest average, may be used.

This method has for its justification a physiological consideration. By definition the basal metabolism is the catabolism in the absence of muscular activity and the stimulatory influence of recently ingested food. Since these are the most potent factors in determining the superbasal metabolism of the individual, the basal metabolism is, practically speaking, synonymous with the minimum metabolism. It is possible, therefore, to consider that the absolute minimum for any individual should be taken as the true basal value. If the mean of all the daily averages of measurements made upon an individual is used as a measure of the basal metabolism a value somewhat higher than the absolute minimum is obtained and we admit that the individual may fall below his own basal value. Against this method is to be urged the criticism that the lowest value may be really subbasal because of errors of measurement. This criticism is in large part met by the concordance of the results for the two or more periods upon which the daily averages are almost invariably based. These minimum values may, like the individual means, be (4) used only once in the calculation of the statistical constant for the population; (5) may be weighted with the number of days observation; or (6) weighted with the square root of the number of days observation.

C. (7) The constants may be computed directly from the whole series of daily means available.

The first group (1 to 3) may be conveniently designated as the *method of individual means*, the second group as the *method of individual minima*, and the third group as the *method of daily means*.

Table VII gives the statistical constants for the daily (24 hours) heat production of the 136 men[18] for whom data (individual means) are given in the protocols of our former publication.[19]

[18] The 103 women considered in our volume were not studied over a period sufficiently long to make it worth while to calculate weighted constants comparable with those for men.

[19] In the full revision of the data for the 863 individual periods a few minor inaccuracies, of no practical importance for the purposes of our earlier volume, were found in the fundamental protocols. The unweighted constants have, therefore, been recalculated for the purposes of this paper.

TABLE VII.

Statistical Constants for Basal Metabolism as Determined from the Means of the Daily Means for Each of 136 Men.

	Total calories per 24 hrs.			Calories per kg.			Calories per square meter.		
	Mean.	Standard deviation.	Coefficient of variation.	Mean.	Standard deviation.	Coefficient of variation.	Mean.	Standard deviation.	Coefficient of variation.
1. Unweighted*	1,630.98±11.86	205.01± 8.38	12.57	25.691±0.139	2.402±0.098	9.35	925.147±3.877	67.038±2.742	7.25
2. Weighted with the square root of the no. of days	1,617.44±10.78	186.43± 7.62	11.53	25.766±0.128	2.219±0.091	8.61	921.715±3.753	64.891±2.654	7.04
Difference (2)-(1)	−13.54±16.03	−18.58±11.33	−1.04	+0.075±0.189	−0.183±0.134	−0.74	−3.432±5.396	−2.147±3.816	−0.21
Diff./$E_{diff.}$	0.84	1.64		0.39	1.37		0.64	0.56	
Percentage difference	0.83	9.49		0.29	7.94		0.37	3.25	
3. Weighted with the no. of days	1,602.60± 9.61	166.22± 6.80	10.37	25.779±0.117	2.030±0.083	7.87	916.272±3.655	63.190±2.584	6.90
Difference (3)-(1)	−28.38±15.26	−38.79±10.79	−2.20	+0.088±0.182	−0.372±0.128	−1.48	−8.875±5.328	−3.848±3.768	−0.35
Diff./$E_{diff.}$	1.86	3.60		0.48	2.91		1.67	1.02	
Percentage difference	1.76	20.90		0.34	16.81		0.96	5.91	
4. Based on daily observation	1,602.57±10.16	175.61± 7.18	10.96	25.780±0.129	2.227±0.091	8.64	916.282±4.100	70.888±2.899	7.74
Difference (4)-(1)	−28.41±15.62	−29.40±11.04	−1.61	+0.089±0.189	−0.175±0.134	−0.71	−8.865±5.643	+3.850±3.990	+0.49
Diff./$E_{diff.}$	1.82	2.66		0.47	1.31		1.57	0.96	
Percentage difference	1.76	15.45		0.34	0.76		0.96	5.58	

* For (1), N = 136; for (2), N = 289.4; for (3), N = 863; for (4), N = 863.

Considering first the mean total daily heat production, which is the fundamental constant for the establishment of a standard value, we note that the mean obtained by weighting is somewhat lower than that secured by giving each individual equal weight, irrespective of the number of days on which observations were made.

The differences are, however, of a low order of magnitude as compared with the average heat production of 1,631 calories. The heat production is on the average 13.54 calories lower when the constants are weighted with the square root of the number of days and 28.38 calories lower when the constants are weighted with the number of days, or based upon the constants for the individual days.[20]

These differences are between total daily heat productions (unweighted) of about 1,631 calories. Thus they are relatively small, only 0.83 and 1.76 per cent[21] by the two methods of weighting. The differences are not merely relatively small as compared with the total heat production but are in all cases less than twice as large as the probable errors of the differences.[22]

Turning now to the results for heat production per kilo of body weight, we have the comparisons set forth in the second section of Table VII.

The means show a slight but wholly insignificant increase in calories per kilo as a result of weighting with the square root of the number of days, or with the number of days, or by using the daily averages in calculating the constants.

Finally consider the results for calories per 24 hours per estimated square meter of body surface.

The heat production is 3.43 calories lower when weighted by the square root of the number of days observation and 8.88 calories lower when weighted with the number of days, than when calculated from the daily averages. The differences are rela-

[20] The means calculated in these two ways should be identical. The slight difference is due to the number of significant figures retained in the calculations.

[21] Percentage differences have been computed by using the average of the two means compared as a base.

[22] The probable errors have in all cases been based on the actual, not the weighted, number of individuals as N.

tively small, being less than 1 per cent in the three comparisons. All differences are less than twice as large as their probable errors.

It is clear from the foregoing constants that *practically it is immaterial whether the population means are calculated from the averages of the individual subjects, from the averages weighted with the number of days, or with the square root of the number of days, or whether they are determined directly from the daily observations.*[23]

From Table VII it appears that the standard deviations obtained by weighting the individual means with the square root of the number of days or with the number of days are lower than those calculated without weighting.

We now have to consider the constants deduced from the minimum values of the daily metabolism. The results are given in Table VIII. Limiting our attention for the moment to a comparison of these constants among themselves we note that, in whatever units measured, the mean metabolism calculated by weighting with the square root of the number of days is always lower than the constant obtained without weighting. When the minima for the individuals are weighted with the number of days instead of with the square root of the number of days the difference between the weighted and the unweighted value is even greater, amounting to −69.6 calories of total daily heat production, −0.579 calories per kilo, and −32.03 calories per square meter of surface area. These differences correspond to relative differences of 4.49, 2.35, and 3.63 per cent of the unweighted constants. *They show that if an absolute minimum, i.e. the one single day with the lowest average of metabolism measurements, for each individual is adopted, the constants for a population will depend to a considerable extent upon the number of days observation for each individual.*

Table IX compares the percentage change in the population constant due to weighting when the population constant is calculated from means and from minima. For all three units of metab-

[23] While this is the result for the large series of data in hand the calculation of the population constant from daily observations by weighting with the number of days is not to be generally recommended since in series in which the number of individuals is small the population average may be too greatly influenced by repeated observations on one or a few intensively studied individuals.

TABLE VIII.

Statistical Constants for Basal Metabolism as Determined from the Minimum Daily Mean for Each of 136 Men.

	Total calories per 24 hrs.			Calories per kg.			Calories per square meter.		
	Mean.	Standard deviation.	Coefficient of variation.	Mean.	Standard deviation.	Coefficient of variation.	Mean.	Standard deviation.	Coefficient of variation.
1. Unweighted*	1,585.11 ± 11.90	205.65 ± 8.41	12.97	24.947 ± 0.139	2.407 ± 0.098	9.65	898.926 ± 3.983	68.870 ± 2.817	7.66
2. Weighted with the square root of the no. of days	1,550.86 ± 11.01	190.38 ± 7.79	12.28	24.686 ± 0.129	2.227 ± 0.091	9.02	883.777 ± 3.955	68.382 ± 2.797	7.74
Difference (2)-(1)	−34.25 ± 16.21	−15.27 ± 11.46	−0.69	−0.261 ± 0.190	−0.180 ± 0.134	−0.63	−15.149 ± 5.613	−0.488 ± 3.970	+0.08
Diff./$E_{diff.}$	2.11	1.33		1.37	1.34		2.70	0.12	
Percentage difference	2.18	7.71		1.05	7.77		1.70	0.71	
3. Weighted with the no. of days	1,515.49 ± 9.91	171.29 ± 7.01	11.30	24.368 ± 0.120	2.068 ± 0.085	8.49	866.893 ± 3.879	67.067 ± 2.743	7.74
Difference (3)-(1)	−69.62 ± 15.48	−34.36 ± 10.95	−1.67	−0.579 ± 0.184	−0.339 ± 0.130	−1.16	−32.033 ± 5.560	−1.803 ± 3.932	+0.08
Diff./$E_{diff.}$	4.50	3.14		3.15	2.61		5.76	0.46	
Percentage difference	4.49	18.23		2.35	15.15		3.63	2.65	

* For (1), N = 136; for (2), N = 289.4; for (3), N = 863.

olism measurement (total calories, C, calories per kilo of body weight, C_k, and calories per square meter of body surface as given by the Du Bois chart, C_D) the percentage differences between the unweighted and the weighted constants are larger when minimum values are used than when individual means are used as a basis for the calculation of the constants.

It is clear, therefore, that the constant representing the mean metabolism of the population is much less dependent on the method of weighting when all daily means are used than when a single minimum value is used.

TABLE IX.

Comparison of Change in Population Metabolism Constant Due to Weighting when Based on Individual Means and Individual Minima.

Measure of metabolism.	Weighted with the square root of the no. of days.			Weighted with the no. of days.		
	Calculated from minima.	Calculated from means.	Difference.	Calculated from minima.	Calculated from means.	Difference.
Total calories, C.....	−2.18	−0.83	−1.35	−4.49	−1.76	−2.73
Calories per kg., C_k..	−1.05	+0.29	−1.34	−2.35	+0.34	−2.69
Calories per square meter, C_D.........	−1.70	−0.37	−1.33	−3.63	−0.96	−2.67

Turning now to a comparison of the constants obtained by the method of means and the method of minima, we have the comparison of the constants calculated by the two methods in Table X.

A comparison of the means shows that the unweighted constants calculated from individual minima are from 2.85 to 2.94 per cent lower than those calculated from individual means. When the constants are weighted with $\sqrt{N}$ the averages calculated from minima are from 4.20 to 4.28 per cent lower than those calculated from individual means. Finally, when the constants are weighted with the number of days the percentage differences of constants calculated in the two ways vary from 5.54 to 5.63 per cent.

That the population constant calculated from minima should be lower than that calculated from individual means is, of course, a necessary result. The fact that the percentage differences are no greater evidences for the *relative* stability of metabolism in the individual and for the accuracy of the series of measurements.

TABLE X.

Comparison of Statistical Constants for Basal Metabolism Based on Individual Means and Individual Minima for 136 Men.

	Unweighted. N = 136			Weighted with the square root of the no. of days. N = 289.4			Weighted with the no. of days. N = 863		
	Mean.	Standard deviation.	Coefficient of variation.	Mean.	Standard deviation.	Coefficient of variation.	Mean.	Standard deviation.	Coefficient of variation.
Total calories.									
From means....	1,630.98 ± 11.86	205.01 ± 8.38	12.57	1,617.44 ± 10.78	186.43 ± 7.62	11.53	1,602.60 ± 9.61	166.22 ± 6.80	10.37
" minima...	1,585.11 ± 11.90	205.65 ± 8.41	12.97	1,550.86 ± 11.01	190.38 ± 7.79	12.28	1,515.49 ± 9.91	171.29 ± 7.01	11.30
Difference.......	+45.87 ± 16.80	−0.64 ± 11.87	−0.40	+66.58 ± 15.41	−3.95 ± 10.90	−0.75	+87.11 ± 13.80	−5.07 ± 9.77	−0.93
Diff./$E_{diff.}$......	2.73	0.05		4.32	0.36		6.31	0.52	
Percentage difference........	2.85	0.31		4.20	2.10		5.59	3.00	
Calories per kg.									
From means....	25.691 ± 0.139	2.402 ± 0.098	9.35	25.766 ± 0.128	2.219 ± 0.091	8.61	25.779 ± 0.117	2.030 ± 0.083	7.87
" minima...	24.974 ± 0.139	2.407 ± 0.098	9.65	24.686 ± 0.129	2.227 ± 0.091	9.02	24.368 ± 0.120	2.068 ± 0.085	8.49
Difference	+0.744 ± 0.197	−0.005 ± 0.139	−0.30	+1.080 ± 0.182	−0.008 ± 0.129	−0.41	+1.411 ± 0.168	−0.038 ± 0.119	−0.62
Diff./$E_{diff.}$......	3.78	0.04		5.93	0.07		8.40	0.32	
Percentage difference........	2.94	0.21		4.28	0.38		5.63	1.88	
Calories per square meter.									
From means....	925.147 ± 3.877	67.038 ± 2.742	7.25	921.715 ± 3.753	64.891 ± 2.654	7.04	916.272 ± 3.655	63.190 ± 2.584	6.90
" minima...	898.926 ± 3.983	68.870 ± 2.817	7.66	883.777 ± 3.955	68.382 ± 2.797	7.74	866.893 ± 3.879	67.067 ± 2.743	7.74
Difference.......	+26.221 ± 5.558	−1.832 ± 3.931	−0.41	+37.938 ± 5.452	−3.491 ± 3.856	−0.70	+49.379 ± 5.330	−3.877 ± 3.768	−0.84
Diff /$E_{diff.}$......	4.72	0.47		6.96	0.91		9.26	1.03	
Percentage difference.....	2.87	2.70		4.20	5.24		5.54	5.95	

The fact that the average metabolism is lower when it is calculated from individual minima than when it is computed from individual means furnishes no argument in favor of either of the methods of computing the metabolism constant. Conclusions in regard to this point must be drawn from the results for weighting discussed above, and from a consideration of the variabilities.

From Table X we note that in whatever units heat production is expressed, the variation in the population metabolism (measured in either the absolute terms of the standard deviation or in the relative terms of the coefficient of variation) is lower when the individual means are employed than when individual minima are used as a basis for calculating the population constants.

If the securing of a constant with the lowest probable error is one of the goals to be attained, the method of means is, therefore, to be preferred over the method of minima.

III. SUMMARY.

In all special investigations in human calorimetry some standard constant measuring the metabolism of the normal individual must be used as a basis of comparison. The selection of this constant presents a problem of considerable difficulty from three sides.

The first is that of the physiological conditions under which the basal metabolism of the individual shall be measured; the second is that of the unit in which the caloric output of the individual shall be expressed; the third is that of the method by which the statistical constants for the standard series shall be obtained.

Basal metabolism measurements are generally made in two or more periods, with the subject in the postabsorptive state and in complete muscular repose, on the same day. Experimental periods which show evidence of muscular activity or of faulty technique in the analyses are discarded. So called minimum periods are utilized for obtaining a mean for the day. This may be designated as the daily mean.

It may be reasonably assumed that the results of the several periods of measurement on a given day stand in the relation of duplicate, triplicate, etc. analyses, and that it is not necessary to

go back of the daily means in determining the constants for the individual or the population.

The purpose of this paper has been to investigate the variability in the basal metabolism of the normal individual, and the method of determining a population mean from a series of individual constants.

The averages of daily periods of observation for individual men who have been studied from 20 to 53 days show statistically significant ranges and standard deviations of metabolism the magnitudes of which depend upon the unit of measurement. When the variabilities are expressed as percentages of the means to show their relative values it appears that the variability is measured by coefficients of variation of about 4 per cent of the average metabolism.

The variability in the metabolism of the individual is positively correlated with the duration of the period of time over which the observations have been distributed. This indicates not merely that the metabolism of the individual under the standard conditions for basal determinations is variable but that the amount of the variation bears a measurable relation to the length of time over which the observations extend.

The correlations between range of observations and variations in metabolism are greater for narrower ranges of observation than for the longer ranges, thus indicating that the greater part of the physiological variations in metabolism will be realized in relatively short periods of time. An examination of the average values of the standard deviations of individuals studied for varying length of time substantiates this conclusion.

It is evident, therefore, that the metabolism of the "normal" subject is not constant even with practically constant body mass but is to some extent in a state of flux. The constants here given furnish some measure of this variation, and may serve as a basis of comparison in future work.

The statistical problem concerning the population constant consists in determining (*a*) whether the individual means or the individual minima (of daily means) shall be used as the basis of calculation, and (*b*) what method of weighting, if any, shall be adopted when repeated observations have been made in varying numbers upon the individuals of a series.

Analysis of the 863 daily means available for 136 men studied at the Nutrition Laboratory shows that in this series the means deduced by weighting do not differ materially from those determined without weighting as in our earlier publication.[24]

Tables VII to X may be consulted for the actual values of these constants.

The results of this study also show that the population constant derived from individual means is less modified by weighting than that deduced from the individual minima. Furthermore, both standard deviation and coefficient of variation are lower when the population constants are based on individual means or upon all daily means than when based on individual minima. Since weighting may logically be demanded, it is clear that the method of means furnishes a more suitable constant for representing the metabolism of a series of individuals than the method of minima.

The method of weighting adopted will depend to some extent upon the personal judgment of the investigator. Weighting with the actual number of days probably attaches relatively too great importance to the observations on certain individuals who happened for one reason or another to be more extensively studied. Probably weighting with the square root of the number of days rather than with the number of days would be the course recommended by most statisticians.

[24] To solve fully the problem of the influence of weighting upon the prediction equations developed in our earlier work would require a full investigation of the influence of weighting upon the constants for stature, body weight, and age, and upon all the possible correlations between these characters and between them and the basal metabolism as well as upon the partial correlations. The results deduced from the present study leave quite unshaken our confidence in the logic of the method used in our earlier treatment of the problem.

THE METABOLISM OF THE EUNUCH.

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With a view to contributing to the knowledge of the fundamental metabolic changes following castration, the experiments here recorded were undertaken upon the oriental eunuch,[1] who affords a unique opportunity for the study of this important subject in its normal setting.

Urine Analysis.

The first specimen of urine (Case 1) was obtained from a hospital patient convalescent from a mild surgical operation. The values obtained for total nitrogen, ammonia, and creatinine, while differing considerably from European standards, were comparable to values obtained from over 100 other estimations of the urine of patients under similar diet and circumstances.[2] The urine of two others was analyzed for total nitrogen, urea, and ammonia. These likewise gave unusually high ammonia values; in fact the results shown resemble those from a cretin or similar case in whom the metabolism is greatly deranged. In these two cases special attention was given to the creatinine determinations and creatine was found in both urines.

Preformed creatinine was estimated by Folin's colorimetric method, and after heating the urines with acid in the usual way, the creatinine was redetermined. As noted by Mathews,[3] creatine is not determined directly but by difference, and by the methods

[1] The characteristics of the Pekin eunuchs have been fully described by Millant (Millant, R., Les eunuques à travers les ages, Paris, 1908).

[2] Read, B. E., and Wang, S. Y., *China Med. J.* (in press).

[3] Mathews, A. P., Physiological chemistry, New York, 2nd edition, 1916, 707.

used striking differences are to be noticed in the values found for preformed creatinine and preformed creatine in the urine of the normal adult male, female, and child. Creatine, in any appreciable amount, does not occur in the urine of male adults, except under special conditions of sickness or diet. Boys secrete creatine up to about 7 years of age. Girls, on the other hand, continue to secrete creatine until puberty, and with women creatine appears in the urine at definite times. Hence one might regard this as infantile, female, or pathological in character. Typical findings are shown in Table I.

TABLE I.

	Age.	Urine.	Creatinine.	Creatine.
	yrs.	*cc.*	*gm.*	*gm.*
Boy	5	620	0.112	0.025
"	11	460	0.157	0.000
Girl	12	1,000	0.224	0.042
Eunuch 3	18	910	0.204	0.424

In order to confirm these values three other samples of urines from eunuchs were obtained and examined. Values were obtained as shown in Table II.

TABLE II.

Case No.	Total creatinine.	Preformed creatinine.	Preformed creatine.
	per cent	*per cent*	*per cent*
2	0.007	Trace.	0.007
3	0.069	0.022	0.047
4	0.099	0.039	0.060
5	0.023	0.006	0.017
6	0.004	0.004	0

Case 6 possibly brings out a distinguishing factor; namely, the influence upon metabolism of such an operation depends upon the age at which the operation is performed. Case 6 was operated at the age of 29 years, when all the male characteristics were well developed. Cases 2 and 3 were operated young enough on the one hand for infantile characteristics to survive, and on the other hand well before the secondary sex characters were developed; and, while the data are somewhat scant, these cases show a

change which follows the physical change from secondary male characters to secondary female characters. Case 4, although 19 years of age when operated, may well be regarded in the same way because the Chinese secondary male characteristics are later in development than in the European. For instance, the writer never remembers seeing a Chinese under 21 years of age with hair on the face, and the voice does not change at puberty so conspicuously as it does in the European. Hence one is led to conclude that removal of the male organs at a suitable age tends to develop chemically as well as physically secondary female characters in the individual. Data obtained in the analyses are given in Table III.

TABLE III.

Case No.	Age.	Age when operated.	Urine.	Specific gravity.	Total nitrogen.	Urea N.	Ammonia N.	Creatinine.	Creatine.
	yrs.	*yrs.*	*cc.*		*gm.*	*gm.*	*gm.*	*per cent*	*per cent*
1	27	(?)	2,020	1.020	9.18		2.39	1.21	
2	18	12	995	1.023	7.12	4.84	2.07	Trace.	0.149
3	18	9	910	1.022	4.09	1.87	1.64	0.204	0.424
4	30	19						0.039	0.060
5	27	(?)						0.006	0.017
6	40	29						0.004	0

THE DETERMINATION OF INORGANIC PHOSPHATE IN URINE BY ALKALIMETRIC TITRATION.

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(Received for publication, February 1, 1921.)

In an investigation of the excretion of phosphate and other substances in short periods (chiefly of 1 hour) now being conducted in this laboratory, it has been necessary to make many inorganic phosphate determinations with limited quantities of urine. In most instances the accuracy possible with colorimetric and nephelometric methods has been sufficient, and, after a considerable amount of time had been spent in attempting to adapt then existing methods to the purpose at hand, the routine determinations were for a time made by precipitating the phosphate with magnesia mixture and determining the phosphate content of the redissolved precipitate nephelometrically (with strychnine molybdate). The necessity of resorting to this none too satisfactory arrangement has now fortunately been removed by the colorimetric method recently devised by Bell and Doisy,[1] and this has since been used instead. In view of the empirical

[1] Bell, R. D., and Doisy, E. A., *J. Biol. Chem.*, 1920, xliv, 55. In connection with this very welcome addition to the supply of micro methods for inorganic phosphate, added emphasis should perhaps be given to the authors' remarks on the necessity of replacing the contents of the standard cup by fresh solution each time the unknown in the other cup is changed. This *must* be done even when several determinations are being made *seriatim* with a single standard. Observing this precaution, I have made several comparisons with the titration method described in this paper, as well as with the von Lorenz method, with a maximum difference of less than 2 per cent. When the rate of phosphate excretion is very small, the amount of urine necessary to give a color comparable with the standard recommended may be sufficient to give a distinct precipitate with the molybdic acid solution, and the error may then be greater. This difficulty may be avoided by repeating the determination with a smaller quantity of urine, using, if necessary, a weaker standard.

character of colorimetric and nephelometric analyses, particularly when they are applied to complex mixtures without any preliminary isolation of the substance to be determined, it seemed unwise to rely exclusively on such methods without confirmation by some procedure relatively free from empirical features. This position has fully justified itself, since the method to be described in this paper (designed partly to obtain such confirmation, and partly to secure more accurate data, when desired, without being obliged to use time-consuming gravimetric methods involving double

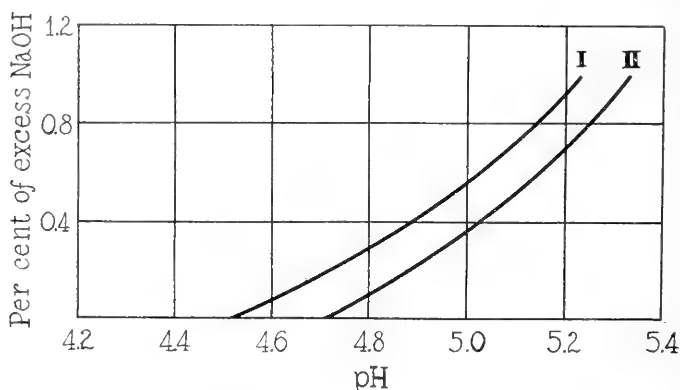

Fig. 1. Titration curves of 0.05 M (I) and 0.01 M (II) monopotassium phosphate solutions (at 20°) on the alkaline side of the end-point. The ordinates represent the excess of alkali in terms of the method described in this paper; *e.g.*, 1 per cent of excess alkali corresponds with the conversion of 2 per cent of the primary phosphate into secondary phosphate.

precipitation) has assisted materially in defining more closely the conditions under which the method of Bell and Doisy is most reliable, and at an earlier date was the means of showing that the direct application to urine of the nephelometric method (as it is used for blood filtrates) gives results that are quite erroneous.

The principle of the present method is the titration, on a small scale, of the magnesium ammonium phosphate precipitate. This titration, introduced many years ago by Stolba,[2] has recently been adapted to urine, on the basis of samples containing about

[2] Stolba, F., *Chem. Zentr.*, 1876, vii, series 3, 727.

40 mg. of phosphorus,[3] by Bauzil[4] and Angiolani.[5] These investigators, as well as others who have proposed modifications of this method, adhere to the use of methyl orange, and it is particularly in this respect that the method requires alteration before it can be used for smaller quantities of material.

Electrometric C_H determinations[6] (Fig. 1) on solutions prepared from purified monopotassium phosphate have shown that the titration curves for 0.05 M and 0.01 M phosphate, in the vicinity of the end-point, are not far from parallel, and that the change in pH is rapid enough to permit the titration of 0.01 M solutions with greater accuracy than is generally stated to be possible with much more concentrated phosphate solutions, provided an indicator with a sufficiently sharp color change is used. 25 cc. of a 0.01 M phosphate solution can in fact be titrated, using methyl red and a standard color for comparison, with an error of only about 0.01 mg. of phosphorus.

The titration curve in Fig. 2 was calculated by means of a formula derived in a manner similar to that proposed by Prideaux,[7] but taking into account the repression of the ionization of phosphoric acid by the large excess of primary phosphate present:

$$R = \frac{(NaH_2PO_4) + 2(Na_2HPO_4)}{(NaH_2PO_4) + (Na_2HPO_4) + (H_3PO_4)}$$

$$= \frac{1 + 2\,\dfrac{(Na_2HPO_4)}{(NaH_2PO_4)}}{1 + \dfrac{(Na_2HPO_4)}{(NaH_2PO_4)} + \dfrac{(H_3PO_4)}{(NaH_2PO_4)}}$$

In this equation, $\frac{(Na_2HPO_4)}{(NaH_2PO_4)}$ may be considered equal to $\frac{k_2}{(H^+)}$, without further correction for incomplete ionization of the two salts, by making use of the apparent dissociation constant of pri-

[3] 1 hour samples of human urine often contain much less than 40 mg.; consequently these methods are useless for short period experiments.

[4] Bauzil, *J. pharm. chim.*, 1917, xvi, 321.

[5] Angiolani, A., *Gior. farm. chim.*, 1917, lxvi, 251; abstracted in *Chem. Abstr.*, 1919, xiii, 27.

[6] With a Clark hydrogen electrode.

[7] Prideaux, E. B. R., *Proc. Roy. Soc. London, Series A*, 1915, xci, 535; The theory and use of indicators, London, 1917, 239.

mary phosphate when the concentration is 0.01 M. Interpolation from the data of Michaelis and Garmendia[8] gives the value 10^{-7} for this constant (k_2).

It has been found experimentally that, in the vicinity of the end-point, the ratio $\frac{(H_3PO_4)}{(NaH_2PO_4)}$ may, with sufficient accuracy, be considered equal to the (H^+) divided by a constant (K). The value of K found for the circumstances under consideration is 4×10^{-3}.

The equation may therefore be written:

$$R = \frac{1 + 2\frac{k_2}{(H^+)}}{1 + \frac{k_2}{(H^+)} + \frac{(H^+)}{K}} = \frac{1 + \frac{2 \times 10^{-7}}{(H^+)}}{1 + \frac{10^{-7}}{(H^+)} + \frac{(H^+)}{4 \times 10^{-3}}}$$

The curve (Fig. 2) was drawn from points calculated on this basis, while the circles represent points determined experimentally with 0.01 M KH_2PO_4 solutions containing various amounts of added NaOH or HCl. The agreement is quite satisfactory.

Entirely apart from the matter of time consumption, titration has one distinct advantage over direct weighing of the magnesium ammonium phosphate (without double precipitation), for most of the substances that are inclined to contaminate the precipitate (calcium phosphate, magnesium phosphate, and magnesium ammonium phosphates other than $MgNH_4PO_4$) do not alter the titration figure, whereas they may seriously affect the weight.[9]

Owing to the small scale of the new method, it has been necessary to devise special means of handling the precipitate. This is accomplished by the use of a filtration tube, consisting simply of a glass tube, about 8 mm. in internal diameter and 120 mm. long, shrunken at the lower end to a bore of 2 mm., and flanged at the upper end. (The capacity of the tube should be well over 5

[8] Michaelis, L., and Garmendia, T., *Biochem. Z.*, 1914, lxvii, 431.

[9] Mathison (Mathison, G. C., *Biochem. J.*, 1909, iv, 237) infers that the presence of citrate prevents contamination with calcium, but there is no difficulty in demonstrating calcium in precipitates from human urine by dissolving in dilute hydrochloric acid, precipitating with oxalate, and applying the microchemical test with sulfuric acid (Chamot, É. M., Elementary chemical microscopy, New York, 1915, 288).

cc.) This device, supported by a rubber stopper in the neck of a suction flask large enough to contain a test-tube with a capacity of about 50 cc., and provided with a thin mat of paper pulp, makes it possible to filter and wash small precipitates in a very short time, and to transfer the washed precipitate (through the hole in the lower end) to a flask for titrating. The use of a proportionately small filtration tube has the further advantage that the amount of washing required by the filter is almost negligible.

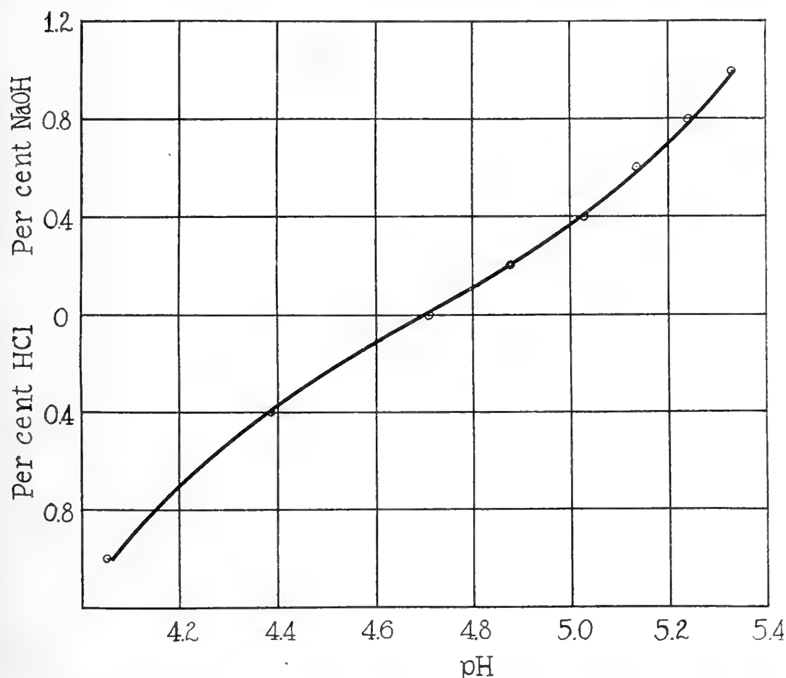

Fig. 2. Calculated titration curve of 0.01 M monopotassium phosphate. For explanation see text.

Special Reagents Required.

Magnesium Citrate Mixture.—Dissolve 80 gm. of citric acid in 100 cc. of hot water. Add 4 gm. of magnesium oxide, and stir until dissolved. Cool, and add 100 cc. of ammonium hydroxide (density 0.90). Dilute to 300 cc., let stand 24 hours, and filter. (If the magnesium oxide contains much carbonate, it should be

freshly ignited.) This is essentially the reagent used by Mathison[9] and others.

95 Per Cent Alcohol.—This reagent must be neutral, since it is used for washing the $MgNH_4PO_4$ precipitate. 5 cc. of it should not more than slightly alter the color of 50 cc. of water containing a little methyl red and previously adjusted with very dilute alkali to an intermediate orange color. Ordinary alcohol will meet this requirement only after distillation from alkali.

Methyl Red Solution.—0.004 per cent solution in 50 per cent alcohol.

Standard Acetate Mixture.—Mix 50 cc. of 2 N acetic acid and 35 cc. of 2 N NaOH (free from carbonate), and dilute to 100 cc.

Standard Color.—To 2 cc. of the above acetate mixture (in a 100 cc. Erlenmeyer flask) add 2 cc. of the methyl red solution and 21 cc. of water.

The Method.

Transfer to a large lipped test-tube (200 $\times$ 20 mm.) an amount of urine containing between 2 and 7 mg. of inorganic phosphorus.[10] Add water, if necessary, to make the total volume about 10 cc.[11] Then add 1 cc. of magnesium citrate mixture and 2 cc. of ammonium hydroxide (density 0.90). Shake the tube until crystallization begins (with the larger amounts this occurs almost immediately), then shake very gently, but continuously, for 15 minutes longer.[12] At least eight samples can be precipitated simultaneously in this way.

[10] Within these limits the method is, of course, more accurate the larger the amount used.

[11] When 10 cc. of urine contain less than 2 mg. of inorganic phosphorus, a larger volume should be used. In this event, the reagents should be increased in proportion, and the same is true when it is desired to use more than 10 cc. of urine for the sake of greater accuracy. If it is necessary to use more than 20 cc. of urine, the prescribed 15 minutes shaking may not be sufficient for complete precipitation. With urines as dilute as this the method is hardly more accurate than Bell and Doisy's.

[12] Merely keeping the mixture in motion (and that is all that is really necessary) tremendously accelerates the precipitation, presumably because of the much larger number of nuclei formed under these conditions. With urine, standing undisturbed over night may not be sufficient for complete precipitation.

With a glass tube transfer to the filtration tube (described above) a sufficient amount of paper pulp[13] to make a mat just thick enough to cover the bottom of the tube, and suck dry. Filter off the precipitate on this mat, using only very gentle suction. Wash with 10 cc. of 2.5 per cent ammonia, and then with four 5 cc. portions of 95 per cent alcohol, making no attempt to dislodge the part of the precipitate that adheres to the wall of the test-tube. Throughout these operations the lower end of the filtration tube should drain into a large test-tube set inside the suction flask, since it is better to keep the flask dry. Between the second and third washings with alcohol, it is well to wash out the test-tube that serves as a receiver.

Remove the filtration tube from the suction flask, and support it (by a clamp) with its lower end inserted into the mouth of a 100 cc. Erlenmeyer flask. Pipette[14] into the test-tube 0.1 N HCl, 1 cc. at a time, until the precipitate dissolves completely on shaking, and pour the resulting solution into the filtration tube. By means of a stiff, sharpened nichrome wire, poke the precipitate and mat through the 2 mm. hole into the Erlenmeyer flask. Rinse with 2 cc. of methyl red solution and 13 cc. of water. To the contents of the Erlenmeyer flask add 0.1 N HCl, 1 cc. at a time, until the solution remains distinctly red after it has been shaken thoroughly. (At least 0.5 cc. of 0.1 N HCl should be added in excess of the amount necessary to decompose the precipitate.) Pour the solution into the lipped test-tube and back until the precipitate is completely dissolved, finally rinsing the few drops remaining in the test-tube into the flask with 5 cc. of water. The solution is now ready for titration[15] with 0.1 N NaOH from a

[13] Shake one 15 cm. ashless filter paper (*e.g.*, Schleicher and Schüll No. 589, black ribbon) with 200 cc. of water in a stoppered bottle until the paper is thoroughly broken up. 2 or 3 minutes vigorous shaking should suffice (Jodidi, S. L., and Kellogg, E. H., *J. Ind. and Eng. Chem.*, 1916, viii, 317).

[14] With a calibrated Ostwald pipette.

[15] If one prefers, more dilute alkali can be used (with an ordinary burette), although it would then be necessary to use less water for rinsing, since the total volume must not be much greater than 25 cc. More dilute alkali offers no particular advantage, and has the disadvantage of being less stable.

micro-burette provided with an accessory tip.[16] Run in the alkali until the color begins to turn, and complete the titration by adding 0.01 cc. of alkali at a time until one such quantity makes the solution definitely yellower than the standard.[17] This method, rather than accurate matching of the colors, is necessary to compensate for the error that would otherwise be introduced because of the fact that the theoretical end-point varies with the concentration of phosphate.

The difference between the volumes of acid and alkali used gives the amount of 0.1 N acid neutralized by the precipitate. The phosphorus content of the sample (in mg.) is obtained by multiplying this figure by 1.552.

Analysis of Phosphate Solution.

A sample of monopotassium phosphate, purified by recrystallization, was dried to constant weight at 110°. A portion of this, ignited to KPO_3, showed the theoretical loss in weight.

TABLE I.

Analysis of Phosphate Solution.

KH_2PO_4 solution used.	0.1 N HCl neutralized by precipitate.	Phosphorus.	
		Present.	Found.
cc.	*cc.*	*mg.*	*mg.*
2.00	0.90	1.40	1.40
	0.91	1.40	1.41
4.00	1.81	2.80	2.81
	1.80	2.80	2.79
6.00	2.71	4.20	4.21
	2.71	4.20	4.21
8.00	3.60	5.60	5.59
	3.61	5.60	5.60
10.00	4.52	7.00	7.02
	4.51	7.00	7.00

Various quantities of a solution, prepared from this material and containing 0.7 mg. of phosphorus per cc., were analyzed by the method described. The results are shown in Table I.

[16] Folin, O., and McEllroy, W. S., *J. Biol. Chem.*, 1918, xxxiii, 517. Folin, O., and Peck, E. C., *J. Biol. Chem.*, 1919, xxxviii, 289.

[17] If a small glass bead is placed in the rubber tube connecting the burette with the accessory tip, the control of the delivery at the end of the titration is made easier.

The Effect of Calcium.

Provided enough citrate is present during the precipitation to prevent the appearance of an amorphous precipitate of calcium phosphate, the results are the same as in the absence of calcium. When the urine contains a large amount of calcium in proportion to the phosphate present, more magnesium citrate must be added, but in doing so it is necessary to increase the volume of the solution and the amount of ammonium hydroxide. If, for example, after the addition of the reagents in the manner stated in the preceding description of the method, the solution immediately develops a turbidity that is obviously not crystalline, a new sample should be measured out, and water added to make the total volume 20 cc. (instead of 10), followed by 2 cc. of the magnesium citrate mixture and 4 cc. of ammonium hydroxide. This has so far never been found necessary with human urines.

On adding the reagents in the usual manner to 6 cc. of the above potassium phosphate solution to which had been added 30 mg. of calcium in the form of calcium chloride, an amorphous precipitate immediately appeared. Nevertheless, on doubling the volume and the quantity of reagents, this precipitate largely disappeared, and triple phosphate crystals began to separate; on continuing the analysis as usual, the result was only slightly wrong (4.22 instead of 4.20 mg. of phosphorus). The determination was then repeated, this time diluting to 20 cc. at the start and adding twice the usual amounts of reagents; no sign of an amorphous precipitate appeared, and the result was entirely correct (4.20 mg.).

Analysis of Urine.

Probably the best evidence of the accuracy of the method with urine is the fact that a second precipitation of the magnesium ammonium phosphate, after dissolving the first precipitate with acid, does not significantly alter the results. The figures obtained after double precipitation do tend to be slightly lower, as may be expected from the additional manipulation necessary, but the difference is only of the magnitude of the difference between duplicates with pure phosphate solutions (Table I).

To show that the method is subject to no gross error, it has been compared with the von Lorenz[18] method. Samples of urine (human) containing about 15 mg. of inorganic phosphorus were precipitated with magnesium citrate mixture and ammonia by shaking for 15 minutes, followed by standing over night (although the last step has never been found to alter the result). The precipitate of magnesium ammonium phosphate was then filtered off, washed with dilute ammonia, dissolved in acid, and precipitated as ammonium phosphomolybdate according to the

TABLE II.

Analysis of Urine.

Urine No.	Titration method.				von Lorenz method.	
	Urine.	0.1 N HCl neutralized.	Inorganic P.		Inorganic P.	
	cc.	*cc.*	*mg. per 100 cc.*	*mg. per hr.*	*mg. per 100 cc.*	*mg. per hr.*
1	10	0.95	14.7		14.9	
2	15	1.15	11.9		11.9	
3	15	2.77	28.7		28.6	
4	35	1.60	7.1		7.1	
5	15	2.86	29.6		29.5	
6	10	4.49	69.7	33.3	69.8	33.4
7	10	1.34	20.8	5.6	20.5	5.5
8	10	2.37	36.8	16.6	36.5	16.5
9	10	4.06	63.0	33.7	62.7	33.6

directions given by von Lorenz. The precipitates were filtered on a platinum mat, as recommended by Neubauer and Lücker.[19] On the basis of preliminary determinations with phosphate solutions, the amount of phosphorus was obtained by multiplying the weight of the precipitate by the factor 0.0144 (the corresponding factor for P_2O_5 would be 0.03296, agreeing with von Lorenz's figure 0.03295).

Table II contains a series of representative results covering somewhat more than the range recommended (2 to 7 mg. of phosphorus). The first five urines were collected without respect to time, but they were obtained during the morning when the

[18] von Lorenz, N., cited in Grünhut, L., *Z. anal. Chem.*, 1907, xlvi, 193.

[19] Neubauer, H., and Lücker, F., *Z. anal. Chem.*, 1912, li, 161.

inorganic phosphate output is likely to be low.[20] Since the amount of phosphate under these circumstances is small compared with other urinary constituents, urines of this sort offer the most rigid test of the method from the standpoint of the possibility of errors due to contamination of the precipitate by other basic substances. Attention is called particularly, in this connection, to Urine 7, which was a 3 hour sample collected during the morning, and shows an average phosphate excretion of only 5.5 mg. of phosphorus per hour.

[20] Fiske, C. H., *J. Biol. Chem.*, 1920, xli, p. lix.

NOTE ON THE FORMATION OF HYDROCYANIC ACID IN PLANTS.

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(Received for publication, January 17, 1921.)

Haas and Hill[1] state that Gautier has put forth the supposition that hydrocyanic acid is formed in plants by the action of formaldehyde on nitrates. They state also that nothing definite is known in regard to its formation in plants.

In order to test out Gautier's supposition, six 500 cc. flasks, each containing 400 cc. of water saturated with carbon dioxide, 2 cc. of 40 per cent formaldehyde, and 1 gm. of potassium nitrate, were treated as follows:

(1) Two flasks were made alkaline to phenolphthalein with sodium carbonate.

(2) Two were made alkaline to methyl orange but acid to phenolphthalein.

(3) Two flasks were made acid to methyl orange. These flasks were stoppered and placed in the sunlight for 1 month. At the end of this time tests were made for hydrocyanic acid by the formation of sulfocyanide.[2]

The results were as follows: In Series 1, no hydrocyanic acid was found; in Series 2, a trace of acid was found; and in Series 3, 6 mg. of the acid were found in each flask. These results, when considered in connection with the fact that the sap of the plant is slightly acid and that nitrates and formaldehydes are present, would indicate that prussic acid may be formed in plants by the action of formaldehyde on nitrates.

[1] Haas, P., and Hill, T. G., An introduction to the chemistry of plant products, London, 2nd edition, 1917, 179.

[2] Menaul, P., and Dowell, C. T., *J. Agric. Research*, 1919–20, xviii, 447.

A COLORIMETRIC METHOD FOR DETERMINING THE HYDROGEN ION CONCENTRATION OF SMALL AMOUNTS OF FLUID.

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(Received for publication, February 1, 1921.)

The importance of the maintenance of the state of neutrality in practically all vital phenomena has become well recognized through the efforts of Sörensen (1), Michaelis and his collaborators (2), Henderson (3), Clark (4), and numerous others. The methods devised by them require the use of a considerable amount of fluid. While this fact is not a disadvantage in most types of investigation in which the pH value is of importance, studies where only single drops of fluid are available render them inadequate. Having in mind an investigation in which only exceedingly small amounts of liquid could be obtained—namely the influence of the H ion concentration on tissue cultures *in vitro*—and also realizing a definite need for other problems, the author has attempted to modify the technique of the colorimetric method that it might be applicable to small quantities of fluid, the gas-chain electrometric method being clearly out of the question. Haas (5) advised the use of indicator papers to meet these requirements. Although this method was not put to a severe test, the inability to secure reliable results and the complexities which arise in the use of such indicators led us to abandon this procedure.

Our first plan was to use tubes of small dimension and employ the dilution method as advised by Clark and Lubs (6) as a practical means by which to titrate bacteriological media. Accordingly, tubes of 2 to 3 mm. were chosen to each of which were added 1 drop of the fluid to be tested, 4 drops of distilled water, and 1 drop of indicator solution. In some of the fluids used there was a very low salt content and buffer action, so that the dilution

with the distilled water changed the pH markedly. Thus it became necessary to perform the determinations with undiluted fluid.

A piece of opal glass had been employed as a background in comparing the colors in the small tubes. This suggested the use of the plate instead of the tubes upon which to make the determinations. The first trial proved its worth, the color tint being just as easily judged as in large tubes and much more readily than in the tubes of 3 mm. diameter. In checking the method with buffer mixtures ranging from pH 2 to 9.6 it was found that an accuracy of at least 0.1 pH could be obtained.

The equipment necessary for performing the test consists of standard buffer solutions, indicators, opal glass plate, and small stirring rods. The buffer solutions used were Clark series kindly furnished by Dr. Clark and the La Motte Chemical Products Company. Although a number of indicators were tried out, the series advised by Clark and Lubs was clearly the most satisfactory. Yet, these indicators have a disadvantage or limitation in that the respective range of a single indicator is relatively short—not short compared to other indicators but in comparison with the possible range of H ion concentration met with in our work. An ideal indicator for our purpose is one that would cover the range from pH 5 to 9. Although it may be impossible to find such an indicator, it occurred to us that a combination could be made of two indicators having opposite color changes; that is, one the color of which became more intense in solutions of increasing H ion concentration and one in which the color intensified as the H ion concentration decreased. Methyl red and brom-thymol blue were tried and found to have a good working range from pH 4.6 to 7.6, the variation in color from pH 5.6 to 6.2 being somewhat indefinite. The combination of methyl red and brom-cresol purple makes a very useful indicator with a range of pH from 4.6 to 7. As an indicator to make only rough estimations of the H ion concentration of any fluid, that is whether it lies between pH 4.6 and 9, a mixture of methyl red and thymol blue is very satisfactory. In like manner a combination of thymol blue and brom-phenol blue makes an exceptionally good double indicator in the range between pH 1.2 and 4.6. The distinct advantage of these combined indicators lies in the fact that they make good "feelers"

for test fluids and, within certain ranges, the exact pH can be determined with a single drop of fluid. These mixtures have been made, and remained unchanged for a period of 3 months.

The concentration of the indicator is important because of the small amount of fluid to be tested. As long as the drop of liquid contains acids of low dissociation and their salts, the strength of the indicator gives a proportionate color intensity of appropriate pH value; if the electrolytic concentration is low and the H ions and the hydroxyl ions are about the same in number, the indicator does not change in color according to the pH of the liquid but is simply diluted with only a partial change. According to Ostwald's theory of indicators, in such cases the H ions are not in sufficient numbers to react with the indicator molecule, the resultant color being a mixture of the unchanged indicator and the part that has reacted with the H ions present in the fluid.

The exact concentration of the indicator varies with the characteristic of the fluid to be tested; highly colored or turbid fluids demand the use of a strong indicator solution, while clear, almost colorless, liquids are best tested with a weaker dilution of the indicator. However, for general work we recommend the concentrations as follows:

Methyl red, brom-cresol purple, phenol red, and cresol red in 0.01 per cent solution in 25 per cent alcohol, while thymol blue, brom-phenyl blue, brom-thymol blue, and thymol blue are made 0.02 per cent. The combined indicators are made by mixing equal parts of a double strength indicator solution; that is, double the concentration employed when the indicators are used singly.

Inasmuch as we are dealing with a final concentration of indicators in the test fluid, three to five times as great as is used in the ordinary test-tube colorimetric method, it should be emphasized that the indicator salts should be very pure.

There is a question as to the stock container for the buffer solutions and indicators for this method. Glass-stoppered bottles with the end of the pipette drawn out to a fine point are perhaps the most convenient. Fig. 1 represents the types of devices tried, of which we think Type C is the most suitable. This particular type was used over a period of 2 months, the buffer solutions remaining unchanged.[1]

[1] The dropper outfit with buffer solution of any pH and indicators can be obtained from the La Motte Chemical Products Company, Baltimore.

Although an opal glass plate was used upon which to perform the test, it is self-evident that any smooth white surface will answer the purpose; the one requisite being the absolute neutrality

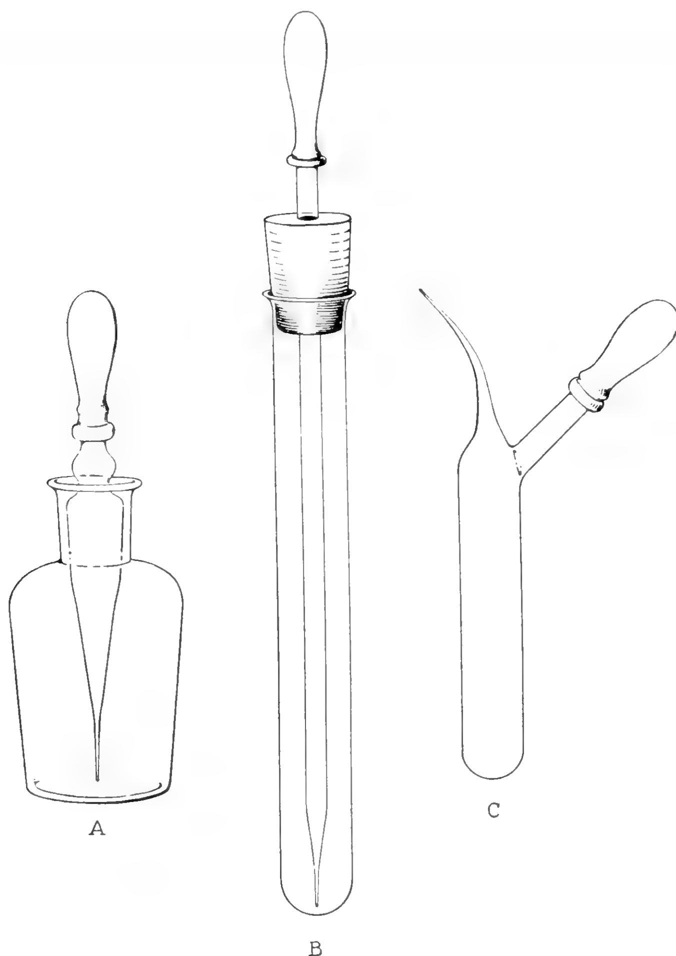

Fig. 1.

of the plate. Ordinary white porcelain pans, evaporating dishes, and China dinner plates were all found to be quite satisfactory. Occasionally a plate which liberates OH ions can be rendered

neutral by soaking in bichromate cleaner for from 24 to 96 hours. It should be rinsed thoroughly with tap water and then with distilled, and finally dried with a clean towel or allowed to dry in an almost vertical position. This procedure for cleaning was used routinely, the bichromate cleanser being necessary only occasionally.

Procedure of the Test.

The method consists simply in mixing a drop of the fluid to be tested and a drop of an appropriate indicator, noting the color, and then placing in close proximity drops of several buffer solutions that are judged to give the same color. The exact match of color can readily be made and the pH determined according to which buffer mixture gave the same color tint. Because of the large relative surface area of a drop, allowing a rapid exchange of gases and evaporation, the reading is made within about 1 minute. Although the alcoholic solution of the indicator facilitates the mixing of the 2 drops, especially if the indicator is allowed to drop on the fluid from a height of several inches, in all fluids of rather high viscosity, it becomes necessary to make mixing complete by means of a small stirring rod.

Theoretically, the drops of indicator and fluid should be of the same size. However, we were able to obtain accurate and consistent results by using only approximate sizes, care being taken to let the drops fall from the pipette held in a vertical position.

In learning the color changes of the indicators, it was found helpful to try all the indicator series with the different buffer solutions. That is, there were placed on a large opal glass plate, in rows, as many drops of the different buffer solutions as indicators to be used. To each drop of the buffer mixtures a drop of one of the indicators was added. This was repeated with each indicator solution. There were, then, on a single plate the various ranges of all the indicators, rendering possible a comparison of their different ranges. For example, a buffer solution of pH 6 is yellow with methyl red, brom-thymol blue, phenol red, etc., but a definite greenish blue tint with brom-cresol purple. This fact has a definite application in making pH determination by the drop method, due to the ease with which one can check results. For instance, if one has 5 drops of a fluid the pH of which is desired, a drop of

each of five indicators may be added, thus getting a series of colors depending on the pH of the fluid. If the colors given by methyl red and thymol blue are yellow, brom-cresol purple is very purple; brom-thymol blue, very blue; phenol red, quite red; cresol red, slightly pink; and one may safely state that the pH is somewhere around 7.6. This checking system cannot be overemphasized in minimizing gross errors.

The method was not standardized by the gas-chain determination except from the fact that the buffer salts furnished by Dr. Clark were checked by him and shown to have maximum errors of not over 0.02 pH. However, all types of bacteriological media, serum, spinal fluid, fruit juices, milk, tissue extracts, etc., were tested both by the test-tube method and drop method and there was practically no difference discernible.

CONCLUSIONS.

A colorimetric method by which H ion concentration can be determined with reasonable accuracy on single drops of fluid has been described. Clark and Lubs series of indicators were used with certain combinations to make double indicators. These combinations make indicators of fairly wide range; thymol blue and brom-phenol blue between pH 1.2 and 4.6; methyl red and brom-thymol blue from pH 4.6 to 7.6; methyl red and brom-cresol purple from pH 4.6 to 7; and for only a very rough "feeler" methyl red and thymol blue from pH 4.6 to 9.

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ON THE PREPARATION OF GALACTONIC LACTONE.

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(Received for publication, February 28, 1921.)

The process of preparation of galactonic lactone is still associated with many difficulties. When the calcium salt of galactonic acid is suspended in water and treated with an equivalent weight of oxalic acid, the filtrate on concentration forms a crystalline deposit which is neither the pure acid nor its lactone, but is a complex mixture of several forms of the acid and of several forms of the lactone. A detailed analysis of the crystalline material was made by Nef.[1]

Pure monomolecular form of lactone is prepared in the following way. The calcium salt is suspended in water, to which an equivalent weight of oxalic acid is added. The filtrate is concentrated under diminished pressure until a crystalline deposit begins to form in the flask. The deposit is redissolved by warming and the syrupy liquid is poured into glacial acetic acid.

The substance which crystallizes out is the hydrated form of the lactone. It analyzed as follows:

0.1099 gm. of air-dried substance lost, on drying under diminished pressure at temperature of water vapor, 0.0114 gm.

0.0985 gm. of the air-dried substance gave on combustion 0.0482 gm. of H_2O and 0.1464 gm. of CO_2.

	Calculated for ($C_6H_{10}O_6+H_2O$). *per cent*	Found. *per cent*
H_2O	10.9	10.38
	Calculated for ($C_6H_{10}O_6$). *per cent*	
C	40.40	40.53
H	5.62	5.47

[1] Nef, J. U., *Ann. Chem.*, 1914, cdiii, 273.

The practically anhydrous form is obtained by a single recrystallization of the original product from 99.5 per cent alcohol. This air-dried substance, on further drying under diminished pressure at the temperature of water vapor, lost but 2 per cent in weight. On titration with 0.1 N alkali, the fresh solution reacts alkaline to phenolphthalein after the addition of the first drop of alkali. When an excess of alkali is added the solution allowed to stand over night, and then titrated back with 0.1 N acid, 0.1000 gm. of the dry substance consumes 5.4 cc. of 0.1 N alkali. The theory requires 5.62 cc. of 0.1 N alkali.

This substance melts at 112° and gave the following optical rotation in water:

$$[\alpha]_D^{20} = \frac{-0.79 \times 100}{1 \times 1.0827} = -73.0°$$

after 96 hours

$$[\alpha]_D^{20} = \frac{-0.76 \times 100}{1 \times 1.0827} = -70.2°$$

after 16 days

$$[\alpha]_D^{20} = \frac{-0.69 \times 100}{1 \times 1.0827} = -63.7°$$

ANTISCORBUTIC POTENCY OF MILK POWDERS.*

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(Received for publication, February 15, 1921.)

The increasing use of milk powders in the home and hospital dietary makes mandatory the accumulation of as complete knowledge of their nutritive value as possible. Such knowledge is especially valuable when the dietary partakes of a restricted character as is the tendency in infant feeding. While it is evident that the method of preparation of milk powders is such as to avoid changes of intensive or extensive nature, yet from what is known of the lability of at least one constituent of milk, *viz.* the antiscorbutic vitamine, it appears very probable that the milk powders will vary considerably in their content of this indispensable constituent with the process of desiccation employed.

From what has been presented in a previous communication (1), it is evident that with fresh milk itself there is introduced a variable nutrient factor due to the fluctuations in its antiscorbutic value with the diet of the lactating animal, which has since been borne out by the work of Hess, Unger, and Supplee (2) and of Dutcher and coworkers (3). Evidently then in a study of the comparative effects of various desiccating processes on the antiscorbutic potency of milk, due consideration must be given to this second factor.

As far as we can learn there are three processes followed in this country of preparing these powders: (*a*) the Merrell-Soule or spray process; (*b*) a spray process in which the milk is not condensed before being dried and in which the powder is cooled and removed a few seconds after being dried; this process was developed at the California Central Creameries; (*c*) the Just process, involving

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the drying of the milk on heated rolls. While other processes or modifications of those mentioned above may be in use, our investigation was confined to the products known to be prepared by the above procedures.

In 1919 (4) we reported observations on a Merrell-Soule powder which did not protect against scurvy when fed to guinea pigs in daily quantities equivalent to 75 to 90 cc. of skimmed milk per individual. At about the same time Hess and Unger (5) reported the successful prevention of scurvy by the use of a milk powder dried by the Just process. From this it appeared very evident that at least one of the factors involved in the preservation of the antiscorbutic potency of milks when converted into powder form was the process of manufacture. There is no doubt that the other factor, namely the initial supply of this vitamine in the milk, is operative in the antiscorbutic vitamine content of the finished powder. Very recently Hess, Unger, and Supplee (2) have given us experimental proof of this fact. They showed that by the use of the Just process a milk powder with potent antiscorbutic properties could be prepared from a green pasture-produced milk, while a milk produced on a diet relatively low in the antiscorbutic vitamine yielded a powder, made by the same method of procedure, of no protective value to a guinea pig. They state that the milk was heated in the Just process for a few seconds to a temperature of 110°C.

EXPERIMENTAL.

In this work as previously, we have used the guinea pig as the experimental animal, feeding it a basal ration of heated alfalfa flour, rolled oats, and common salt, to which was added the milk powder to be studied. The alfalfa flour was heated in an autoclave at 15 pounds pressure for $\frac{1}{2}$ hour. Such a ration, with the milk powder displaced by casein, is an excellent scorbutic diet and it is not open to the objection that can be raised against scorbutic rations made from grains only or grains and some hay. These latter rations—so often used—are deficient in other nutritive factors, such as adequate proteins and adequate mineral matter and may, therefore, complicate the scurvy symptoms.

Usually in our ration the milk powder was used in the proportion of 40 per cent of the ration. With an intake per day of 18

to 20 gm. of the ration, this would mean a daily consumption of milk powder equivalent to an intake of 70 to 80 cc. of liquid milk. We have already shown (1) that milk produced on dry feeds will contain sufficient of the antiscorbutic vitamine to protect a

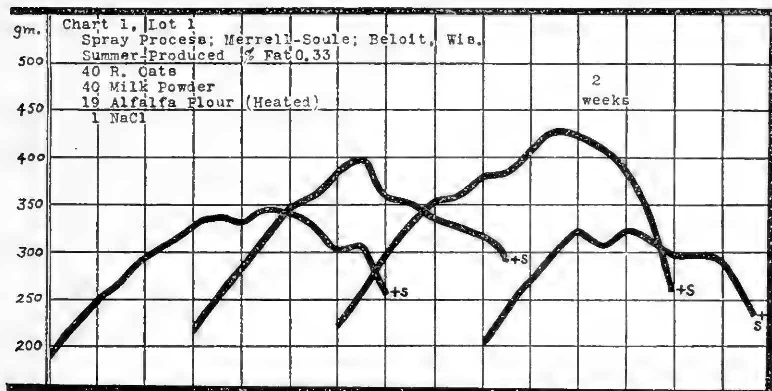

CHART 1. Failure of a skimmed milk powder made by the Merrell-Soule process from summer-produced milk to protect against scurvy.

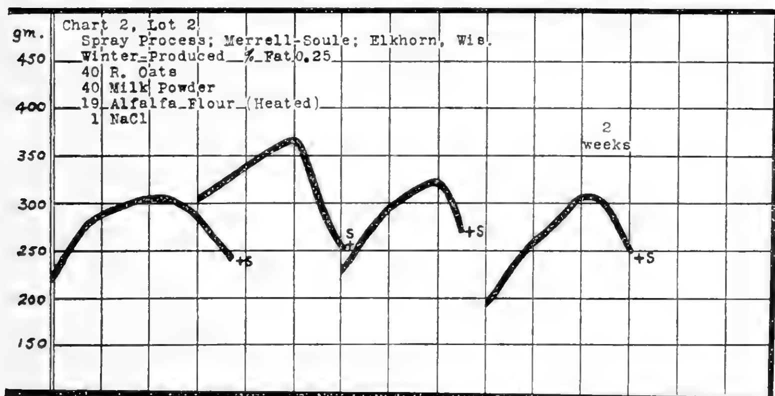

CHART 2. Another failure of skimmed milk powder made by the Merrell-Soule process to protect against scurvy. This was winter-produced milk.

guinea pig fully when allowed this amount per day. In some instances the proportion of milk powder in the ration was either raised or lowered for the purpose of determining more fully the comparative antiscorbutic potency of a particular powder.

In Charts 1 and 2 are shown results secured with powder made by the Merrell-Soule process.[1] The powder made from summer-produced milk (Chart 1) no doubt contained some of the antiscorbutic vitamine, but not in sufficient quantity to give adequate protection against the development of the disease. A winter-produced milk converted into powder by the Merrell-Soule process was less protective against scurvy than the summer-produced milk (Chart 2).

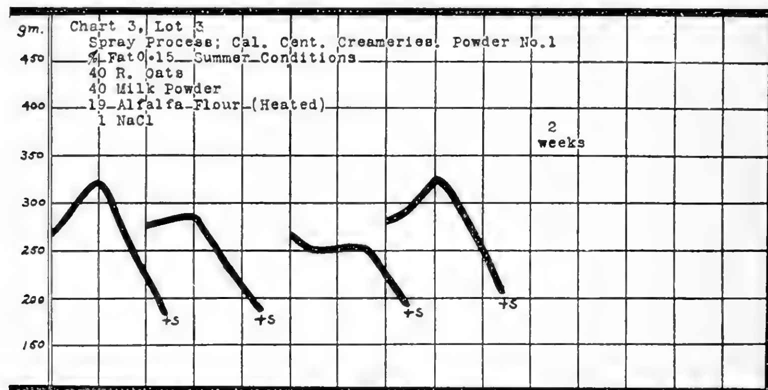

CHART 3. Failure of a skimmed milk powder made by the California Central Creameries spray process to protect against scurvy. Green grass was available in the production of this milk, which was pasteurized at 80°C. before being dried.

In Charts 3 to 7, inclusive, are given the results secured with the spray process as developed at the California Central Creameries, San Francisco. These milk powders were made from milks produced under northern California conditions where the cows received some green grass the entire year, and in addition, beets, carrots, and a grain mixture.[2] Powder 1, Chart 3, had been pre-

[1] These powders were kindly furnished us by The Sturtevant, Wright, and Wagner Company, Beloit, Wisconsin, and by the Wisconsin Dairy and Cheese Company, Elkhorn, Wisconsin. Our thanks are due these firms for their courtesy.

[2] These powders with descriptive matter were kindly furnished us by Mr. C. E. Gray of the California Central Creameries, San Francisco. Our thanks are due him for this courtesy.

pared from milk pasteurized at 80°C. for a few seconds, and after desiccation the products had been cooled and removed from the drying chamber within a few seconds. The desiccating tempera-

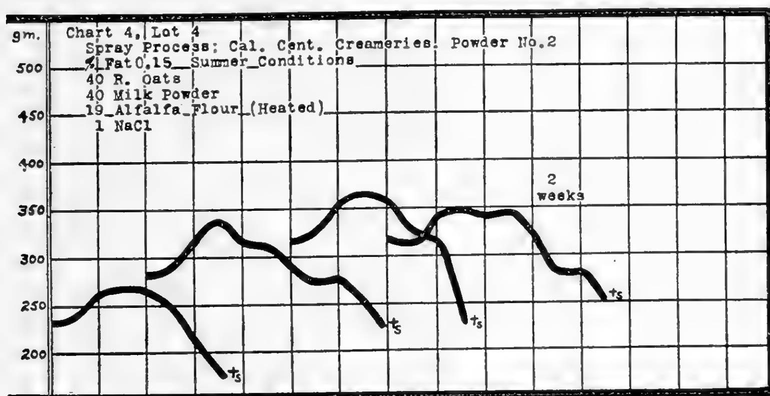

CHART 4. Failure of a skimmed milk powder made by the California Central Creameries spray process to protect against scurvy. The milk was pasteurized at 62.5°C. for 25 minutes before being dried. This milk was also produced where some green grass was available.

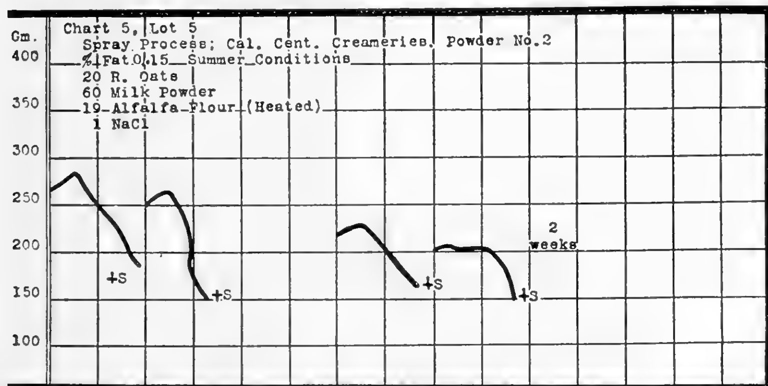

CHART 5. Another failure to protect against scurvy with the same milk powder as used with Lot 4, Chart 4, but fed at a higher level.

ture unfortunately was not available in the records. This powder had lost its antiscorbutic properties.

Powder 2, Chart 4, was pasteurized at 62.5°C. for 25 minutes before drying. It also had little protective value in the pro-

portion used in our ration. When we increased the proportion of this powder in the ration from 40 per cent to 60 per cent (Chart 5) the results were not different from those secured on the 40 per

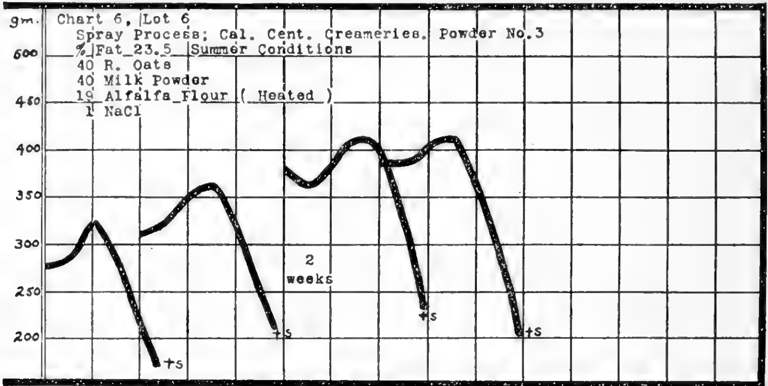

CHART 6. Failure of a whole milk powder made by the California Central Creameries spray process to protect against scurvy. This milk was pasteurized at 62.5°C. for 25 minutes.

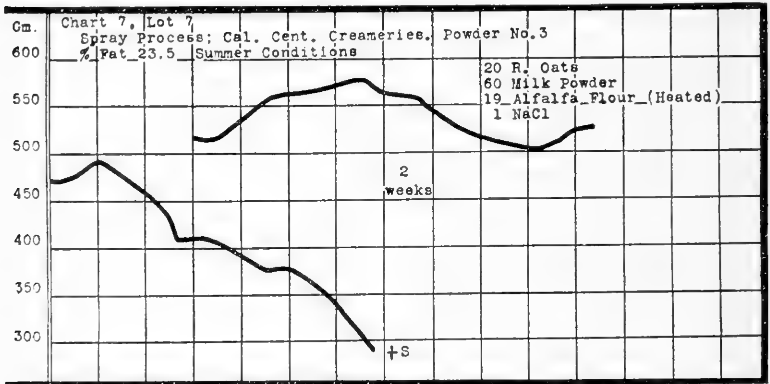

CHART 7. The use of a higher level of the same milk powder as used with Lot 6, Chart 6. This powder still retained some of the antiscorbutic vitamine but not enough, even at this high level of feeding, to protect all individuals.

cent level. Both the above powders were skimmed milk powders. A whole milk powder made by the California Central Creameries process was likewise impotent with respect to its ability to

protect against scurvy. In Chart 6 are shown the results secured with a whole milk powder made from a milk which was pasteurized at 62.5° for 25 minutes and then dried without further treat-

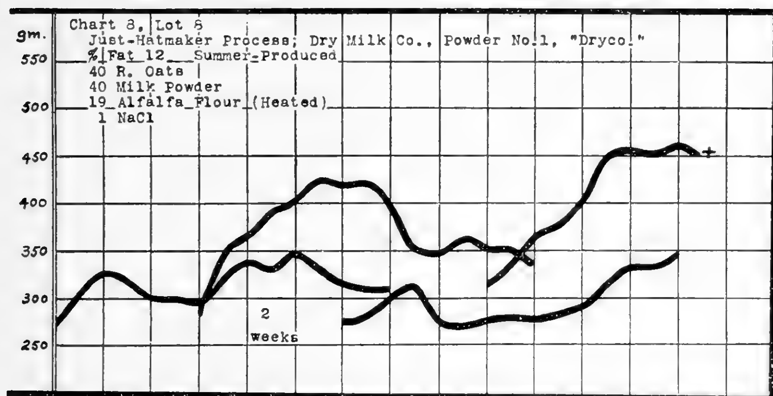

Chart 8. Full protection against scurvy was offered by this milk powder containing 12 per cent of fat and made by the Just process. One animal died from unknown causes at the end of 9 weeks restriction to this ration.

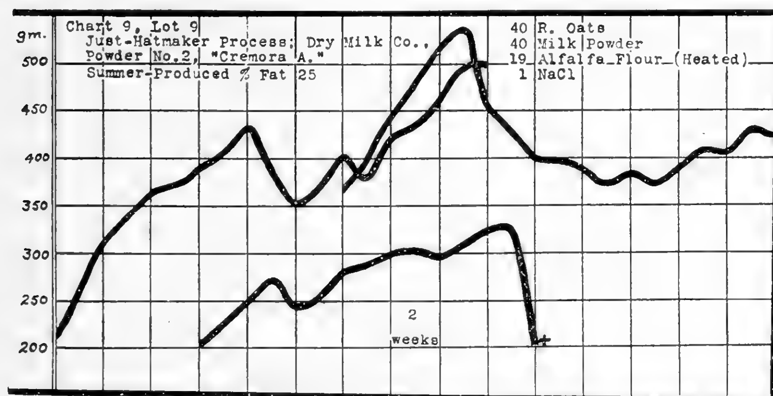

Chart 9. Protection against scurvy by a whole milk powder made from a summer-produced milk and prepared by the Just process.

ment. With this powder as 40 per cent of the ration, all the animals succumbed to scurvy in 5 to 6 weeks. When fed at a 60 per cent level (Chart 7) one animal was protected against scurvy for 17 weeks, while the other animal developed symptoms

early and slowly declined. Evidently there was some of the antiscorbutic factor left in this milk powder.

Milk powders made by the Just process[3] (roll process) are as a class more effective antiscorbutics than those made by the spray

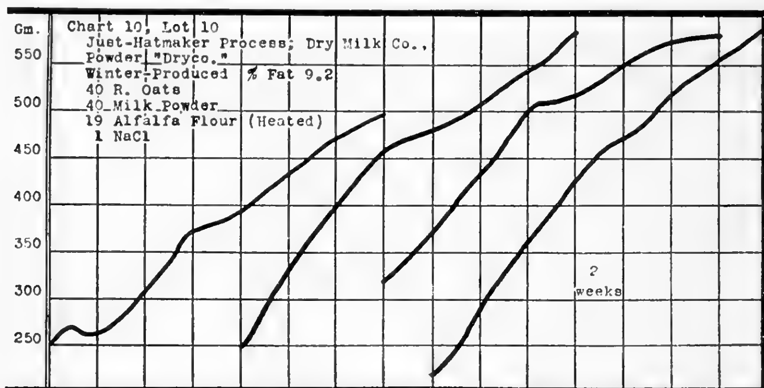

CHART 10. This chart illustrates the antiscorbutic efficiency of a winter milk converted to powder by the Just process and fed in quantities equivalent to 75 to 80 cc. of liquid milk per individual per day. This powder contained 9.2 per cent of fat.

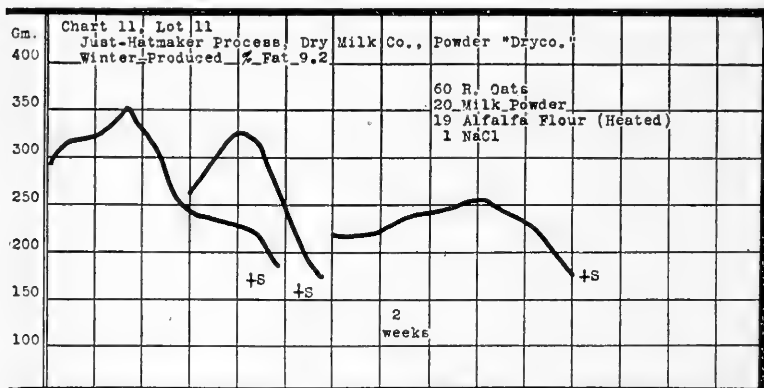

CHART 11. A reduction of the quantity of milk powder used with Lot 10, Chart 10, to a liquid milk intake of from 35 to 40 cc. per individual per day resulted in the production of scurvy.

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[3] These powders were kindly furnished by the Dry Milk Company, New York. Our thanks are due them for this courtesy.

processes. Charts 8 to 12, inclusive, illustrate this fact. Even these powders may not always be effective antiscorbutics, as for example, the sample of "Milcora" brand made from winter-produced milk. This was a skimmed milk powder (Chart 12). Other powders made by the Just process were excellent protectives against scurvy, as for example, those illustrated in Charts 8 and 9. A powder manufactured by the Just process from winter-produced milk (Chart 10) and containing 9.2 per cent of fat possessed excellent antiscorbutic properties. If we contrast this result with the result secured with the skimmed milk brand "Milcora" (Chart 12), we are led to raise the question as to the possible rôle of fat

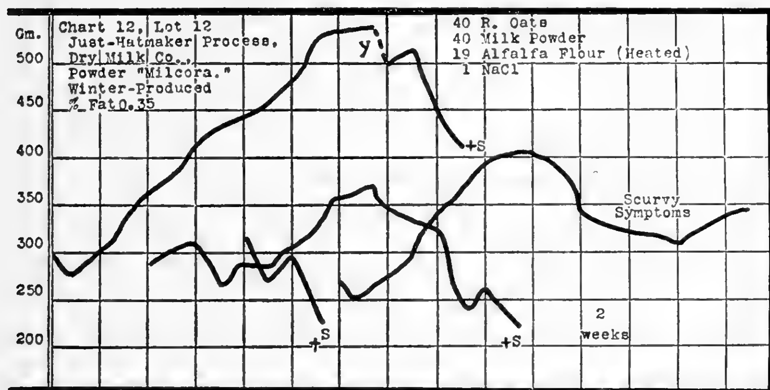

CHART 12. This winter-produced skimmed milk, converted to a powder by the Just process, had lost in a considerable measure its antiscorbutic properties. In most cases the onset of scurvy was greatly delayed The powder contained 0.35 per cent of fat.

as a protective agent against the destruction of the antiscorbutic in the drying processes. However, it is true that in the spray process (California Central Creameries) the fat did not become a factor in lessening the destruction of the antiscorbutic vitamine during the processing (see Chart 6).

SUMMARY.

1. Milk powders vary in their antiscorbutic properties. Aside from the factor of the initial quantity of this vitamine in the milk as influenced by feed, the powders vary in their potency with the

process used in their manufacture, the spray processes of manufacture being more destructive of the antiscorbutic vitamine than the Just process.

2. These results should in no way condemn the milk powders made by spray processes. They only point out their limitations when used as the sole source of nutrients in infant feeding.

3. Probably with all milk powders, irrespective of method of manufacture, the safest procedure in a restricted dietary, particularly in infant feeding, is to supplement them with some potent source of the antiscorbutic vitamine. A possible exception to this statement would apply to the powders made by the Just process from summer-produced milks or even winter-produced milks where the cow's ration is made rich in the antiscorbutic vitamine by the proper selection of roots and tubers.

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THE RELATION OF LACTIC ACID BACTERIA TO CORN SILAGE.*

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Recently there have appeared many reports concerning the effect of inoculation on corn silage (1). In general, these reports claim that the seeding of corn fodder with certain bacteria causes the formation of a higher grade product. According to some of the statements inoculation with selected lactic acid bacteria prevents spoilage to a large degree, reduces alcohol formation, and improves flavor and palatability. Although much has been written concerning the effect of bacteria on ensiled corn a review of the reports shows but little data to support most of the statements.

No doubt the seeding of corn fodder with certain of the lactic acid bacteria will hasten fermentation processes, but that this is of any advantage to the farmer is not yet established. As a rule, properly ensiled corn keeps well and is ready to feed within about 2 weeks. Spoiling of corn silage except in the upper layer exposed to the air almost never occurs. The loss in this way is small and depends to a large degree on the moisture of the silage and on how well it is packed.

From the results of preliminary tests Gorini concluded that the seeding of corn forage with lactic acid bacteria improves the keeping qualities of the silage and that the different types act differently upon silage. He emphasized the importance of these bacteria in the making of good silage, and suggested the use of selected lactic acid bacteria for certain types of forage.

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The experiments described in this paper represent an attempt to determine the effect of inoculation on the composition of silage together with a study of the normal flora of green corn and of silage. Concerning the mechanism of silage fermentations there are at present many different views (2). Some investigators look upon the plant cell or enzymes as the chief agents, while others attribute to bacteria the principal rôle in the formation of silage. It may be safely stated that accumulating data tend more and more to indicate the dominant influence of bacteria in the production of silage.

EXPERIMENTAL.

The silage was made from corn grown on the University Farm and though the ears were well matured the leaves and stalks were still green. The corn was cut by a large power cutter into small pieces and packed tightly into the silo by continuous tramping.

For each ton of green fodder 5 liters of a 48 hour culture were used. These cultures were made by growing the desired organism in yeast water, and were diluted, and sprinkled over the green fodder as it was packed in the silo. The lactic acid-producing bacteria used were in one case the organisms of the *Lactobacillus pentoaceticus* group, and in another, a mixed culture of *Bacillus lactis acidi* and *Bacillus bulgaricus*.

The first series of experiments was set up as follows: one large silo was filled with uninoculated corn fodder as a control, and two tanks about 8 feet high and 4 feet in diameter were filled with inoculated corn fodder. Although the conditions in the tanks were not exactly comparable with those in the large silo, it was found that the silage from these tanks had the general appearance, odor, and taste of normal silage.

Samples of 10 to 20 pounds were removed for analysis at intervals of 12 to 65 days after inoculation. To obtain representative samples the first silage for analysis was taken at a total depth of about 3 feet, or at least more than 1 foot below the zone of spoiled silage at the top. Just as soon as the sample was obtained the top silage that had been removed was returned to its proper container and tightly repacked. In this way it was hoped to prevent as much as possible the passage of air into the fermenting

silage. Samples for subsequent analysis were drawn from greater depths, always well below the level at which the previous sample had been taken.

The silage was ground in a meat chopper, a uniform sample of the ground tissue was taken for the moisture determination, and the juice was expressed from the remainder with a strong hand press. When 400 cc. of this juice were expressed, a portion of it was diluted with sterilized water, and plated with glucose-yeast water agar. Attempts to estimate the bacterial flora by direct microscopic count failed to give satisfactory results. The remaining juice was used in the determination of volatile acid, non-volatile acid, and alcohol.

The treatment of the corn fodder and the results of the chemical and bacteriological analyses at different times are given in Table I. An examination of this table shows that inoculation of corn fodder with lactic acid-producing bacteria regardless of types of bacteria results in a slightly lowered production of volatile acid. This decrease in volatile acid as compared with that of the control is noted with the lactic acid bacteria in all three analyses.

It is in the horizontal columns of this table, Nos. 4, 5, and 6, non-volatile acid, that the effect of inoculation is most clearly shown. The culture of *Lactobacillus pentoaceticus* retards the production of non-volatile acid while the mixed culture of lactic acid organisms favors this process. The analyses after 12, 28, and 54 days show a high lactic acid content of the silage inoculated with the mixed culture as compared with that of the control. Except in the very early stages of fermentation, the silage inoculated with *Lactobacillus pentoaceticus* is lower in total non-volatile acid than the control. It will be seen from Nos. 7, 8, and 9 that inoculation brings about a change in the alcohol content of silage. The *Lactobacillus pentoaceticus* organisms favor alcohol production, while the mixed cultures of lactobacilli tend to retard alcohol formation.

Although the moisture content of the green corn was somewhat lower than is commonly found in silage, the fermentation processes proceeded rapidly. The chief point of interest in these determinations is the gradual increase in moisture as the silage becomes older.

TABLE 1.

Effect of Inoculation on the Composition of Silage from Large Containers.

No.	Products.	Time after inoculation.	Untreated control.	Inoculated with *L. pentoaceticus.*	Inoculated with *B. lactis acidi* and *B bulgaricus.*
		days			
1	Volatile acid as acetic, *per cent of dry silage*	12	0.930	0.721	0.650
2	" " " " " " " " "	28	0.926	0.876	0.674
3	" " " " " " " " "	54	1.235	1.046	1.000
4	Non-volatile acid as lactic, *per cent of dry silage*	12	1.980	2.702	4.199
5	" " " " " " " " "	28	5.360	4.948	6.491
6	" " " " " " " " "	54	6.857	5.904	7.566
7	Alcohol as ethyl, *per cent of dry silage*	12	0.830	0.670	0.520
8	" " " " " " " "	28	0.630	0.684	0.526
9	" " " " " " " "	54	0.764	0.973	0.650
10	Moisture, *per cent of moist silage*	12	65.85	58.62	60.48
11	" " " " " "	28	67.07	61.80	62.68
12	" " " " " "	54	67.13	62.14	63.47
13	Bacteria in 1 gm. of dry silage	12	81,400,000	81,400,000	323,000,000
14	" " 1 " " " "	28	8,160,000	97,000,000	487,400,000
15	" " 1 " " " "	54	1,080,000	86,660,000	161,500,000

For additional work with inoculated silage, barrels of 50 gallon capacity were filled with some of the same corn fodder as was used in the other experiments. To make the barrels air-tight and also to prevent any absorption of the juice by the wooden staves they were coated on the inside with paraffin. In these miniature silos it was planned to study the types of fermentation and to compare the silage in the barrels with that in the large silo. The cut corn was inoculated uniformly and packed into the barrels. The tops of the barrels were sealed with a layer of paraffin 2 inches thick, and, except for lack of pressure, the conditions in these miniature silos were similar to those in the large silo. The barrel series was treated as indicated below:

1. Control, no inoculation.
2. Inoculated with *Lactobacillus pentoaceticus*.
3. Inoculated with *Bacillus lactis acidi* and *Bacillus bulgaricus*.
4. Inoculated with *Lactobacillus pentoaceticus*, *Bacillus lactis acidi*, and *Bacillus bulgaricus*.

In appearance and odor this silage resembled that of the large silo. The inoculated silage gave a more pleasant aroma than the uninoculated. The samples of silage were analyzed as previously described. The results of the chemical and bacteriological analyses are given in Table II.

The silage in these barrels on analysis showed approximately the same products as those found in the larger containers. Apparently the lack of pressure did not result in a great change in the chemical composition of the silage. From the figures of the table it is clear that the bacteria are present in great numbers as late as 68 days. The seeding of silage with selected bacteria of the lactic acid group influences the silage in many ways. Except in one case that of silage with *Lactobacillus pentoaceticus* 21 days, the inoculated silage showed a greater number of bacteria than the control.

The results of the chemical analyses agree in general with those reported in Table I. There is a slight increase in volatile acid in the corn inoculated with *Lactobacillus pentoaceticus;* with lactobacilli, and lactobacilli plus *Lactobacillus pentoaceticus*, there is little if any difference. There is no well defined difference in the amount of the non-volatile acid in the treated and untreated corn. In the corn inoculated with the mixed culture of lacto-

TABLE II.

Effect of Inoculation on the Composition of Silage from Barrels.

No.	Products.	Time after inoculation.	Untreated.	Inoculated.		
			Control.	*L. pentoaceticus.*	*B. lactis acidi* and *B. bulgaricus.*	*B. lactis acidi, B. bulgaricus,* and *L. pentoaceticus.*
		days				
1	Volatile acid as acetic, *per cent of dry silage*	21	1.081	1.218	1.166	1.130
2	" " " " " " " " "	47	0.858	1.296	0.923	1.113
3	" " " " " " " " "	68	0.973	1.375	1.106	1.151
4	Non-volatile acid as lactic, *per cent of dry silage*	21	4.848	5.357	6.226	3.115
5	" " " " " " " " "	47	5.328	6.549	6.019	5.989
6	" " " " " " " " "	68	6.180	6.362	6.783	5.980
7	Alcohol as ethyl, *per cent of dry silage*	21	0.881	0.733	0.934	0.655
8	" " " " " " " "	47	0.726	1.138		0.892
9	" " " " " " " "	68	0.645	1.047		0.889
10	Moisture, *per cent of moist silage*	21	61.00	66.50	65.00	64.85
11	" " " " " "	47	62.29	67.12	63.21	64.07
12	" " " " " "	68	62.88	67.01	63.55	61.97
13	Bacteria in 1 gm. of dry silage	21	256,000,000	240,000,000	353,000,000	
14	" " 1 " " " "	47	173,500,000	247,000,000		440,400,000
15	" " 1 " " " "	68	189,750,000	272,200,000	327,750,000	660,000,000

bacilli there is a somewhat greater amount of lactic acid. It is in the analyses for alcohol that the effect of inoculation is most marked; the *Lactobacillus pentoaceticus* series shows the highest alcohol content and the mixed culture of *Lactobacillus pentoaceticus* and lactobacilli rank next in order. It has been noted (3) that *Lactobacillus pentoaceticus* ferments hexose sugars with the production of considerable amounts of alcohol and acids, and the pentose sugars with volatile acid but no alcohol; the lactobacilli on the other hand do not ferment pentoses to any extent and do not produce appreciable amounts of alcohol or volatile acid from hexoses.

Bacteriological Analyses.

1 cc. of the juice expressed as described above was diluted and plated with 1 per cent glucose-yeast water agar. After several agar media were tested, *e.g.* nutrient, litmus-lactose, and Nährstoff-Heyden agars, it was found that 1 per cent glucose-yeast water agar pH 6.8 gave the highest count and apparently the greatest number of different types of colonies. All plates were incubated at 28°C. for 1 week. Although 37°C. has been used by various investigators it was found that plates incubated at 28°C. gave greater numbers and a greater variety of colonies.

To gain some idea of the fate of the organisms normally present on corn when ensiled, examinations were made of the types of bacteria on plates from the green corn and from the silage at different stages in the fermentation. The colonies on the plates were studied as to structure and form, and the different types picked off into litmus milk and into a measured amount of 1 per cent xylose-yeast water. The percentage of the various types of colonies on the plates was noted in all cases. The litmus milk cultures were examined regularly; and after 10 days at 28°C. the xylose-yeast water cultures were titrated with 0.1 N barium hydroxide. Stained mounts were made from the xylose-yeast water cultures after they were neutralized, and also from the litmus milk. Whenever an organism produced a characteristic colony on the plate and high acid in the xylose-yeast water, and showed the characteristic bacillus form in the stained mount, it was classed as one of the *Lactobacillus pentoaceticus* type. In

glucose-yeast water agar the colony is deep, small, lance-shaped, yellowish, and opaque. The edge is usually smooth and uniform, but sometimes has a woolly appearance. The organism is a Gram-positive, blunt-ended rod, which does not form spores. It is a fairly large organism, 0.6 to 0.7μ wide and 1.6 to 3.0μ long under different conditions. It occurs singly or in short filaments, with a characteristic grouping in bundles of parallel organisms.

The results of the plate counts show that the corn at the time it was placed in the silo had about 86,000,000 bacteria in 1 gm. of dry tissue, while there were 81,400,000 in 1 gm. of dry silage after 12 days. 16 days later the total number of bacteria had decreased to 8,160,000, and after 54 days there were only 1,080,000. This rapid decrease in the bacterial flora is in agreement with the findings of earlier investigators. Counts made under similar conditions have shown that within 1 to 3 days after ensiling there is an enormous increase in the total number of bacteria followed by a rapid decrease.

After 12 days the count in the inoculated silage had reached a figure at which it remained practically constant for 53 days instead of steadily decreasing as in the count on the uninoculated silage. The increase in numbers usually noted at first is largely due to the presence of a large supply of fermentable carbohydrates. After the consumption of most of this available food and after the production of acid, unfavorable conditions for the growth of many types of organisms are produced. The bacteriological examination indicates that the fermentation activity is soon narrowed down to a few aciduric types, with a consequent reduction in total numbers as the other types rapidly disappear.

The bacterial flora of the fresh green corn was noteworthy in respect to variety of forms, but most of these organisms persisted for only a short time. There were very few organisms that produced acid from lactose; and none was found that produced high acid from xylose broth. After 12 days the *Lactobacillus pentoaceticus* type in particular had begun to assume a prominent place in the flora. Inoculation with *Lactobacillus pentoaceticus* apparently caused this type of organism to predominate earlier with a greater percentage of the type at the final examination than in the silage from the other silos. In both inoculated and

uninoculated silage the final examination after 8 or 10 weeks shows that the *Lactobacillus pentoaceticus* type formed 50 per cent or more of the flora. Accompanying it was found a short round-ended rod, often in pairs, which resembled *Bacillus lactis acidi*, but was iridescent on a glucose agar slant. *Lactobacillus pentoaceticus* and this short rod both gave a negative test for catalase, indicating that they belong to the lactic group.

SUMMARY.

An examination of the bacteriological and chemical analyses of inoculated and uninoculated silage indicates:

1. That the *Lactobacillus pentoaceticus* type plays an important part in the chemical changes produced. These organisms are present throughout the fermentation and in the last stages are the predominant type. An increase in *Lactobacillus pentoaceticus* means an increase in alcohol and volatile acid with a decreased amount of lactic acid.

2. That the organisms of the *Bacillus lactis acidi* type persist and act only during the first days of the fermentation.

3. That the production of alcohol is undoubtedly due in part to the action of the predominant pentose-fermenting type of lactic organism.

4. That inoculation may hasten and intensify the production of certain products during the early stages of the fermentation, but that in the later stages both chemical and bacteriological analyses of all inoculated and uninoculated silage show approximately the same chemical composition and the same kinds of microorganisms.

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THE DESTRUCTION OF PENTOSANS IN THE FORMATION OF SILAGE.*

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The formation of silage is attended by a considerable loss of dry matter. The extent of this loss has been determined at a number of experiment stations and ranges from 10 to 30 per cent, depending on such factors as variety, maturity, moisture content, and packing of the corn, and size and construction of the silo. In the majority of cases the loss has been more than 15 and less than 20 cent. The results of 8 years work at three experiment stations, Wisconsin (1), Vermont (2), and Pennsylvania (3), give an average loss of 18.4 per cent. Under the best conditions attainable, King (4) found an average loss of 6.38 per cent in a silo lined with galvanized iron.

The part of the dry matter upon which the heaviest loss falls is the nitrogen-free extract. In the reports of the experiment stations already quoted the average loss in nitrogen-free extract varied from 10 to 42 per cent. The loss of nitrogen-free extract results mainly from the destruction of the sugars by fermentation processes. Since the sugars do not as a rule comprise more than 15 per cent of the nitrogen-free extract a loss of 20 to 30 per cent must involve a destruction of other carbohydrates, such as starch, pentosans, or celluloses.

The only data on the destruction of pentosans in corn silage that have been found in the literature are those recorded by Russell (5), who reported a loss of 32 per cent of the furfural-yielding

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substances and 55 per cent of the nitrogen-free extract. From this figure it appears that pentosans are readily destroyed in silage fermentation.

In a recent paper on the starch content of experimental silage Dox and Yoder (6) report that "the starch content remains constant throughout the fermentation." It is not apparent from this paper whether the loss in dry matter was considered in calculating the starch content of the silage during the different stages of fermentation. A loss of dry matter and of starch might occur in such proportions that the percentage of starch in the residual material is the same as that of the original corn forage. Additional evidence that starch is not attacked was found in the unchanged physical appearance of the starch granules when examined under the microscope.

A comparison of the dry matter of corn fodder with that of silage shows that the products of fermentation, volatile at 100–105°C., are included in the losses. Volatile acids, alcohols, esters, and ammonia are the chief compounds of this type. A part of the loss in dry matter is accounted for by the carbon dioxide evolved during silage fermentation. The quantity of carbon dioxide produced has been determined in but few cases. From silage made in the laboratory, Lamb (7) found from 0.315 to 0.508 per cent of carbon dioxide produced in 7 days. From sterilized corn fodder inoculated with pentose-fermenting bacteria Peterson and Fred (8) obtained from 0.88 to 1.09 per cent of carbon dioxide in 21 day silage. Neidig (9) found carbon dioxide to predominate in the gases present in a silo, but no quantitative measurement of the amount of carbon dioxide evolved can be obtained from his data.

Since the production of silage is probably in large part the result of bacterial action it is reasonable to expect a considerable destruction of pentosans. The destruction of pentosans in the intestinal tract of farm animals has been found by different investigators to be a matter of considerable amount. Figures ranging from 50 to 100 per cent have been reported at various times. On a ration made up entirely from the corn plant, McCollum and Brannon (10) found a destruction of 66.8 per cent. Many of the same kinds of microorganisms found in the digestive tract of herbivora are also found in silage and it is not improbable that in

both places they are the chief agents in the destruction of pentosans. Likewise the rapid destruction of pentosans in the decay of vegetable matter in nature suggests the occurrence of somewhat similar processes in the fermentation of silage.

EXPERIMENTAL.

The silage analyzed was made in several different ways. In the first series it was made in a large silo under the usual conditions and this was taken as a control. Two galvanized tanks 8 feet high and about 4 feet in diameter were filled at the same time and with the same kind of corn fodder as the large silo. One of these tanks was inoculated with the pentose-fermenting bacteria, *Lactobacillus pentoaceticus*, whose activities and products have been previously described (11), and the second tank was inoculated with a mixture of lactic acid bacteria, *Bacillus lactis acidi* and *Bacillus bulgaricus*.

In a second series filled at the same time as the silo and tanks, a number of barrels of 50 gallon capacity were used. The top of the silage was covered with cloth and about 2 inches of paraffin to exclude the air as much as possible. A third series with milk bottles as containers was set up in the laboratory at the same time as the tank and barrel series. The results of the chemical and bacteriological analyses are incorporated in the preceding paper (12) to which reference is made for these data.

Samples of the silage formed in the tank and barrel series were taken and analyzed at different times during the fermentation. About 10 pounds were taken at each sampling, dried at 60–70°C., ground in a mill, and a subsample of this ground to a fine powder. This powder was dried to constant weight at 105°C. and a suitable weight, about 1 gm., was used for distillation with hydrochloric acid, as described in the Kröber method (13) for the determination of pentosans. With the above methods of sampling, grinding, and drying, duplicate determinations checking to within 5 mg. of phloroglucide were obtained without difficulty. If a difference greater than 5 mg. between duplicates was found, the determination was repeated. In a number of determinations the phloroglucide was extracted with 95 per cent alcohol at 60°C. to determine the presence of methyl pentosans but little or no decrease in the

weight of phloroglucide was obtained. The analyses of the silage from the control silo and from the tanks (Series 1) were made at successive intervals and the data obtained are given in Table I. These data show a decrease in the pentosan content of the silage after 12 days of from 1.3 to 4.1 per cent. The figures at the end of 28 and 54 days show approximately the same results with the exception of the large silo. Here the pentosan content was highest at the time of the last analysis. As corn from different fields

TABLE I.

Pentosans in Silage at Various Times During the Fermentation.

Treatment.	Age of silage.	Calculated on the dry basis.	
		Water-soluble substances yielding furfuraldehyde, calculated as pentosans.	Total pentosans.
	days	*per cent*	*per cent*
Untreated corn	0	0.30	21.8*
Uninoculated silage	12	0.50	17.7
Inoculated with *L. pentoaceticus*	12	0.49	20.5
" " lactobacilli†	12	0.63	19.4
Uninoculated silage	28	0.74	18.4
Inoculated with *L. pentoaceticus*	28	0.53	20.1
" " lactobacilli	28	0.73	18.7
Uninoculated silage	54	1.22	21.3
Inoculated with *L. pentoaceticus*	54	0.62	20.9
" " lactobacilli	54	0.90	19.9

* Average of five determinations ranging from 21.24 to 22.32 per cent.

† A mixed culture of *B. lactis acidi* and *B. bulgaricus*.

was used in filling the large silo it is possible that this sample which was taken about 15 feet below the top of the silo represents a different lot of corn from that at the top.

No such uncertainty enters into the first two analyses or into the analysis of the tank, barrel, and bottle silage. All these were filled from the same lot of corn. The decrease of 1.3 to 4.1 per cent of pentosan does not represent the entire loss of pentosans as no account of the loss in dry matter is considered in calculating these figures. The actual loss of pentosans for all the series will be considered at the end of this paper in connection with Table V.

It is noteworthy that a small but fairly constant amount of water-soluble pentosans is present at each time of analysis. This indicates a slow hydrolysis of the pentosans, either by the bacteria in the silage, or by the acids formed in the fermentation of

TABLE II.

Pentosans in Barrel Silage Analyzed at Successive Intervals during the Fermentation.

Treatment.	Age of silage.	Calculated on the dry basis.	
		Water-soluble substances yielding furfuraldehyde, calculated as pentosans.	Total pentosans.
	days	*per cent*	*per cent*
Untreated corn	0	0.30	21.8
Uninoculated silage, Control 1	21	0.85	21.2
" " " 2	21	0.92	20.9
Inoculated with *L. pentoaceticus*	21	0.53	18.3
" " lactobacilli	21	0.76	18.3
" " *L. pentoaceticus* and lactobacilli	21	0.56	20.3
Uninoculated, Control 1	47	0.85	20.9
" " 2	47	0.76	19.4
Inoculated with *L. pentoaceticus*	47	0.50	18.3
" " lactobacilli	47	0.73	16.2
" " *L. pentoaceticus* and lactobacilli	47	0.62	17.6
Uninoculated, Control 1	66	0.84	22.3
" " 2	66	0.80	20.0
Inoculated with *L. pentoaceticus*	66	0.55	19.2
" " lactobacilli	66	0.75	18.8
" " *L. pentoaceticus* and lactobacilli	66	0.56	19.6

the sugars. In both the tank and barrel series, the silage inoculated with the pentose fermenter shows at all times the lowest percentage of water-soluble pentosans.

In the barrel series the analyses at different times may properly be compared as all the silage was made from the same lot of corn. The inoculated and uninoculated barrels are also comparable

since all conditions except that of inoculation were the same. The data show a smaller percentage of pentosans in the silage than in the corn fodder. This decrease is greater at the end of 47 days than at the time of the first analyses. The percentage of pentosans dropped from 21.8 in the corn fodder to as low as 16.2 in the 47 day barrel, inoculated with the lactic acid bacteria. An actual decrease is shown in all the silage irrespective of inoculation but the greatest decrease took place in the inoculated silage.

The small amount of water-soluble pentosans noted in connection with the tank silage is again apparent in the barrel series. The smallest amount is present in the silage inoculated with the pentose fermenter. This result might be expected as it is a rapid fermenter of xylose. As shown by bacteriological analysis pentose-fermenting organisms are also present in the other barrels, but probably not to the same extent. The data are given in Table II.

The Pentosan Content of Silage Made in Milk Bottles.

At the time that the tank and barrel series were set up a number of quart milk bottles were filled from the same batch of corn

TABLE III.

Pentosans in Silage Made in Bottles.

Treatment.	Age of silage.	Calculated on the dry basis.	
		Water-soluble substances yielding furfuraldehyde, calculated as pentosans.	Total pentosans.
	days	*per cent*	*per cent*
Untreated control	41	0.42	21.8
Inoculated with *L. pentoaceticus*	41	0.49	21.9
" " lactobacilli	41	0.52	21.6
" " *L. pentoaceticus* and lactobacilli	41	0.54	20.8

fodder as was used in filling the tanks and barrels. These were closed with one-hole rubber stoppers and Bunsen valves.

At the end of 41 days the bottles were opened and disclosed a strong butyric acid fermentation. This is not the normal type of fermentation in silage so the data are hardly comparable with

the data obtained in the tank and barrel series. The percentage of pentosans in the silage is almost exactly the same as that of the original corn fodder. In view of the extensive fermentation that had taken place these high results are best explained on the assumption that the pentosans were more resistant to the action of the bacteria than some of the other plant constituents and hence increased on a percentage basis. A similar effect was noted in the barrel series where the percentage of pentosans is higher at the time of the third analysis than at the time of the second analysis. The data are given in Table III.

The Water-Soluble Pentosans of Corn Forage and Silage.

As has already been noted there is present in corn forage and in silage a small amount of material which is soluble in water, and gives furfural on distillation with hydrochloric acid. In the

TABLE IV.

Pentosans Extracted from Corn Forage by Water and Diluted Acids.

Treatment.	Solvent.	Time of extraction.	Pentosans calculated on dry basis.
			per cent
Unsterilized.	Water.	3 hrs. at 20°C.	0.64
Sterilized.	"	3 " " 20 "	0.57
"	Acids and toluene.	20 days " 28 "	0.69
"	Water.	20 " " 28 "	0.69
"	Acids.	20 " " 28 "	0.73

calculations these furfural-yielding substances are expressed as pentosans although some of these substances are probably present in the fodder and silage as free pentoses. A sample of very green corn analyzed for pentoses according to the method of Davis and Sawyer (14) gave 0.62 per cent in the stalk and 0.60 per cent in the immature cob. The silage in all three series contained more of this water-soluble substance at each analysis than was found in the corn forage. The persistence of this water-soluble material suggests a slow hydrolysis of the pentosans either by the action of the bacteria or by the acids present in the silage. An attempt was made to determine if the degree of acidity present in silage is sufficient to hydrolyze the pentosans.

Dried samples of the corn forage used in making the silage were sterilized and then subjected to the action of a solution containing 2 per cent acetic acid and 5 per cent lactic acid at different temperatures for 20 days. The samples were then analyzed for soluble pentosans, but no appreciable increase in the amount of soluble pentosans was found. From these data it appears that the hydrolysis of pentosans and the maintenance of pentoses in the silage were due to the action of microorganisms. The data are given in Table IV.

The Percentage of Methyl Pentosans in Corn Fodder and Silage.

The precipitate of phloroglucide was extracted with 95 per cent alcohol according to the method of Ellett and Tollens (15) for the determination of methyl pentosans. In twenty-five determinations of total pentosans the decrease in the weight of the phloroglucide (about 0.200 gm.) varied from 0.000 to 0.014 gm. with an average of 0.0034 gm. These determinations were made on 1 gm. of dried material and therefore indicate the presence of about 0.34 per cent of methyl pentosans in corn fodder and silage. The water-soluble pentosans likewise showed no departure from the above figures.

Loss of Pentosans Calculated on the Basis of the Original Corn Fodder.

If the data found in Tables I and II are recalculated to the original corn fodder a higher figure for the loss in pentosans is obtained. It is assumed that this may properly be done since there is unquestionably a loss in dry matter during the process of fermentation. As has already been pointed out, this loss varies from 10 to 30 per cent. In a large silo this loss according to King (4) would be about 10 per cent while in small tanks and barrels the losses would be much greater. With a tank somewhat larger than those employed in this experiment Woll (16) found a loss of 26.1 per cent.

In the following calculations an average loss of 10 per cent has been assumed in order to give a minimum figure for the loss in pentosans. The percentages found by actual determination and

calculated on this basis together with the proportion of the total pentosans disappearing during a silage fermentation are given in Table V. The silage contained from 1 to 4 per cent less pentosans than the corn fodder from which it was made. Calculated on the original fodder this decrease becomes from 2 to 5 per cent. Of the total pentosans present in the corn fodder, from 10 to 22 per cent may be destroyed in the silo.

TABLE V.

Summary of the Loss of Pentosans in the Tank and Barrel Series of Silage after Allowing for the Loss of Dry Matter.

Material.	Age of silage.	Calculated on the dry basis.		
		Total pentosans.		Proportion of original pentosans lost.
		Found.	Calculated.*	
	days	*per cent*	*per cent*	*per cent*
Corn fodder	0	21.8	21.8	0
Tank series.				
Uninoculated silage	54	21.3	19.2	12
Inoculated with *L. pentoaceticus*	54	20.9	18.8	14
" " lactobacilli	54	19.9	17.9	18
Barrel series.				
Uninoculated silage, Control 1	66	22.3	20.1	8
" " " 2	66	20.0	18.0	17
Inoculated with *L. pentoaceticus*	66	19.2	17.3	12
" " lactobacilli	66	18.8	17.0	22
" " *L. pentoaceticus* and lactobacilli	66	19.6	17.7	19

* Calculated on the original dry corn fodder assuming a loss of 10 per cent of dry matter.

SUMMARY.

The total amount of pentosans in corn fodder, as the result of a number of determinations, was found to be 21.8 per cent. Only a trace (0.34 per cent) of methyl pentosans was found. During the fermentation of corn fodder some of the pentosans are destroyed. The percentage of pentosans in silage varies at different times due to variations in the loss of dry matter. At the end of 50 days the percentage was found to range from 17.6 to

20.9 per cent. If the loss of dry matter is taken into account the decrease is from 2 to 5 per cent. Assuming a loss of 10 per cent of dry matter it is calculated that from 15 to 20 per cent represents the minimum loss of pentosans in the fermentation of corn silage.

Pentoses or other furfural-yielding substances, soluble in water, are present in the silage throughout the fermentation. The production of these substances is probably due to the action of the microorganisms in the silage. A mixture of 2 per cent acetic and 5 per cent lactic acid failed to bring about any appreciable hydrolysis of the pentosans in 20 days at 28°C. A sample of immature corn contained 0.62 per cent of free pentoses in the stalk and 0.60 per cent in the immature cob.

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A CLINICAL METHOD FOR THE QUANTITATIVE DETERMINATION OF POTASSIUM IN SMALL AMOUNTS OF SERUM.

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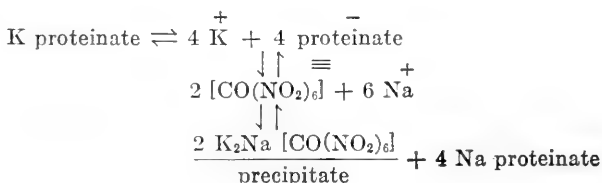
A method for the quantitative determination of potassium in blood has already been described (1). In this method the blood or serum is ashed in platinum, the ash is dissolved in dilute acetic acid, and the potassium precipitated by means of the well known sodium cobalti-nitrite reagent. The precipitate is then separated from the supernatant fluid by filtering through a thin Gooch pad and washed with cold water. The pad and precipitate are transferred to a small beaker and the amount of 0.01 N potassium permanganate required to oxidize the precipitate is determined. From this the amount of potassium present in the sample is calculated. We have found it possible to eliminate the ashing and thus materially shorten the procedure. Other changes in technique have been introduced so that only 1 cc. of serum is required for a single determination.

As early as 1871 Pribram (2) showed that calcium may be precipitated quantitatively from alkaline serum by adding an excess of ammonium oxalate. The following year Gerlach and Drechsel (3) demonstrated that this precipitate is purer when precipitation is accomplished by adding ammonium oxalate to serum previously acidified with acetic acid. De Waard (4) claims to have precipitated calcium quantitatively from serum by merely adding an excess of a saturated solution of ammonium oxalate. Such a solution we have found has a pH of 6.4 (dibrom-cresol), thereby preventing the precipitation of calcium phosphate. We have applied the McCrudden technique for the precipitation of calcium, directly to serum and the results found are identical with those obtained when a similar technique is applied to the ash

of the same serum (5). Marriott and Haessler (6) have shown that the so called inorganic phosphorus may be precipitated from unashed serum as ammonium magnesium phosphate. Magnesium may be quantitatively precipitated from serum as the same compound.

Preliminary qualitative tests showed that sodium cobalti-nitrite does not precipitate creatine, creatinine, or urea. It does, however, form an insoluble compound with ammonia, but this is present in serum only in minute amounts, if at all. These facts suggested to us the possibility of precipitating potassium quantitatively from unashed serum by adding an excess of a sodium cobalti-nitrite reagent so adjusted as regards its pH that the proteins of the serum remain in solution.

The fact that potassium and calcium may be precipitated quantitatively from serum does not necessarily contradict the theory of the presence of compounds of negatively charged proteins with cations, such as calcium or potassium proteinate. The studies of Loeb (7) have made the existence of such compounds in normal serum highly probable since the pH of the isoelectric point of the proteins of serum varies from 4.7 to 5.4 (8) and therefore at a pH of 7.6, the reaction of normal serum, such compounds must exist. Nevertheless if the laws that are applied to equilibria of solutions of electrolytes and their component ions hold true for these protein cation compounds, there should exist an equilibrium between the undissociated potassium proteinate, the free negatively charged protein ions, and the positively charged potassium ions. The precipitation of the latter by the sodium cobalti-nitrite reagent would result in a further dissociation of undissociated potassium proteinate. The liberated potassium would then in turn be precipitated and hence the quantity of potassium finally remaining in combination with protein would be indeed very small at pH 5.7 (the pH of the reagent).



Principle of the Method.

The potassium is precipitated in undiluted serum by adding an excess of a sodium cobalti-nitrite reagent, the pH of which is about 5.7 (methyl red) and therefore is more alkaline than the isoelectric point of the proteins of serum. This reagent precipitates all the potassium while the proteins remain in solution. The precipitate is centrifuged and washed repeatedly with water and then titrated as in the original method.

The Material to Be Used.

Most of our determinations have been made on serum. The blood is collected by venous puncture in the usual manner. The needle and syringe should be chemically clean and absolutely dry to avoid hemolysis. The serum should be separated from the clot as soon as possible. The analysis may be made any time within the next 48 hours, during which time the serum should be kept in the ice chest. The method as described for solutions of blood salts may be applied to solutions of ashed serum.

The Method.

1 cc. of serum is measured into a 15 cc. graduated centrifuge tube. 2 cc. of the sodium cobalti-nitrite reagent are then slowly added drop by drop and the contents of the tube thoroughly mixed. For the estimation of known solutions of potassium or ashed specimens it is advisable to make the volume of the solution to be estimated up to 2 cc. and then slowly add 1 cc. of the reagent. At the end of 45 minutes 2 cc. of water are added, and the contents are again mixed and centrifuged at a speed of 1,300 to 1,400 revolutions per minute for $\frac{1}{2}$ hour. All but 0.3 cc. of the supernatant fluid is syphoned off by the use of a syphon tube, the lower end of which is curved so that the opening is directed upward. Care must be taken that the precipitate is not disturbed. 5 cc. of water are then allowed to run down the side of the tube which is then gently agitated so that the added water is thoroughly mixed with the residual reagent. Care should be taken that the precipitate itself is disturbed as little as possible. This may be accomplished by holding the tube vertically and

gently hitting the lower end with a circular motion. The brown fluid may be seen to rise and mix with the supernatant fluid. The tube is then centrifuged for 5 minutes. The procedure is repeated three times so that the precipitate is washed four times in all. The supernatant fluid from the last washing should be perfectly clear. After this has been syphoned off, an excess of 0.02 N potassium permanganate is added (2 cc. are sufficient for normal serum) followed by 1 cc. of approximately 4 N sulfuric acid. The precipitate is then thoroughly mixed with the fluid by means of a glass rod. The sample is heated in a boiling water bath until no further change in color can be observed. This should not exceed $1\frac{1}{2}$ minutes even if large quantities of precipitate are present. For small quantities 1 minute will suffice. An amount of 0.01 N sodium oxalate sufficient to decolorize the solution completely (generally 2 cc.) is then added. The excess of oxalate is then determined by titrating to a definite pink color with 0.02 N potassium permanganate.

Calculation.

The total number of cc. 0.01 N potassium permanganate required to oxidize the potassium cobalti-nitrite $\times$ 7.1 = mg. potassium in 100 cc. serum. 1 cc. of 0.01 N potassium permanganate will oxidize a quantity of potassium cobalti-nitrite corresponding to 0.071 mg. of potassium. Thus if 2 cc. of 0.02 N potassium permanganate are originally added and 0.43 cc. of the same solution is used in the final titration and 2 cc. of 0.01 N sodium oxalate are required to decolorize the sample after the first oxidation, then 2.43 cc. − 0.03 (the blank) $\times$ 2 (to convert 0.02 N to 0.01 N) − 2.00 (cc. 0.01 N sodium oxalate added to decolorize the sample) $\times$ 7.1 = 19.9 mg. K per 100 cc. serum.

Reagents and Apparatus.

1. Sodium Cobalti-Nitrite Reagent.—Solution A.—25 gm. of cobalt nitrate crystals (J. T. Baker) are dissolved in 50 cc. of water and to this solution are added 12.5 cc. of glacial acetic acid.

Solution B.—120 gm. of sodium nitrite (potassium-free) (Merck) are dissolved in 180 cc. of water. This gives a total volume of about 220 cc. To all of Solution A are added 210 cc. of Solu-

tion B. An evolution of nitric oxide gas occurs at once. Air is drawn through the solution until all the gas has passed off. The reagent[1] is placed in the ice chest and filtered each time before using. It will keep at least 1 month.

2. Approximately 4 N *Sulfuric Acid.*—20 cc. of concentrated sulfuric acid are diluted to 100 cc. with distilled water.

3. 0.02 N *Potassium Permanganate.*—This is prepared by diluting N or 0.1 N potassium permanganate and is standardized after each series of determinations against the 0.01 N sodium oxalate (Sörensen).

4. 0.01 N *Sodium Oxalate.*—This is prepared from 0.1 N sodium oxalate. The latter is made by dissolving 6.7 gm. of Sörensen's sodium oxalate in a liter of water with the aid of 5 cc. of concentrated sulfuric acid.

Details of the Method.

Centrifuge Tubes.—These tubes must be carefully cleaned. They should be thoroughly scrubbed with the aid of a brush and washed with freshly prepared cleaning fluid (concentrated commercial H_2SO_4 and potassium dichromate). The tubes are then thoroughly rinsed with distilled water. Unless this procedure is followed, the precipitate will adhere to the walls of the tube and low results will be obtained.

Precipitation.—The reagent should be added to the serum very slowly and the tube continually shaken throughout this procedure. Although there is only slight danger of occlusion of the sodium cobalti-nitrite reagent by the potassium precipitate formed in the serum, occlusion undoubtedly takes place in solutions of potassium amd other salts when the reagent is added too rapidly. This is shown by the results recorded in Table II.

Washing the Precipitate.—The precautions to be observed have been described above.

The Titration.—It is important to add an excess of the permanganate solution before the heating is commenced. The order in which these solutions are added should be carefully observed. The end-point is reached when a definite pink color persists for 1 minute.

[1] The reagent thus prepared has a pH of 5.7 (Clark, W. M., The determination of hydrogen ions, Baltimore, 1920, 57).

Apparatus.—The apparatus required consists of a 1 cc. and a 2 cc. Ostwald pipette, a graduated centrifuge tube, the lower end of which should have a diameter of approximately 3 to 4 mm., a 5 cc. micro-burette graduated in 0.02 cc., a syphon tube, the lower end of which is curved so that the opening is directed upward, and a high speed centrifuge.

TABLE I.

Analysis of Solutions Containing Known Amounts of Potassium as KCl.

Sample No.	K present.	K found.	Error.
	mg	*mg.*	*per cent*
1		0.205	+1.5
2		0.213	+5.4
3		0.207	+2.5
4		0.199	−1.5
Average......	0.202	0.206	+2.0
5		0.405	+0.2
6		0.410	+1.5
7		0.399	−1.2
8		0.401	−0.7
Average......	0.404	0.404	±0.0
9		0.617	+1.8
10		0.625	+3.1
11		0.599	−1.2
Average......	0.606	0.613	+1.2

TABLE II.

Occlusion of Sodium Cobalti-Nitrite by Too Rapid Addition of the Reagent to 1 Cc. of Solution B.

2 cc. of reagent added rapidly. K found.	2 cc. of reagent added drop by drop. K found.	K present.
mg.	*mg.*	*mg.*
0.258	0.241	
0.271	0.231	
0.260	0.234	
0.264	0.241	0.237
Average..0.263	0.236	0.237

TABLE III.

*Analysis of Samples of Solution B.**

Sample No.	K present.	K found.	Error.
	mg.	*mg.*	*per cent*
1		0.230	−2.9
2		0.237	±0.0
3		0.230	−2.9
4		0.235	−0.8
5		0.241	+1.7
6		0.237	±0.0
Average......	0.237	0.235	−0.8
7		0.343	−3.4
8		0.343	−3.4
Average......	0.355	0.343	−3.4
9		0.481	+1.5
10		0.474	±0.0
Average......	0.474	0.477	+0.6

* Composition of Solution B.

NaCl	7.739	gm.
$Na_2HPO_4 + 2H_2O$	2.005	"
KCl	0.453	"
$CaCO_3$	0.250	"
$MgSO_4 + 7H_2O$	0.189	"
Concentrated HCl	10.0	cc.
Water to	1,000.0	"

1 cc. of this solution contained 0.237 mg. K.

TABLE IV.

Potassium Content of Normal Adult Serum Determined Directly.

Serum No.	K per 100 cc.
	mg.
1	20.0
2	19.6
3	20.0
4	19.2
5	20.0
6	19.0
7	19.7
8	19.6
9	19.3
10	19.7
Average	19.6

TABLE V.

K Found in Normal Adult Serum after Ashing.

Serum No.	K per 100 cc.
	mg.
1	19.9
2	17.9
3	21.3
4	19.7
5	20.0
6	20.3
7	19.5
8	18.9
9	20.3
10	20.5
11	19.0
12	20.3
13	20.2
14	20.2
15	19.0
16	19.5
Average	19.8

TABLE VI.

Comparison of Amount of K Found in Ashed and Unashed Sera.

Serum No.	K per 100 cc	
	Serum ashed.	Serum unashed.
	mg.	*mg.*
1	20.5	20.0
2	19.0	19.6
3	20.3	20.0
4	20.2	19.2
5	20.2	20.0
6	19.0	19.0
7	19.5	19.7
Average...........	19.8	19.6

TABLE VII.

Recovery of Potassium Added to Serum.

Serum No.	K present.	K added.	Total.	K found.
	mg.	*mg.*	*mg.*	*mg.*
1	0.219	0.101	0.320	0.327
	0.219	0.202	0.421	0.435
	0.219	0.202	0.421	0.414
2	0.220	0.101	0.321	0.331
	0.220	0.202	0.422	0.413
	0.220	0.202	0.422	0.430
3	0.190	0.118	0.308	0.313
	0.190	0.166	0.356	0.360
	0.190	0.237	0.427	0.432

TABLE VIII.

Potassium Content of Normal and Pathological Sera.

Serum No.	Diagnosis.	Age.	K per 100 cc.
			mg.
1	Normal.	Adult	19.5
2	"	11 yrs.	18.2
3	"	2 "	20.0
4	Epidemic influenza, secondary pneumonia.	1 "	48.2
5	" " " "	8 "	43.0
6	" " " "	8 "	51.0
7	" "	6 "	42.0
8	Measles, secondary pneumonia.	5 "	46.3
9	Primary pneumonia.	3 "	64.6
10	Burn.	18 mos.	50.0
11	Acute gastrointestinal indigestion.	9 "	23.0
12	" " "	3 "	25.0
13	Typhoid fever, hereditary syphilis.	7 yrs.	43.5
14	" " acute otitis media.	7 "	41.7
15	Acute rhinopharyngitis.	1 yr.	35.6
16	Scarlet fever.	10 yrs.	38.0
17	" "	9 "	70.0

Protocols.

The results of the estimation of potassium in solutions containing known amounts of potassium are given in Tables I and III. Tables IV, V, and VI show that the potassium content of normal adult serum is singularly constant and that the values obtained on ashed and unashed serum are practically identical. Table VII demonstrates that known amounts of potassium added to serum may be quantitatively recovered. The amounts of potassium found in the serum of infants and children suffering from various pathological conditions are given in Table VIII. An enormous increase in the potassium content of serum is to be noted. The significance of this increase is being investigated and the results will be reported at a later date. When serum remains in contact with the clot potassium ions migrate from the corpuscles into the serum.

CONCLUSIONS.

1. A simple method for the determination of potassium directly in 1 cc. of serum has been described.

2. The same technique applied to a solution of blood salts containing known amounts of potassium gives results generally within 3 per cent of the amount actually present.

3. Known amounts of potassium added to serum may be recovered quantitatively.

4. In normal serum no discrepancy between the results obtained on the serum directly and on the ash of the serum was found.

5. The results obtained on serum are accurate to within ± 5 per cent of the amount of potassium actually present.

6. The potassium content of the serum of both normal children and adults is singularly constant, the maximum variation being from 18 to 21 mg. per 100 cc.

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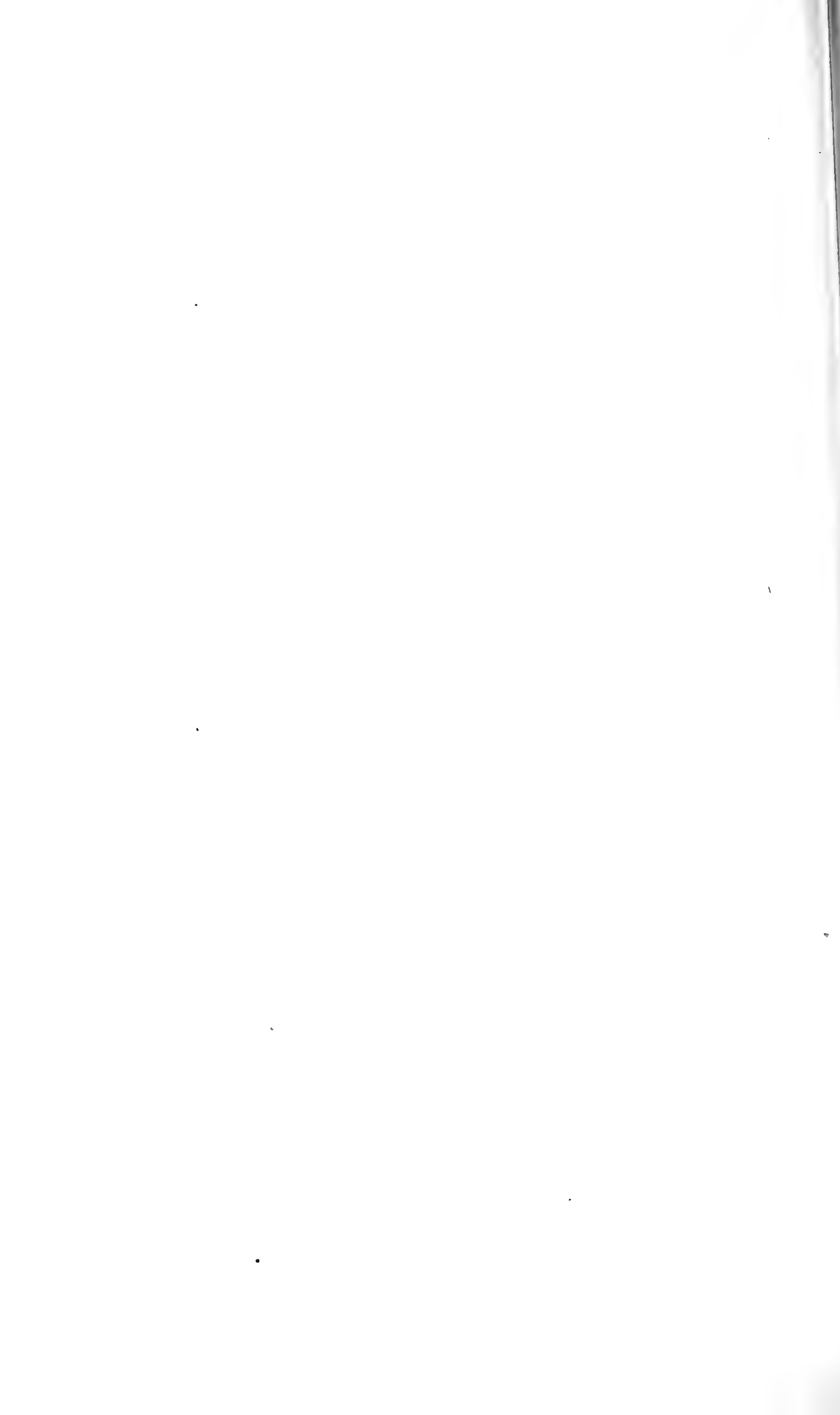

A NOTE ON A MODIFICATION OF THE VAN SLYKE METHOD OF PROTEIN ANALYSIS.

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In some work on the analysis of the protein of the pecan by the Van Slyke method (1), it was found that the precipitation of the humin by the calcium oxide was incomplete and interfered with the subsequent isolation of the amino-acids precipitated by phosphotungstic acid. For this reason the regular procedure was modified somewhat, which greatly shortened the time required for the analysis.

This modification consists in precipitating the humin and ammonia from the protein hydrolysate by phosphotungstic acid (15 gm. for 3 gm. of protein), in 150 cc. of boiling solution containing about 10 per cent H_2SO_4 or HCl, and allowing to stand for several hours, until cold. The phosphotungstates of the basic amino-acids are brought into solution by again heating to boiling for a few minutes on a sand bath and the insoluble phosphotungstates of ammonia and humin filtered off and washed with 50 cc. of boiling 10 per cent acid. Enough phosphotungstic acid, 5 to 10 gm., is now added to the filtrate to precipitate completely the hexone bases and the solution is set aside for these to precipitate. The usual procedure for the determination of the hexone bases is followed from here on. The ammonia remaining with the hexone bases was determined in eight analyses, by distillation *in vacuo* with excess calcium hydroxide just before the determination of arginine. In no case were more than 2 or less than 1 mg. of ammonia N found.

The ammonia is determined by making the residue alkaline and distilling. The humin may be determined by making a Kjeldahl determination on the residue from the ammonia, but as there is often a great excess of inorganic residue which causes violent bumping during the digestion I have found it more satisfactory to

precipitate the humin in an aliquot of the original hydrolysate by adding, while stirring, a 10 per cent solution of sodium tungstate to the hot 10 per cent acid solution. The sodium tungstate is added slowly till the humin is precipitated as a black curdy mass and the supernatant liquid is clear. No definite amount of sodium tungstate can be stated, as the quantity needed depends largely on the amount of humin; usually 2.5 to 10.0 cc. of sodium tungstate are required to 2 gm. of hydrolyzed protein. The humin is then filtered off, washed with 10 per cent acid, and the nitrogen determined by the Kjeldahl method. Since the hexone bases do not precipitate quantitatively from this filtrate, it is discarded.

TABLE I.

Nitrogen.	Analysis I.	Analysis II.	Per cent of total N_2.		Van Slyke (1).	Osborne, Van Slyke, Leavenworth, and Vinograd (2).
			I	II		
	mg.	*mg.*	*per cent*	*per cent*	*per cent*	*per cent*
Total N_2	640.0	640.0				
Humin N_2	5.04	5.5	0.787	0.86	0.86	0.58
NH_3N_2	165.4	165.0	25.84	25.78	25.52	24.61
Hexone bases N_2*	71.7	72.0	11.20	11.25	12.9	10.97
Ammonia N_2 remaining with hexone bases	1.4	1.0	0.22	0.16		
Monoamino and non-amino N_2	400.8	401.5	62.62	62.73	60.5	62.65
Total N_2			100.667	100.78	99.78	98.81

* Corrected for solubility of bases.

Gliadin, in which the maximum of ammonia is found, was analyzed by this procedure and the results agree well with the published data (2).

The analysis of gliadin by the foregoing method gave results as shown in Table I. Another sample of gliadin analyzed 25.03 and 25.17 per cent of nitrogen as ammonia.

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LECITHIN.

IV. LECITHIN OF THE BRAIN.

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It may seem unwarranted to reopen the question of brain lecithin. Does brain contain lecithin? Does this, if present, differ from lecithins of other organs? These questions at this date seem peculiar when one takes into consideration that Gobley,[1] who discovered lecithin in the egg yolk in 1846, reported its isolation from the brain in 1847. Later workers, still of the group of pioneers, substantiated these early observations. Among them the affirmative work of Thudichum[2] carries most weight since no other single worker has devoted as much energy and care to the isolation of the brain components.

In recent years, Koch[3] has claimed to have isolated lecithin from the brain tissue. On the other hand, Fränkel and Linnert[4] deny the presence of lecithin in the brain tissue of man. True, these authors are inclined to see in their finding a point of differentiation in the chemical structure of the human brain from that of other species. On the other hand, older workers never recorded any differences in the lipoids of the brain derived from different species, and the question naturally arose, does the negative finding of Fränkel and Linnert apply also to the brain of other species than of man? In the days of Gobley, and even in those of Thudichum, the methods of identification of individual lipoids were very imperfect and, *a priori*, one would naturally be inclined to attribute more weight to results obtained by more recent work.

[1] Gobley, M., *J. pharm. chim.*, 1847, xi, 409; xii, 1.

[2] Thudichum, J. L. W., A treatise on the chemical constitution of the brain, London, 1884.

[3] Koch, W., *Z. physiol. Chem.*, 1902, xxxvi, 134.

[4] Fränkel, S., and Linnert, K., *Biochem. Z.*, 1910, xxiv, 268; xxvi, 44.

But, even granting the existence of brain lecithin, one is still in the dark on the question of its structure, relying only on the experimental data of the older workers. Very recently, and only in this laboratory were hydrolyses made on lecithins which did not contain considerable admixtures of cephalin. This work has brought out the fact that lecithins differ in the nature of the fatty acids entering into their structure. Thus it was found that while the unsaturated acid of the egg yolk was oleic,[5] that of the liver[6] was of the linolic series. As to the saturated acids, the lecithin of the egg yolk contained two acids, palmitic and stearic, while that of the liver seemed to contain only stearic. In view of our latest experience on the egg lecithin, this latter point concerning the liver lecithin is in need of reinvestigation. At any rate, the work on the egg lecithin and that on the liver lecithin suggest that there may exist a difference between individual lecithins. Thus the present work was directed towards the solution of two questions. First, of the existence of lecithin in the brain tissue, and, as this has been answered in the affirmative, there arose the second question as to the nature of its fatty acids.

As regards the preparation of lecithin from brain tissue, our experience is in accord with that of Thudichum. This author writes: "Lecithin . . . . is only with difficulty evolved from the brain, on account not only of the many stages of the processes necessary for its isolation, but also on account of its readiness to decompose under certain conditions." Early in our work, we obtained evidence of the presence of lecithin in the brain tissue. However, analytically pure lecithin was isolated only after a process of purification was evolved in the course of the work on the unsaturated lipoids of the liver.

This method is based on the observation that some impurities (cerebrosides) are insoluble in cold glacial acetic acid, others in a mixture of a glacial acetic and alcohol, whereas lecithin is soluble in both reagents. Hence, the crude material is dissolved in warm glacial acetic acid and the solution is allowed to cool. On cooling there is formed a precipitate consisting chiefly of cerebrosides. To the filtrate from this material ten volumes of 95 per cent alcohol are added. This treatment brings down a pre-

[5] Levene, P. A., and Rolf, I. P., *J. Biol. Chem.*, 1921, xlvi, 193.

[6] Levene, P. A., and Ingvaldsen, T., *J. Biol. Chem.*, 1920, xliii, 359.

cipitate consisting mainly of cephalin. The filtrate from this second precipitate yields a product consisting of nearly equal proportions of lecithin and cephalin. Thus a sample consisting of 55 per cent of lecithin and 45 per cent of cephalin had the following elementary composition:

	per cent
C	65.87
H	10.45
N	1.91
P	3.89

For convenience of analysis and purification this sample was hydrogenated by Paal's method. It then had the following composition:

	per cent
C	65.69
H	10.94
N	1.92
P	3.79

$$\text{Ratio}\ \frac{NH_2\ N}{\text{Total N}} = \frac{38}{100}$$

This sample has approximately the same composition as the corresponding material prepared from the egg yolk. This finding is important in its bearing on the composition of brain cephalin. A brain cephalin having the elementary composition required by the theory is thus indicated and, it is hoped, will soon be prepared.

For the separation of lecithin from cephalin one still has to resort to its cadmium chloride salt. Again in preparing lecithin from the brain as from the egg yolk, one obtains a pure product more readily from the acetone-soluble fraction. The analysis of a sample of dihydrolecithin prepared from this fraction is reported in the experimental part of this paper. It had an elementary composition required by theory, and in other properties was identical with hydrolecithin prepared from egg yolk. Thus the existence of lecithin among the lipoids of brain tissue may be regarded as definitely established.

Regarding the chemical relationship of the brain lecithin to other lecithins, it was found that it had the same composition as lecithin from the egg yolk. The distinction between lecithins lies apparently in the differences of the character of their fatty

acids. The fatty acids isolated from the egg lecithin are oleic, palmitic, and stearic acids. The same acids were also isolated from the brain lecithin. This finding suggests the possibility of the existence in the brain tissue also, of more than one lecithin.

EXPERIMENTAL.

1. Isolation of Lecithin from the Ethereal Extract of Ox Brains.

Ethereal extracts of desiccated ox brain tissue were used as the source of lecithin. The desiccated tissue was extracted with acetone prior to its extraction with ether containing 5 per cent of water. After concentration, the residual ethereal extract was poured into acetone. The resulting precipitate was redissolved in ether and allowed to stand over night at 10°C. Generally a sediment of "white matter" formed, which was removed by centrifugalization. The ethereal solution was again concentrated and precipitated by acetone. This operation was repeated until the ethereal solution on standing over night no longer gave a white precipitate. The ethereal solution was precipitated with cold alcohol and the precipitate was again dissolved in ether and again precipitated from alcohol. After a third such precipitation the combined alcoholic solutions were concentrated to a small bulk, under diminished pressure, the solution being kept below room temperature during the process. The residual material was precipitated from acetone. From 200 ox brains 400 gm. of this lecithin fraction were obtained, having at this stage the following composition:

No. 282. 2 gm. of substance were hydrolyzed with 10 per cent HCl, neutralized, and concentrated to 25 cc.

5 cc. of this solution for Kjeldahl determination required 2.31 cc. of 0.1 N HCl, equivalent to 0.003234 gm. of nitrogen.

2 cc. of this solution for Van Slyke determination gave 1.04 cc. of nitrogen at $T = 23°$ and $P = 757$ mm., equivalent to 0.0005819 gm. of nitrogen.

$$\text{Ratio } \frac{NH_2\ N}{\text{Total } N} = \frac{44}{100}$$

0.1958 gm. of substance used for Kjeldahl determination required 2.70 cc. of 0.1 N HCl, equivalent to 0.0378 gm. of nitrogen.

0.2937 gm. of substance gave 0.0296 gm. of $Mg_2P_2O_7$.

0.1030 gm. of substance, dried under diminished pressure at temperature of water vapor gave on combustion 0.0992 gm. of H_2O, 0.2518 gm. of CO_2, and 0.0082 gm. of ash.

	Calculated for $C_{41}H_{78}O_9NP$ (cephalin).	Calculated for $C_{43}H_{86}O_9NP$ (lecithin).	Found No. 282 (calculated ash-free).
	per cent	*per cent*	*per cent*
C	66.17	65.26	67.70
H	10.57	10.95	11.07
N	1.88	1.77	1.93
P	4.17	3.92	2.79

100 gm. of No. 282 were dissolved with very gentle warming in 500 cc. of glacial acetic acid. This solution, on standing over night in the refrigerator, deposited a fine white precipitate (Precipitate I), consisting apparently of cerebrosides which had not been entirely removed by the previous treatment. The insoluble precipitates from two distinct lecithin fractions, both of which were isolated by the same method, amounted, respectively, to 10 and 15 per cent of the lecithin fraction from which they were extracted.

The mother liquor of this precipitated material was poured into ten volumes of 95 per cent alcohol. After standing over night in the refrigerator, a fine, flocculent, light-colored precipitate was formed (Precipitate II). On exposure to air, it coalesced to a sticky, dark-colored, gummy mass. In two experiments, this fraction consisted, respectively, of 8 and 6 per cent of the weight of the starting material.

The alcoholic solution was concentrated under diminished pressure, without heat, to dryness. This semisolid material (Precipitate III) was emulsified with water, and precipitated with acetone. 100 gm. of the original material yielded 65 gm. of a golden yellow, plastic, buttery mass, which showed no tendency to darken on exposure to air, and even after prolonged drying under diminished pressure did not become either hard or brittle.

The analytical data of Precipitates I, II, and III, follow:

Precipitate I.

No. 290. 2 gm. of substance were hydrolyzed with 10 per cent HCl, neutralized, and concentrated to 25 cc.

5 cc. of this solution for Kjeldahl determination required 28 cc. of 0.1 N HCl, equivalent to 0.000392 gm. of nitrogen.

2 cc. of this solution for Van Slyke determination gave 0.11 cc. of nitrogen at $T = 25°$ and $P = 752$ mm., equivalent to 0.0000603 gm. of nitrogen.

$$\text{Ratio } \frac{NH_2\ N}{\text{Total N}} = \frac{40}{100}$$

0.1500 gm. of substance containing 3.43 per cent moisture used for Kjeldahl determination required 1.62 cc. of 0.1 N HCl.

It contained no phosphorus.

0.1012 gm. of substance, dried under diminished pressure at temperature of water vapor gave on combustion 0.1030 gm. of H_2O, 0.2722 gm. of CO_2, and 0.0012 gm. of ash.

	Found No. 290.
	per cent
C	73.34
H	11.38
N	1.17
P	0.00

Precipitate II.

No. 291. 2 gm. of substance were hydrolyzed with 10 per cent HCl, neutralized, and concentrated to 25 cc.

5 cc. of this solution for Kjeldahl determination required 2.46 cc. of 0.1 N HCl, equivalent to 0.003444 gm. of nitrogen.

2 cc. of this solution for Van Slyke determination gave 2.06 cc. of N at $T = 25°$ and $P = 252$ mm., equivalent to 0.00113 gm. of nitrogen.

$$\text{Ratio } \frac{NH_2\ N}{\text{Total N}} = \frac{82}{100}$$

0.1939 gm. of substance used for Kjeldahl determination required 1.96 cc. of 0.1 N HCl.

0.2909 gm. of substance gave 0.0394 gm. of $Mg_2P_2O_7$.

0.1010 gm. of substance dried under diminished pressure at temperature of water vapor gave on combustion 0.0902 gm. of H_2O, 0.2388 gm. of CO_2, and 0.0122 gm. of ash.

	Calculated for $C_{41}H_{78}O_8NP$ (cephalin).	Calculated for $C_{43}H_{86}O_9NP$ (lecithin).	Found No. 291 (calculated ash-free).
	per cent	*per cent*	*per cent*
C	66.17	65.26	65.80
H	10.57	10.95	10.37
N	1.88	1.77	1.41
P	4.17	3.92	3.77

Precipitate III.

No. 247. 2 gm. of substance were hydrolyzed with 10 per cent HCl, neutralized, and concentrated to 25 cc.

5 cc. of this solution for Kjeldahl determination required 1.91 cc. of 0.1 N HCl, equivalent to 0.00267 gm. of nitrogen.

2 cc. of this solution for Van Slyke determination gave 0.85 cc. of N at $T = 21°$ and $P = 764.2$ mm., equivalent to 0.00048 gm. of nitrogen.

$$\text{Ratio } \frac{NH_2\,N}{\text{Total N}} = \frac{45}{100}$$

0.1943 gm. of substance used for Kjeldahl determination required 2.83 cc. of 0.1 N HCl.

0.2914 gm. of substance gave 0.0388 gm. of $Mg_2P_2O_7$.

0.1082 gm. of substance dried under diminished pressure at temperature of water vapor gave on combustion 0.0994 gm. of H_2O, 0.2570 gm. of CO_2, and 0.0116 gm. of ash.

No. 303. 2 gm. of substance were hydrolyzed with 10 per cent HCl, neutralized, and concentrated to 25 cc.

5 cc. of this solution for Kjeldahl determination required 3.26 cc. of 0.1 N HCl, equivalent to 0.003164 gm. of nitrogen.

2 cc. of this solution for Van Slyke determination gave 1.04 cc. of N at $T = 26°$, and $P = 742.8$ mm., equivalent to 0.00056 gm. of nitrogen.

$$\text{Ratio } \frac{NH_2\,N}{\text{Total N}} = \frac{46}{100}$$

0.1947 gm. of substance used for Kjeldahl determination required 2.66 cc. of 0.1 N HCl.

0.2921 gm. of substance gave 0.0408 gm. of $Mg_2P_2O_7$.

0.1078 gm. of substance dried under diminished pressure at temperature of water vapor gave on combustion 0.1007 gm. of H_2O, 0.2603 gm. of CO_2, and 0.0094 gm. of ash.

	Calculated for $C_{41}H_{78}O_8NP$ (cephalin).	Calculated for $C_{43}H_{86}O_9NP$ (lecithin).	Found No. 247 (calculated ash-free).	Found No. 303 (calculated ash-free).
	per cent	*per cent*	*per cent*	*per cent*
C	66.17	65.26	66.25	65.87
H	10.57	10.95	10.53	10.45
N	1.88	1.77	2.04	1.91
P	4.17	3.92	3.80	3.89

On reduction by Paal's method, rapid absorption of hydrogen occurred, and the dihydrolecithin crystallized on cooling. 10 gm. of No. 247 on hydrogenation gave 5 gm. of dihydrolecithin after recrystallization from methyl ethyl ketone. This substance gave the following rotation and analysis:

$$[\alpha]_D^{20} = \frac{+0.20 \times 100}{1 \times 4} = +5.00°$$

No. 244. 2 gm. of substance were hydrolyzed with 10 per cent HCl, neutralized, and concentrated to 25 cc.

5 cc. of this solution for Kjeldahl determination required 2.66 cc. of 0.1 N HCl, equivalent to 0.003724 gm. of nitrogen.

2 cc. of this solution for Van Slyke determination, gave 1.02 cc. of N at $T = 23°$, and $P = 755$ mm., equivalent to 0.000569 gm. of nitrogen.

$$\text{Ratio } \frac{NH_2\ N}{\text{Total N}} = \frac{38}{100}$$

0.1932 gm. of substance used for Kjeldahl determination required 2.70 cc. of 0.1 N HCl.

0.2898 gm. of substance gave 0.0396 gm. of $Mg_2P_2O_7$.

0.1026 gm. of substance dried under diminished pressure at temperature of xylene vapor gave on combustion 0.0988 gm. of H_2O, 0.2436 gm. of CO_2, and 0.0104 gm. of ash.

	Calculated for $C_{41}H_{82}O_8NP$ (hydrocephalin).	Calculated for $C_{43}H_{88}O_9NP$ (hydrolecithin).	Found No. 244 (calculated ash-free).
	per cent	*per cent*	*per cent*
C	65.81	65.10	65.69
H	11.05	11.18	10.94
N	1.87	1.77	1.92
P	4.15	3.91	3.79

2. Isolation of Lecithin from the Acetone Extract of Ox Brains.

Acetone-Soluble Fraction.—The acetone-soluble material from ox brains, a semisolid mass consisting principally of cholesterol with a small admixture of lecithin and cephalin, was repeatedly extracted with large quantities of warm alcohol, until the alcoholic solution after concentration gave no precipitate when treated with cadmium chloride solution. The alcoholic extract thus obtained was cooled to 0°C. and the cholesterol which crystallized on standing separated by filtration. The mother liquor was concentrated under diminished pressure to a smaller volume, and the cholesterol removed as completely as possible by crystallization. After filtration a saturated alcoholic solution of cadmium chloride was added to the filtrate until the precipitation of the lecithin was complete. The precipitate was then washed by decantation with acetone, until it settled to the bottom of the jar in a finely divided white, easily filtered powder. The cadmium chloride salt of lecithin thus isolated contained, as indicated by a Van Slyke amino determination on the hydrolyzed material, from 30 to 40 per cent of cephalin.

This material was further purified by suspension in ether, adding water to the suspension until a clear solution was obtained, and reprecipitating the salt by pouring the solution into alcohol. If the material had not been sufficiently well washed with acetone after its original precipitation, filtration at this point would leave a rather gummy mass, the character of which could be markedly improved by using acetone instead of alcohol as a precipitant from the ether-water solution.

After three or four such precipitations from alcohol, the amino content usually fell to approximately 15 per cent. Its further purification was effected by dissolving the salt in toluene and precipitating it with ether.[5]

Several samples of material isolated in this way were combined and again precipitated by alcohol from an ether-water solution. About 125 gm. of material (No. 185) were obtained and analyzed as follows:

No. 185. 2 gm. of substance were hydrolyzed with HCl, neutralized, and concentrated to 25 cc.

5 cc. of this solution for Kjeldahl determination required 3.05 cc. of 0.1 N HCl, equivalent to 0.004270 gm. of nitrogen.

2 cc. of this solution for Van Slyke determination gave 0.1 cc. of nitrogen at $T = 23°$, and $P = 752$ mm., equivalent to 0.00005545 gm. of nitrogen.

$$\text{Ratio}\ \frac{NH_2\ N}{\text{Total N}} = \frac{3.2}{100}$$

This lecithin cadmium chloride was decomposed in 50 per cent alcoholic solution with ammonium carbonate in accordance with the method of Bergell.[7] Further purification was effected by emulsification with water, and subsequent precipitation by acetone (MacLean). It was then reduced by Paal's method, and the hydrolecithin thus obtained, after one recrystallization from methyl ethyl ketone, gave the following analysis:

0.1443 gm. of substance used for Kjeldahl determination required 1.83 cc. of 0.1 N HCl.

0.2646 gm. of substance gave 0.0378 gm. of $Mg_2P_2O_7$.

0.1065 gm. of substance dried under diminished pressure at temperature of xylene vapor gave on combustion 0.1040 gm. of H_2O, 0.2561 gm. of CO_2, and 0.0105 gm. of ash.

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[7] Bergell, P., *Ber. chem. Ges.*, 1900, xxxiii, 2584.

	Calculated for $C_{43}H_{88}O_9NP$ (hydrolecithin).	Found No. 188.
	per cent	*per cent*
C	65.10	65.57
H	11.18	10.92
N	1.77	1.78
P	3.91	3.98

Three observations of the optical rotation of this material were made in chloroform solutions of various concentrations. These were as follows:

$$[\alpha]_D^{20} = \frac{+0.23 \times 100}{2 \times 2} = +5.75°$$

$$[\alpha]_D^{20} = \frac{+0.33 \times 100}{1 \times 6} = +5.5°$$

$$[\alpha]_D^{20} = \frac{+0.30 \times 100}{0.5 \times 10} = +6.0°$$

3. Fatty Acids of Hydrolecithin.

By boiling 5 gm. of this material with ten parts of 10 per cent HCl for 8 hours, the fatty acids were isolated. The ether-soluble material from this hydrolysis mixture was precipitated by the addition of lead acetate. This salt, in benzene solution, was decomposed with hydrogen sulfide and the mixture of acids crystallizing from the concentrated benzene solution after two recrystallizations from respectively acetone and ethyl acetate, gave the following analysis:

0.0993 gm. of substance dried by fusion, gave on combustion 0.1125 gm. of H_2O and 0.2749 gm. of CO_2.

	Calculated for $C_{18}H_{36}O_2$.	Found No. 197.
	per cent	*per cent*
C	75.99	75.50
H	12.70	12.67

The melting point of this material was lower than those of samples of Kahlbaum's "K" palmitic and stearic which had been purified for purposes of comparison. Melting point determinations of these three substances were carried out simultaneously.

The following (corrected) melting points, the time interval averaging 7 seconds per degree, were obtained.

Palmitic acid. °C.	No. 197. °C.	Stearic acid. °C.
63–64	62–63	69–71

4. Fatty Acids of Lecithin Cadmium Chloride.

A. Saturated Fatty Acids.—A sample of amino-free lecithin cadmium chloride was boiled for 8 hours with methyl alcohol containing 5 per cent sulfuric acid. The esters of the saturated fatty acids crystallized from the hydrolysis mixture on cooling. After recrystallization they were fractionally distilled under a diminished pressure of 2.8 mm. These fractions were hydrolyzed and the elementary analyses, molecular weights as calculated from their titration values, and melting points of the respective acids are indicated below.

All samples of fatty acids were dried by fusion on a hot plate and to insure absolute freedom from moisture in the material used for elementary analysis were remelted under diminished pressure at the temperature of xylene vapor until constant weight was obtained. The molecular weights were calculated by the titration of approximately 1 gm. of acid dissolved in 10 cc. of toluene and 25 cc. of methyl alcohol (neutral to phenolphthalein) with 0.5 N NaOH using phenolphthalein as an indicator.

The melting points as given are corrected and were taken at such a rate that the time interval per degree rise in temperature was 6 seconds.

First fraction, No. 185. B.P. = 166–168°C.

After hydrolysis to the acid:

0.0894 gm. of substance gave on combustion 0.1039 gm. of H_2O and 0.2460 gm. of CO_2.

1.0010 gm. of substance required for neutralization 7.70 cc. of 0.5 N NaOH corresponding to a molecular weight of 260.

M.P. = 61–62°C.

No. 185 was refractionated. The lower fraction was:

No. 192. B.P. = 156–159°C.

After hydrolysis to the acid:

0.1000 gm. of substance gave on combustion 0.1150 gm. of H_2O and 0.2754 gm. of CO_2.

0.8846 gm. of substance required for neutralization 6.9 cc. of 0.5 N NaOH corresponding to a molecular weight of 256.

M.P. = 63–64°C.

Second fraction, No. 186. B.P. = 168–170°C.

After hydrolysis to the acid:

0.9602 gm. of substance required for neutralization 7.25 cc. of 0.5 N NaOH corresponding to a molecular weight of 265.

M.P. = 59–60°C.

Third fraction, No. 187. B.P. = 170–180°C.

After hydrolysis to the acid:

1.0617 gm. of substance required for neutralization 7.70 cc. of 0.5 N NaOH corresponding to a molecular weight of 275.

M.P. = 59–60°C.

Fourth fraction, No. 188. B.P. = 180–190°C.

After hydrolysis to the acid:

0.1006 gm. of substance gave on combustion 0.1162 gm. of H_2O and 0.2784 gm. of CO_2.

0.9566 gm. of substance required for neutralization 6.81 cc. of 0.5 N NaOH corresponding to a molecular weight of 281.

M.P. = 63–64°C.

Fifth fraction, No. 189. B.P. = 190–200°C.

After hydrolysis to the acid:

0.1004 gm. of substance gave on combustion 0.1148 gm. of H_2O and 0.2794 gm. of CO_2.

0.8955 gm. of substance required for neutralization 6.30 cc. of 0.5 N NaOH corresponding to a molecular weight of 284.

M.P. = 70–71°C.

Sample.	Boiling point of ester. $P = 2.8$ mm.	Analysis of acid.		Molecular weight of acid.	Melting point of acid.
		H	C		
	°C.	*per cent*	*per cent*		°C.
First fraction, No. 185	166–168	13.00	75.02	260	61–62
Refractionated lower fraction, No. 192	156–159	12.87	75.10	256	63–64
Second fraction, No. 186	168–170			265	59–60
Third " " 187	170–180			275	59–60
Fourth " " 188	180–190	12.92	75.47	281	63–64
Fifth " " 189	190–200	12.79	75.89	284	70–71
Required for $C_{16}H_{32}O_2$ (palmitic acid)		12.58	74.92	256	63–64
Required for $C_{18}H_{36}O_2$ (stearic acid)		12.76	75.93	284	70–71

B. Unsaturated Fatty Acid.—The unsaturated ester was isolated from the mother liquor from which the saturated ester had crystallized, by extraction of the neutralized, concentrated residue with ether. Hydrolysis and subsequent decomposition of the sodium salt gave an acid with an iodine value of 88.

0.2446 gm. of substance absorbed 0.21664 gm. of iodine when titrated according to the method of Wijs.

	Calculated for $C_{18}H_{34}O_2$ (oleic acid).	Found No. 181.
Iodine number	90	88

Reduction of this acid by Paal's method yielded an acid, the analysis of which corresponded to that of stearic acid.

No. 182. 0.1004 gm. of substance gave on combustion 0.1161 gm. of H_2O and 0.2810 gm. of CO_2.

1.3300 gm. of substance required for neutralization 9.40 cc. of 0.5 N NaOH corresponding to a molecular weight of 284.

	Analysis.		Molecular weight.	Melting point.
	H	C		
	per cent	*per cent*		*°C.*
No. 182	12.93	76.32	284	70–71
Required for $C_{18}H_{36}O_2$ (stearic acid)	12.76	75.93	284	70–71

SOME OBSERVATIONS ON THE STABILITY OF THE ANTISCORBUTIC VITAMINE AND ITS BEHAVIOR TO VARIOUS TREATMENTS.*

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Delf and Skelton (1) and Givens and Cohen (2) have reported that the drying of cabbage even at relatively low temperatures resulted in an almost complete destruction of its antiscorbutic properties. Givens and McClugage (3) have shown that orange juice dried at 55–60°C. for 50 hours was even less effective in preventing the onset of scurvy in the guinea pig than that dried at 75–80°C. for a relatively short time. That a material can be dried with only a partial destruction of this vitamine, if the temperature is sufficiently low and the time sufficiently short, is apparent from this work of Givens and McClugage, as well as from recent work by Harden and Robison (4).

The behavior of the vitamines to heat has been summarized by Drummond (5) to be approximately as follows: both the water-soluble vitamine and the fat-soluble vitamine are stable up to 100°C. and only slowly destroyed above this temperature, while the antiscorbutic vitamine is gradually destroyed above 50°C. and rapidly above 80°C.

The differential solubility of the antiscorbutic vitamine has not been extensively studied. Drummond (5) and his associates, in their classification of the vitamines, speak of the antiscorbutic as water-soluble C. Its water-soluble nature seems likely since it is contained in the watery extraction of fruits, vegetables, and green plants. However, salts and organic compounds occurring with it in these materials may play a considerable part in its solu-

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bility. Hess and Unger (6) reported it to be soluble in 80 per cent alcohol, by which it was extracted from orange juice. Unpublished data accumulated in this laboratory confirm this fact. The water-soluble vitamine is also soluble in water and 95 per cent alcohol (7) but insoluble in ether. The fat-soluble vitamine is soluble in fats and can be extracted with ether from butter, milk, and animal tissues containing it (8). Some plant tissues, as alfalfa, yield this vitamine to extraction with ether, while others may not (9).

The use of fullers' earth and dialyzed iron for separating the water-soluble from the antiscorbutic vitamine has been employed by Harden and Zilva (10). From a mixture of yeast and orange juice the water-soluble vitamine was removed by these materials but not the antiscorbutic factor. Harden also found that the latter vitamine was filterable through a Berkefeld filter.

Concerning the action of the common reagents, about the only information at hand is the behavior of the vitamine toward alkalies. Both Hess and Unger (11) and Harden and Zilva (12) reported that, when orange juice was made 0.05 N alkaline with sodium hydroxide and left to stand in the cold for 24 hours, destruction of the antiscorbutic vitamine was practically complete. The water-soluble vitamine (antineuritic) is not stable toward alkalies, while the fat-soluble vitamine is fairly stable in this reaction. In acids, apparently all three vitamines are comparatively stable, particularly at room temperature (5).

The experiments reported in this paper are concerned with the properties of the antiscorbutic vitamine, its destructibility by various processes, and its behavior to some common reagents and solvents. This work is entirely preliminary in character. The first part is devoted to an inquiry relative to its stability to heat and fermentation. Cabbage, both in the undried state and as sauerkraut, and corn in the form of silage were studied. The second part of the paper deals with various treatments to which orange juice was exposed.

EXPERIMENTAL.

The scorbutic diet used in all these experiments consisted of 69 parts of rolled oats, 25 parts of ground alfalfa hay (autoclaved for 30 minutes at 15 pounds pressure), 5 parts of casein, and 1 part

of common salt. These materials furnish a ration which satisfies, as far as known, all the factors of nutrition for the guinea pig, excepting the antiscorbutic. In the experiments reported the various materials tested were either incorporated with the above ration or were given separately. In all instances the quantities given daily were closely controlled.

The development of the course of scurvy in the guinea pig is shown in Chart 1, Lot 1. These animals received the scorbutic ration and succumbed to scurvy in 30 to 35 days. The symptoms exhibited by these animals agreed with the symptomatology of guinea pig scurvy described by Cohen and Mendel (13) and by other workers.

Effect of Heat and Fermentation on Cabbage and Corn (Silage).

In the methods thus far employed for drying antiscorbutic substances, such as cabbage, orange juice, and tomatoes, oxygen of the air has been present. It was believed that if the oxygen was displaced by an inert gas such as carbon dioxide, the destruction of the antiscorbutic vitamine could be prevented. Therefore, cabbage was dried in a vacuum oven in an atmosphere of carbon dioxide at 65°C. It required 35 hours to dry the material thoroughly. It was then gradually incorporated with the scorbutic ration and fed to the animals in Lot 2, Chart 2. They received the equivalent of 1.5 gm. of raw cabbage daily. The minimum amount of raw cabbage needed for protection against scurvy in the guinea pig has been found to be a daily allowance per individual of 1 gm. (14). Practically no protection was offered by our preparation, showing that for the time of exposure the carbon dioxide atmosphere had had no apparent effect in lessening the destruction of this vitamine.

Lot 3, Chart 3, received sauerkraut in the amounts indicated in the chart. At the time of commencing the experiment the sauerkraut, which was obtained at a local grocery store, was about 4 weeks old. Although larger and more mature animals were used, scurvy developed at an early date. No appreciable degree of protection was secured even with an allowance of 5 gm. per individual per day.

This destruction of the antiscorbutic vitamine during the fermentation of the cabbage in the making of sauerkraut suggested the necessity of testing silage to see if there was a similar destruction of the antiscorbutic vitamine during silage making. Barnes and Hume (15) expressed the opinion that milk produced by cows on summer pasture would be more highly antiscorbutic than that produced by winter feeding. This supposition was confirmed by work done at this station (16) and elsewhere. The presence or absence then of this vitamine in silage would throw light on this problem, since silage would seem to be the most likely of winter feeding stuffs to possess marked antiscorbutic properties. Lot 4, Chart 4, received an allowance of 20 gm. per individual per day of a silage of very inferior quality. Silage A was made from dry corn fodder and water had been added to it at the time of filling the silo. When used in these experiments it was rather dry and mouldy. Lot 5, Chart 5, received a somewhat better grade, Silage B, which was of good quality when fed. The third group of animals in this set of experiments, Lot 6, Chart 6, received the best silage to be had from the silos of the University Farm. It was made from corn still green when placed in the silo and had kept without spoilage. The curves of the animals' weights in Charts 4, 5, and 6 are practically identical. The last group survived a few days longer, but considering the difference in the maturity of the corn and the quality of the silage, the variation in the antiscorbutic properties was exceedingly slight. From the standpoint of winter-produced milk, it would appear that cows derive little of the antiscorbutic factor from silage, and consequently, it is easily understood why such milk would be low in its content of this vitamine. However, further work is necessary to make clear the factors involved in the destruction of the antiscorbutic vitamine during fermentation. It is possible that the temperature attained during the fermentation of large masses of plant material may be the deciding factor. Esten and Mason (17) report that the temperature of silage during fermentation is usually 26–30°C. A temperature of 30°C. is very often maintained in a silo for a month or more and could easily account for the destruction of the antiscorbutic vitamine.

Behavior of the Antiscorbutic Vitamine of Orange Juice to Various Treatments.

Chart 7 shows the growth curve of guinea pigs and the prevention of scurvy by the daily administration per individual of 1 cc. of orange juice. This amount has been taken as the minimum (18) for complete protection from scurvy with the ration used in this laboratory.

For a number of years organic solvents have been employed in the separation of the fat-soluble and the water-soluble vitamines from materials containing them, but little work has been done on the solubility of the antiscorbutic vitamine. Its solubility in alcohol has already been noted. The two animals in Lot 8, Chart 8 (Nos. 29 and 30), received orange juice which had been extracted with ether. The orange juice and ether were shaken together in a separatory funnel and the ethereal layer was separated. A second extraction was made and any residual ether was removed by evaporation at room temperature from a shallow dish. In this as well as in the following experiments the juice was prepared daily. Both animals showed normal behavior without any signs of scurvy. Apparently the antiscorbutic vitamine behaves like the antineuritic vitamine, being insoluble in ether.

Lot 9, Chart 8, received orange juice to which ether had been added, and then evaporated. The purpose here was to determine whether or not ether had any destructive action on this vitamine. The results were entirely negative.

Aeration of orange juice was next tried to determine whether or not there was any removal of the antiscorbutic factor by volatilization. The juice was placed in an aeration bottle and aerated for 2 hours. Lot 10, Chart 9, shows the results of this work on aeration. Apparently there was no removal of the antiscorbutic vitamine by such treatment.

Oxidizing agents appear to bring about a rapid destruction of the antiscorbutic vitamine. Hydrogen peroxide was first tried. When added to orange juice it reacted readily at room temperature and when allowed to stand in the light for some time, the excess of peroxide was destroyed. The addition of one volume of H_2O_2 to two volumes of orange juice when allowed to react for an hour at room temperature and in the diffused light of the room,

resulted in a marked destruction of this vitamine.[1] Reference to Chart 10 shows that there must have been a very considerable destruction since all the animals died from scurvy in 10 to 13 weeks. Potassium permanganate, Lot 12, Chart 10, was quite as drastic a destructive agent, as far as the protection from scurvy was concerned, as was the peroxide. An amount of permanganate solution just sufficient to leave a permanent tinge of color to the orange juice had been added and then allowed to react for an hour before being fed.

Later in the course of the disease, Nos. 41 and 42 were given, in addition, 1 cc. of untreated orange juice. They revived and lived considerably longer than the other two animals, but were too far advanced with scurvy to be saved. The results obtained here indicate that the antiscorbutic vitamine is readily destroyed by oxidation.

The only ready means of testing the effect of a reducing agent on the antiscorbutic vitamine was by means of molecular hydrogen generated with zinc. Chart 11 shows the curves of four animals given 1 cc. of orange juice treated with hydrogen for 1 hour. All grew at a normal rate. The treatment given the juice in this case was very mild. The manner of exposure to hydrogen gas could hardly be expected to cause much change and experiments with nascent hydrogen should be conducted. In studies on the effect of hydrogenation on the other vitamines (8, 19) the material had been kept at high temperatures to aid the reaction.

Investigation by Harden and Zilva (10) on the absorption by fullers' earth of the antineuritic and non-absorption of the antiscorbutic vitamines has already been mentioned; also filtration of the latter through a Berkefeld filter. Charcoal absorption was tried by us with the result of a partial removal of the antiscorbutic vitamine. All the animals (Chart 12) showed signs of scurvy by the 12th week. However, only one individual, No. 47, died, and this was due to a secondary cause, probably pneumonia. The other three animals showed typical semiadvanced stages of scurvy at the end of 18 weeks, indicating that the charcoal had removed some of this vitamine from orange juice.

[1] The destruction of the antiscorbutic vitamine by H_2O_2 has also been reported recently by Hess (Hess, A. F., *J. Am. Med. Assn.*, 1921, lxxvi, 693).

Lot 15, Chart 13, shows the result of a repetition of the work of Harden and Zilva (10) on filtration through a Chamberlain candle. The animals behaved normally for 10 weeks and then developed scurvy. Two died with this disease. Starting at the 14th week, the other two animals were given untreated orange juice and survived. These results indicate that there is a partial retention of the antiscorbutic vitamine by the Chamberlain candles we used, differing in this respect from the results reported by Harden and Zilva. The minimum quantity of orange juice used by Harden and Zilva in their experiments was 3 cc. per individual per day, which no doubt is not low enough to decide whether this vitamine is partially held by the Chamberlain candle or not. Possibly the size of the pores in the candle will determine the amount of vitamine passing through. It probably would be difficult, however, to secure a filter sufficiently fine to retain all this vitamine, judging from the experience gained in this experiment.

SUMMARY.

1. Desiccation of cabbage in an atmosphere of carbon dioxide for 35 hours at 65°C. did not prevent the destruction of the antiscorbutic vitamine.

2. Fermentation processes involved in the making of sauerkraut from cabbage and silage from corn result in a destruction of the antiscorbutic factor. What part heat played in this destruction is as yet undetermined.

3. This vitamine was not removed from orange juice by either ether or by aeration. Oxidizing agents such as hydrogen peroxide and potassium permanganate caused its destruction, while the mild reducing action of molecular hydrogen was without effect.

4. Blood charcoal and the Chamberlain filter removed a measurable amount of this vitamine from orange juice. No doubt size of pores and character of material are factors in such absorptions, and quantitative separation by such means from the water-soluble vitamine is not to be expected.

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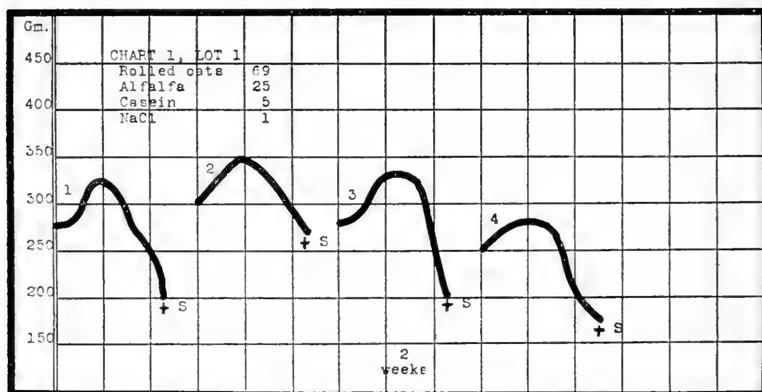

CHART 1. The animals in this lot received the scorbutic diet of rolled oats, alfalfa autoclaved for 30 minutes at 15 pounds pressure, casein, and common salt. This was fed as the basal ration in all the experiments of this series. All the animals gave the typical picture of guinea pig scurvy.

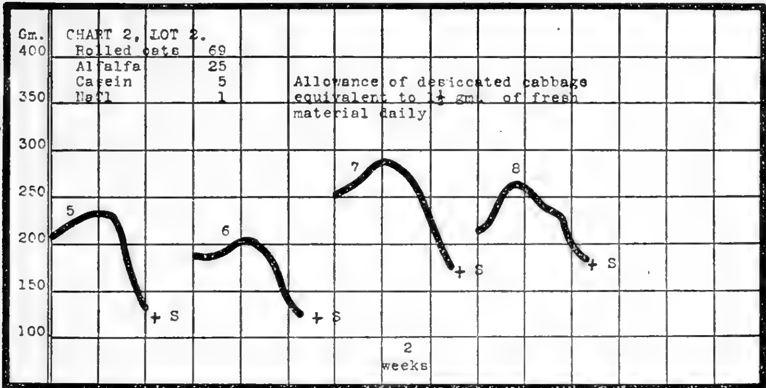

CHART 2. In addition to the basal ration, the four animals in this lot received cabbage desiccated in a vacuum oven in an atmosphere of CO_2. A temperature of 65°C. maintained for 35 hours was necessary to reduce the cabbage to dryness. The daily allowance was the equivalent of 1.5 gm. of fresh cabbage, the amount needed to give normal growth and protection. Absolutely no protection was offered indicating complete destruction of the antiscorbutic.

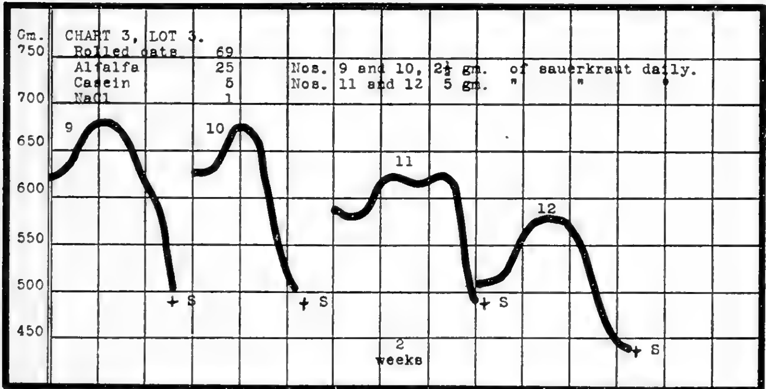

CHART 3. Sauerkraut used in this experiment was fresh material which had undergone normal fermentation. Animals 9 and 10 received 2.5 gm. a day while Nos. 11 and 12 received 5 gm. Since 1 gm. of fresh cabbage is sufficient for protection, it is apparent that during fermentation the vitamine was destroyed.

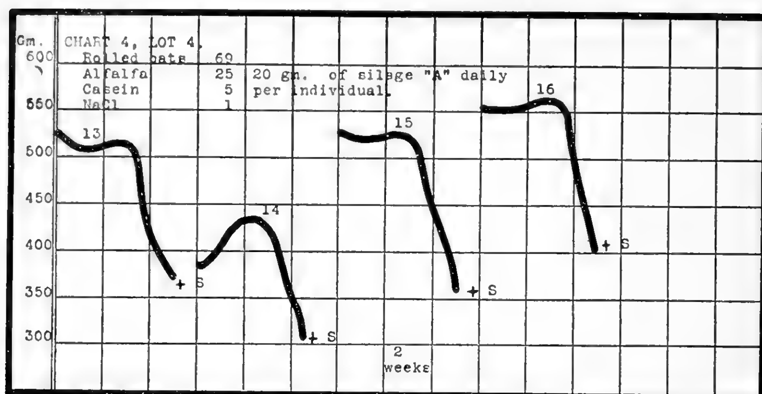

Chart 4. Silage A was made from dry corn fodder to which water had been added at the time of ensiling. When used, the material was rather dry and mouldy. The daily allowance of 20 gm. was about all the animals would consume. All died of scurvy within 5 weeks.

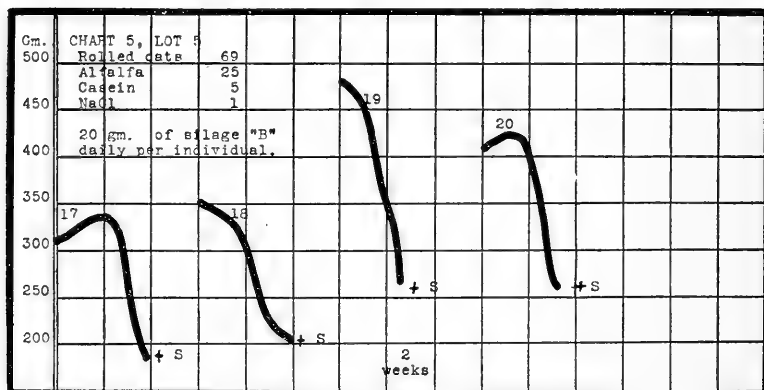

Chart 5. Silage B was made from greener corn than Silage A. It was of fair quality and appeared quite fresh in the middle of the winter. No protection was offered since the animals succumbed within 25 days.

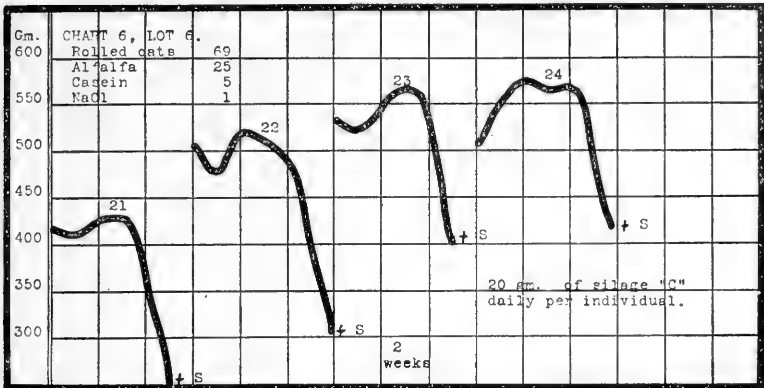

CHART 6. Silage C constituted the best grade of silage used. The corn at the time of ensiling was at the proper stage of maturity. It was green and fresh when fed the animals in the above lot. A slight degree of protection was apparently offered, yet it was so slight as to be practically negligible. Evidently, during fermentation of the silage there is a destruction of the antiscorbutic vitamine.

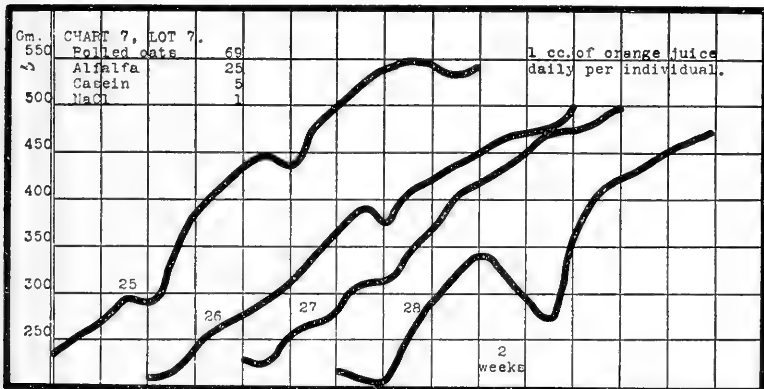

CHART 7. These animals received 1 cc. of orange juice daily. This amount has been found to be the minimum for adequate protection. The juice after being squeezed out was either centrifuged or strained through a linen cloth. No symptoms of scurvy were observed during the 18 weeks the animals were on the experiment.

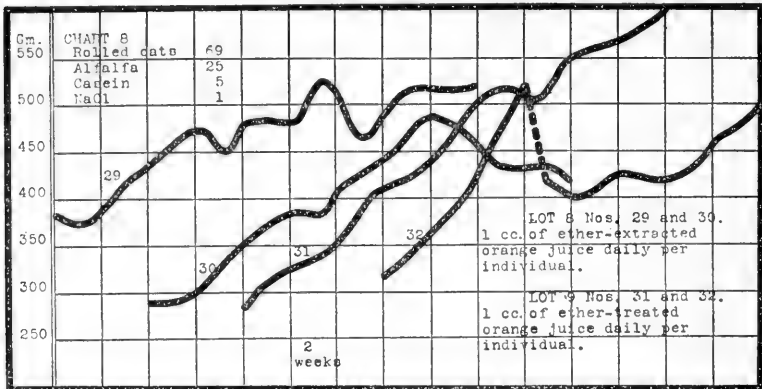

Chart 8. Lot 8. The orange juice given the two animals in this lot was extracted with ether to remove any ether-soluble materials present. Two successive extractions of equal quantities of juice and ether were made in a separatory funnel and the residual ether was evaporated off. The two guinea pigs grew normally showing that no appreciable amount of the vitamine was removed by the ether.

Lot 9. The ether and orange juice were merely mixed and the ether was evaporated off. Both animals made normal growth. No. 32 gave birth to one guinea pig which grew well until weaned.

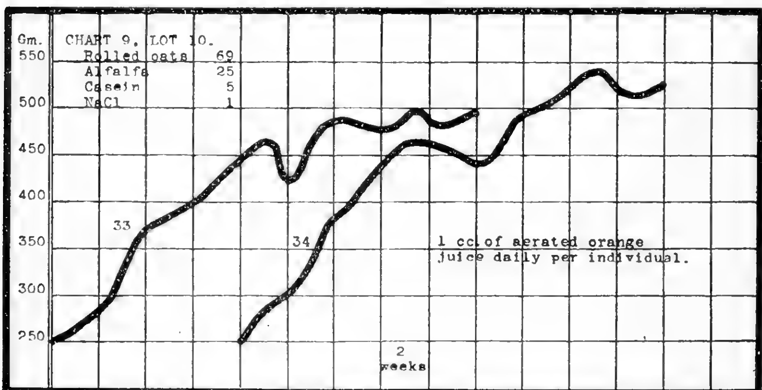

Chart 9. The aerated orange juice used in this experiment was prepared by aerating the material at room temperature in an aeration bottle for 2 hours. That there was no loss by aeration is indicated by the normal growth made. Non-volatility at room temperature, evidently, is an additional property of the antiscorbutic vitamine.

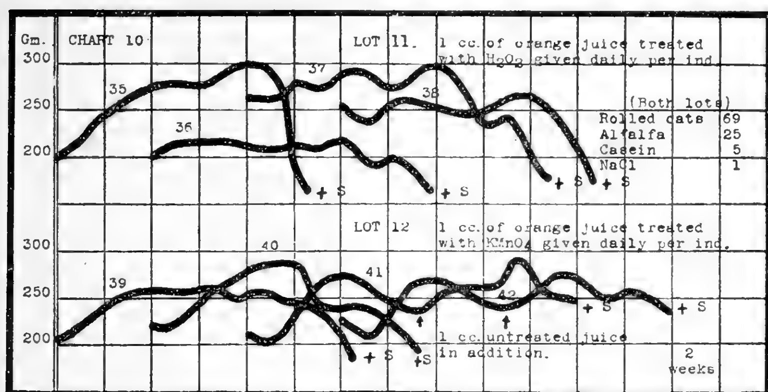

CHART 10. Lot 11. One volume of H_2O_2 was added to two volumes of orange juice and allowed to react for an hour. After 8 weeks all four animals developed scurvy and in 13 weeks all were dead. The typical scurvy picture was observed in each case. The destruction of the vitamine was apparently brought about by the oxidizing action of the peroxide.

Lot 12. The oxidizing reagent used here was $KMnO_4$. An amount of permanganate solution just sufficient to leave a permanent tinge of color to the orange juice was added and then allowed to react for an hour. No marked harmful effects of the permanganate were observed. Addition of 1 cc. of untreated juice to the daily allowance of Nos. 41 and 42 prolonged their lives over a month longer than the other two. About the same degree of destruction of the vitamine was observed as with the previous lot.

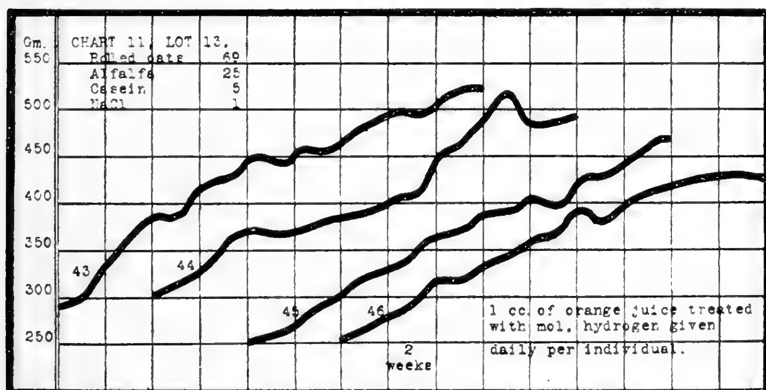

CHART 11. These animals received hydrogenated orange juice. The "hydrogenation" consisted of bubbling molecular hydrogen into the juice. This very mild treatment had no effect on the antiscorbutic property of the material since all four animals grew normally.

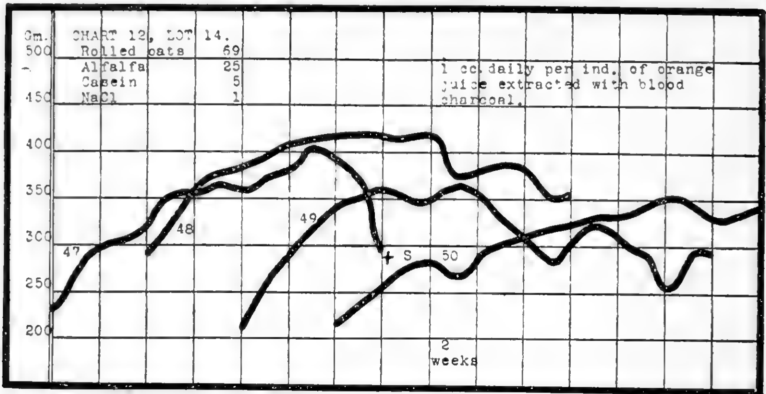

CHART 12. Ground blood charcoal was added to orange juice, and the mixture shaken at intervals for 15 minutes and then filtered. Animal 47 died of scurvy at the end of the 14th week. The other three were well advanced with scurvy at the end of 18 weeks when the experiment was stopped. The charcoal evidently removed quite a portion of the vitamine.

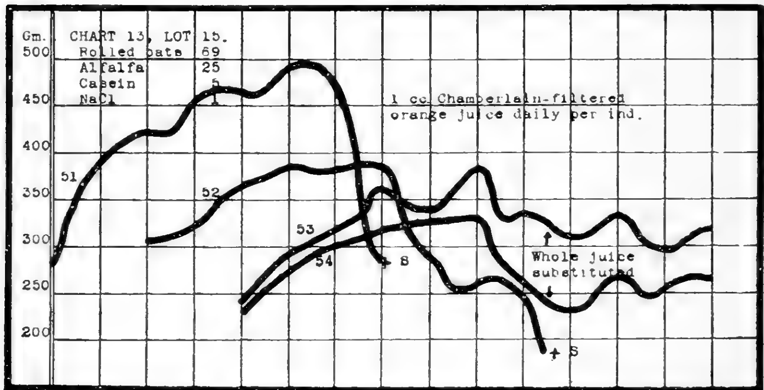

CHART 13. The orange juice was filtered through a Chamberlain filter under an air pressure of 15 pounds. The filtrate was a clear amber-colored liquid. By the 10th week, all four animals had developed scurvy. Nos. 51 and 52 died while the other two were saved by substituting whole juice for the filtered. Even more of the antiscorbutic vitamine was removed by this treatment than by the charcoal absorption. The use of a finer pored candle for the filtering would have probably removed more.

BLOOD SUGAR REGULATION AND THE ORIGIN OF THE HYPERGLYCEMIAS.

I. GLYCOGEN FORMATION AND GLYCOGENOLYSIS.

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(Received for publication, February 2, 1921.)

The normal blood sugar concentration is from 0.09 to 0.10 per cent with a physiological latitude between 0.07 to 0.11 per cent. In a number of mammals the blood sugar concentration is also found to be 0.09 to 0.10 per cent (1).

This blood sugar concentration is constant in normal individuals, and the regulation is very finely adjusted. Normal feeding does not cause any change. By administering glucose the concentration is increased rapidly within a few minutes, whereafter it quickly decreases to the normal. Values more than 0.12 per cent are generally referred to as hyperglycemias.

The disclosure of the mechanism of this normal regulation and of the causes of its disorder is indispensable for the apprehension of the nature of diabetes.

The first workers on these problems considered the appearance of sugar in the circulating blood as a pathological phenomenon. The blood serum in diabetics was found to taste sweet (2). When it was proved that starch by digestion in the intestine forms sugar (3), and that sugar occurs in the blood of normal animals after feeding carbohydrates (4), the blood sugar was considered as physiological, but, however, as accidental and being closely connected with the digestion.

This was the opinion when Bernard (5) commenced the series of investigations which proved that sugar occurs normally in the blood and independently of the digestion. His statements that the vena hepatica in a meat-eating dog also contains sugar, that the isolated and washed liver produces sugar, that this

sugar formation is of a fermentative nature, that the puncture of the medulla oblongata at the base of the fourth ventricle may produce hyperglycemia and glycosuria, and that the origin of this sugar is a polysaccharide (glycogen), which disappears after piqûre, are the epochal discoveries which form the starting point for modern research on diabetes.

Bernard supposed that blood sugar was secreted by the liver. This sugar secretion was, according to his opinion, regulated by the nervous system. He supposed that sugar combustion took place in the lungs. Between sugar combustion in the lungs and sugar secretion in the liver was a nervous connection by means of the vagus, *via* the medulla oblongata. The combustion in the lungs caused a reflex increase in secretion in the liver.[1] Later, when sugar was proved to exist in all circulating blood, Bernard in his theory put the capillaries in place of the lungs, but did not, however, alter the theory as to blood sugar regulation by reflex. By a nerve path *via* the medulla oblongata, the liver received impulses to increase sugar secretion, and in this manner the blood was supplied with sugar in proportion to the requirement.

According to this theory Bernard considered diabetes as a nervous disease. And this view on diabetes as a disease of nervous origin, as well as his opinion of the blood sugar regulation as governed by the requirement of the organs for sugar, is still accepted by many clinicians. Von Noorden (6), however, introduced a change in this theory. He still assumed, as did Bernard, that the transformation of glycogen into glucose was governed by the requirement of the organs for sugar. But he supposed that this transformation took place on a chemical signal from the muscles to the liver,[2] passing through the blood stream.

Many attempts have been made to examine the nature of the process that takes place by the transformation of glycogen in the liver. Bernard supposed that the diastase, found by him and later stated by many other investigators, might be an important factor in this process. The diastase occurs *in vivo* in the liver cells. This fact does not make these processes any easier to explain. The question is: How is it possible for the glycogen, which also occurs intracellularly, to exist in the same cell as the

[1] Bernard (5), p. 321ff.

[2] von Noorden (6), p. 153.

diastase without at once being hydrolyzed to glucose. Bang (7) holds that the pure liver diastase is inactive and that an activator must be supplied. As far as another diastase, the ptyalin, is concerned, it has been proved that such activators exist. The dialyzed, inactive ptyalin is activated, as shown by Michaelis and Pechstein (8), by a number of neutral salts. Bang (1, 7) has, by experimenting on surviving frog liver, found that it is the same case with the liver diastase, as far as can be concluded from the increased sugar formation. Before the diastase can become active and before sugar really can be formed, the diastase must be in contact with sodium chloride. Normally, however, the resting normal cell is impermeable to sodium chloride. By the secretion of bile the liver cells absorb and excrete sodium chloride, and it is this process, which, according to Bang, maintains the normal blood sugar concentration. Bang has also found that adrenalin in the isolated frog liver surviving in Ringer solution, causes a heavy formation of sugar, and the mechanism of the adrenalin hyperglycemia is, therefore, according to Bang (1), established in that way; that is, the adrenalin makes the liver cells permeable to sodium chloride, which thereafter activates the diastase. The hypothesis is attractive, but the proofs are still missing that the adrenalin changes the permeability.

Another suggestion, made by Grode and Lesser (9) is that the glycogen and the diastase exist separately in the same cell, but under certain influences these two substances may come in contact.

Both of these hypotheses try to explain how it is possible for the glycogen to exist in liver cells together with the diastase. It can be explained according to both why sugar formation takes place in the surviving liver and especially quickly in the ground liver. But neither of them gives a satisfactory explanation of the mechanism of glycogen formation and of glycogenolysis. They do not explain how sugar is secreted apparently according to requirement. Neither do they explain how it happens that the blood sugar concentration is constant, even if the liver is free from glycogen, nor what causes the liver cells, apparently at the right moment, to transform the right quantity of glycogen into glucose and secrete it into the blood. A treatment of these questions and an investigation regarding the nature of these processes will always be incomplete, provided that they deal only with glycogenolysis.

The processes taking place are of two kinds, namely a glycogen formation and a glycogenolysis, and both participate in the blood sugar regulation. The conditions under which these processes normally take place and the influences, which under physiological and pathological circumstances may be prevailing, must be examined separately.

In this paper the conditions of glycogen formation and of glycogenolysis are dealt with. In Paper II the experimental data are given, and in Paper III an effort has been made to correlate the facts.

Glycogen Formation.

Bernard believed, as is well known, that glycogen is formed only from protein. The deposit of glycogen in the liver also, after feeding with carbohydrates, was, in his opinion, caused by the protein-sparing effect of carbohydrates. By the investigations of Pavy (10), E. Voit (11), and C. Voit (12) it was proved that animals fed carbohydrates form glycogen directly, even in a higher degree than proteins.

In 1903 Grube (13) commenced experiments to determine whether carbohydrates form glycogen in the artificially perfused liver. Grube experimented on turtles and is the first who has been able to prove a glycogen formation by the method of surviving organs, in which Martz (14) a few years earlier was not successful. Grube supposed that the reason why Martz was unable to observe any glycogen formation was that the latter used too strong concentrations of glucose in his transfusions, namely 2 and 2.6 per cent, and that these concentrations harmed the liver cells. However, it is difficult to agree with Grube in this objection, when the positive result which Doyon and Morel (15) obtained by injection of a 30 per cent solution of glucose into the vena mesenterica in a dog is taken into consideration. That the concentration is without importance was further clearly demonstrated by the formation of glycogen which Freund and Popper (16) observed by injection into the vena mesenterica in dogs of 1, 2, 5, 10, and 25 per cent solutions of glucose, and also by Parnas and Baer (17) by injections of 0.5 and 2.5 per cent solutions. Ishimori (18) also observed glycogen formation in a rabbit by injection of a 20 per cent solution of glucose into the

vena mesenterica. In numerous experiments on rabbits Barrenscheen (19) obtained formation of glycogen in the isolated liver by 2 per cent solution of glucose. Barrenscheen also made the observation that 2 to 5 days after *total* pancreatectomy the dog liver cannot form glycogen in transfusion experiments; but after *partial* pancreatectomy the glycogen formation takes place normally.

It is therefore interesting to see what method is used by the different investigators. Grube in all his investigations used a liver-pancreas preparation and obtained positive results. Martz, however, who did not observe any glycogen formation in the liver perfused with solutions of glucose, experimented on isolated liver without pancreas. The other investigators quoted, all of whom obtained positive results, did not experiment on isolated livers, but only injected the sugar solution into the vena mesenterica, and the liver thus received the pancreas hormone in a normal manner.

By critical examination of the literature on this subject it is concluded that the pancreas is necessary for glycogen formation in the liver, an opinion already put forward by de Meyer (20) in 1909.

This opinion is further supported by other investigations made by Langfeldt (21). During examinations on alimentary hyperglycemia in normal dogs it was observed that blood sugar concentration increased rapidly; the maximum was reached after about 1 hour, whereafter the concentration again decreased quickly. The curve was pointed. The sugar concentration in the blood might increase to 0.17 per cent without sugar passing into the urine. As the sugar rapidly disappeared from the blood and was not excreted in the urine, we were forced to believe that it was either burned or deposited as glycogen. Combustion cannot explain the disappearance of such large quantities of glucose which have been used in these experiments, up to 30 gm. per kilo of body weight, in such a short time, and there is no other explanation possible than that it is deposited as glycogen. That a diffusion of glucose as such into the tissues should take place and that it should remain there in crystalloid state is impossible on account of the increase in the osmotic pressure which would occur. As long as the maximum glycogen formation in the body,

namely 37 gm. per kilo (Schöndorff (22)) is not reached, and since the presence of other carbohydrates is not proved, it is to be assumed that the glucose normally is rapidly converted into glycogen.

In partially depancreatized dogs we observed by similar alimentary tests that the rate at which the blood sugar concentration decreased lessened shortly before diabetes was manifest. A long time after the operation the animals were normal with regard to this point. The pancreas remnant secreted sufficiently to secure a quick glycogen formation. But gradually as the pancreas remnant sclerotized, this ability was reduced; that is, after the maximum was reached the blood sugar concentration did not decrease so rapidly by the tolerance tests as before. Otherwise the dogs were normal in this period, they did not have spontaneous glycosuria when fed normally, and the blood sugar concentration was normal at the beginning of the tolerance test. The only difference in the condition was that more time was required before the blood sugar concentration again became normal. Gradually, less and less glucose was required to produce a more lasting hyperglycemia. These alimentary hyperglycemias of long duration in fasting, partially depancreatized dogs cannot be caused by anything else but a reduced ability for glycogen formation—an ability which normally depends upon a sufficient pancreas.

On the basis of these experiments and of the experiments by other investigators *it is therefore concluded that the formation of glycogen depends upon the presence of the pancreas hormone.* There is probably also a formation of glycogen by means of the diastase as enzymatic glycogenolysis, like all enzymatic processes, is reversible. However, this process is, from a quantitative standpoint, subordinate compared with the specific glycogen formation by means of the pancreas hormone. But it explains why the liver never is entirely free from glycogen even after total pancreatectomy.

Glycogenolysis.

That glycogen is transformed into glucose post mortem has been known since the days of Bernard; likewise that this postmortem transformation is due to a diastatic enzyme, which can

be extracted with water and precipitated with alcohol.[3] Bernard supposed that this enzyme also appears *in vivo* and that it acts in the sugar formation *in vivo*.

Not every one agreed with this. Thus Schiff (23) supposed that the liver normally was entirely sugar-free and that the diastase was formed post mortem in the blood. Tiegel (24) was of the same opinion and supposed that the diastase was formed by destruction of the erythrocytes. Pavy (25) at first was of the opinion that the sugar was formed post mortem and in such a way that the glycogen diffused into the blood, which had diastatic ability. The pathological sugar formation *in vivo* depended upon the contact of the glycogen with the blood. Seegen (26) did not acknowledge the existence of a diastatic liver enzyme, and Bial (27) denied that the liver itself produced a diastase, suggesting that it appeared to have immigrated from the blood.

Thus, while a number of investigators identified the liver diastase with the blood diastase, a number of other investigators, as Dastre (28), Paton (29), Cavazzani (30), and others, suggested that sugar formation was not of enzymatic nature, but was caused by a specific action of protoplasm.

There was no general agreement with Bernard's theory of glycogenolysis. Nor was von Wittich's statement (31) that glycerol extract from the washed, blood-free liver had diastatic effect, regarded as convincing. When Tebb (32) had shown that liver powder could be kept in alcohol for 6 months without losing its diastatic effect, and when Wohlgemuth (33), using the Buchner method, had produced a liquid with heavy diastatic effect, the enzymatic nature of sugar formation was considered as proved. Zegla (34), Macleod and Pearce (35), and others demonstrated the enzyme by the same method.

The existence of the diastase in the liver cells was now to be considered as a fact. For the further investigation of normal and pathological sugar formation it was necessary to know whether sugar formation in the liver really was a vital function of the liver cells, or whether it was independent of the nervous system and of the internal secretion.

[3] Bernard (5), p. 182.

The proof was given by Bang (7). In experiments on isolated frog livers surviving in Ringer solution, Bang proved that sugar formation in the liver is a vital and independent process.

A heavier sugar formation as a result of an increased glycogenolysis may take place, however, under different influences. Glycosuria, after administration of inorganic acids *per os* or by intravenous injection, was recorded by Naunyn, von Frerichs, Pavy, and Külz (cf. Elias (36)). Ehrlich and von Frerichs (37) observed that the glycogen disappeared when frogs were put into water containing acetic acid.

The effect of acids on the metabolism of carbohydrates was examined by Elias (36), by Elias and Kolb (38), and by Elias and Schubert (39) in an extensive manner. They observed that small amounts of acids *per os* (rabbits and dogs) as well as by transfusion of the liver (turtles) caused glycogenolysis, which was followed by hyperglycemia and glycosuria.

One may conclude from observations made on partially depancreatized dogs by Langfeldt (21) that an increase of the hydrogen ion concentration in the blood of the portal veins is able to produce a heavy glycogenolysis. On account of the absence of alkaline pancreatic juice in these dogs the acid gastric juice will not be neutralized in the intestine and consequently the hydrogen ion concentration in the blood of the portal veins is increased, when the gastric juice or the contents of the stomach are absorbed in the intestine.

In these dogs it was observed that the secretion of gastric juice itself, caused by giving water or bouillon, was able to produce a considerable hyperglycemia. In depancreatized, but yet not diabetic animals, a smaller, but distinct hyperglycemia likewise was produced in the same way, while a normal dog did not show any change in the blood sugar concentration.

Corresponding observations were made by Murlin and Sweet (40), who delayed the appearance of the glycosuria by ligation of the pylorus or by gastrectomy before pancreatectomy.

When all these facts are taken into consideration, the question arises: What is the nature of this acid action? Considering the known influence of hydrogen ion concentration on enzyme action in general, we are led to expect that the acid supply affects the diastase by altering the reaction of the mixture.

As far as all diastases hitherto examined are concerned, it has, as previously mentioned, been proved that pure diastase is inactive. Together with neutral salts, or rather, with the anions of these salts, the salivary diastase forms complex compounds, and these compounds have diastatic effect (Michaelis and Pechstein (8), Norris (41)). These different salt diastases have their optimum of action at different hydrogen ion concentrations; the nitrate diastase at pH 6.7, the chloride diastase at pH 6.9, the sulfate and phosphate diastases at pH 6.1.

If it should be proved that the liver diastase also forms such complex compounds with different hydrogen ion optima, it would remain to be determined whether the endocrinogenous hyperglycemias, the adrenalin, and the thyroiodine hyperglycemias —which are the only experimental hyperglycemias—are perhaps due to the formation of similar complex compounds with the liver diastase, resulting in a change in the conditions of reaction.

The present problem thus consists of an examination of the conditions of action of the liver diastase, acting on glycogen, the influence of the most important physiological anions, and of extracts from the endocrine organs.

The examination of these problems forms the experimental part of this work, and the results obtained form together with the reflections in this paper the basis for that theory on the blood sugar regulation and on the mechanism of the origin of the hyperglycemias, which will be presented in Paper III.

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BLOOD SUGAR REGULATION AND THE ORIGIN OF THE HYPERGLYCEMIAS.

II. CONDITIONS OF ACTION OF LIVER DIASTASES.

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(Received for publication, February 2, 1921.)

In this communication the experimental data concerning the problems mentioned in Paper I are given.

Methods.

The enzyme was produced by the method described by Wiechowski (1). A calf liver was washed through the portal vein with distilled water. After the larger part of the connective tissue had been removed the liver was put through a meat chopper and dried quickly in thin layers on glass plates in an air current. The dried liver was then ground in a mill after adding five times its volume of toluene, the toluene filtered off, the sediment washed with toluene, and extracted with three times its volume of absolute alcohol. After filtrating off the alcohol, the sediment was dried in the air. Wiechowski used an emulsion of this powder in water. In our experiments the liver powder was extracted in a thermostat for 5 days at 37°C. with ten times its weight of a 50 per cent solution of glycerol in chloroform-water. Then it was centrifuged, the liquid poured out and kept in a brown bottle with a glass stopper. 0.5 cc. of this liquid was used.

Hydrolysis of the glycogen took place in 25 cc. measuring flasks. In all experiments the same quantity of glycogen was used, namely 5 cc. of a 1.5 per cent solution (= 75 mg.), and the same quantity of enzyme, 0.5 cc.

The different hydrogen ion concentrations were produced by means of the Sörensen phosphate solutions (2). In each test 5 cc. were used.

After the addition of the substance to be examined in concentrations which will be mentioned in connection with the experiments, the flask was filled to the mark with chloroform-water.[1] The temperature was maintained at 37°C. in all experiments.

The hydrogen ion concentration was controlled by the colorimetric method of Sörensen (2).

The quantity of glycogen, which was hydrolyzed, was examined by the method of Hagedorn and Jensen (3). For the analysis, 0.1 cc. was taken with a capillary pipette. The quantity of glucose found, multiplied by 0.927, gives the quantity of glycogen hydrolyzed.

EXPERIMENTAL.[2]

Phosphate Diastase.

After it was found that pure diastase (free from chlorides) had no hydrolytic effect on glycogen in distilled water, the effect of the enzyme in Sörensen's phosphate solutions was examined. The results thus give a view of the action of the phosphate diastase at different hydrogen ion concentrations. Each test-tube contained 5 cc. of 1.5 per cent solution of glycogen, 5 cc. of phosphate solution, 0.5 cc. of enzyme, and chloroform-water to the mark.

The liquids were examined after 4, 19, and 48 hours with results as given in Table I.

The optimum is at pH 6.2. The curve sinks quickly on the acid side and falls asymptotically on the alkaline side. At the pH of the blood the hydrolysis is inconsiderable (Fig. 1). The optimum of the phosphate diastase lies so far on the acid side that this compound under physiological conditions never can have any influence in eventual alterations in the regulation of the blood sugar content.

Chloride Diastase.

In the experiments on the action of the chloride ion, 1 cc. of 15 per cent solution of sodium chloride was added. Thus the

[1] It is not advisable to put chloroform directly into the liquid in the flask, because the enzyme is precipitated by shaking with chloroform.

[2] Every experiment was repeated several times and with the same result. The sterility of the solutions was controlled.

TABLE I.

Reaction.	Glycogen hydrolyzed.		
	After 4 hrs.	After 19 hrs.	After 48 hrs.
pH	*per cent*	*per cent*	*per cent*
5.9	16.43	17.01	21.88
6.23	17.59	21.88	26.48
6.46	14.24	14.82	20.56
6.64	13.76	14.24	17.59
6.81	12.02	13.18	14.69
6.97	10.28	10.28	12.02
7.16	8.54	8.54	9.4
7.38	6.38	7.38	8.54
7.73	5.64	5.64	7.38
8.04	5.64	5.64	7.38

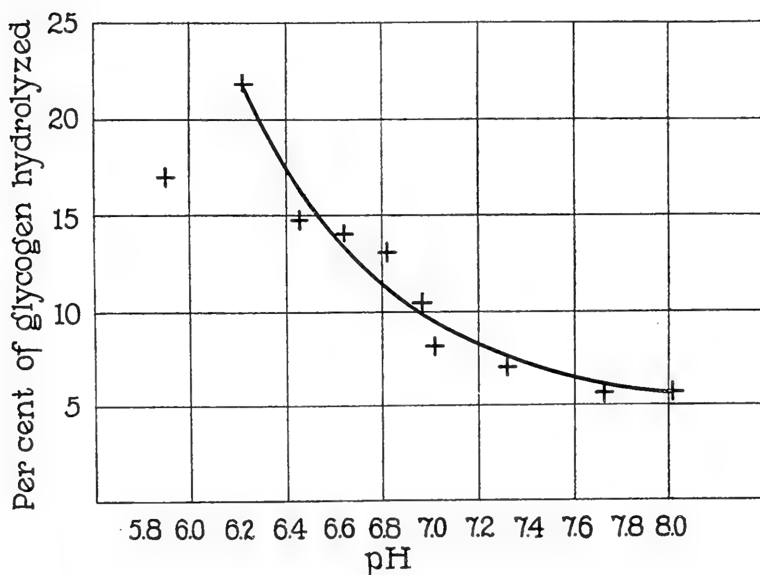

Fig. 1. The action of phosphate diastase after 19 hours.

NaCl concentration in the flasks was 0.6 per cent. Each flask thus contained 5 cc. of 1.5 per cent solution of glycogen, 5 cc. of phosphate solution, 0.5 cc. of enzyme, 1 cc. of 15 per cent NaCl

solution, and chloroform-water to the mark. The results are given in Table II.

TABLE II.

Reaction.	Glycogen hydrolyzed.		
	After 4 hrs.	After 19 hrs.	After 48 hrs.
pH	*per cent*	*per cent*	*per cent*
5.90	9.7	21.3	28.8
6.23	14.8	23.0	31.7
6.46	13.5	22.4	30.0
6.64	11.9	20.1	25.9
6.81	16.0	27.0	34.0
6.97	9.7	17.8	21.8
7.16	8.2	16.6	21.3
7.38	7.6	13.1	17.2
7.73	6.0	11.4	14.3
8.04	5.8	11.4	14.3

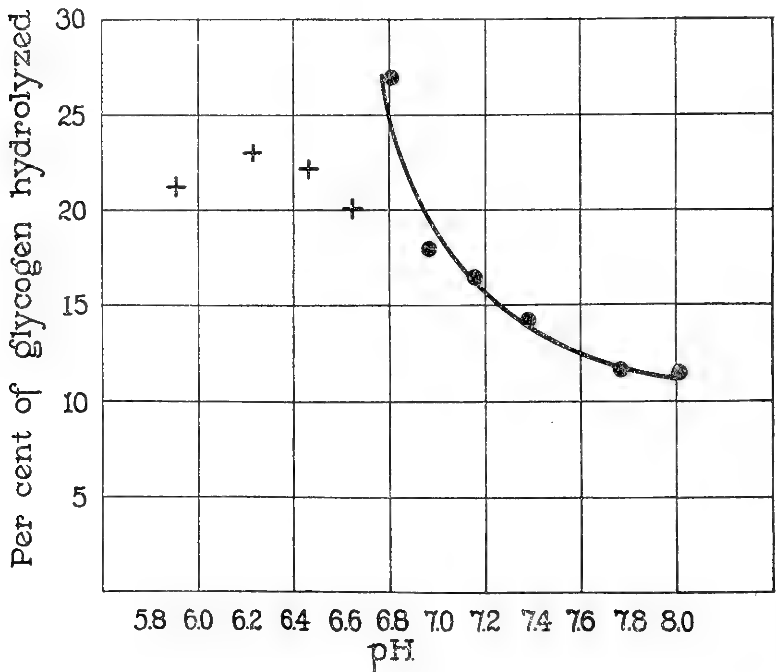

Fig. 2. The action of chloride diastase after 19 hours.

The optimum is at pH 6.8. As in the case of the phosphate diastase this curve also falls asymptotically to the alkaline side (Fig. 2). At the pH of the blood it lies a little higher than the phosphate curve. By more acid reaction than pH 6.8 the curve sinks more quickly until it meets with the asymptotically falling phosphate curve. By examining the chloride diastase two optima are found, the one representing the chloride compound, the other the phosphate compound.

It is thus proved that the liver diastase acts in agreement with the salivary diastase, having an optimum of about pH 6.2 for the phosphate diastase and about pH 6.8 for the chloride diastase. By using electrometric methods it would probably be possible to determine these optima more exactly. Thus it is possible that these optima lie a little on one or the other side of pH 6.2 and 6.8. For the physiological consideration of the results, however, such a small displacement is of no importance.

Experiments with Adrenalin.

Adrenalin is the most active and most extensively examined of the endocrine organ extracts, which may influence the metabolism of the carbohydrates. Usually 1 mg. of adrenalin, injected subcutaneously, is sufficient to produce hyperglycemia and glycosuria. Bang found that adrenalin added to the isolated surviving frog liver was able to produce increased glycogenolysis when diluted 1:200,000 and 1:400,000.

The method in the adrenalin experiments was as follows: To the glycogen in the phosphate mixtures was added at the same time as the enzyme a solution of adrenalin (Parke, Davis and Company) of such a concentration that the final concentrations of adrenalin were 1:125,000, 1:500,000, and 1:5,000,000. The quantities of the other substances were as given before. Each flask thus contained 5 cc. of 1.5 per cent glycogen solution, 5 cc. of phosphate mixture, 0.5 cc. of enzyme, 0.5 cc. of adrenalin solution, and chloroform-water to the mark.

The adrenalin solutions, of which 0.5 cc. was taken for each test, were prepared in the following manner: 1:1,000 adrenalin was diluted with water two and one-half, ten, and 100 times, thus giving solutions of adrenalin 1:2,500, 1:10,000, and 1:100,000.

From these solutions was taken 0.5 cc. and added to the test-tubes, which then were filled to the 25 cc. mark with water, thus giving adrenalin concentrations of 1:125,000, 1:500,000, and 1:5,000,000.

The solutions were examined after 19 hours with results as given in Table III.

TABLE III.

Concentration of adrenalin..........	1:125,000	1:500,000	1:5,000,000
Reaction.	Glycogen hydrolyzed.		
pH	*per cent*	*per cent*	*per cent*
6.64	24.7	20.6	14.2
6.81	24.7	19.5	14.2
6.97	24.7	20.1	11.1
7.16	21.8	21.2	9.4
7.38	25.3	16.0	7.3
7.73	29.6	12.6	6.2
8.04	21.8	10.2	6.2

The optimum action of the diastase after addition of adrenalin lies more on the alkaline side. In the solutions here examined this effect is very noticeable in the dilution 1:125,000 and can be observed also in the dilution 1:500,000. The hydrolysis is also much heavier in these tests than in the dilution 1:5,000,000, where no effect of the adrenalin is detectable. The result in this test is identical with the result in that of the phosphate diastase (Fig. 3).

Experiments with Thyroiodine.

The thyroiodine extract was produced from thyroiodine tablets which for this purpose were extracted with water for 5 days. The extract was added to the tests in dilutions of 1:15,000 and 1:50,000 counted as extract of the fresh gland. The conditions otherwise were as before. The sugar was determined 4, 19, and 48 hours later with results as shown in Table IV.

The experiment shows no change in the optimum of the diastase. Because of the sodium chloride content of the tablets the optimum is that of the chloride diastase.

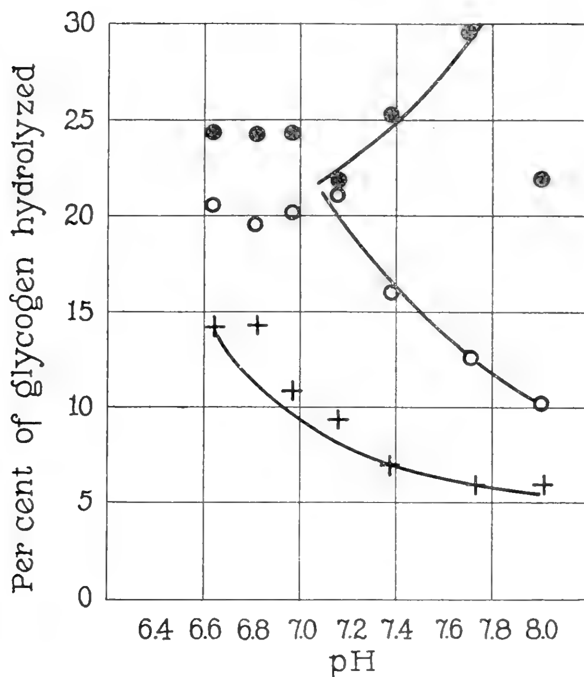

FIG. 3. The action of adrenalin diastase after 19 hours. + = dilution of adrenalin 1:5,000,000. ○ = dilution of adrenalin 1:500,000. ● = dilution of adrenalin 1:125,000.

TABLE IV.

Reaction.	Glycogen hydrolyzed.		
	After 4 hrs.	After 19 hrs.	After 48 hrs.
pH	*per cent*	*per cent*	*per cent*
5.9	7.9	17.2	36.3
6.23	6.8	11.4	21.8
6.46	3.8	10.2	25.3
6.64	7.9	16.0	30.5
6.81	13.1	16.6	32.8
6.97	10.2	13.7	29.3
7.16	7.9	11.4	23.6
7.38	7.9	9.7	18.4
7.73	7.9	7.9	13.7
8.04	3.8	7.3	9.1

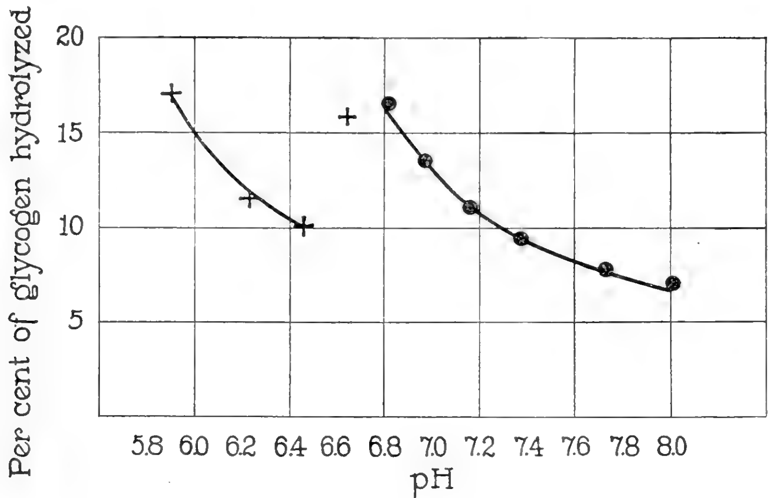

FIG. 4. The action of liver diastase under the influence of thyroiodine extract after 19 hours. The experiment shows that there is no influence of this extract on glycogenolysis. There are two optima, representing the phosphate and the chloride optima.

TABLE V.

Reaction.	Glycogen hydrolyzed.	
	Anterior lobe.	Posterior lobe.
pH	*per cent*	*per cent*
5.9	23.0	21.8
6.23	23.0	26.4
6.46	21.3	20.5
6.64	16.4	17.5
6.81	16.4	14.6
6.97	12.6	12.0
7.16	9.1	9.4
7.38	8.5	8.5
7.73	6.2	7.3
8.04	5.6	7.3

Experiments with Extracts of Hypophysis.

In these experiments extracts of the posterior and of the anterior lobes of fresh ox hypophysis were used. After dissection of the organ each lobe was weighed separately, then ground with sand,

and thereafter extracted for 5 days with 100 times its quantity of water. Of this extract 1 cc. was added for each test. The flasks thus contained 5 cc. of 1.5 per cent solution of glycogen, 5 cc. of phosphate mixture, 0.5 cc. of enzyme, 1 cc. of hypophysis extract, and chloroform-water to the mark. The determinations were performed 36 hours later and showed results as given in Table V.

The experiment shows no effect of the extracts from the anterior and posterior lobes of the hypophysis. In both experiments the optimum corresponds to that of the phosphate diastase.

The effect of combination of organ extracts was next examined.

Adrenalin and Thyroiodine.

As mentioned before, the thyroiodine diluted 1:50,000 did not cause any displacement of the optimum of the curve. Adrenalin

TABLE VI.

Reaction.	Glycogen hydrolyzed after 24 hrs.
pH	*per cent*
5.9	12.2
6.23	31.5
6.46	23.4
6.64	20.8
6.81	28.2
6.97	29.5
7.16	38.4
7.38	38.5
7.73	40.0
8.04	40.7

diluted 1:500,000 had a slight though noticeable influence. Table VI shows that simultaneous addition of adrenalin and thyroiodine in dilutions as just mentioned had a very strong effect.

The curve here falls asymptotically from pH 8.4 towards the neutral point (Fig. 5). 48 hours later, the alkaline solutions were entirely clear and it was possi' le to read the curve directly with the eye from the appearance of the liquids.

However, to make it possible to conclude something from this experiment about the physiological processes, the experiment must be performed under physiological conditions. The concen-

tration of adrenalin thus must not be greater than 1: 5,000,000 (corresponding to 1 mg. of adrenalin in 5 liters of blood), and the concentration of sodium chloride must be 0.6 per cent.

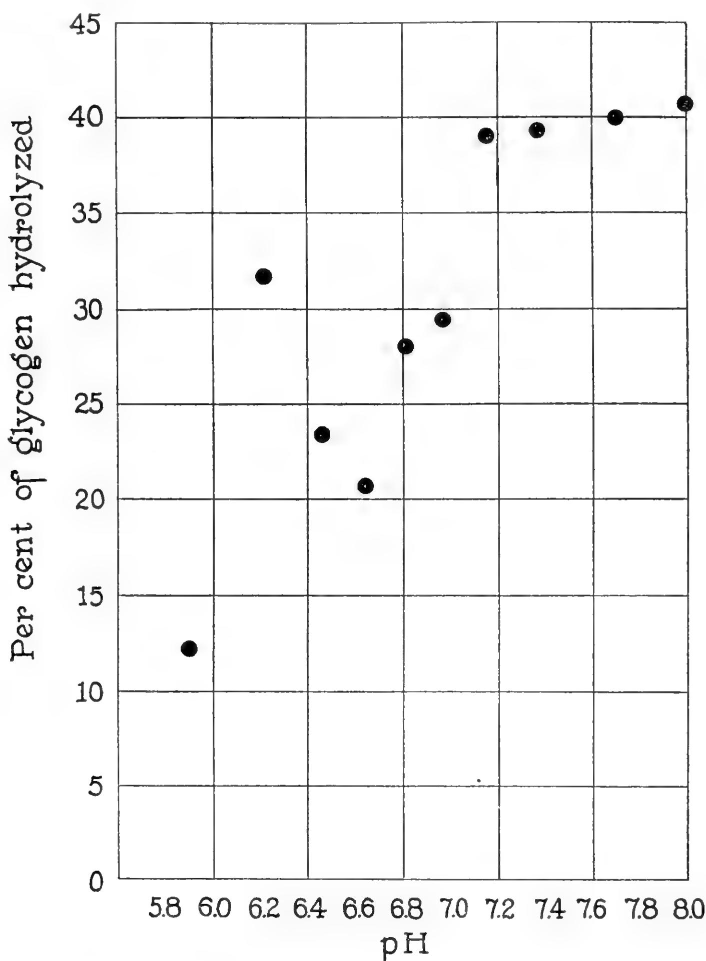

Fig. 5. The action of liver diastase under the influence of adrenalin in a concentration of 1:500,000 and thyroiodine extract in a concentration of 1:50,000, after 24 hours.

In this experiment the flasks thus contained 5 cc. of 1.5 per cent solution of glycogen; 5 cc. of phosphate mixture; 0.5 cc. of

TABLE VII.

Reaction.	Glycogen hydrolyzed.	
	After 4 hrs.	After 19 hrs.
pH	*per cent*	*per cent*
5.9	10.2	16.6
6.23	13.7	20.7
6.46	11.4	17.8
6.64	12.6	21.3
6.81	16.0	25.3
6.97	13.7	25.2
7.16	14.3	24.7
7.38	16.0	26.0
7.73	17.2	27.6
8.04	19.5	29.9

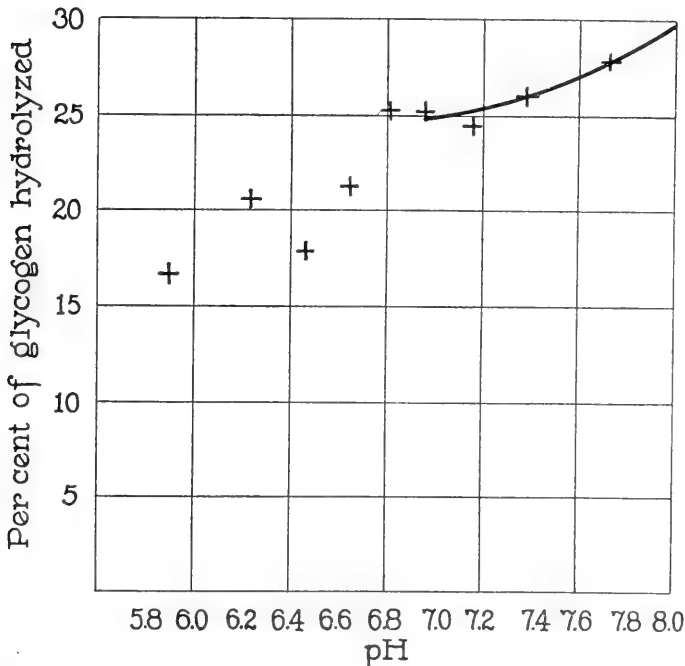

Fig. 6. The action of liver diastase after 19 hours under the influence of adrenalin in a concentration of 1:5,000,000, and thyroiodine extract in a concentration of 1:50,000.

enzyme; 1 cc. of 15 per cent NaCl solution; 0.5 cc. adrenalin solution, diluted 1:100,000 (final dilution 1:5,000,000); 0.5 cc. of thyroiodine solution, diluted 1:1,000 (final dilution 1:50,000); and chloroform-water to 25 cc. Table VII and Fig. 6 show that the experiment had the same result in this dilution.

During these experiments it was further observed that adrenalin, or adrenalin and thyroiodine, added to the mixture already started with pure phosphate solution (without any other substance) had little or no influence. It is therefore to be concluded that the combination between the diastase and the different anions is not a very easily dissociable combination.

Furthermore, experiments were performed with extracts of the anterior and posterior lobes of the hypophysis with addition of adrenalin in dilution 1:5,000,000. Such an addition had no influence. Neither did an addition of the hypophysis extracts to the adrenalin-thyroiodine mixture produce any change of the characteristic effect of these substances.

SUMMARY.

1. The optimum action of the liver diastase in the presence of phosphate anions is at pH 6.2. The optimum of the chloride diastase is at pH 6.8.

2. When adrenalin is added the optimum is shifted to the alkaline side. The optimum is at pH 7.73.

3. Thyroiodine when added alone has no influence on the hydrolysis of glycogen.

4. Simultaneous addition of adrenalin and thyroiodine has a very strong effect. Adrenalin solutions, which are so diluted that they have no influence, are active in combination with thyroiodine. This effect is noticeable also in experiments performed under physiological conditions; *i.e.* with adrenalin concentration not stronger than 1:5,000,000.

5. Extracts of the anterior and posterior lobes of hypophysis have no influence on glycogenolysis.

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BLOOD SUGAR REGULATION AND THE ORIGIN OF THE HYPERGLYCEMIAS.

III. THEORY.

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(Received for publication, February 2, 1921.)

On the basis of the experimental results and of the previous exposition of the other facts concerned, an attempt is made to explain the blood sugar regulation and the mechanism of the origin of the hyperglycemias.

Normal Blood Sugar Regulation.

As previously mentioned, Paper I, it is usually supposed that sugar formation in the liver is regulated by circumstances *outside* the liver. The requirement of the organs for sugar is the discharging moment and in a nervous or chemical way the liver then is influenced to form glucose from glycogen.

In opposition to this opinion we may, according to our investigations, explain the maintenance of the constant blood sugar concentration in a simpler way. It is not necessary to suppose any cause at a distance, and the requirement of the organs for sugar may be disregarded.

We do not know of nor have we proved the existence of any glycogenolytic agency in the liver other than the diastase. As the action of this diastase depends on precise hydrogen ion concentrations, we may suppose that the glycogenolysis is regulated by the hydrogen ion concentration of the liver tissue. The hydrogen ion concentration of the blood is pH 7.33 at 37°C.; that of the tissues is probably a little less alkaline, on account of the continuous formation of carbonic acid, but still on the alkaline side of the neutral point.

According to this opinion sugar formation should be constant, not inconstant as would be the case if the regulation took place according to the requirement. Under normal conditions the liver always discharges the same quantity of glucose.

One may also conclude from the experiments that the blood sugar concentration probably would be higher if the optimum of the chloride diastase lay closer to the hydrogen ion concentration of the liver tissue. We may therefore consider the location of this optimum suitably away from the hydrogen ion concentration of the tissue as an *arrangement of security*. Here we have an example of an enzyme, which acts not under conditions most favorable to the enzymatic action, but on the contrary in a manner limited by the milieu wherein it is situated.

The maintenance of the blood sugar concentration is considered to take place in the following manner:

Glycogenolysis is governed by the hydrogen ion concentration in the liver cells (*a*, Fig. 1). With a constant blood flow and constant temperature glucose is discharged at a constant rate from the liver to the blood. A part of this glucose is burned (*b*), another part is stored in the muscles and cells as glycogen (*c*), and the remainder returns to the liver, where it again forms glycogen (*d*). Here glycogen formation depends upon the presence of the pancreas hormone (*e*), and the material for this formation is chiefly the carbohydrates and the proteins of the food (*f*).

This theory also explains why the blood sugar concentration is constant when the liver is exhausted of glycogen, and in starvation. In this case the organism is supported from its own stores, from the glycogen of the cells, from protein, and from fat. These substances are broken down in starvation, carried to the liver as dissociated products, and the process is then the same as in the case of the food supply (*g*).

The conclusion is reached that glycogen formation and glycogenolysis are consecutive processes. The diastase acts continuously and glycogen formation takes place rapidly.

Origin of the Hyperglycemias.

It is evident from Fig. 1, which demonstrates the above hypothesis of blood sugar regulation, and from Fig. 2, in which the experimental results are presented, that hyperglycemia may arise in three ways:

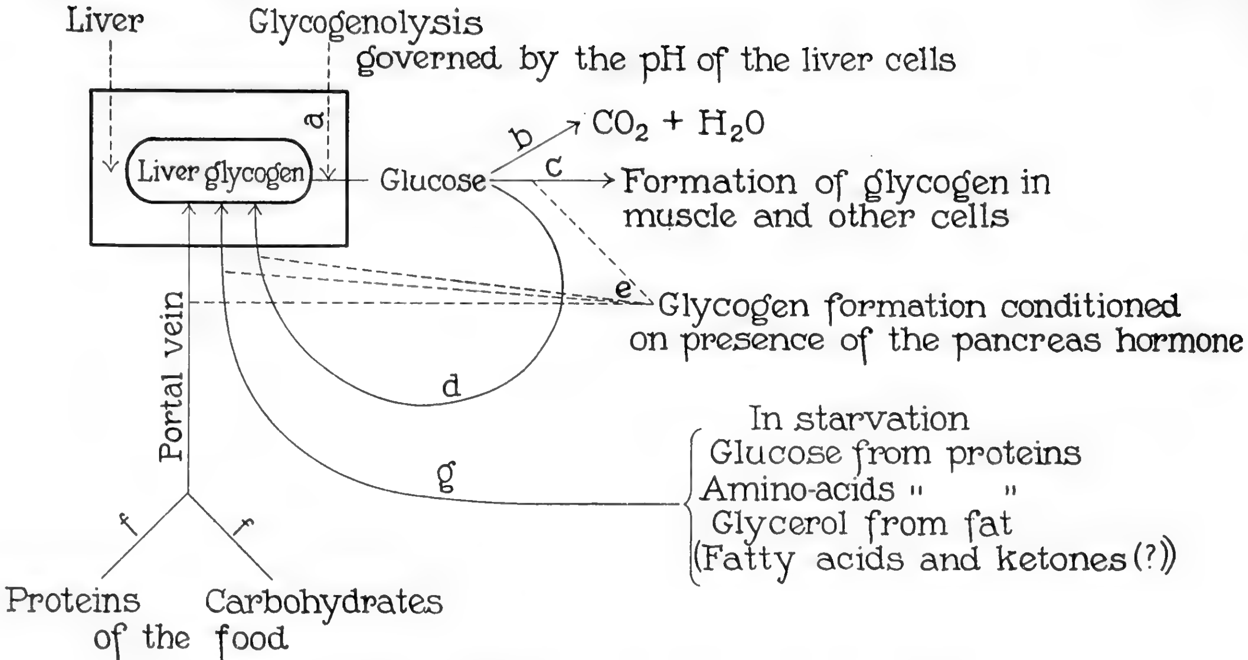

FIG. 1. Schematic representation of the theory on blood sugar regulation.

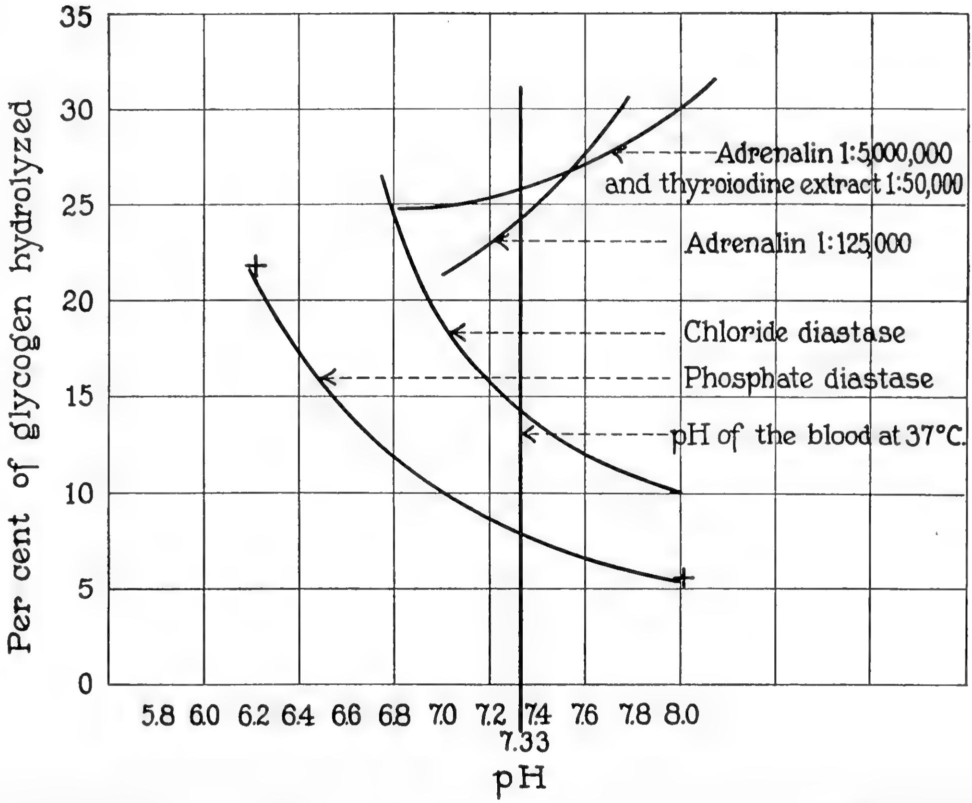

Fig. 2. Action of liver diastase after 19 hours under different influences.

1. By a change of the hydrogen ion concentration of the liver tissue so that the pH is identical with or close to the optimum of one of the curves.

2. Or by a displacement of the curve of action of the liver diastase in such a way that the optimum corresponds or is close to the pH of the tissue.

3. Or by a lack in the formation of glycogen on account of the insufficient functioning of the pancreas, which would cause a superfluity of glucose in the blood.

Under normal conditions practically only the phosphate and chloride diastases are of importance, and of these the chloride diastase is more important on account of the greater affinity of the Cl ion for diastase. The optimum of the chloride diastase lies at pH 6.8. This hydrogen ion concentration represents neutral reaction at 37°C. Therefore, to obtain maximum glycogenolysis, the milieu of the diastase in the liver must be neutral.

The action on the blood sugar concentration of acids given *per os* or by transfusion of the liver, the influence of the gastric juice in depancreatized dogs, and the postmortem glycogenolysis are thus explained.

The second way in which hyperglycemia may arise is by displacement of the optimum of the liver diastase to or close to the hydrogen ion concentration of the liver tissue.

Such a displacement takes place under the influence of adrenalin or, in a higher degree, by simultaneous influence of adrenalin and thyroiodine. It may be that the diastase forms a complex compound with these substances, as it does with phosphates, chlorides, and many other substances, and that the affinity between diastase and adrenalin-thyroiodine extract is greater than between diastase and chloride ion. The new complex compound has its optimum at pH 7.73. The glycogenolysis is very intensive, and even at pH 7.33 it is just as heavy as the Cl diastase at pH 6.8.

That the adrenalin alone, when much diluted, is inactive, but very active when thyroiodine is added, is consistent with the action of subcutaneously injected adrenalin on the blood sugar. When thyroidectomy is performed previous to the adrenalin injection, the adrenalin is either inactive or much larger doses are required (1). This is completely explained by these experiments.

Thyroiodine, when added alone, is without effect; only with adrenalin has it any influence on the glycogenolysis. By this experiment the variable and inconstant results, which are obtained in investigations regarding the effect on the blood sugar concentration by feeding thyroiodine, are explained. The effect depends mainly on the secretion of adrenalin under the influence of the sympathetic nervous system, which does not react in the same manner in every case of hyperthyroidism.

That the extracts of hypophysis are without effect on glycogenolysis is also in agreement with the biological experiments on the action of these substances on the blood sugar. It has not been possible to produce experimentally hyperglycemia or glycosuria by injection of extracts of hypophysis. Nevertheless spontaneous glycosuria or genuine diabetes is observed not infrequently in the case of acromegalia. This glycosuria may be due to a temporary disturbance of function in any of the other endocrine organs. A qualitative alteration of the function of the anterior lobe of hypophysis is also possible. However, the normal hypophysis has no direct influence on glycogenolysis.

The third way in which a hyperglycemia may arise is by a lack in the formation of glycogen, such as very probably takes place in pancreatic diabetes, to which diabetes mellitus is due. The *experimental* pancreatic diabetes belongs partially also to the first group, as there is not only a lack in the glycogen formation, but there also undoubtedly takes place an increased glycogenolysis in the liver on account of the absence of the neutralizing pancreatic juice.

All the experimental hyperglycemias belonging to the first and second group and owing to increased glycogenolysis are of a transitory nature. The former theories on this problem are based mainly on experiments regarding transitory hyperglycemias and glycosurias. Thus the theory of nerve control is based on piqûre and on numerous clinical observations on the influence of the nervous system and nervous disturbances on the glycosuria in diabetes. Characteristic of these experiments and observations was the transitory nature of the glycosuria or of the increase of an already existing glycosuria. The same is the case in the adrenalin-thyroiodine experiments of Eppinger, Falta, and Rudinger (1), which resulted in the "Wechselwirkung" theory of

diabetes. All these glycosurias are transitory glycosurias, which disappear when the glycogen is exhausted. None is chronic and it is therefore not logical to draw from these, conclusions about chronic diabetes.

In opposition to hyperglycemias caused by increased glycogenolysis and limited in duration by the stores of glycogen, hyperglycemias due to insufficient formation of glycogen are chronic. Here the blood is always supplied in some way with sugar, and thus there is no limit for the duration of the hyperglycemia.

Where the glycosurias which occur in some infectious diseases and in some intoxications shall be placed in this classification must be determined in future investigations.

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BLOOD AS A PHYSICOCHEMICAL SYSTEM.*

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(Received for publication, January 25, 1921.)

It is now apparent that, in the respiratory changes of the blood, at least six important simultaneous variables are involved (1–4). These may be conveniently taken as the free and combined oxygen of the whole blood, [O_2] and [HbO_2]; the free and combined carbonic acid of the serum, [H_2CO_3] and [$BHCO_3$]; the hydrogen ion concentration of the serum, expressed as pH; and the chloride concentration of the serum, [BCl].

Such a choice of variables is, indeed, a simplification of the facts, for the obscure problem of the combination of carbonic acid within the corpuscles is thereby avoided, as well as the problem of the movement of water and other substances, accompanying that of chloride, between cells and serum. Evidently, therefore, the simplification leads to approximations rather than to exact representations of the equilibrium. In the present paper, however, no attempt is made to consider the theoretical interpretation of the very complex physicochemical processes involved in the system, or, since the state of our knowledge forbids it, to express the relations with great accuracy. All considerations of the present paper apply to defibrinated blood.

It is a matter of common experience that, if the concentrations of free oxygen and free carbonic acid are fixed, the whole equilibrium is determined. Further, it is evident that if this is true for any pair of the six variables it must be true for every pair, since they are all related according to the ordinary relations of physicochemical equilibrium. Moreover, it is known from researches of Barcroft and his associates (4) that if definite values

* A paper presented to the (British) Physiological Society, October, 1920. The nomogram was described at the Physiological Congress, Paris, July, 1920.

are assigned to any two of the three variables, free oxygen, combined oxygen, and free carbonic acid, the condition of equilibrium is defined; the investigation of Christiansen, Douglas, and Haldane (2) established the same fact for the three variables, free and combined carbonic acid, and combined oxygen; and the same relation holds for the three variables, free and combined carbonic acid,

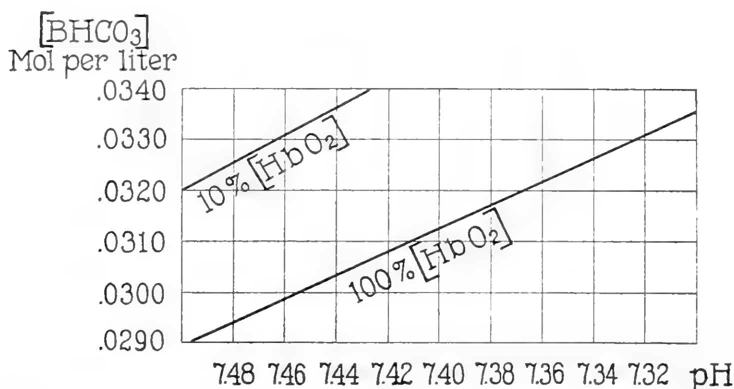

Fig. 1.

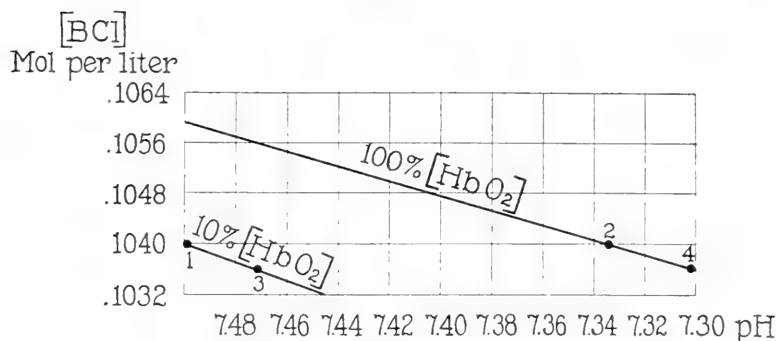

Fig. 2.

and hydrogen ion concentration (5). Finally, an investigation now in progress proves that a similar connection exists between serum chloride concentration and any two of the five variables, free and combined oxygen, free and combined carbonic acid, and hydrogen ion concentration.

In seeking an expression of these facts, it may be noted, first, that, since the equilibrium is determined by assigning values to

any two variables, a two dimensional diagram should suffice. We may therefore begin with Fig. 1, which expresses graphically the values of pH and [$BHCO_3$] for blood in which the saturation of hemoglobin with oxygen is 100 and 10 per cent, and with Fig. 2, which gives similar relationships between pH and [BCl]. These two figures represent the averages of certain preliminary observa-

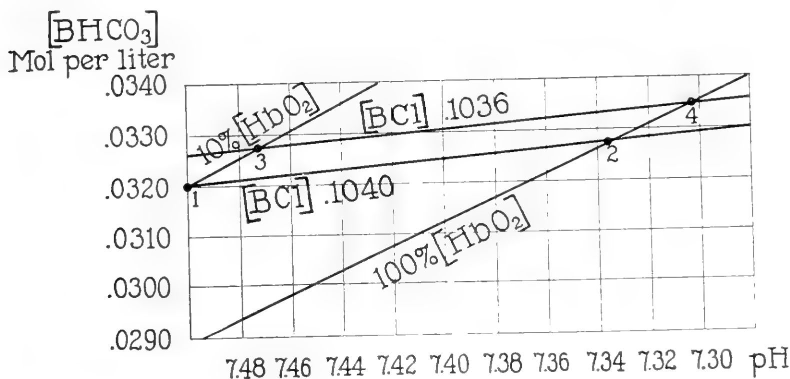

Fig. 3.

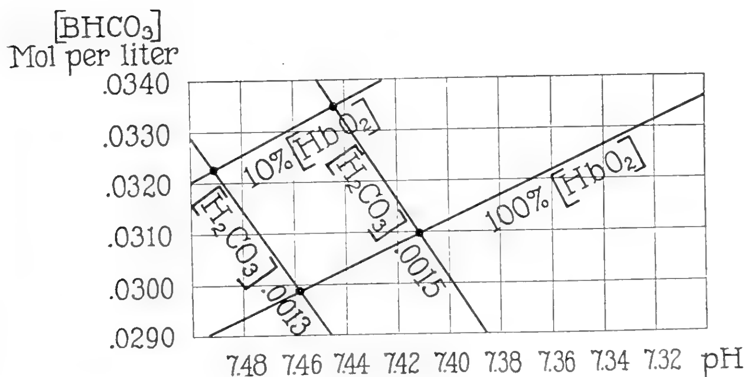

Fig. 4.

tions on five different samples of defibrinated ox blood, obtained in the course of the investigation mentioned above. No claim is here made for their accuracy. But since Fig. 1 is consistent with the research of Christiansen, Douglas, and Haldane (2), and Fig. 2 with that of Hasselbalch and Warburg (6), as explained below, it is evident that the figures are adequate as a means to

discover the general form of the geometrical representation of the equilibrium. Indeed the data of Christiansen, Douglas, and Haldane and of Hasselbalch and Warburg might have been used without modifying the final result (Fig. 5).

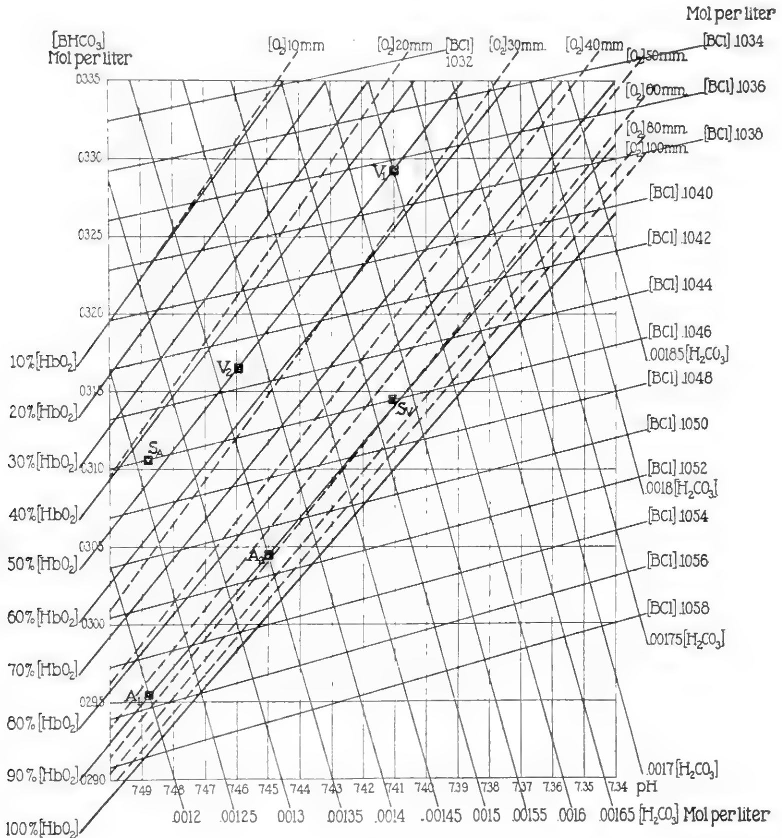

Fig. 5.

Fig. 2 may now be employed in order to discover the values of [BCl] for points on the two curves of Fig. 1, and, if the points on the two curves corresponding to equal concentrations of chloride are joined, Fig. 3 is obtained. This gives simultaneous values of pH, [$BHCO_3$], and [BCl] for oxygenated and reduced blood.

Again beginning with Fig. 1, we may mark on the two curves points of equal carbon dioxide tension, and join them. The result is Fig. 4, which shows simultaneous values of pH, $[BHCO_3]$, and $[H_2CO_3]$ for reduced and oxygenated blood.

There is nothing to forbid the combination of Figs. 3 and 4 into one diagram showing the coordinates of the four variables, pH, $[BHCO_3]$, $[BCl]$, and $[H_2CO_3]$ for reduced and oxygenated blood. In this diagram we may next (somewhat arbitrarily in the present state of knowledge) interpolate lines for the degree of saturation of hemoglobin with oxygen. This gives a fifth system of coordinates. And, finally, if we assume the equation

$$\frac{[H_2CO_3]+7.7}{0.014} = \frac{[Hb]\cdot[O_2]^{2.5}}{[HbO_2]}$$

which holds for Barcroft's blood,[1] to apply to this case, we are enabled to calculate points corresponding to equal tensions of oxygen, and thus to introduce a set of coordinates for free oxygen. The final result, Fig. 5, is the desired geometrical expression, where the background of rectangular coordinates corresponds to values of pH and $[BHCO_3]$. $[BHCO_3]$, $[H_2CO_3]$, and $[BCl]$ are expressed in units of N/10,000 concentration,[2] $[O_2]$, in the usual manner as mm. of tension, $[HbO_2]$, in percentage saturation.

As the method of construction shows, this nomogram expresses at once the results of Barcroft upon the oxygen dissociation curve of blood, and of Christiansen, Douglas, and Haldane on the carbon dioxide dissociation curve, as well as the peculiarities of the acid-base equilibrium, and of the distribution of chlorides. Obviously it has the property that if values are assigned to any two of the variables, all six are determined.

There are certain reservations, however, which must be clearly expressed regarding the present nomogram. First, the lines represent data of varying degrees of accuracy, obtained partly for ox blood and partly for human blood. Accordingly, the quantitative relationships are no doubt slightly different from those which any one specimen of blood would manifest. Second, it is quite

[1] *Cf.* Henderson (1), p. 403.

[2] Values of $[H_2CO_3]$ in units of N/10,000 may be converted approximately into mm. of tension by dividing by 0.4.

possible that the true [HbO_2] coordinates are more nearly straight lines when drawn against a background in which not only the hydrogen ion concentrations but also the bicarbonate concentrations are plotted logarithmically. Third, the correct coordinates of [H_2CO_3] and [BCl] are not straight lines, but possess a very slight curvature. All these, however, are matters of detail, which will require consideration only when more accurate data are at hand. Finally, it should be noted that all data employed in constructing the nomogram have been obtained with defibrinated blood *in vitro*.

The significance of the nomogram is most easily appreciated by considering particular points, for example A_1 and V_1, which may be taken to represent the cases of arterial and venous blood respectively. The coordinates of these points are as follows:

	pH	[O_2]	[HbO_2]	[H_2CO_3]	[$BHCO_3$]	[BCl]
		mm.	*per cent*			
A_1...	7.488	56	90	12×10^{-4} N	295.4×10^{-4} N	$1{,}055.5 \times 10^{-4}$ N
V_1...	7.411	26	50	16×10^{-4} N	329.3×10^{-4} N	$1{,}036.5 \times 10^{-4}$ N

Evidently these coordinates give a fair representation of the difference between arterial and venous blood.

The difference between arterial and venous serum bicarbonate concentrations is 33.9×10^{-4} N. This may be contrasted with the difference between the bicarbonate concentrations corresponding to two points, S_A and S_V, which both fall upon the coordinate $1{,}046 \times 10^{-4}$ N for [BCl], and which have the coordinates for pH, 7.488 and 7.411 respectively, of the points A_1 and V_1. Since S_A and S_V have the same coordinate for [BCl], it follows that in passing from one to the other, and in general in passing from any point to any other point with the same value of [BCl], there can be no exchange of electrolyte between serum and corpuscles. Therefore, the increase of [$BHCO_3$] in passing from S_A to S_V is due to the reaction of the constituents of the serum under the influence of increasing concentration of carbonic acid. In short, for such a case, the serum behaves as if isolated from the corpuscles.

This conclusion is confirmed by the observation of Hasselbalch and Warburg upon the relationship between pH and [$BHCO_3$] for

isolated serum (6). It should be noted, however, that the work of the Danish investigators does not indicate the relative importance of movement of chloride, of water, and of other substances between cells and serum, in the case of whole blood. Hence the work of Hasselbalch and Warburg confirms the form of the isopleths of [BCl], but leaves uncertain the numerical values which should be assigned to them.

The coordinates of [$BHCO_3$] for S_A and S_V are 310.6 × 10^{-4} N and 314.4 × 10^{-4} N respectively. The difference between these concentrations is 3.8 × 10^{-4} N, in contrast with 33.9 × 10^{-4} N for the difference between A_1 and V_1. Thus it is evident that the escape of carbonic acid in the lung and its absorption in the tissues must depend chiefly upon the corpuscles. Even in the serum, and presumably therefore in the plasma, much the greater part of the *variation* in bicarbonate concentration is the result of a heterogeneous reaction with the corpuscles and only a small amount, if the present estimate is quantitatively correct approximately 10 per cent, of the loading and unloading of carbonic acid in the serum depends upon a reaction exclusively within the serum. It is hardly necessary to point out that the serum constituents in question are mainly the proteins (3), and that it is the peculiarity of hemoglobin in its equilibrium with base which is responsible for the larger part of the phenomenon (1).

Not all the other isopleths have the same real significance as those of chloride concentration. We shall therefore postpone further considerations of this kind until more data have been collected, so as to make possible precise quantitative discussions.

A further and more direct proof of the variable affinity of hemoglobin for base, as a function of its saturation with oxygen, may be deduced from a consideration of the points A_2 and V_2. The coordinates of these points are as follows:

	pH	[O_2]	[HbO_2]	[H_2CO_3]	[$BHCO_3$]	[BCl]
		mm.	*per cent*			
A_2...	7.450	58	90	13.5 × 10^{-4} N	304.4 × 10^{-4} N	1,051.3 × 10^{-4} N
V_2...	7.459	25	50	13.75 × 10^{-4} N	316.6 × 10^{-4} N	1,043.0 × 10^{-4} N

In spite of the fact that A_2 is relatively arterial and V_2 relatively venous in all other respects, the reaction of V_2 is slightly more

alkaline than that of A_2. In other words it is possible to find conditions such that the simultaneous addition of carbonic acid and removal of oxygen from blood will change the reaction in the alkaline direction. It is hard to imagine another explanation of this fact than that oxyhemoglobin is more acid (or, conceivably, less alkaline) than reduced hemoglobin.

It remains to say a word regarding the general significance of the nomogram. Previous investigations have led to the proof of a relationship, in certain cases, between three of the six variables in question. This relationship always has the character of an ordinary algebraic equation in three unknowns, corresponding to a contour line chart where all three variables are determined if definite values are assigned to any two. Such is the case for $[\overset{+}{H}]$, $[H_2CO_3]$, and $[BHCO_3]$ (5, 7); for $[O_2]$, $[HbO_2]$, and $[H_2CO_3]$ (4); or for $[H_2CO_3]$, $[BHCO_3]$, and $[HbO_2]$ (2); and only lack of adequate experimental data has heretofore prevented the establishment of such a relationship between [BCl], $[H_2CO_3]$, and any one of the other four variables.

All these relationships are expressed by the nomogram. But, among six variables, taking three at a time, there are twenty different combinations. Hence, in addition to the three familiar cases above mentioned, the nomogram expresses seventeen other similar relationships.

This is, however, but another way of saying that the real physicochemical equilibrium of the blood is a more complex phenomenon than anything that has been previously considered. The laws of the acid-base equilibrium, the oxygen dissociation curve, and the carbon dioxide dissociation curve are all true, but they are all incomplete, and each is a different aspect of one larger phenomenon. The most general characteristic of this equilibrium, and one of the most familiar characteristics of all physiological phenomena, is that no change can be made at any point without producing changes at all points, including changes which, at first sight, have no apparent physicochemical connections with the original change. Nevertheless, since the number of degrees of freedom in the system is less than would be supposed, these changes are very restricted, and among the conceivable configurations of the system only an infinitesimal fraction is actually possible.

SUMMARY.

The concentrations of free and combined oxygen, of free and combined carbonic acid of the serum, of serum chloride, and of the hydrogen ion may be represented upon a two dimensional nomographic chart. The relationship between the six variables is such that, for a given blood, when values are assigned to any two, the values of the other four are determined and the condition of equilibrium unequivocally defined.

The nomogram illustrates the known facts regarding the acid-base equilibrium of blood, the oxygen dissociation, the carbon dioxide dissociation, the distribution of chlorides, and the influence of oxygen upon the affinity of hemoglobin for base.

Hence it may be concluded that all six variables are involved in a single physicochemical equilibrium.

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STUDIES ON ORGANIC REGULATION.

I. THE COMPOSITION OF THE URINE AND THE BLOOD OF THE HIBERNATING FROG, RANA VIRESCENS KALM. (PIPIENS GM.)

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The work in the present paper is preliminary to an investigation on the resorption of material during the involution of the tadpole's tail. The relative importance of autolysis and phagocytosis in this process has been studied by Morse,[1] but studies on the economy of the resorbed substances are lacking.

My experiments were carried out with hibernating frogs. The composition of the urine of the summer frog has been studied by Toda and Taguchi,[2] but they have dealt only with the inorganic constituents. Except for some occasional remarks, *e.g.* by Nebeltau[3] and Poulsson,[4] nothing has been done to determine the organic material of frog urine.

One of the reasons for this is the fact that for a long time the accurate analysis of the urine of small animals was practically impossible. The methods of Folin and Denis[5] and Folin and Wu,[6] however, have removed this difficulty.

Another reason for the lack of data is perhaps the difficulty in collecting frog urine in sufficient quantities to make an analysis worth while. The method which Toda and Taguchi used at the suggestion of Aldehoff[7] consisted in simply binding off the skin around the cloaca for 24 hours with a strong but soft string. This

[1] Morse, W., *Biol. Bull.*, 1918, xxxiv, 149.

[2] Toda, S., and Taguchi, K., *Z. physiol. Chem.*, 1913, lxxxvii, 371.

[3] Nebeltau, E., *Z. Biol.*, 1889, xxv, 123.

[4] Poulsson, E., *Arch. exp. Path. u. Pharmakol.*, 1891, xxix, 244.

[5] Folin, O., and Denis, W., *J. Biol. Chem.*, 1916, xxvi, 473.

[6] Folin, O., and Wu, H., *J. Biol. Chem.*, 1919, xxxviii, 81.

[7] Aldehoff, G., *Z. Biol.*, 1891, xxviii, 303.

may perhaps be the reason why their figures show such a marked and enviable regularity, which I could not get in my experiments, notwithstanding the fact that they were working on summer frogs so that complications due to the intake of food might be expected. By their procedure one must get the urine as concentrated as possible.

The purpose of my investigation—comparison with tadpoles—made it desirable to use another method. Not only the urinary excretion, but also that of the skin, which, as will be pointed out, may have considerable importance, had to be determined. For this purpose I put ten or fifteen frogs into a small aquarium with some water. As it appeared in some preliminary experiments that the water intake was of considerable importance, care was taken to keep this quantity of water constant in the various experiments. The animals were kept in the aquarium for 24 hours (in preliminary experiments it appeared that after a longer time much of the urea had been converted into ammonia). The feces and other solid constituents were removed by means of a strong centrifuge, the quantity of liquid was measured, and the determinations were made by the methods of Folin and Denis[5] and Folin and Wu.[6]

Another series of experiments was made with pure urine obtained by "milking out" the animals. In the early morning the bladder of the frog is almost always full, and by a slight pressure one can expel the urine into a test-tube. By milking out several animals a considerable quantity of urine can thus be collected.

The Properties and Composition of Frog Urine.

According to Toda and Taguchi, pure frog urine is as clear and colorless as water. Even on prolonged standing it does not get turbid. In thick layers it develops, according to these authors, a faint yellow color. (I never have observed this in the urine which I obtained from the frogs in the vivarium, where the average temperature never exceeded 10°. In other experiments, however, which will be reported elsewhere, a yellow color was observed in the urine of frogs which had been kept for some time at higher temperatures.) The specific gravity of frog urine is, according to Toda and Taguchi, 1.0009 to 1.0018 at 23–26°. The reaction of the urine—all these data are from summer frogs—is weakly acid to litmus. (The urine of winter frogs is, in my experience, neutral to litmus.) The average osmotic pressure is 1.27 atmospheres. The average electrical conductivity is 0.78

$\times 10^{-3}$. The urine of the summer frogs contains 0.193 per cent of organic constituents and 0.053 per cent of ash. In the ash, phosphoric acid and sodium are present in large quantities.

Our own figures indicate the extreme simplicity of composition of the urine of the winter frog. In the first place, testing for inorganic constituents, we did not succeed in demonstrating the presence of phosphates either with magnesia mixture or with uranium acetate applied directly to the urine. Moreover, the quantity of chlorides is extremely small; after removal of the uric acid by means of acids, silver nitrate in several cases did not give any precipitate and when a precipitate appeared it was slight.

Of the organic constituents almost all the total nitrogen is recovered as urea and ammonia. The quantity of colloidal nitrogen seems to be exceedingly small. Uric acid is present but only in small quantities; this may perhaps be the reason why some authors did not detect it. Creatinine and creatine cannot be demonstrated by the very sensitive Jaffé test (1:5,000). This result is in agreement with the results of Denis on fish urine. Allantoin could not be demonstrated. Neither sugar nor albumin is present.

Methods.

The determinations of total nitrogen, urea N, and ammonia N were made within 24 hours. Total nitrogen was determined by digestion of 1 or 2 cc. of urine by means of Folin's digestion mixture (sulfuric-phosphoric acid mixture and $CuSO_4$)[8] and direct Nesslerization of the digest. In the neutralization of the acid it was found advisable to add the Nessler solution first. From a burette I added 10 per cent KOH till color appeared. After that the addition of KOH was continued till the color remained constant. This technique prevents precipitation.

Urea N together with ammonia N was determined by urease decomposition after some pyrophosphate-phosphoric acid buffer mixture had been added. The ammonia was again determined by direct Nesslerization.

[8] Folin and Denis,[5] pp. 474–478.

The preformed ammonia N was determined by using Folin's permutit method and subtracted from the urea + ammonia N figure.

Uric acid was determined in the pure urine by the method used by Grigaut[9] for blood filtrates. Its special feature—it is only a slight modification of Folin's method—is the fact that the coloring reagent is applied directly. I was obliged to use this method because several times no precipitate was obtained when the acid solution of silver lactate was added nor even when silver nitrate was added after the urine had been acidified with nitric acid.

The results are given in Table I.

TABLE I.

Content of 100 Cc. of Frog's Urine.

Experiment No.	Total N.	Urea.	Urea N.	Ammonia.	Ammonia N	Uric Acid.	Uric Acid N.
	mg.	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*
1	18.5	34.1	15.9	0.87	0.72	0.14	0.047
2	17.6	31.0	14.4	1.13	0.92	0.14	0.047
3	14.0	26.9	12.5	0.20	0.17	0.19	0.066
4	32.3	62.7	29.2	2.9	2.4	0.22	0.073
Average..	20.6	38.7	18.0	1.27	1.05	0.17	0.058

Table I shows the importance of the water intake through the skin.[10] Frog urine appears to be about ten times as dilute as that of man, as shown also by the figures of Toda and Taguchi. While human urine contains, according to the figures given by Mathews,[11] 0.69 per cent of inorganic and 0.39 per cent of organic solids, frog urine, even though by the ligation of the cloaca was obtained as concentrated as possible, contains only 0.053 per cent of inorganic and 0.193 per cent of organic solids. This process of water intake seems to be dependent on temperature. In 1904 Overton[12] reported that "when a frog is kept in water so that its

[9] Grigaut, A., *Compt. rend. Soc. biol.*, 1920, lxxxiii, 1273.

[10] It must be stated that in the frog vivarium this factor is very irregular and beyond the possibility of any control as many men in the laboratory are using the same material.

[11] Mathews, A. P., Physiological chemistry, New York, 3rd edition, 1920.

[12] Overton, E., *Vorl. Mitt. Verh. physik. med. Ges. Würzburg*, 1904, xxxvi, 282.

head is above the surface of the water and drinking is made impossible, a continuous inflow through the skin takes place." The velocity of this process seems to be very highly dependent on temperature and is at 30–33° four to five times as large as at 0°. The difference in concentration of the four samples in Table I shows clearly that a standard for the composition of frog urine cannot be given. Nevertheless the average of these four determinations is as follows: 20.6 mg. of total N, 38.7 mg. of urea, 1.27 mg. of ammonia, and 0.17 mg. of uric acid for 100 cc.

It should be noted as will be discussed later that the concentration of the uric acid in the urine is less than in the blood.

When we compare our average figures with those observed in man (2.13 mg. of urea, 0.46 mg. of ammonia, and 0.046 mg. of uric acid per cc. according to Mathews), we see that the urine of

TABLE II.

Comparison of Average Urine Compositions.

Animal.	Total N.	Urea.	Ammonia.	Uric acid.
	mg. per 100 cc.	*mg. per 100 cc.*	*mg. per 100 cc.*	*mg. per 100 cc.*
Man	181	213	46	4.6
Frog	20.6	38.7	1.27	0.17
Dogfish	420	729	3.75	—
Goosefish	830	258	28.5	2.15

the frog contains relatively more urea, less ammonia, and less uric acid. Denis[13] has given a series of figures on fish urine. In her earlier paper an analysis is given of a composite sample of the urine of ten fasting dogfish (*Mustelis canis*). In calculating her figures we find that 1 cc. of urine contains: total N 4.2 mg., urea 7.3 mg., and NH_3 0.0375 mg., while no figure for uric acid is given. In another paper this author gives figures on a composite sample of the urine of six goosefish (*Lophius piscatorius*). By calculation we find that 1 cc. contains: total N 8.30 mg., urea 2.58 mg., ammonia 0.285 mg., and uric acid 0.0215 mg. In Table II these four urines are presented in figures representing the content in mg. per 100 cc.

In the first place we see that man, frog, and dogfish show a small margin between total nitrogen and urea nitrogen. The

[13] Denis, W., *J. Biol. Chem.*, 1912–13, xiii, 225; 1913–14, xvi, 389.

large margin between these two in the goosefish is still unexplained. It is not due to amines (Denis). In the second place we see that frog and dogfish urine show a very high percentage of urea, higher than is found in man and much higher than in goosefish. The quantity of uric acid seems to be low in frog urine; later on we will see that perhaps part of it is excreted through the skin in the frog.

The Daily Excretion.

The methods used in obtaining the daily urine have been described in the introduction. After the feces had been removed

TABLE III.

24 Hr. Excretion of the Frog.

Experiment No.	Total N.	Urea N + ammonia N.	Urea.	NH_3 . N	Ammonia.	Uric acid.	Uric acid N.
	mg.	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*
1	1.21	1.08	2.32	0.55	0.67	0.033	0.011
2	1.26	1.04	2.17	0.33	0.40	0.046	0.015
3	1.74	(2.75)	—	0.32	0.39	0.021	0.0069
4	1.81	1.65	2.94	0.28	0.34	0.020	0.0066
5	2.28	2.05	3.35	0.49	0.59	0.027	0.009
6	2.49	2.49	4.77	0.27	0.33	0.0255	0.0083
7	2.62	2.24	3.46	0.63	0.76		
Average..	2.02		3.17		0.50	0.0290	

the determinations were made as in the pure urine except in the determination of the uric acid. For this I could not use the method of Grigaut, as this urine was always slightly turbid. I therefore determined it as done by Folin and Wu in blood filtrates. My trust in all my uric acid figures is not very great; the color was so faint that an exact reading was not possible. Nevertheless the figures give an idea of the order of magnitude of the uric acid concentration. The results are presented in Table III arranged according to the quantities of total nitrogen; it need scarcely be stated that in reality they did not come out in this regular way.

It is clear that there is a marked irregularity in the figures, which is the more noticeable because a winter frog does not have any nitrogen intake, so that one would expect the figures to be

more regular. The water intake is not responsible for this fact because, as I stated before, I tried to keep the quantity of water as constant as possible. It occurred to me that perhaps the temperature, which varied over a large range, might be responsible for it. A special investigation, which will be published elsewhere, showed that I was right. According to the figures obtained in these experiments, the range of variation in temperature in the laboratory was at least 13–22.5°, which was in fact the case.

From our figures we can calculate the average for the daily excretion of the hibernating frog: 2.02 mg. of total N, 3.17 mg. of urea, 0.50 mg. of ammonia, and 0.029 mg. of uric acid.

Comparing these figures with those for pure urine (Table IV), we see that the daily quantity of urine excreted by the frog must be about 10 cc. Though no trial has been made to determine this directly, *e.g.* by inserting a cannula into the urethra, the

TABLE IV.

	Total N.	Urea.	Ammonia.	Uric acid.
	mg.	*mg.*	*mg.*	*mg.*
Daily urine excretion.........	2.02	3.17	0.50	0.029
Pure urine by milking out 10 cc.	2.06	3.87	0.127	0.017

average of a great many experiments indicates the correctness of this conclusion.

Moreover, we see that in the total excretion there is relatively less urea, more ammonia, and more uric acid than in the pure urine. This may partly be due to water-soluble components of the feces determined in the total nitrogen; the margin between total nitrogen and urea plus ammonia N is in fact larger in the daily urine than in the pure urine. The chief reason is presumably the ammoniacal decomposition of the urea by bacteria during the time of the experiment. As far as the uric acid figures are concerned it should be noted that they have been obtained by different methods, so that the difference between urinary excretion and total excretion may be due to errors in method (see the remarks of Grigaut). On the other hand, however, the following observation deserves attention. In making a watery extract of 32 gm. of frog skin (five animals) I found

4.2 mg. of total non-protein N, of which 1.7 mg. was NH_3 N, 0.5 mg. urea N, no creatinine or creatine, and 0.44 mg. uric acid. The figures for uric acid and ammonia are high enough to indicate that perhaps part of these substances is excreted through the skin. In this connection it is of interest to recall that excretion is, in its primitive form, a function of the skin and its morphological derivatives.

Moreover, a comparison between the frog and man as far as their daily nitrogen output is concerned may be made. The average weight of 55 frogs of our material was 60.2 gm. In reducing the figures on the average human output as they are given by Mathews, to this weight, we get the figures of Table V.

TABLE V.

Excretion per 24 Hrs. and 60 Gm. Body Weight.

	Total N.	Urea.	Ammonia.	Uric acid.
	mg.	*mg.*	*mg.*	*mg.*
Frog................	2.02	3.17	0.50	0.029
Man................	9.72	17.20	0.51	0.400

In considering these figures of man we must keep in mind that 11.55 mg. (proportionally reduced) of nitrogen were ingested every day, but on the other hand that in the frog which is a much smaller animal we may expect a more important metabolism. In this connection it is of interest to recall that some of the figures given by Lusk on fasting men show a reduction of the nitrogen output of 50 per cent. A comparison, though it is not of great value as absolutely no food is taken in by the winter frog, shows that the frog's metabolism is extremely reduced. Definite statements must be delayed till figures on summer frogs have been obtained.

Non-Protein Nitrogenous Constituents of the Blood.

The blood was obtained by puncturing the heart with a pipette with a narrow capillary end. A bulb immediately behind this capillary served to collect the blood. Before the puncture the apparatus was rinsed in 1 per cent oxalate solution. Some oxalate was put into the collecting cylinder in which the quantity of blood was measured.

The proteins in the blood were precipitated according to Folin and Wu's directions. In the protein-free blood filtrate the total nitrogen, urea nitrogen, and uric acid nitrogen were determined by the methods of these authors. Ammonia appeared not to be present in determinable quantities. The results are given in Table VI.

The irregularities are doubtless chiefly due to differences in water intake, which appear to play an important rôle even in the concentration of the waste constituents of the blood.

TABLE VI.

Non-Protein Nitrogenous Constituents in 100 Gm. of Frog Blood.

Experiment No.	Total non-protein N.	Urea N.	Uric acid N.
	mg.	*mg.*	*mg.*
1	14.7	14.0	—
2	21.6	20.4	0.47
3	17.1	16.3	0.37
4	15.7	14.7	0.46
Average......	17.3	16.3	0.43

TABLE VII.

Comparative Amounts of Non-Protein Nitrogenous Constituents in 100 Gm. of Blood.

Animal.	Total non-protein N.	Urea.	Uric acid.
	mg.	*mg.*	*mg.*
Man 1..................	32	15	2.0
Man 2..................	34	16	2.5
Frog....................	16	35	1.3
Dogfish.................	1,000	1,720	0.0
Goosefish...............	40	17	0.9

Amino-acids are present in the blood, as shown by a positive ninhydrin, but the amount is too small to be estimated quantitatively.

In Table VII is a comparison of the blood analysis of two men (Man 1 on a low nitrogen diet with a N output of 6.2 gm.; Man 2 on a high nitrogen diet with a N output of 24 gm.), of the frog, of the dogfish as an example of an elasmobranch, and of the goosefish as a teleost. The figures for man have been taken from Folin and Denis,[14] and the figures for fish from Denis.

[14] Folin, O., and Denis, W., *J. Biol. Chem.*, 1914, xvii, 488.

The amount of urea in frog blood is relatively high. In this connection it is very remarkable that a large amount of urea is also found in the blood of all investigated elasmobranch fish and not in the Teleostii, especially when the low concentration of urea in frog urine is considered. In connection with what I have pointed out for the urine this fact shows that a certain analogy between the urine and the blood of the elasmobranch and the frog exists.

Uric acid is extremely low in all fishes, and in the elasmobranch fishes Denis did not even find any. Baglioni,[15] however, stated that in the blood of the dogfish more uric acid is found than in the urine and because uric acid is a threshold substance and is found in the urine of these fishes, this seems to be rather probable. While in frog blood practically no ammonia can be detected, this substance is found in all fishes, but relatively more in the Elasmobranchii than in the Teleostii. Neither creatinine nor creatine is present in amounts which can be estimated in frog blood. The sensitive test of Jaffé gave a negative result. They are present in large quantity in fish blood, but we must again remember that our frogs were hibernating and for this reason cannot be compared with alive and constantly moving fishes.

SUMMARY.

The present paper is an introduction to a study of the resorption of material during involution of the tadpole's tail. Figures are given on the composition of the urine of the hibernating frog. On the average 100 cc. of urine were found to contain: 20.6 mg. of total nitrogen, 38.7 mg. of urea, 1.27 mg. of ammonia, and 0.17 mg. of uric acid. The water intake has great influence on the concentration of the urine. The total daily excretion, from kidneys and skin, has also been studied. A frog of about 60 gm. excretes daily 2.0 mg. of total nitrogen, 3.2 mg. of urea, 0.50 mg. of ammonia, and 0.029 mg. of uric acid. Temperature has a tremendous influence on the figures.

In 100 gm. of frog blood I found an average of 17 mg. of total non-protein nitrogen, 3.5 mg. of urea, no ammonia, and 1.3 mg. of uric acid. All determinations have been made by the methods of Folin, Denis, and Wu. The data are compared with those of Denis on fish urine and blood, and with the figures for man.

[15] Baglioni, S., *Zentr. Physiol.*, 1906–07, xx, 105.

THE EFFECT OF AGE ON PANCREATIC ENZYMES.

By FREDERIC FENGER and MARY HULL.

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(Received for publication, March 7, 1921.)

The present investigation was carried out for the purpose of obtaining definite information regarding deterioration of the principal enzymes in desiccated fat-free powdered preparations of hog pancreas. Full details of the samples, their source, preparation, and composition together with the methods of analysis, were given in a previous report.[1] The samples were stored in well stoppered but only partly filled amber-colored bottles at room temperature, thereby insuring conditions comparable with those occurring in commerce. After the samples had stood 12 months, they were tested a second time. For the purpose of comparison the original activity of the freshly prepared samples is given in Table I, together with the present enzymic values.

It will be seen from the tabulated results that all the samples have deteriorated materially in diastatic and lipolytic activity. The loss in diastase ranges from about 50 per cent to almost complete inactivity while that of the lipase is from 10 to 90 per cent. The greatest loss in diastatic power occurs in samples in which attempts had been made to activate the proteolytic enzyme by duodenal mucosa. The lipase deteriorates with such irregularity that we are not warranted in making any statement as to possible causes. It is interesting to note that the proteolytic activity of all samples remained practically unchanged at the end of a year. In one pancreas preparation 5 years old (not of this group) the proteolytic activity remained quite constant while the diastatic and lipolytic enzymes had become practically inert. This stability is similar to that of the gastric proteolytic enzyme. We have samples of dry scale pepsin which have shown no perceptible proteolytic loss in 7 years.

[1] Fenger, F., and Hull, M., *J. Biol. Chem.*, 1919, xxxviii, 487.

TABLE I.

Experiment No.	Composition of samples.	Date of preparation.	Diastase.			Trypsin.			Lipase.		
			Activity.		Loss.	Activity by Fuld-Gross method.		Loss.	Activity 0.1 N NaOH to neutralize acid from 10 cc. neutral olive oil emulsion and 0.10 gm. enzymic powder.		Loss.
		1919	*1919*	*1920*	*per cent*	*1919*	*1920*	*per cent*	*1919* cc.	*1920* cc.	*per cent*
1.	Entire hog pancreases, desiccated whole.	Feb. 4	1:91	1:17	81.3	1:27	1:28	0*	33.8	8.93	73.6
		" 6	1:95	1:18	81.0	1:16	1:16	0	34.5	7.93	77.0
		" 19	1:87	1:18	79.3	1:28	1:31	0*	22.8	8.43	63.0
		Mar. 4	1:87	1:14	83.9	1:17	1:17	0	20.4	7.10	65.2
		" 12	1:100	1:8	92.0	1:20	1:20	0	20.6	4.40	78.6
	Average.		1:92	1:15	83.5	1:22	1:22	0	26.4	7.36	71.5
2.	Minced hog pancreases.	Feb. 4	1:62	1:21	66.1	1:19	1:19	0	24.4	6.10	75.0
		" 19	1:57	1:22	61.4	1:24	1:24	0	24.8	6.85	72.4
		Mar. 4	1:49	1:14	71.4	1:20	1:20	0	27.5	5.15	81.3
		" 12	1:60	1:25	58.3	1:19	1:19	0	21.4	6.20	71.0
		Apr. 1	1:50	1:10	80.0	1:19	1:21	0*	23.2	6.18	73.4
	Average.		1:56	1:18	67.4	1:20	1:21	0	24.3	6.10	74.6
3.	Minced hog pancreases, 99.0 per cent.	Feb. 4	1:100	1:24	76.0	1:11	1:10	0*	30.5	5.83	80.9
		" 19	1:83	1:20	75.9	1:26	1:25	0*	26.1	5.80	77.8
	Sodium chloride, 1.0 per cent.	Mar. 4	1:100	1:22	78.0	1:18	1:18	0	29.0	4.65	84.0
		Apr. 1	1:87	1:18	79.3	1:17	1:16	0*	26.7	7.90	70.4
	Average.		1:92	1:21	77.3	1:18	1:17	0	28.1	6.05	78.3

4.	Minced hog pancreases, 90.0 per cent. Liquid hog bile, 10.0 per cent.	Feb. 19 Apr. 1	1:51 1:61	1:22 1:11	56.9 72.0	1:15 1:16	1:15 1:16	0 0	45.3 45.0	9.18 8.90	79.7 80.2
	Average.		1:56	1:17	64.5	1:15	1:15	0	45.2	9.04	80.0
4a.	Minced hog pancreases, 90.0 per cent. Distilled water, 10.0 per cent	Mar. 4	1:25	1:11	56.0	1:0.5	1:0.5	0	28.5	2.78	90.25
5.	Minced hog pancreases, 89.0 per cent. Liquid hog bile, 10.0 per cent. Sodium chloride, 1.0 per cent.	Feb. 19 Mar. 4 Apr. 1	1:105 1:86 1:105	1:36 1:36 1:31	64.0 58.1 70.5	1:14 1:17 1:17	1:14 1:17 1:16	0 0 0*	44.9 41.2 41.0	8.60 8.83 9.30	80.9 78.6 77.3
	Average.		1:99	1:34	64.2	1:16	1:16	0	42.4	8.91	78.9
5a.	Minced hog pancreases, 89.0 per cent. Distilled water, 10.0 per cent. Sodium chloride, 1.0 per cent.	Mar. 4	1:59	1:21	64.4	1:2	1:2	0	29.1	5.03	82.6
6.	Minced hog pancreases, 75.0 per cent. Minced hog duodena, 25.0 per cent.	Feb. 4 " 19	1:35 1:24	Trace. "	Approximately 98.0. 98.0	1:100 1:117	1:105 1:115	0* 0*	7.8 12.5	5.65 8.53	27.6 31.8
	Average.		1:30			1:109	1:110	0	10.2	7.09	29.7

* Variation probably due to experimental differences.

TABLE I—*Concluded.*

Experiment No.	Composition of samples.	Date of preparation.	Diastase.			Trypsin.			Lipase.		
			Activity.		Loss.	Activity by Fuld-Gross method.		Loss.	Activity 0.1 N NaOH to neutralize acid from 10 cc. neutral olive oil emulsion and 0.10 gm. enzymic powder		Loss.
		1919	*1919*	*1920*	*per cent*	*1919*	*1920*	*per cent*	*1919* *cc.*	*1920* *cc.*	*per cent*
7.	Minced hog pancreases, 74.0 per cent. Minced hog duodena, 25.0 per cent. Sodium chloride, 1.0 per cent.	Feb. 4 " 19	1:57 1:53	Trace. Less than 1:5.	Approximately 98.0. More than 99.0.	1:89 1:111	1:87 1:111	0* 0	8.8 12.5	7.93 8.90	10.0 28.8
	Average.		1:55			1:100	1:99	0	10.6	8.42	19.4
8.	Minced hog pancreases, 65.0 per cent. Minced hog duodena, 25.0 per cent. Liquid hog bile, 10.0 per cent.	Mar. 12 Apr. 1	1:63 1:49	Trace. "	More than 99.0. Approximately 98.0.	1:111 1:105	1:105 1:105	0* 0	38.6 40.0	5.88 6.70	84.8 83.25
	Average.		1:56			1:108	1:105	0	39.3	6.29	84.03
9.	Minced hog pancreases, 64.0 per cent. Minced hog duodena, 25.0 per cent. Liquid hog bile, 10.0 per cent. Sodium chloride, 1.0 per cent.	Feb. 19	1:50	Trace.	More than 99.0.	1:111	1:111	0	40.2	8.28	79.4

CONCLUSION.

Storing powdered pancreas preparations under ordinary conditions for 1 year reduces their diastatic and lipolytic activities very materially, certain samples having become almost completely inert. The proteolytic activities of the same samples, however, remain quite constant. This clearly indicates that trypsin, occurring both in the desiccated natural gland and in the activated preparations, is by far the most stable of the pancreatic enzymes.

NITROGEN DISTRIBUTION OF THE PROTEINS EXTRACTED BY DILUTE ALKALI FROM PECANS, PEANUTS, KAFIR, AND ALFALFA.

By C. T. DOWELL and PAUL MENAUL.

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(Received for publication, February 25. 1921.)

We have taken advantage of the fact that all proteins are soluble in basic solution to separate them from the other substances in foods and feeds which make it impracticable to apply the Van Slyke method to determine the nitrogen distribution. We found that the proteins of pecans and peanuts could be extracted practically completely by dilute solution of sodium and barium hydroxide, and precipitated from the alkaline solution by acidifying with acetic acid. In the proteins of kafir and alfalfa the extraction was not so complete, nor were the precipitated proteins as pure as in the case of pecans and peanuts. However, since all the proteins were soluble in alkali, it seems reasonable to suppose that if the material was finely ground the part of the proteins which was extracted was a representative sample of the total. In order to determine whether or not this assumption was true, we made extractions in which different percentages of the protein were dissolved and the nitrogen distribution of the precipitate was determined.

EXPERIMENTAL PART.

Proteins of Pecans.—Pecan flour was prepared by pressing out the greater part of the oil with a laboratory beet press and extracting the rest with petroleum ether. Afterwards the residue was ground so it would pass a wire screen of 100 mesh. We found that 92 per cent of the nitrogen compounds of the pecan flour was soluble in 0.2 per cent sodium hydroxide. The extraction with the 5 per cent solution of barium hydroxide was not so good for some reason which was not clear to us. The akaline solution was made neutral with acetic acid. The precipitated proteins were dried

and the nitrogen was determined by the Kjeldahl method. The precipitate from the sodium hydroxide extract contained 85 per cent protein, calculated by the nitrogen factor 6.25. The per cent of protein in the precipitate from the barium hydroxide extract averaged about 100, using the usual factor 6.25. We do not claim that this precipitate was pure even in the case of barium hydroxide extract, but we think it reasonable to assume that we come nearer meeting the correct results by the Van Slyke method using this preparation than we would if we had hydrolyzed the unpurified flour.

These protein preparations were hydrolyzed in an autoclave at 30 pounds pressure for 3 hours using sulfuric acid of 25 per cent concentration. The nitrogen distribution in the resulting hydrolysates is shown in Table I.

TABLE I.

Preparation	$Ba(OH)_2$			NaOH
	I	II	Average.	
Humin	5.56	5.6	5.58	10.15
Amide	8.23	8.51	8.37	8.5
Cystine	0.8	0.8	0.8	0.8
Arginine	23.5	23.28	23.39	23.00
Histidine	3.81	4.11	3.96	3.75
Lysine	5.64	5.61	5.62	5.6
Monoamino N	52.0	52.2	52.1	47.55
Total	99.54	100.11		99.35

Two points deserve notice in connection with Table I. The first is the larger amount of humin formed in the sodium hydroxide preparation, and the second is that the monoamino nitrogen is less in the case of the sodium hydroxide preparation. This might indicate that the humin was formed from one or more of the monoamino-acids. The fact that the humin appears to come from the monoamino-acids is in harmony with the claim made by Gortner and Holm.[1] We were interested to find that the impure sodium hydroxide preparation gave a test for pentosans which might account according to our work[2] for the smaller amount of monoamino-acids in the sodium hydroxide preparation. It is interesting to note also that no insoluble humin was formed in

[1] Gortner, R. A., and Holm, G. E., *J. Am. Chem. Soc.*, 1917, xxxix, 2477.
[2] Dowell, C. T., and Menaul, P., *J. Biol. Chem.*, 1919, xl, 131.

the barium hydroxide preparation. This, it seems to us, indicates that the barium hydroxide preparation was practically pure.

Proteins of Peanuts.—The nitrogen distribution of the proteins of peanuts was determined by Nollau.[3] Some individual proteins of peanuts have been studied by Johns and Jones.[4] It was our purpose here as in the case of pecans to extract the crude flour with dilute alkali and determine the nitrogen distribution of the practically pure proteins.

The method of preparing the flour of peanuts, the extraction, and the preparation of the proteins are the same as already described for the pecans. It was found that about 70 per cent of the proteins of the peanut could be extracted with the 5 per cent solution of barium hydroxide and that the purity of the

TABLE II.

Preparation	$Ba(OH)_2$		NaOH
	I	II	
NH_3	11.46	11.5	11.65
Humin	1.00	1.4	2.5
Arginine	17.6	17.55	17.2
Histidine	1.77	2.00	2.75
Cystine	0.77	0.77	0.77
Lysine	6.45	6.00	5.67
Monoamino N	61.25		59.58
Non-amino N	1.6		1.58

proteins using the factor 6.25 was 103 per cent. It is not claimed that the proteins thus prepared were pure because we are not justified in assuming the factor 6.25 to be the correct one, but as in the case of proteins of pecans it must be granted that the preparation is relatively pure. The per cent of protein extracted by 0.2 per cent sodium hydroxide was 91 and the purity was 88 per cent. These preparations were hydrolyzed as in the case of the pecan protein and nitrogen distribution was determined by the Van Slyke method. This distribution is shown in Table II.

It is seen in Table II that the per cent of protein extracted by sodium hydroxide was 91 and that extracted with barium hydrox-

[3] Nollau, E. H., *J. Biol. Chem.*, 1915, xxi, 611.

[4] Johns, C. O., and Jones, D. B., *J. Biol. Chem.*, 1918, xxxvi, 491.

ide was 70 per cent. This fact together with the similarity in the nitrogen distribution indicates that either a representative sample is obtained or that there is but one protein in peanuts.

Proteins of Alfalfa and Kafir.—Alfalfa which was ground to pass a 40 mesh sieve was extracted with a 0.3 per cent sodium hydroxide, and 62 per cent of the nitrogen compounds were extracted. 61 per cent of the nitrogen extracted was precipitated when the solution was made slightly acid with acetic acid. The purity of the precipitated protein was found to be 85 per cent, using the factor 6.25.

Kafir which had been ground to pass a 60 mesh sieve was extracted with 0.2 per cent sodium hydroxide. The maximum extraction of the nitrogen was 38 per cent and the purity of the

TABLE III.

Proteins of	Alfalfa.	Kafir.	
	Average of two analyses.	I (52 per cent).	II (83 per cent).
NH_3	6.8	8.46	8.8
Humin	7.8	4.5	4.3
Arginine	11.01	2.46	2.35
Histidine	6.26	1.77	1.8
Cystine	0.85	0.96	0.96
Lysine	5.26	1.1	1.0
Monoamino N	53.53	74.95	
Non-amino N	8.48	7.2	

protein was found to be only 53 per cent. For some reason it has not been possible for us to get a high degree of extraction of the proteins of kafir with any one solution. We did not make a nitrogen distribution of the sodium hydroxide extract because so small a percentage of the nitrogen compounds was extracted. We found that by using 0.5 per cent of sodium hydroxide in 70 per cent alcohol we could extract 83 per cent of the proteins of kafir. The alcoholic solution was neutralized with acetic acid after which the alcohol and most of the water were distilled off and absolute alcohol was added to the residue. The protein thus prepared gave a 100 per cent purity, using the factor 6.25. The nitrogen distribution of the proteins extracted from alfalfa and kafir is shown in Table III.

The nitrogen distribution of the proteins of kafir given in Table III leads us to conclude, as in the case of the proteins of peanuts, that either a representative sample is extracted in both cases or there is but one protein present. From our knowledge of the proteins of grains in general it would not seem reasonable to assume that there is but one protein in kafir. This leads us to think that the extraction of the proteins with dilute alkaline solutions may enable us to obtain the amino-acid composition of foods and feeds by means of the Van Slyke method of analysis. It seems to us that it is much more important to determine the amino-acid composition of a food or feed than it is to isolate and analyze their individual proteins. We realize, however, that further work will have to be done before we can say definitely that the extraction of proteins by alkaline solutions will enable us to apply the Van Slyke method of analysis to all classes of foods and feed substances.

AMINO-ACIDS IN NUTRITION.*

III. IS PROLINE A GROWTH-LIMITING FACTOR IN THE PROTEINS OF PEAS (VICIA SATIVA)?† WHAT NUCLEUS IN ZEIN IS RESPONSIBLE FOR SUPPLEMENTING THESE PROTEINS?

By BARNETT SURE.

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McCollum, Simmonds, and Parsons (1) have recently demonstrated that zein supplements the proteins of peas (*Vicia sativa*). Zein is entirely lacking in tryptophane and lysine and probably

* Published with the permission of the Directors of the Experiment Stations of the University of Wisconsin, Madison, and the University of Arkansas, Fayetteville.

An abstract of this paper was presented at the Chicago meeting of the American Society of Biological Chemists, December 28, 1920.

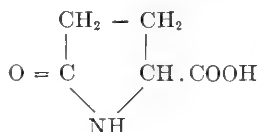
† Lot 73 (Chart VII) received peas which were identified as *Pisum sativum*. This experiment was carried out at the Experiment Station of the University of Arkansas with a new lot of peas shipped from Madison, where the rest of the peas (*Vicia sativa*) were procured. The author, however, was surprised to find that several individuals out of a dozen, when the peas were fed at a 45 per cent level, made almost normal growth for a period of 3 months. The rest of the individuals grew at a rate about one-half normal.

At the recent Chicago meeting of the American Society of Biological Chemists, Johns and Finks reported that the proteins of the dried yellow pea (*Pisum sativum*), fed at a 75 per cent level, are satisfactory for normal growth without any amino-acid additions. The author was then interested to find out the classification of the last lot of peas he worked with at the Arkansas Experiment Station, and Dr. Tracey of the Office of Horticultural and Pomological Investigations, Bureau of Plant Industry, identified them as *Pisum sativum*.

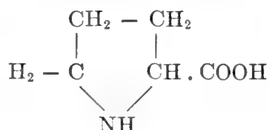
It should, therefore, be noted that in this paper the term "peas" is used for two different plants, one *Vicia sativa* and the other *Pisum sativum;* also that Lot 73 (Chart VII) is the only experiment in which the *Pisum sativum* peas were used.

has very little, if any, cystine; and since that protein belongs to the class called prolamines, it was thought that zein might possess its supplementing value by virtue of its high proline content. It is true, zein is also high in leucine and has a satisfactory tyrosine content. If proline should not be the nucleus of the supplementary character, then either tyrosine or leucine or a combination of these amino-acids might be, if implicit faith is to be placed in chemical methods of analysis.

Although McCollum, Simmonds, and Parsons found that gelatin does not supplement peas (gelatin being high in proline), it was thought that, since it is so indigestible (2), the fragment containing the proline nucleus may escape digestion and hence fail to bring about response. Therefore, peas were chosen as a further basis for exploring the capacity of the animal body to synthesize the pyrrolidine nucleus of the protein molecule (3). Before proline was introduced in these experiments, an experiment was started with 1 per cent of the ration in the form of pyrrolidone carboxylic acid as a preliminary trial to test the possible response to the pyrrole nucleus. In case a positive response of proline might be the outcome of the following experiments, it was thought that it might be of interest in physiology to determine whether pyrrolidone carboxylic acid,

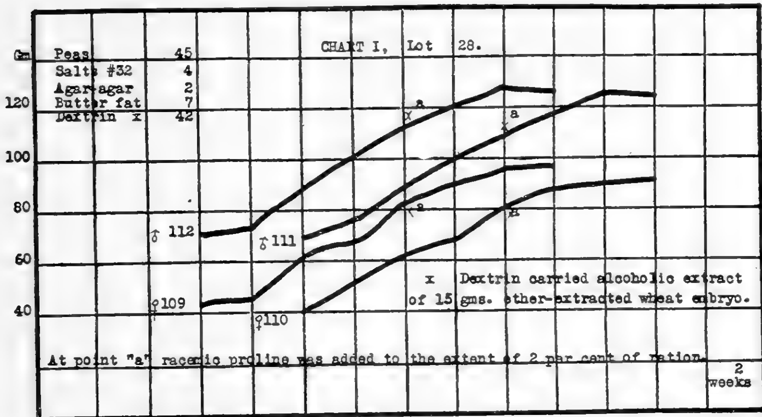


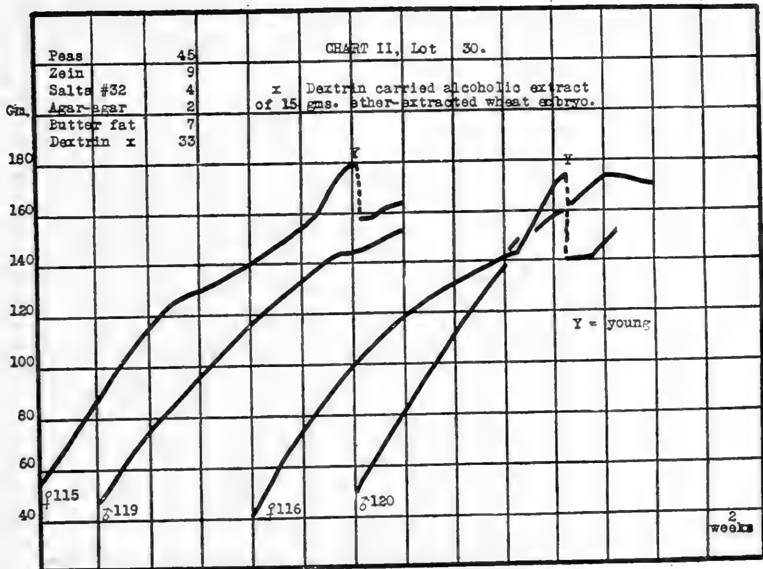
being very similar in composition and structure to proline,



but differing in the ketonic group, might be transformed into proline. The plane of intake was 45 per cent, which is comparable with that used by McCollum, Simmonds, and Parsons, so as to eliminate the level of protein ingested as a limiting factor. The addition of 9 per cent of zein was repeated, so as to have compar-

able results. Corroborative evidence of the supplementing effect of zein to peas has been obtained. The results of the experiments are indicated in Charts I to IX.[1]





[1] The peas used in these experiments were autoclaved for 1 hour at 15 pounds pressure and dried on a steam bath.

Chart I, Lot 28.—During the first 8 weeks the animals received 45 per cent of peas as the source of protein and made very little growth during that period. At point "a" racemic proline containing 12 per cent of amino nitrogen, prepared by the Fischer ester method, was added to the extent of 2 per cent of the total ration. This should be equivalent to 1 per cent optically active proline. After being used 2 weeks, this preparation was exhausted and another preparation of optically active proline, containing 5 per cent of amino nitrogen, prepared by the method of Fischer and Boehner (4), was introduced to the extent of 1 per cent of the total ration. It will be noted no response was obtained after 6 weeks. Proline, therefore, is not the primary limiting factor in the proteins of peas.

Chart II, Lot 30.—This chart shows that, as McCollum, Simmonds, and Parsons have reported, zein supplements the proteins of peas to a considerable extent.

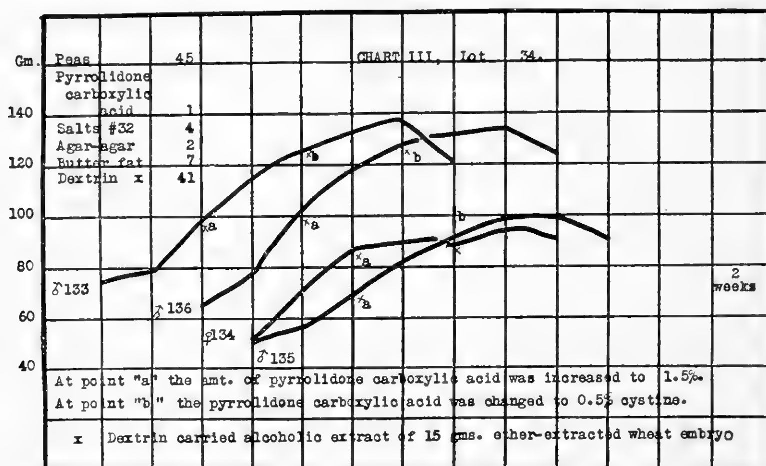
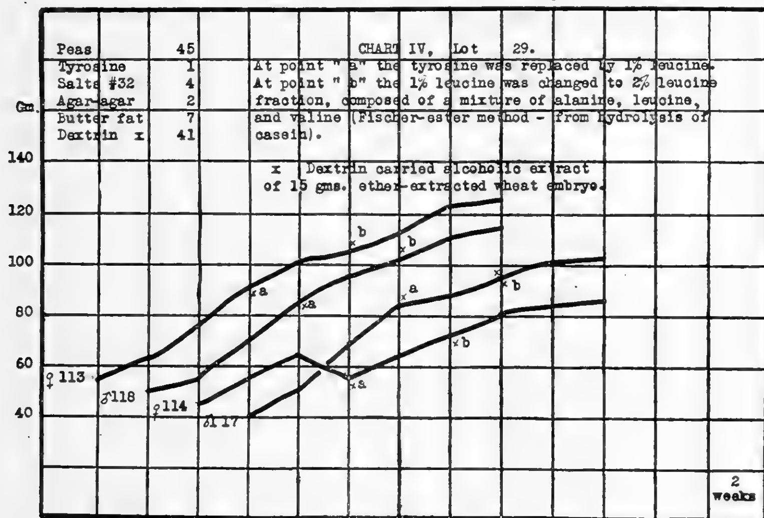


Chart III, Lot 34.—This lot had 1 per cent of the total ration in the form of pyrrolidone carboxylic acid containing 8 per cent of amino nitrogen coming from glutamic acid. At point "a" the amount was increased to 1.5 per cent. It will be noted that two animals started growing at a fair rate, so that at first it seemed as if this might be an indication of the possible response to the pyrrole ring. Judging from later periods of growth, however, the response must be looked upon as negative. At point "b" the 1.5 per cent of pyrrolidone carboxylic acid was changed to 0.5 per cent of cystine and the administration of this amino-acid was continued for a month, but even in this case there was no response. Cystine, therefore, is not the limiting factor in the proteins of the peas.

Chart IV, Lot 29.—This chart indicates that animals will not respond to additions of tyrosine as high as 1 per cent of the total ration to the proteins of peas at the level indicated. At point "a" the tyrosine was replaced by an equivalent amount of leucine, but there was no response to the latter addition.



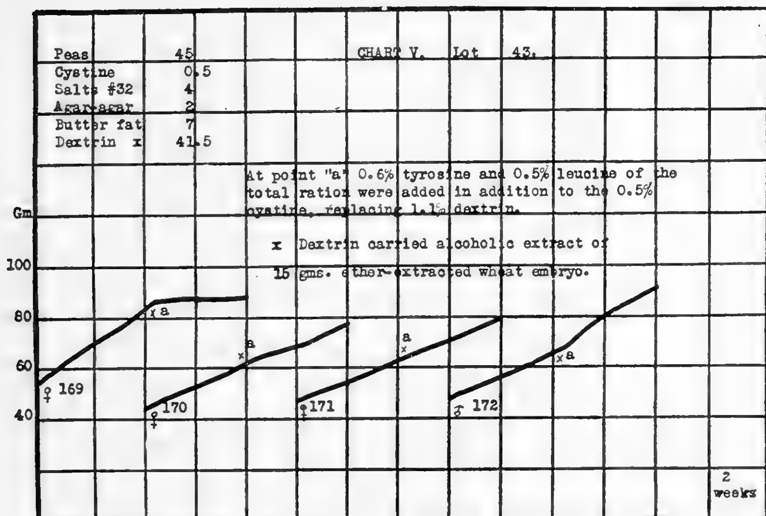


Chart V, Lot 43.—This ration had 0.5 per cent of cystine of the total ration, and the object of the experiment was to check up the negative results obtained after additions of cystine to peas at later periods of growth, as indicated after point "b" in Chart III. After 4 weeks of growth 0.6 per cent of tyrosine and 0.5 per cent of leucine of the total ration were added in addition to the 0.5 per cent of cystine, with a view of finding out whether the three amino-acids together may improve the nutritive quality of peas; but after 4 weeks no beneficial results could be detected, as the curves indicate.

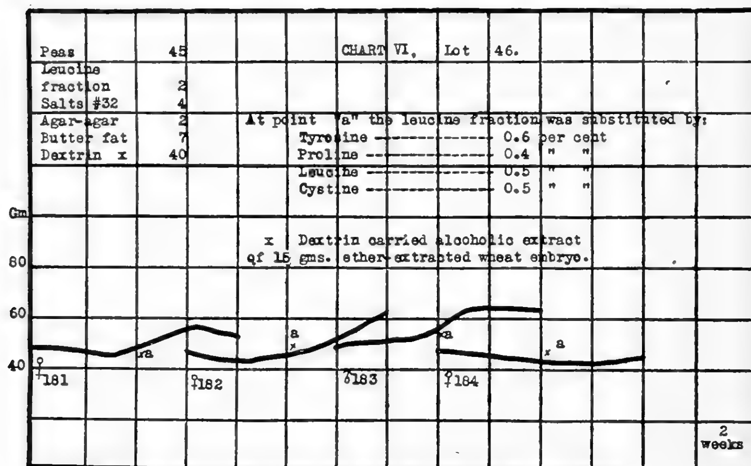
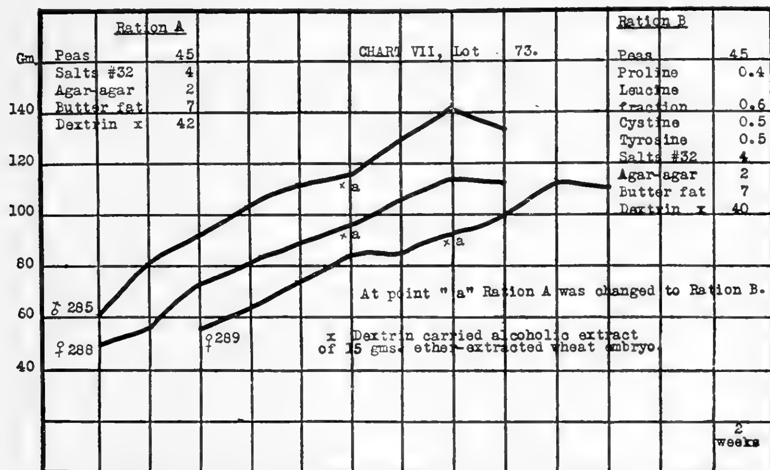


Chart VI, Lot 46.—This chart shows that the leucine fraction (alanine, leucine, and valine) is not the fragment of the molecule of the proteins of peas that is responsible for their deficiency. At point "a" amino-acid additions were made with a view of determining whether response to proline might be obtained when administered in the presence of cystine, tyrosine, and leucine, Lot 43 (Chart V), being the control. After 4 weeks, however, which is ample time for response to amino-acids, the animals remained as stunted as before. It must be concluded then that neither alanine, leucine, valine, tyrosine, cystine, nor proline are the amino-acids deficient in the proteins of peas (*Vicia sativa*).

Chart VII, Lot 73.—This experiment is a duplication of the experiment on Lot 46 (Chart VI), with the exception that the leucine fraction was used instead of isolated leucine, so as to find if a response to proline could be obtained in the presence of alanine, leucine, valine, cystine, and tyrosine.

Although there was some little growth during the first 10 weeks of experimentation, the curves indicate that there was no appreciable change in the character of growth after the addition of these amino-acids.



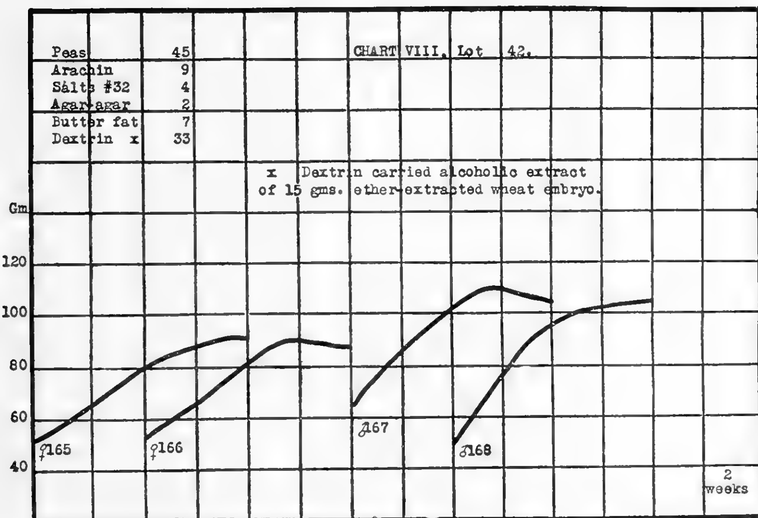


Chart VIII, Lot 42.—This lot had 9 per cent of the ration added in the form of arachin, one of the globulins of the peanut, but, as it will be noted, there is little, if any, supplementing value.

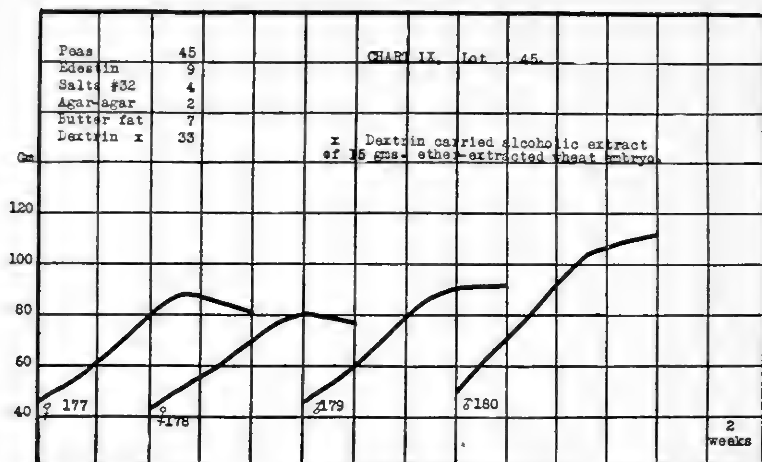


Chart IX, Lot 45.—The peas in this ration were fortified with 9 per cent of edestin, the globulin from hemp seed, but the curves indicate very little, if any, response.

DISCUSSION.

McCollum, Simmonds, and Parsons have shown that when 45 per cent of the ration is introduced in the form of peas (*Vicia sativa*) the proteins therein are deficient in character, as rats will only maintain their body weights on such a ration, even though all the other factors in the diet are rendered satisfactory for growth. They also found that neither lactalbumin nor gelatin will improve the quality of the proteins of peas, but that zein supplements the pea proteins to a considerable extent. The author corroborates these latter findings, and would add that neither arachin nor edestin improves the nutritive value of the proteins of peas. An examination of the composition of zein, analyzed by Osborne and Clapp and Osborne and Liddle (5), shows that this alcohol-soluble protein is fairly abundant in leucine, 19.6 per cent; in the benzene nuclei, phenylalanine, 6.6 per cent, and hydroxyphenylalanine or tyrosine, 3.6 per cent; in proline, 9.0 per cent; and that zein is also high in glutamic acid, 26.2 per cent, but that it is absolutely deficient in tryptophane and lysine, that it is quite low in arginine, 1.6 per cent, and histidine, 0.6 per cent, there

being no analysis for cystine reported. The analysts have recovered 85.4 per cent of the total nitrogen of this protein, and the author, therefore, placed considerable faith in the accuracy of this analysis, and used it as a guide for investigating biologically what nucleus or nuclei there may be in zein that improves the deficient character of the pea proteins. Special attention was given to the pyrrolidine nucleus, proline, but no response was obtained even when introduced as high as 1 per cent of the total ration in optically active form. Although it was found impossible in this laboratory[2] to isolate cystine from zein, and since zein has a total sulfur content of 0.6 per cent, the possibility of the transformation of this sulfur or part of it into cystine suggested itself and cystine additions were tried, but no improvement in growth was observed. Since no response was obtained to isolated leucine nor to the leucine fraction, composed of alanine, leucine, and valine, we must conclude that none of these amino-acids is a limiting factor in the proteins of the peas (*Vicia sativa*). No response was obtained by tyrosine additions alone or when added together with leucine and cystine; so the benzene ring must be eliminated as the nucleus responsible for the deficiency of the character of the proteins in question. Proline, even in the presence of alanine, leucine, valine, cystine, and tyrosine, did not supply the missing link furnished by zein in improving the poor quality of the proteins of peas.

This eliminates, then, *alanine, leucine, valine, tryptophane, lysine, cystine, tyrosine,* and *proline* as being the amino-acids that are responsible for the deficient character of the proteins of peas (*Vicia sativa*), and since zein, which supplements the proteins of these peas, is very low in arginine and histidine, it is here suggested, particularly in view of the recent work of Dakin on protein analysis (6), that zein may contain one or more amino-acids necessary for growth as yet not isolated by previous methods of technique.

[2] Mr. A. Koehler, of the Laboratory of Agricultural Chemistry of the University of Wisconsin, Madison, has very carefully analyzed zein when purified by reextracting three times with 70 per cent alcohol and could find no cystine in it.

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SYNTHESIS OF INOSITE HEXAPHOSPHORIC ACID.

By S. POSTERNAK.

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(Received for publication, January 24, 1921.)

Some time ago I published a method for the synthetic preparation of inosite hexaphosphoric acid and proved that this acid is identical with the phosphoorganic reserve material of green plants.[1] My process utilizes as the dehydrating agent phosphorus pentoxide for the esterification of inosite with *o*-phosphoric acid. The reaction is not quantitative, since phosphorus pentoxide condenses also *o*-phosphoric acid and thus eliminates it partly from the esterification process. Under the experimental conditions selected a state of equilibrium results, in which, among lower esters of inosite, about 8 per cent of inosite hexaphosphoric acid can be isolated. In the research cited the yield was stated to be 3 to 5 per cent. Since then I have been able, by changing the experimental conditions slightly, to increase the yield.

For the isolation of the inosite hexaphosphoric acid out of the complex reaction mass, which contains large quantities of *m*-phosphoric acid, I first convert the latter, by heating with an excess of sodium hydroxide in sodium pyrophosphate, which is difficultly soluble in cold water. Smaller quantities of *o*-phosphate are simultaneously formed. I then crystallize out systematically as far as possible the mineral phosphates. There results finally a syrup consisting for the greater part of sodium salts of the esters of inosite. After having been acidulated with acetic acid it is precipitated with calcium acetate. The insoluble calcium salts are transformed into water-soluble acid salts and finally into a solution of calcium-sodium double salts. This process leads to the crystallization of the characteristic calcium-

[1] Posternak, S., *Compt. rend. Acad. Sc.*, 1919, clxix, 138.

sodium salt of the phosphoorganic reserve material. Dried at 120°C. the composition of this salt corresponds to the formula $C_6H_{12}O_{27}P_6Ca_2Na_8$.[2]

The properties of the free acid, as well as those of the different salts of the inosite hexaphosphoric acid occurring in nature, were described by me explicitly in 1903, as well as all the reactions which are usually employed to identify this substance, including the precipitability of protein.[3] In the course of time I have however arrived at the conclusion that these reactions are not to be regarded as characteristic and specific; they belong to the majority of polyphosphoric acid esters so that recourse must always be had to the crystallization of the above named calcium-sodium salt in order to speak with certainty of the presence of organic reserve material. In view of the importance of this salt for the identification of inosite hexaphosphoric acid I have endeavored to make this crystallization easily practicable so that it might be carried out quickly and with small quantities. The method was described in 1919 and at the same time the important fact was communicated, that the completely saturated sodium salt can be separated out in well developed and definite clinorhombic prisms, corresponding to the formula $C_6H_6O_{24}P_6Na_{12}.3\,H_2O + 35H_2O$, which are adapted for crystallographic measurements.[4,5] The method of preparing this salt was described, the crystals depicted, and the results of the measurements given.

[2] In his last paper on the presence of inosite hexaphosphoric acid in the seeds of *Acer saccharinum* (Anderson, R. J., *J. Biol. Chem.*, 1920, xliii, 469) Anderson opposes the formula of the water-free acid $C_6H_{18}O_{24}P_6$ to my formulation $C_6H_{24}O_{27}P_6$ or $C_6H_{18}O_{24}P_6.3H_2O$; as if there was a fundamental difference between his and my conception of the constitution of the phosphoorganic reserve material of green plants.

Inasmuch as the synthesis of hexaphosphorus acid has now been accomplished, there can be no possibility of doubt concerning the real composition of this material. The addition of $3H_2O$ in the formula has only this one purpose, to call attention to the fact, that in the acid itself as in most of its better known salts, the removal of these 3 molecules of water seems impossible without disintegration of the compound (Posternak, S., *Compt. rend. Acad. Sc.*, 1919, clxix, 37).

[3] Posternak, S., *Compt. rend. Acad. Sc.*, 1903, cxxxvii, 337.

[4] Posternak, S., *Compt. rend. Acad. Sc.*, 1919, clxviii, 1216.

[5] Posternak, S., *Compt. rend. Acad. Sc.*, 1919, clxix, 337.

To return to the synthesis of inosite hexaphosphoric acid, I may mention that after crystallization from the above described mixture of the calcium-sodium salt of the inosite ester, the latter was converted into the saturated sodium salt, which was also obtained in beautiful clinorhombic prisms. Compared with the corresponding natural salt crystallographically, they proved absolutely identical; the elementary composition and other properties were also the same. Consequently the question of the synthesis of inosite hexaphosphoric acid and the constitution of the organic reserve material of green plants were definitely solved when Anderson,[6] in a research recently published, questions and negatives my results after having tried to repeat my work. In reality he has studied the reaction between inosite and *o*-phosphoric acid in presence of phosphorus pentoxide retaining the experimental conditions prescribed by me, but working up his reaction mass in quite a different manner. Anderson has evidently not clearly grasped the final purpose of the various operations and steps described by me for the isolation of inosite hexaphosphoric acid. Otherwise he would not have followed a method according to which the acid formed is subjected to decomposition and cannot be separated from the lower inosite esters.

Anderson neutralizes the aqueous suspension of his reaction mass with sodium hydroxide, using litmus as an indicator. By this procedure only about two-thirds of the acid equivalents of the esters formed are neutralized; *m*-phosphoric acid is thereby not converted into pyrophosphate, which makes the almost quantitative separation of the mineral phosphates, as attained by my method, impossible. He then adds sulfuric acid and heats for 1 hour at 100°. If now the excess of sodium hydroxide does not at this temperature perceptibly decompose inosite hexaphosphoric acid, then more or less mineral phosphoric acid is split off from the compound under acid reaction. A 2 N solution of free inosite hexaphosphoric acid, according to my findings, splits off at 96° within 1 hour 2.3 per cent mineral phosphoric acid, which makes possible the decomposition of 13.8 per cent of the organic phosphorus compound.

[6] Anderson, R. J., *J. Biol. Chem.*, 1920, xliii, 117.

For the isolation of this compound Anderson converts the mixture of the inosite esters into acid barium salts and lets these separate spontaneously out of the freshly prepared solution in hydrochloric acid neutralized more or less exactly with barium hydroxide, or he precipitates them with alcohol. This method seems to have given good results in Anderson's hands as long as it was a question of liberating the natural product from concomitant mineral phosphates or other impurities. In the presence, however, of pyrophosphates or other inosite phosphoric acid esters whose barium salts show similar solubility, it is impossible to separate the inosite hexaphosphoric acid thus. After painstaking manipulations Anderson succeeds in isolating from an experiment with 12 gm. inosite, two barium salts. The first, weighing 7.3 gm., corresponds to the formula $C_{12}H_{18}O_{32}P_8Ba_3$ or calculated for free acid, $C_6H_{12}O_{16}P_4$; and represents, according to Anderson "inosite dihydrophosphoric acid in which two hydroxyls of each molecule of the pyrophosphoric acid have reacted with two alcoholic hydroxyls of the inosite." The second, for which he gives no formula, but analytical data, is regarded by Anderson as "a mixture of inosite phosphoric acids, but the nature of these esters could not be determined."

We will return later to this mixture. Here at this point I would only remark, that 12 gm. inosite should yield theoretically approximately 70 gm. acid barium salts of the esters. Of this quantity only 11.3 gm., that is 16 per cent, were isolated and analyzed. *Therefore, 84 per cent of the derivates of inosite remained uncharacterized and undetermined.*

If Anderson infers from his experiments, notwithstanding the precise facts described by me, that "the synthesis of phytic acid or inosite hexaphosphoric acid cannot be considered as accomplished and it appears doubtful if this substance can be successfully synthesized by the methods heretofore employed," this is certainly an unwarranted and inadmissible conclusion. Anderson should only have followed exactly the method described by me and he would have obtained positive results. His failure proves the superiority of my method of isolating and identifying inosite hexaphosphoric acid and clearly shows to what extent my researches have shed light upon the problem concerning the nature of phosphoorganic reserve material of green plants.

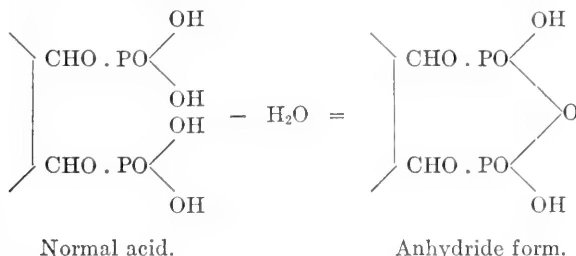
And finally, Anderson himself, without noting the fact, has made it probable that my method gives the desired results. The second barium product, which he has treated rather lightly, can also be formulated. From his analyses can be calculated for the free acid the formula $C_6H_{16}O_{20}P_5$ or $C_6H_{15}O_{20}P_5$ on account of the usually too high water content. This formula could very well correspond to a mixture of dipyrophosphoric ester and "phytic acid"

$$2\ C_6H_{15}O_{20}P_5 = C_6H_{12}O_{16}P_4 + C_6H_{18}O_{24}P_6$$

Anderson's research containing the proof of the presence in the mixture of dipyrophosphoric acid ester, which is a tetra-basic acid is of some interest. I also have isolated out of the ester mixture beside inosite hexaphosphoric acid, an inosite tetraphosphoric acid. This was normally octo-basic corresponding to the formula

$$C_6H_8O_6[PO(OH)_2]_4$$

It appears to me very plausible, that the normal inosite tetraphosphoric acid first formed and perhaps also the inosite hexaphosphoric acid, is on further addition of phosphorus pentoxide converted into an anhydride by elimination of one molecule of water out of two neighboring rests of phosphoric acid:



Normal acid. Anhydride form.

Heating with an excess of sodium hydroxide according to my process apparently reconverts this anhydride form into the normal ester. More detailed experimental and the analytical data are to be found in a paper which has been published elsewhere.[7]

[7] Posternak, S., *Helv. Chim. Acta*, 1921, iv, 150.

THE BASIC AMINO-ACIDS OF GLYCININ, THE GLOBULIN OF THE SOY BEAN, SOJA HISPIDA, AS DETERMINED BY VAN SLYKE'S METHOD.

By D. BREESE JONES and HENRY C. WATERMAN.

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(Received for publication, March 21, 1921.)

The high value of the soy bean as a protein food makes the composition of its protein with regard to the hexone bases and cystine a matter of particular interest. Either as the sole source of protein in the diet, or as a supplement to other foods whose proteins show a quantitative or qualitative deficiency, the soy bean has shown a remarkable efficiency (1). In view of this well known fact, it is somewhat surprising that no analysis of the soy bean protein by Van Slyke's (2) method has been published. The monoamino-acids of glycinin have been determined in an hydrolysis by Osborne and Clapp (3); and the same investigators also determined the hexone bases by the direct isolation method of Kossel and Kutscher. Cystine was not isolated. No determination of the basic amino-acids by the more recent method of Van Slyke seems to have appeared.

The first investigation of the soy bean protein seems to have been that of Meissl and Böcker (4). It was again investigated, in the light of later methods, by Osborne and Campbell (5) in 1898. These investigators used 10 per cent sodium chloride solution as their extractant and fractionated the proteins of the saline extract by dialysis. In this manner the globulin, *glycinin*, the principal protein of the soy bean, and a very small amount of another protein, were isolated. We have made an examination of the 10 per cent sodium chloride extract of the ground soy bean by the ammonium sulfate method of fractionation; and our results confirm the conclusion of Osborne and Campbell, arrived at by dialytic fractionation; *i.e.*, that but one globulin is extracted in

significant quantities by sodium chloride solution from ground soy bean.

The glycinin used in this analysis was prepared, therefore, in accordance with the procedure of Osborne and Campbell. The whole beans were ground to a fine meal and this meal was extracted with aqueous 10 per cent sodium chloride. To prevent the meal from settling to the bottom of the container, a stirrer consisting essentially of an upward-acting propeller turning within a perforated cylinder was used. The meal was drawn upward through the cylinder as fast as it settled, and discharged through the upper perforations and over the top into the surrounding liquid. The extraction was continued for about 2 hours. The remaining operations in the preparation of the glycinin were carried out exactly as prescribed by Osborne and Campbell. The desiccated preparations were exposed in thin layers to a slow current of filtered air, in an apparatus devised and used by us for this purpose in a previous investigation (6), for 48 hours in order that the powder might absorb moisture to equilibrium with that of the atmosphere; dried protein preparations are too hygroscopic to permit of accurate weighing. Moisture (loss at 110°C.) and ash were determined, and the analysis calculated on the moisture- and ash-free basis. The material used contained 16.94 per cent of nitrogen.[1]

Samples of about 3 gm. each of the protein were hydrolyzed by boiling with 100 cc. of 20 per cent hydrochloric acid for 24 hours. The phosphotungstate precipitate was decomposed by the amyl alcohol and ether method (7). On account of the greater accuracy obtainable in the measurement of dilute solutions, we prefer to make up the basic amino-acids to 100 cc. rather than to 50 cc. as originally directed. The aliquots taken for the various determinations must be also doubled, of course, if this be done.

As might be expected, the results given by this analysis for the hexone bases are all somewhat higher than those obtained by the direct isolation of these amino-acids. The value found for lysine (9.06 per cent) is particularly striking in this connection, while the histidine figure, on the other hand, agrees well with that

[1] The preparations of glycinin used in this determination were made, and the elementary analysis was performed by Mr. S. Phillips of this Laboratory.

obtained by the Kossel and Kutscher method. The cystine figure is unquestionably low, of course, since a significant proportion of this amino-acid is destroyed in the hydrolysis. The nitrogen distribution figures are given in Table I. The percentages of the

TABLE I.

*Analysis of Glycinin by Van Slyke's Method.**

Sample I, ash- and moisture-free protein, 2.8283 gm.; nitrogen, 0.4791 gm.†
Sample II, " " " " 2.8248 " ; " 0.4785 "

	I	II	I	II	Average.
	gm.	*gm.*	*per cent*	*per cent*	*per cent*
Amide N	0.0581	0.0586	12.13	12.25	12.19
Humin N	0.0048	0.0045	1.00	0.94	0.97
Arginine N	0.0731	0.0738	15.27	15.42	15.35
Cystine N	0.0039	0.0038	0.81	0.79	0.80
Histidine N	0.0106	0.0123	2.21	2.57	2.38
Lysine N	0.0503	0.0480	10.50	10.03	10.27
Amino N of filtrate	0.2642	0.2642	55.14	55.21	55.18
Non-amino N of filtrate	0.0138	0.0142	2.88	2.97	2.93
Total N recovered	0.4788	0.4794	99.94	100.18	100.07

* Nitrogen figures corrected for solubilities of the phosphotungstates of the basic amino-acids.

† Nitrogen content of protein 16.94 per cent.

TABLE II.

Basic Amino-Acids in Glycinin.

Amino-acid.	I	II	Average.	Kossel and Kutscher method.*
	per cent	*per cent*	*per cent*	*per cent*
Arginine	8.02	8.11	8.07	5.12
Cystine	1.17	1.18	1.18	
Histidine	1.38	1.60	1.44	1.39
Lysine	9.26	8.85	9.06	2.71
Ammonia	2.27	2.29	2.28	2.56
Tryptophane†	1.41	1.32	1.37	

* Values of Osborne and Clapp (3).

† Calculated according to Gortner's (8) observation, that 86.5 per cent of the tryptophane N is converted into humin N on hydrolysis in the presence of excess of carbohydrate; as none was added in this case, except that present in the preparation used, this is to be regarded as minimal.

amino-acids, calculated on the ash- and moisture-free sample, are given in Table II; and, for comparison, Osborne and Clapp's figures for the hexone bases are here quoted. We have added an estimation of tryptophane calculated from the total humin nitrogen according to Gortner and Blish's (8) observation that 86.5 per cent of the tryptophane nitrogen is converted into humin nitrogen in an acid hydrolysis in the presence of carbohydrate. This figure is to be regarded as a minimal inasmuch as no carbohydrate was added previous to the hydrolysis. A determination of the monoamino-acids of glycinin is in progress, and it is intended to attempt the isolation of tryptophane in a separate hydrolysis, with alkali or with trypsin, in this connection.

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A HYDROGEN ELECTRODE VESSEL ADAPTED FOR TITRATIONS.

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(Received for publication, March 9, 1921.)

In the course of recent investigations, it became necessary to devise a hydrogen electrode which would be serviceable for measuring hydrogen ion concentrations and making electrometric titrations of small samples of material. At times it was not desirable to permit the escape of gases or volatile matter from the electrode vessel. This could not be accomplished in the usual type of electrode vessel, such as McClendon's,[1] where a continual stream of hydrogen is necessary. The apparatus pictured in Fig. 1 met our requirements. Because of its simplicity and the inexpensiveness of its construction we believe that it would be of service to other investigators.

The electrode vessel, the only part requiring the services of a glass-blower, consists of an upper stationary portion, *A*, consisting of a glass tube which carries by means of a rubber stopper the electrode, burette, and gas inlet and outlet tubes; and a lower rotating portion, *B*, which contains the substance under examination. A mercury seal makes possible a gas-tight chamber. The various parts of the electrode are shown in outline in Fig. 1. In order to illustrate the actual position of the component parts of *A*, the stationary portion of the electrode, a cross-section is given in Fig. 2. Of the various parts carried by *A*, the metallic electrode requires special description.

The glass tube, *a*, is permanently fixed in *A*. At its lower extremity, a short detachable electrode, *b*, is attached to it by

[1] McClendon, J. F., *J. Biol. Chem.*, 1918, xxxiii, 19.

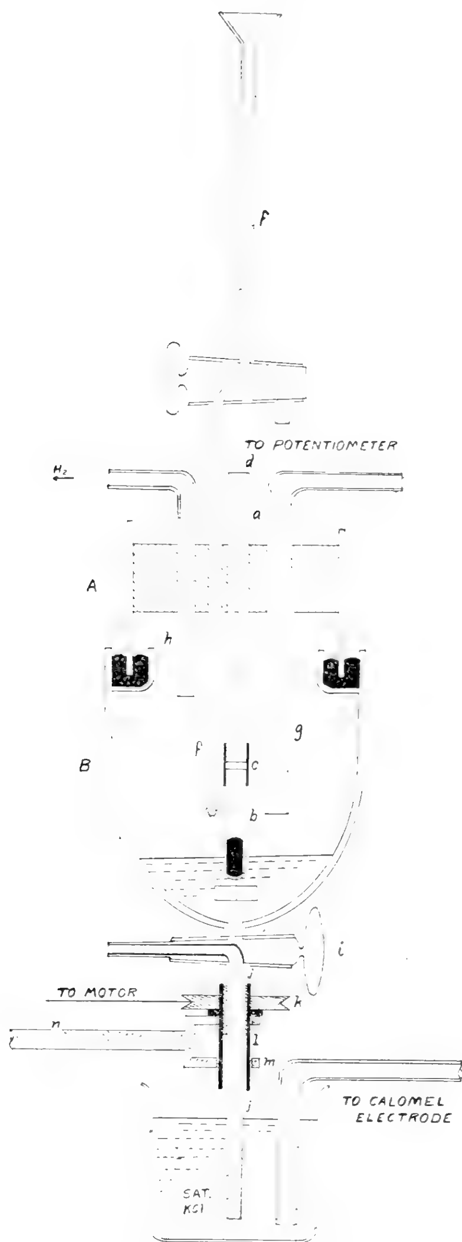

Fig. 1.

Fig. 2.

means of rubber tubing, *c*. The short electrode, *b*, consists of a small platinum wire, one end of which is fused into the glass tube and to the other end of which has been fused a bit of gold or platinum, beaten out in the form of a paddle. It is by means of this paddle-shaped electrode that stirring is effected. Furthermore on account of its being detachable, it can be easily removed for recoating with palladium or platinum at any time. A copper wire, *d*, extending the length of tube *a*, and dipping into the mercury contained in *b*, affords connection with the potentiometer.

To *f*, a 1 cc. Mohr pipette, graduated to hundredths of a cc. is sealed a small funnel at the upper end and a 3-way stop-cock at the lower end. Connection with a piece of capillary tubing which extends through *A*, is made by means of rubber tubing or by sealing the ends together. The 3-way stop-cock is necessary at this point because it was found that fluid would not enter the burette from the funnel unless a larger opening than that afforded by the capillary tubing was provided.

The remaining parts carried by *A*, namely the inlet tube, *g*, and the outlet tube, *h*, require no explanation save that a trap should be provided at the outlet in order to prevent the backflow of air. No trouble with leaks has been experienced up to the present time.

Part *B*, the lower rotating portion of the electrode vessel, carries a stop-cock, *i*, with a right-angled bore which can alternately connect the vessel or the long glass stem, *j*, with the exterior. Rotation of *B* can be effected in any convenient manner. We have provided for this in the following way. The stem, *j*, is held in a wooden pulley, *k*, by means of a rubber stopper. Within and attached to the pulley is a brass tube, *l*. This rotates within the brass sleeve, *m*, whose side arm, *n*, is clamped to a ring stand.

Manipulation of the electrode is as follows. The vessel is filled with hydrogen. The solution to be investigated is admitted to the chamber *B* by a pipette attached to stop-cock, *i*, by means of a short piece of rubber tubing. This can be washed out and renewed as often as necessary. The stop-cock, *i*, is now turned so that the stem, *j*, is connected with the exterior and the saturated solution of potassium chloride into which *j* dips, is drawn

up and out of the stop-cock by means of the pipette which is still attached. The stop-cock is then turned off and the pipette removed. Rotation is begun and continued until equilibrium is reached. Since a central band of the stop-cock, *i*, is left ungreased, contact between the hydrogen and calomel electrodes is effected. Electrical connection made in this way has never been broken although rotation has been continued for an hour. The technique of taking readings and making titrations is so well known that further explanation of the method of operation is unnecessary.

Potential measurements made through a closed stop-cock cannot be made with as great accuracy as those made through an open system. We have found that hydrogen ion determinations of standard solutions made simultaneously with the Clark electrode and our apparatus agreed within one millivolt. Since this error lies within the limits in which hydrogen ion determinations of biological materials can be reproduced, we believe that for the present purposes at least, it does not detract significantly from the accuracy of the results obtained.

A SIMPLE METHOD FOR THE DIRECT QUANTITATIVE DETERMINATION OF SODIUM IN SMALL AMOUNTS OF SERUM.

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(From the Department of Pediatrics, the Johns Hopkins University, Baltimore.)

(Received for publication, March 18, 1921.)

Method.

2 cc. of serum are transferred to a platinum dish. To these are added 10 cc. of the potassium pyroantimonate reagent followed by 3 cc. of 95 per cent alcohol. The alcohol should be added drop by drop and the specimen stirred with a rubber-tipped rod. After standing 45 minutes, the precipitate is transferred to a weighed Gooch crucible and washed with 8 to 12 cc. of 30 per cent alcohol. The crucible is dried at 110°C. for 1 hour,[1] cooled in a desiccator for 30 minutes, and weighed. The weight of the precipitate divided by 11.08 equals the number of mg. of sodium present in the sample.

Preparation of the Potassium Pyroantimonate Reagent.

500 cc. of distilled water are heated to boiling in a Pyrex flask and approximately 10 gm. of potassium pyroantimonate (J. T. Baker) are added. The boiling is continued from 3 to 5 minutes, the flask immediately cooled under running water, and when the contents are cold 15 cc. of 10 per cent KOH (alcohol-washed) are added. The reagent is then filtered through ash-free filter paper into a paraffined bottle. We have found that frequently some of the undissolved potassium pyroantimonate will pass through even the best filter paper. If the reagent is allowed to stand 24 hours

[1] The crucible is placed in the oven the temperature of which is *gradually* raised to 110°C.

after filtering, all the undissolved potassium pyroantimonate will settle to the bottom. The supernatant fluid is then clear and may be used as long as it remains so. The reagent keeps perfectly well at room temperature for at least 1 month. 10 cc. of this reagent will precipitate 11 mg. of sodium. The 10 per cent KOH should also be kept in a paraffined bottle.

Before the reagent is used for the first time, it should be tested for the presence of sodium and also the fact ascertained that none of the potassium pyroantimonate is precipitated by the addition of alcohol in the proportion used in the method. This is accomplished by adding to 10 cc. of the reagent in a platinum dish 2 cc. of distilled water and 3 cc. of 95 per cent alcohol.

Details of the Method.

Serum.—The serum may be separated from the clot any time within 24 hours after collection of the sample as we have found that the sodium content of normal serum is not changed during this time by contact with the clot.

Platinum.—The platinum dishes must be scrupulously clean, otherwise the precipitate has a tendency to adhere to the sides. The dishes are cleaned with fine sand, then rinsed with distilled water.

Addition of Reagent and Alcohol.—No special precautions are necessary for the addition of the reagent. The 95 per cent alcohol has to be added drop by drop while the mixture is stirred with a rubber-tipped rod. Redistilled alcohol should be used.

Precipitation.—Precipitation is complete from 30 to 45 minutes after the addition of the alcohol.

Gooch Crucibles.—The Gooch crucibles are prepared by placing one layer of No. 40 Whatman filter paper in the bottom, on top of this a thin layer of asbestos, then a second layer of filter paper, and finally a second layer of asbestos. The precipitate is so fine, however, that for the first four or five determinations results will be obtained which are from 3 to 10 per cent low. After this the results are generally accurate within 1 or 2 per cent. We would therefore recommend that four crucibles be prepared as outlined above, and before any quantitative determinations are made, between 300 and 400 mg. of sodium pyro-

antimonate be filtered through each pad, thus clogging the pores of the filter. The sodium pyroantimonate may be prepared by the addition of 10 cc. of the reagent and 3 cc. of 95 per cent alcohol to 2 cc. of a sodium chloride solution containing from 3 to 5 mg. of sodium per cc. After standing about 5 minutes 60 to 100 mg. of precipitate will be obtained. This is transferred to the Gooch crucible and washed with 30 per cent alcohol. The procedure is repeated until 300 to 400 mg. have been transferred. We have used one set of crucibles over 25 times and at the end of that time only moderate suction was necessary to produce the optimum rate of filtration. Thus, if four crucibles are prepared in the manner described above, over 100 sodium determinations may be made before it is necessary to make a new set.

Filtration.—The precipitate is transferred to the Gooch crucible with the aid of a rubber-tipped rod. After the pad has become soaked, moderate suction is used so that the fluid goes through at the rate of 10 to 15 drops per minute. When all the fluid has passed through the filter, the rubber-tipped rod is washed off, and the small amount of precipitate remaining in the platinum dish transferred to the Gooch by means of 8 to 12 cc. of 30 per cent alcohol.

Drying.—The drying[1] is carried out at 110°C. for 1 hour and then the crucible and contents are placed in a desiccator to cool. At the end of 30 minutes the crucible is weighed.

Protocols.

Table I gives the results of a series of sodium determinations on samples of a known solution of blood salts. The maximum deviation from the theoretical is ± 2.5 per cent.

The data given in Table II indicate that known amounts of sodium added to serum, the sodium content of which has been previously determined, may be quantitatively recovered.

The results given in Table III demonstrate that the amount of sodium found in serum is the same whether the determination be done on the serum directly or on the ash of the same serum. This would indicate that normal serum contains no organic substances and no volatile inorganic substances in appreciable amounts which are precipitated by the reagent.

TABLE I.

*Estimation of Sodium in Samples of Solution B.**

	Na present.	Na found.	Error.
	mg.	*mg.*	*per cent*
		3.52	−1.1
		3.62	+1.7
	3.56	3.65	+2.5
Average.......	3.56	3.60	+1.1
		7.02	−1.4
		7.13	+0.1
	7.12	7.04	−1.1
Average.......	7.12	7.06	−0.8
		10.78	+0.9
		10.61	−0.7
	10.68	10.82	+1.3
Average.......	10.68	10.73	+0.5

* Composition of Solution B.

NaCl..	7.739 gm.
$Na_2HPO_4 + 2 H_2O$..............................	2.005 "
KCl..	0.453 "
$CaCO_3$..	0.250 "
$MgSO_4 + 7 H_2O$................................	0.189 "
Concentrated HCl..................................	10 cc.
H_2O to..	1,000 "

TABLE II.

Recovery of Sodium Added to Serum.

Serum.	Na present.	Na added.	Total Na found.	Total Na present.	Error.
	mg.	*mg.*	*mg.*	*mg.*	*per cent*
1	6.76	3.56	10.20	10.32	−1.0
1	6.76	7.12	13.75	13.88	−1.0
1	6.76	10.68	17.54	17.44	+0.6
2	6.85	3.56	9.90	10.41	−4.9
2	6.85	7.12	13.97	13.97	±0.0
2	6.85	10.68	17.08	17.53	−2.5

TABLE III.

Comparison of Amount of Sodium Found in Ashed and Unashed Sera of Normal Adults.

Serum.	Na per 100 cc. serum.	
	Ashed.	Direct unashed.
	mg.	*mg.*
1	336	324
2	338	337
3	334	342
4	334	338
5	345	339
6	334	344
7	333	338
8	340	323
Average..............	336	335

TABLE IV.

Sodium Content of Serum in Normal and Pathological Conditions.

Case No.	Age.	Diagnosis.	Na per 100 cc. serum.
			mg.
1	Adult.	Normal.	335
2	11 yrs.	"	326
3	11 "	"	339
4	10 "	"	350
5	5 "	"	331
6	5 "	"	334
7	4 "	"	346
8	2 "	"	334
9	6 "	"	330
10	9 "	"	340
11	10 "	Hereditary syphilis.	338
12	10 "	Epilepsy.	331
13	11 mos.	Tetany.	323
14	5 "	"	324
15	6 "	"	337
16	6 yrs.	Chronic nephritis.	315
17	10 "	" "	341
18		Pneumonia.	321
19	2 yrs.	Acute intestinal indigestion, acidosis 29 volumes per cent serum bicarbonate.	218
20	1 yr.	Acute intestinal indigestion, acidosis 20 volumes per cent serum bicarbonate.	270

In Table IV are given a number of determinations on sera of normal adults and children. A few determinations on pathological sera are also included.

DISCUSSION.

A method for the direct determination of sodium in the ash of serum or whole blood has already been described.[2] In this procedure the ash is dissolved in dilute HCl, the solution made just alkaline with 10 per cent KOH, and the sodium ions precipitated by adding an excess of a solution of potassium pyroantimonate. Since sodium pyroantimonate is quite soluble in water, precipitation is hastened and made complete by adding absolute alcohol up to 30 per cent of the volume of the sample. The precipitate is then separated by filtering through a weighed Gooch crucible. The crucible is dried at 110°C., cooled, dried in a desiccator, and again weighed.

We have found that with a slight modification of this technique sodium may be completely precipitated directly from serum by means of the same reagent. Although this reagent forms an insoluble compound with NH_4 ions, the amount of this ion in serum is insignificant. There is no doubt that the addition to serum of this definitely alkaline reagent ($pH > 8$) precipitates calcium phosphate but the total amount of this salt present in 2 cc. of serum does not exceed 0.5 mg. Since this amount of serum generally yields about 75 mg. of sodium pyroantimonate precipitate, the error is a negligible one. It was thought that the addition of alcohol would precipitate the proteins present, but this does not occur because the serum is first diluted five times and to this volume of fluid (usually 12 cc.) not more than 3 cc. of 95 per cent alcohol are added drop by drop with stirring. In the actual determination the precipitate settles promptly and is crystalline. The supernatant fluid is a clear straw-colored liquid.

In the article referred to above,[2] the literature dealing with direct methods for the determination of sodium is reviewed. In this review, the method described by Ball,[3] which is based upon the formation of an insoluble sodium cesium bismuthi-nitrite com-

[2] Kramer, B., *J. Biol. Chem.*, 1920, xli, 263.

[3] Ball, W. C., *J. Chem. Soc.*, 1910, xcvii, 1408.

pound, is discussed. Certain objections to the method are given; namely, that the reagent is expensive, does not keep well, and large quantities must be used for each determination. The precipitation must be accomplished in the absence of air and requires 24 to 48 hours for completion.

Recently Doisy and Bell[4] have described a method which is an adaptation of the method of Ball. None of the objections given above has been overcome in the more recently described method.

A complete review of the literature dealing with the preparations and the properties of sodium pyroantimonate has been published elsewhere.[5]

CONCLUSIONS.

1. A simple gravimetric method for the quantitative estimation of sodium in 2 cc. samples of serum has been described.
2. Determinations on solutions containing known amounts of sodium yield results that are within ± 2.5 per cent of the actual amount present.
3. Sodium added to serum may be recovered quantitatively.
4. The results obtained when the determinations are done on the serum directly are practically identical with those found when the determination is made on the ash of the same serum.

[4] Doisy, E. A., and Bell, R. D., *J. Biol. Chem.*, 1921, xlv, 313.

[5] Friedheim, C., Gmelin-Kraut's Handbuch der anorganischen Chemie, Heidelberg, 7th edition, 1912, iii, *2te Abt.*, 816.

ON THE RATE OF NITROGEN ELIMINATION.

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Studies on the rate of nitrogen elimination have been made by a large number of investigators for the purpose of studying the metabolic behavior of various proteins. The products of absorption following the ingestion of one protein may differ considerably from others due to the wide variation in the chemical composition of the proteins, as indicated by the qualitative and quantitative differences in the amino-acids obtained upon hydrolysis. Therefore, variations in the rate of deamidization of the various amino-acids may alter the curve of nitrogen excretion following the ingestion of different proteins.

As indicated by Mendel and Lewis,[1] however, there are other important considerations quite unassociated with the metabolic behavior of the amino-acids which play an important part in the rate of nitrogen elimination following the ingestion of various proteins. Variations in the rate of gastric digestion; passage of the food material down the alimentary tract; pancreatic and intestinal digestion; the rate and degree of absorption; and the effect of non-nitrogenous foodstuffs; all play an important part. Finally there is another consideration upon which much stress has not been laid; namely, the functional state of the kidneys.

The great influence of the above factors on the rate of nitrogen elimination is indicated by the results obtained by Mendel and Lewis in a large series of experiments. They attribute the variations in the rate of nitrogen excretion in their own experiments and in the experiments of previous investigators to variations in the rate of alimentary, rather than metabolic processes. It is

[1] Mendel, L. B., and Lewis R. C., *J. Biol. Chem.*, 1913, xvi, 19.

significant in this connection that Van Slyke and White[2] used the rate of nitrogen elimination as an index of the rate of absorption from the alimentary tract.

On the other hand, variations in the rate of nitrogen elimination have been attributed by others to differences in the rate of metabolism of different proteins caused by the absorption of larger or smaller cleavage products.

Most of the previous workers have determined only the nitrogen output in the urine for 2 or 3 hour periods through the 24 hours. Pepper and Austin,[3] however, have determined simultaneously the curve of non-protein nitrogen in the blood, together with the curve of nitrogen excretion, and their evidence on the alimentary factors as effecting the rate of nitrogen elimination is essentially similar to ours.

In a series of experiments on kidney function we have observed both the non-protein nitrogen and urea in blood simultaneously with the rate of nitrogen elimination and report the results in the present paper.

EXPERIMENTAL.

Our observations were made upon adult female dogs raised in our own kennels. The animals received no food for 24 hours previous to the experiment. In the morning they were fed a weighed amount of protein in the form of lean beef. This they ingested voluntarily together with a measured volume of water. The amounts of meat fed varied from 10 to 50 gm. per kilo of body weight. Immediately after feeding, the animal was catheterized. The external saphenous vein on the hind leg was exposed under local anesthesia, through a small skin incision about ½ inch long. 6 or 7 cc. of blood were drawn from the vein using a 10 cc. glass syringe and transferred to a 50 cc. Erlenmeyer flask containing potassium oxalate to prevent clotting. As the needle was withdrawn from the vein a gauze or cotton compress was applied and held in place by a bandage. This effectively controlled the hemorrhage. Subsequent samples of blood were drawn from the same vein. If care is taken to introduce the needle into the same opening in the vein each time many blood samples can be obtained. In case clotting in the vein occurs it may be necessary to thrust the needle down through the clot until the point is well below it.

The first catheterization was done between 8 and 9 a.m. and repeated at 2 hour intervals throughout the day until 11 p.m. Blood samples were drawn at 1 hour intervals immediately after each catheterization and in

[2] Van Slyke, D. D., and White, G. F., *J. Biol. Chem.*, 1911, ix, 219.

[3] Pepper, O. H. P., and Austin, J. H., *J. Biol. Chem.*, 1915, xxii, 81.

the middle of the 2 hour urine periods. After the last catheterization and blood sample the animal was placed in a metabolism cage in order to collect the night urine. The animal was removed from the cage the next morning and catheterized at the end of the 24 hour period. A blood sample was drawn at the same time. In most of the experiments one or two additional 2 hour observations were made immediately following the 24 hour period.

The analyses included the determination of the total nitrogen, urea, and ammonia in the various samples of urine and the non-protein nitrogen and urea in the bloods. For the determination of the total nitrogen,[4] urea,[5] and ammonia[6] in urine the methods of Folin were used. The non-protein nitrogen and urea in blood were determined by the methods of Folin and Wu.[7]

Results.

Our findings represent the results obtained from a series of 25 experiments. Instead of including the individual protocols and data of all experiments, which are voluminous, we have charted (Charts 1 and 2) the results obtained from two experiments which illustrate to a maximum degree the cardinal points mentioned. The dogs used in these two experiments were of approximately the same age and weight and received the same amount of meat and water. The abscissæ represent equal increments of time; the ordinates, gm. of nitrogen for the total and urea nitrogen in the urine and mg. per 100 cc. for the non-protein and urea nitrogen in the blood.

As the blood was drawn at 1 hour intervals, the curves for urea and non-protein nitrogen are plotted at hourly intervals. The urinary nitrogen is plotted from the 2 hour output of urea and total nitrogen up to the last catheterization at night. This represents the excretion for the first 14 hours. From the last catheterization at night to the termination of the 24 hour period the next morning, the urinary nitrogen is plotted from the average 2 hour output calculated from the total output for the 10 hour night period.

Our results are in general agreement with the findings of many previous workers; namely, that a large part of the protein nitro-

[4] Folin, O., and Wright, L. E., *J. Biol. Chem.*, 1919, xxxviii, 461.

[5] Folin, O., and Youngburg, G. E., *J. Biol. Chem.*, 1919, xxxviii, 111.

[6] Folin, O., and Bell, R. D., *J. Biol. Chem.*, 1917, xxix, 329.

[7] Folin, O., and Wu, H., *J. Biol. Chem.*, 1919, xxxviii, 106.

gen consumed is rapidly transformed in the body and excreted in the urine, largely as urea. There is an hourly increase in the nitrogen excretion beginning shortly after the meal, increasing to a maximum and returning approximately to the fasting or original level within 24 hours.

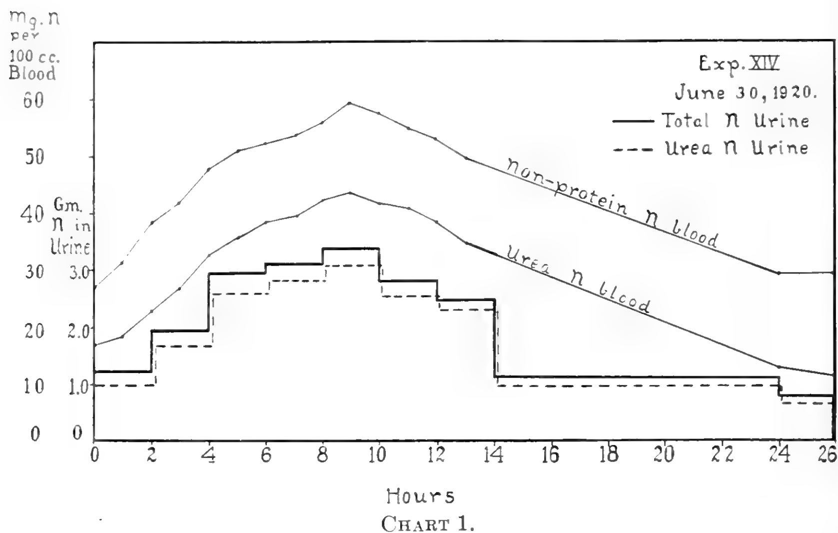

Chart 1.

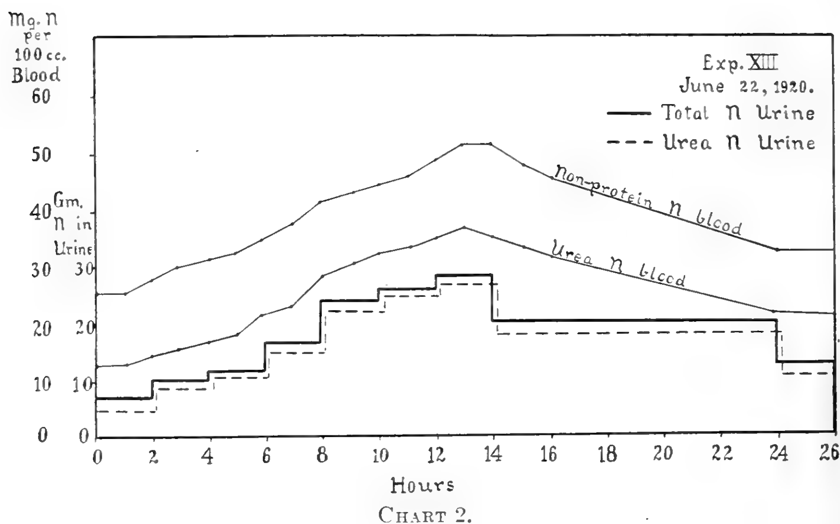

Chart 2.

The increase in nitrogen excretion began $1\frac{1}{2}$ to 2 hours after feeding. Beginning within the second 2 hour period there was a rapid rise in the nitrogen excretion, reaching a maximum in 4 to 12 hours and returning approximately to the original level within 24 hours. The character of the curve of excretion was influenced by the amount of protein fed. When moderate amounts were ingested, that is 15 to 25 gm. of meat per kilo of body weight, the maximum excretion of nitrogen usually occurred about 4 to 8 hours after feeding and returned to the original level within 20 to 24 hours. With larger amounts however, 35 to 45 gm. of meat per kilo of body weight, the maximum excretion of nitrogen usually occurred about 6 to 12 hours after feeding, the peak of the curve was more prolonged and usually a longer time was required to return to the original level.

We have observed variations in the curve of nitrogen elimination, not only following the ingestion of varying amounts of meat, but also when the same amount of meat per kilo of body weight was fed, not only to different animals, but to the same dog on different occasions. The cause for the variations in the curves of nitrogen excretion becomes more apparent when compared with the curves for the non-protein nitrogen and urea nitrogen in the blood. Beginning 1 to 2 hours after the ingestion of the meat there was a rise in the non-protein nitrogen of the blood and almost simultaneously a similar increase in the urea. The non-protein nitrogen and urea nitrogen in blood increased to a maximum within 4 to 12 hours and then gradually returned to the original level as in the case of the urine nitrogen. In a given experiment the curves of non-protein and urea nitrogen in blood, and the total nitrogen and urea in the urine run closely parallel. As a result the same variations were found in the case of the blood nitrogen as were obtained for the nitrogen output in the urine. In our experiments apparently the factor determining to a large extent the changes in nitrogen output in the urine was the variation in the concentration of the urea in blood, for as the concentration of urea in blood rises and falls the total nitrogen output in the urine, which is largely urea nitrogen, rises and falls simultaneously. We must look then to the urea and non-protein nitrogen of the blood in seeking the explanation of the variations in the rate of nitrogen excretion. Upon examining the curves

for the non-protein and urea nitrogen in the blood in the series of experiments the following points are particularly striking.

Beginning 1 to 2 hours after the ingestion of the meat there was an hourly increase in the non-protein nitrogen of the blood, which rose to a maximum and gradually returned to the original level usually within 24 hours. This rise and fall in the non-protein nitrogen in a given experiment was associated with a similar and almost simultaneous rise and fall in the urea nitrogen in the blood which closely paralleled the curve for non-protein nitrogen.

Although the curves for non-protein and urea nitrogen in blood closely paralleled each other in a given experiment, nevertheless in different experiments the character of the curves varied considerably. In some the rise was rapid, reaching a maximum within 4 to 8 hours and then receding to about the original level within 20 hours. In others the rise was more gradual, reaching a maximum within 6 to 12 hours after feeding and usually returning to the original level within 24 to 28 hours. Based upon the work of Folin and Denis[8,9] and Van Slyke,[10] we attribute the increase in the non-protein nitrogen of the blood to the absorption of the amino-acids from the alimentary tract and the simultaneous rise in the urea in the blood to the rapid urea formation from the absorbed amino-acids. In this connection it should be remembered that the blood was drawn at 1 hour intervals. Had the blood been obtained at shorter intervals no doubt the rise in the urea would not have occurred simultaneously with that of the non-protein nitrogen, but would have lagged behind somewhat.

The variations in the character of the curves for the non-protein and urea nitrogen in the different experiments we attribute to variations in the rate of absorption of the amino-acids from the alimentary tract. The early and rapid rise in the blood nitrogen noted in certain experiments is due, we believe, to the early and rapid absorption of the amino-acids, and the delayed and gradual rise to delayed and slower rate of absorption. The rate of absorption reflects the rate of the sum total of the various alimentary processes mentioned before.

[8] Folin, O., and Denis, W., *J. Biol. Chem.*, 1912, xi, 87.

[9] Folin, O., and Denis, W., *J. Biol. Chem.*, 1912, xii, 141.

[10] Van Slyke, D. D., and Meyer, G. M., *J. Biol. Chem.*, 1912, xii, 399.

Since in a given experiment the curve of nitrogen excretion closely follows and parallels the blood nitrogen, we attribute the variations observed in the rate of elimination of nitrogen in the series of experiments to the same factors responsible for the variations observed in the curves for the blood nitrogen; namely, to variations in the rate of the various processes of alimentation and absorption.

SUMMARY.

1. Studies on the nitrogen elimination at 2 hour intervals for 24 hours, and the urea and non-protein nitrogen of the blood at 1 hour intervals, were made on dogs after feeding lean meat.

2. The results indicate that the rate of nitrogen elimination is an index of the rate of digestion and absorption.

A STUDY OF THE CHLORINE CONTENT OF MILK AND BLOOD AFTER THE INGESTION OF SODIUM CHLORIDE.

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(Received for publication, March 21, 1921.)

In a recent study[1] of the chlorine content of human milk, we noted that there was a great variation in the concentration of this constituent, both in the chlorine content of milk of different mothers and in specimens obtained from individual women at intervals during the day. This observation was based on the analyses of a large number of specimens obtained from women in all stages of lactation and living under various conditions.

In seeking an explanation for these variations we had occasion to consider among other factors, the influence of food intake. As far as we were able to determine from observations on human subjects the ingestion of salt caused no effect on the output of chlorine in the milk.

The lactating human mother is not, however, an ideal experimental subject and in order to obtain more definite evidence on the point in question we have carried out a series of experiments on goats with the object of ascertaining the effect of feeding sodium chloride upon the chlorine content of milk.

The elaborate experiments of Forbes and his collaborators[2] would lead us to believe that practically no demonstrable effect is produced on the sodium chloride concentration of the milk of cows by variations in the salt content of the diet. These conclusions were based on the analyses of composite samples covering experimental periods of about 20 days.

[1] Sisson, W. R., and Denis, W., *Am. J. Dis. Child.*, in press.

[2] Forbes, E. B., *Ohio Agric. Exp. Station, Bull. 295*, 1916; *Bull. 308*, 1917.

In a study[3] of the chlorine content of cows' milk and cows' milk modifications commonly used in infant feeding, we gave sodium chloride to cows in single doses as high as 500 gm. without producing any appreciable change in the chlorine content of the milk. Although marked variations were noted in specimens of cow's milk, there was no evidence that the amount of salt in the diet in any degree affected the chlorine content of the milk.

The experiments of Forbes, as stated above, cover rather long periods and include no data on the early effects produced by high sodium chloride dosage given over a limited time. In our work we have, therefore, attempted to throw light on this phase of the problem and have directed our inquiries along two general lines, first a study of the morning and evening milk of animals on diets without added sodium chloride and later on rations to which had been added varying amounts of salt, and second a series of hourly observations on the milk and blood of animals that were receiving large doses of sodium chloride.

The goat was selected as the most suitable experimental animal for such a study. The three females used were of different breeds, but all were in the early part of their lactating period. The animals were brought to the laboratory at least a week before any experimental work was undertaken. By the end of this time they had become accustomed to their surroundings and new mode of life and had entirely recovered from the mental effects incident to their introduction into a new environment; in view of the undoubted effects of psychic stimuli on the quality of the mammary secretion we feel that this precaution is essential in a study of this nature.

The daily ration consisted of hay and 400 gm. of a mixture of equal parts of oats and cracked corn. On the days on which samples of milk and blood were obtained at frequent intervals, the animals were kept in the laboratory and supplied with freshly cut grass or hay in addition to the grain. At other times the goats were allowed to graze for about 7 hours a day. Water was allowed in unlimited amounts. The animals were housed in stalls in the laboratory where they could be carefully observed.

Our experimental procedure was as follows: The dose of sodium chloride was dissolved in about 300 cc. of water and administered

[3] Unpublished data.

by pouring it into the animals' throat by means of a long necked bottle. Precautions were taken in milking the goats to avoid all contaminations from the excreta. All samples of blood were obtained by venous puncture from the external jugular vein.

The determination of milk chlorides was carried out by the method described by Van Slyke and Donleavy,[4] for use with blood plasma. As goats' milk contains only one-third as much chloride as blood plasma it was necessary to make a few changes in the concentration of the reagents. Therefore, although no claim for originality is made in this connection it has seemed desirable to furnish a brief description of our technique. Attempts to employ the Van Slyke-Donleavy technique without change to human milk were unsuccessful as it was found impossible to obtain a satisfactory precipitation of the proteins.[1] Our procedure was as follows.

To 10 cc. of milk (goat or cow) were added 20 cc. of a 1.2 per cent solution of picric acid and 20 cc. of standard silver nitrate solution. Precipitation of the proteins was instantaneous and complete, and after standing for 10 minutes the liquid was poured on a chlorine-free filter. To 10 cc. of the clear filtrate are added 2 cc. of the starch indicator, and the excess of silver then titrated back with potassium iodide solution.

As it seemed desirable to express our results in terms of mg. of chlorine per 100 cc. of milk we have, in order to avoid the use of a factor, used a solution of silver nitrate of a slightly different concentration[1] than that specified by McLean and Van Slyke. Check analyses indicating the correctness of our results have been reported elsewhere.[1] Plasma chlorides were determined by the method of Rappleye.[5]

Experiment 1.—The animal used in this experiment was a common American goat (Goat 1), that weighed 31.8 kilos. She was in the third month of lactation and her average yield of milk for 24 hours was only 150 cc. The average chloride concentration of the milk of specimens obtained a few days after the goat was brought to the laboratory was 165 mg. per 100 cc. of milk. The experiment was divided into three periods. Period 1 from

[4] Van Slyke, D. D., and Donleavy, J. J., *J. Biol. Chem.*, 1919, xxxvii, 551.

[5] Rappleye, W. C., *J. Biol. Chem.*, 1918, xxxv, 509.

Mar. 16 to Apr. 16, 1920. No sodium chloride was added to the ration. Analyses of the milk made at frequent intervals gave the results shown in Table I.

TABLE I.

Effect of Sodium Chloride-Free Ration on the Chlorine Content of the Milk.

Date.	Time.	Chlorine per 100 cc. milk.
1920		*mg.*
Mar. 16	9.00 a.m.	169
" 16	5.00 p.m.	165
" 17	8.00 a.m.	169
" 20	8.00 "	189
Apr. 1	8.30 "	167
" 2	8.00 "	168
" 2	4.00 p.m.	161
" 9	9.00 a.m.	153

Period 2 from Apr. 17 to 27. The dose of sodium chloride was 30 gm. daily. This was given in 15 gm. doses after the morning and the evening milking. Examination of the mixed milk for each 24 hour period gave the results in Table II.

TABLE II.

Effect of Ingestion of 30 Gm. of Sodium Chloride Daily on the Chlorine Content of Milk.

Date.	Volume of milk.	Chlorine per 100 cc.
1920	*cc.*	*mg.*
Apr. 17	105	162
" 18	120	148
" 19	100	148
" 20	108	148
" 21	112	153
" 22	75	157
" 23	76	165
" 24	65	167
" 25	50	174
" 26	50	165
" 27	78	174

Period 3. As the results obtained in Period 2 appeared to indicate that the administration of relatively large doses of sodium chloride for a period of 11 days is without effect on the chlorine content of the milk we decided to continue the experiment, but to increase the dose to 20 gm. (two doses per day), and to adopt the practice of making separate analyses of the morn-

ing and evening milk and of administering the sodium chloride 30 minutes before instead of after the milking time as had been done in Period 2. The results of this analysis are shown in Table III.

TABLE III.

The Chlorine Content of Morning and Evening Milk on Ration with 20 Gm. Sodium Chloride Given before Collection of Specimen.

Date.	Volume of milk.	Chlorine per 100 cc
1920	*cc.*	*mg.*
Apr. 28, a.m.	75	168
p.m.	17	210
" 29, a.m.	35	199
p.m.	15	212
" 30, a.m.	50	204
p.m.	28	187
May 1, a.m.	80	170
p.m.	25	
" 2, a.m.	75	184
p.m.	75	196
" 3, a.m.		196
p.m.		156
" 4, a.m.		147
p.m.		166
" 5, a.m.		172
p.m.		162

This experiment shows that the chlorine concentration of the milk of a goat giving a relatively small volume of milk and with a normally high salt content, is not changed after a period of 24 days on a "salt-free" ration. The average chlorine concentration of the milk of this goat before the experimental period was 165 mg. per 100 cc. of milk and the average of the 24 days was 167.6 mg.

The results obtained in the second period of this experiment indicate that the administration of relatively high doses of sodium

chloride for a period of 11 days causes essentially no change in the chlorine concentration in the daily output of milk. The average concentration of this period was 165.5 mg. When the dose of sodium chloride was increased from 0.50 gm. per kilo of body weight to 0.78 gm. as was done in Period 3 of this experiment, and the specimens of milk collected 30 minutes after the administration of the salt, a higher chlorine concentration of the milk was obtained. The average for this period of 8 days on excessively high doses of sodium chloride was 182.6 mg. of chlorine per 100 cc. of milk. The results of the analyses as shown in Table III indicate that this increased chlorine concentration varied considerably in the morning and evening milk.

It is, therefore, apparent from the results of this experiment that a high salt diet can be taken without affecting the chlorine content of the milk. The experiment suggests however that a change may be produced if the dosage of salt is sufficiently high. From these findings it is apparent that the salt can easily be transferred to the muscles and subcutaneous tissues or excreted by the kidneys without affecting the mammary secretions. We made no chloride determinations on the blood plasma in this experiment, an omission that is to be regretted. It has, however, been shown by Austin and Jonas[6] that the blood of dogs fed for relatively long periods on high salt diets contained no higher concentration of plasma chlorides than before the period of salt feeding.

The three experiments, the results of which are presented below, were undertaken to determine the effect on the chlorine concentration of the milk when doses of sodium chloride were given sufficient to raise the concentration of the plasma chloride.

Experiment 2.—May 13 to 14, 1920. Goat 1, used in the former experiment, was given 80 gm. of sodium chloride in four doses as shown in Table IV. The average daily milk production of this goat previous to the experiment was about 150 cc.

Experiment 3.—Absorption of sodium chloride. June 15, 1920. Goat 2, weight 22.5 kilos. This animal was in the first month of lactation. The average daily yield of milk was about 1 liter, see Table V.

Experiment 4.—Absorption of sodium chloride. June 15, 1920. Goat 3, weight 36 kilos. An old animal that had kidded a few weeks before being brought to the laboratory. The average daily milk production was about 700 cc., see Table VI.

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[6] Austin, J. H., and Jonas, L., *J. Biol. Chem.*, 1918, xxxiii, 91.

It will be noted from these experiments that a very marked increase in the chlorine concentration of the blood took place after the ingestion of the sodium chloride. We were unable to

TABLE IV.

The Effect of Ingestion of 80 Gm. of Sodium Chloride Given in Four Doses of 20 Gm. Each on the Volume and Chlorine Concentration of Milk.

Time.	Sodium chloride administered.	Volume of milk.	Chlorine per 100 cc. milk.
1920	*gm.*	*cc.*	*mg.*
May 13, 8.15 a.m.	0	95	168
8.30 "	20		
9.30 "	20	10	144
10.30 "	20	7	138
11.30 "	20	4	177
2.30 p.m.		6	198
May 14, 8.30 a.m.		56	216

TABLE V.

The Effect of Ingestion of 75 Gm. of NaCl Given in Four Doses upon the Volume of Milk and Chlorine Concentration of Blood Plasma and Milk.

Time.	Sodium chloride fed.	Volume of milk.	Chlorine per 100 cc.	
			Milk.	Plasma.
1920	*gm.*	*cc.*	*mg.*	*mg.*
June 15, 9.05 a.m.		650	140	350
9.15 "	15			
10.15 "	20			
11.15 "	20			
12.05 p.m.		90	174	
12.15 "	20			468
3.00 "		60	234	514
8.00 "		60	218	543
June 16, 10.00 a.m.		24	245	
3.30 p.m.		10	322	
June 17, 9.00 a.m.		29	245	
6.00 p.m.		75	205	
June 18, 8.00 a.m.		200	166	
4.00 p.m.		190	163	

bleed the goat in Experiment 2, but in Experiments 3 and 4, increases in plasma chlorine of 36 and 13 per cent respectively were obtained. In Experiments 3 and 4 more complete

data were obtained and coincident with the rise in plasma chlorides, there was a very great increase in the chlorine concentration of the milk, as is seen in Figs. 1 and 2. This amounted to 56.5 per cent in Experiment 3 and to 17.6 per cent in Experiment 4.

The toxic symptoms following the administration of the sodium chloride, in the case of Goat 2, Experiment 3, were noteworthy. Shortly after the second dose, the goat became manifestly restless, the ears were held close to the head and there developed a marked tremor and spasticity of the extremities. Soon the prostration became so severe that the animal was unable to stand, and had

TABLE VI.

The Effect of Ingestion of 60 Gm. of NaCl Given in Four Doses Upon the Volume of Milk and Chlorine Concentration of Blood Plasma and Milk.

Time.	Sodium chloride fed.	Volume of milk.	Chlorine per 100 cc.	
			Milk.	Plasma.
1920	*gm.*	*cc.*	*mg.*	*mg.*
June 15, 9.20 a.m.		200	182	366
9.25 "	15			
10.20 "	15			
11.20 "	15			
12.20 p.m.	15		195	432
3.00 "		35	221	426
8.30 "		60	213	432
June 16, 8.30 a.m.		350	171	360
11.00 "		65	186	
3.30 p.m.		125	180	

frequent convulsive movements, involving particularly the muscles of the neck and the extremities. The condition of the goat became so extreme 7 hours after the salt had been given that its recovery seemed improbable. During the 4 hours when its symptoms were most extreme it drank about 10 liters of water, an amount that represented about one-half its body weight. The abdomen became greatly distended and there developed a very marked diarrhea which lasted for a number of hours. The acute toxic symptoms disappeared in about 24 hours, however, the goat became emaciated, and its milk production fell from 750 to about 30 cc. a day.

SUMMARY.

A series of experiments has been carried out to determine the effect of diet upon the chlorine concentration of milk. It has been shown that on a salt-free diet for a period of 24 days no change took place in the chlorine concentration of milk from a goat giving about 150 cc. a day, also that the ingestion of 1.2 gm. of sodium chloride per kilo of body weight, daily, over a period of 6 days, causes no change in chlorine concentration of the milk.

Further experiments were undertaken with goats to show the effect of increasing the chlorine concentration of the blood plasma upon the chlorine content of the milk. It was noted that by the administration of an amount of sodium chloride sufficiently large to produce an 18 per cent increase in the concentration of this salt in the blood plasma, the chlorine content of the milk is also raised. Coincident with this increase in the chlorine concentration of the milk and blood plasma the volume of milk became greatly diminished.

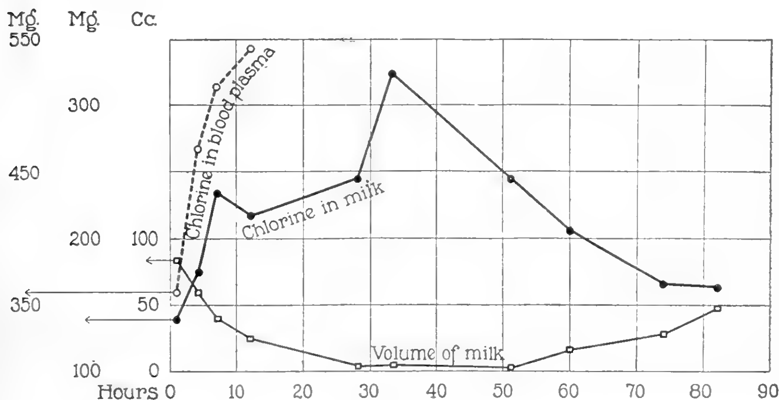

Fig. 1. Effect of ingestion of 75 gm. of sodium chloride given in four doses (15, 20, 20, and 20 gm.), at hourly intervals, upon the chlorine concentration of the milk and blood plasma and the volume of milk. The volume of milk is represented on the basis of the amount secreted at 2 hour intervals.

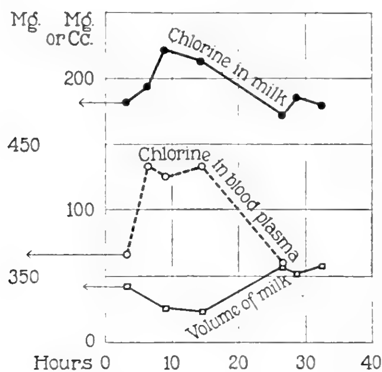

Fig. 2. Effect of ingestion of 60 gm. of sodium chloride, given in four equal doses at hourly intervals upon the chlorine concentration of milk and blood plasma and volume of milk. The volume of milk is represented on the basis of the amount secreted at 2 hour intervals.

BLOOD BICARBONATE LEVELS FOLLOWING ADMINISTRATION OF SODIUM BICARBONATE.

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(Received for publication, March 18, 1921.)

It is clinically desirable at times to increase the bicarbonate content of the blood of certain patients. The administration of sodium bicarbonate accomplishes this purpose. Since, however, too much alkali is harmful, and too little fails of its purpose, the question of dosage is very important. The formula evolved by Palmer and Van Slyke[1] can be used with sufficient accuracy at least in normal individuals. They point out some considerations which must be remembered in using the well known bicarbonate administration test for acidosis.

The work of Palmer and Van Slyke was repeated on twenty-five normal individuals—laboratory workers, nurses, and students. The technique was to draw blood with proper precautions, to centrifuge under oil, and to determine the carbon dioxide capacity of the plasma in a Van Slyke burette after equilibration with the analyst's alveolar air. A weighed quantity of sodium bicarbonate was then given by mouth with about half a tumbler of water. This procedure was performed sufficiently long after a meal (breakfast) to have presumably an empty stomach. Preliminary experiments indicated that the highest point in the blood bicarbonate occurred in approximately $1\frac{1}{2}$ to 2 hours. Accordingly, after resting, a second specimen of blood was taken in 2 hours and another analysis made. Table I gives the extreme departures from the calculated and ten examples around the medium. The observed increase is surprisingly close to the calculated one.

[1] Palmer, W. W., and Van Slyke, D. D., *J. Biol. Chem.*, 1917, xxxii, 499.

The same procedure was adopted in a series of twenty-five patients, the diagnosis of whom is given in Table II. It is apparent at once, that deviation of observed from calculated

TABLE I.

Weight.	Dose of $NaHCO_3$.	Blood CO_2 before.	Blood CO_2 after.	Actual increase.	Theoretical increase.	Difference.
kg.	*gm.*	*vol. per cent*	*vol. per cent*	*vol. per cent*	*vol. per cent*	*vol. per cent*
48.9	10	61.7	74.0	12.3	7.7	+4.6
61.7	10	65.5	65.5	0	6.1	−6.1
56.2	10	63.6	65.5	1.9	6.7	−4.8
52.1	10	61.6	63.4	1.8	7.2	−5.4
81.7	10	59.5	64.3	4.8	4.6	+0.2
84.4	10	66.2	69.1	2.9	4.5	−1.6
43.6	10	60.7	67.3	6.6	8.7	−2.1
60.8	10	59.8	64.5	4.7	6.2	−1.5
71.2	4	62.4	63.3	0.9	2.1	−1.2
56.7	10	66.2	73.4	7.2	6.8	+0.4
56.2	10	56.0	62.9	6.9	6.9	0.0
59.4	10	63.4	68.0	4.6	6.4	−1.8
68.3	10	61.2	64.9	3.7	5.7	−2.0
81.3	10	59.5	65.0	5.5	4.7	+0.8

TABLE II.

Weight.	Disease.	Dose of $NaHCO_3$.	Blood CO_2 before.	Blood CO_2 after.	Actual increase.	Theoretical increase.	Difference.
kg.		*gm.*	*vol. per cent*	*vol. per cent*	*vol. per cent*	*vol. per cent*	*vol. per cent*
58.9	Cardiorenal.	15	62.4	68.1	5.8	9.7	−3.9
71.2	Mild diabetes.	15	60.5	67.2	6.7	8.0	−1.3
63.9	12 hrs. after herniorraphy.	15	66.2	66.2	0	8.9	−8.9
64.0	Pulmonary tuberculosis.	15	64.3	66.2	1.9	8.9	−7.0
73.5	Cardiorenal.	15	65.3	70.0	4.7	7.7	−3.0
52.2	Gastroptosis.	10	69.1	66.2	−2.9	7.2	−10.1
47.0	Pyloric obstruction.	15	66.2	67.3	1.1	12.1	−11.0
56.1	Cardiorenal.	10	62.6	76.8	14.2	6.7	+7.5
57.2	Chronic cystitis.	10	65.5	76.8	11.3	6.7	+4.6
62.5	" cholecystitis.	10	65.3	67.2	1.9	6.0	−4.1

values is greater than in the normals. The reasons are probably numerous, and the number of cases of any particular disease is too small for discussion and conclusions.

A number of obviously influencing factors in the calculation of such data are pointed out by Palmer and Van Slyke. The rapidity with which the bicarbonate leaves the stomach is one. Another is the acidity of the gastric juice; a third, rapidity of absorption of the bicarbonate, or in case it is neutralized by the acid of the stomach, its "sparing effects" on the alkali which would have been poured into the duodenum. The amounts of acid formed in the body during the period of observation is important. This can be somewhat controlled in normals by rest; in those in whom there is excess acid formation, *e.g.* ketones in diabetes, this factor can hardly be estimated. It was at first thought that in such individuals, it might be possible to estimate at least the relative amount of acid formed during any given time. That is, by giving a weighed amount of bicarbonate and estimating the increase in blood bicarbonate which should occur within reasonable error, and observing the actual rise, the difference would represent the relative amount of acid. Obviously, there are many sources of error, and our clinical material has been too limited. The possibility, however, seems to deserve further study. The factor, 70 per cent for fluid weight, seems to be the best adapted for these calculations. In Vierordt's "Daten und Tabellen,"[2] 65 per cent is given.

In a number of patients, it is often thought advisable to give sodium bicarbonate solution intravenously. In patients on whom this procedure was done for eclampsia, diabetic coma, uremia, etc., it was impossible, on account of the extreme gravity of the situation, to study its effects on the blood bicarbonate. Therefore, normal dogs were used.

Technique.

The weight of the animals was regarded as the variable, while the dose of sodium bicarbonate, intravenously injected in 4 per cent solution, was kept constant at 5 or 10 gm. In the first experiments, blood was withdrawn from a heel vein through needle and syringe. The syringe was disconnected, and without removing the needle, warm sodium bicarbonate solution was run

[2] Vierordt, H., Anatomische, physiologische, und physikalische Daten und Tabellen zum Gebrauche für Mediziner, Jena, 3rd edition, 1906, 378.

in from a suitable glass vessel. Calculations of the theoretical increase in blood bicarbonate were made on the assumption that blood weight was approximately one-thirteenth of the body weight. The fluid increase calculation was made in the usual way. It was soon found necessary to discover what occurs in as short intervals as possible, not only after the injection was completed, but also during the course of the injection (Collip and Backus[3]). Therefore, in later experiments, blood was taken at 5 to 15 second intervals through a cannula placed in the carotid artery as close to its origin as possible. Before each sample was

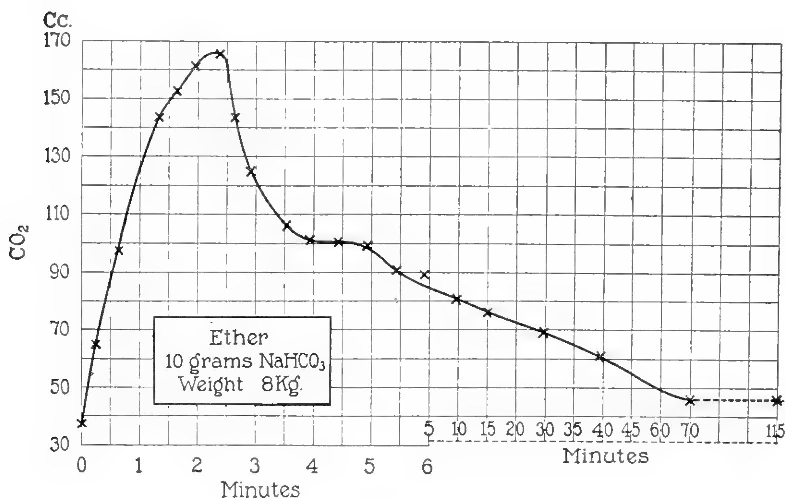

Fig. 1.

taken, a spurt was allowed to waste so that no stasis occurred. Collections were made before, during, and after, the administration of the solution. The total number in all the cases investigated was four in earlier, and six in later experiments. Some were anesthetized by ether. This is deprecated in this type of experiment by certain investigators. Accordingly, four animals were operated under local anesthesia with cocaine. The curves, Figs. 1 to 4, however, in both types of animals are essentially the same. The left leg of the curve represents the period during which the solution

[3] Collip, J. B., and Backus, P. L., *Am. J. Physiol.*, 1920, li, 551.

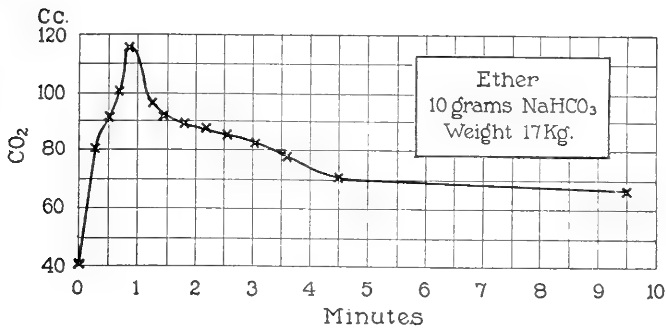

Fig. 2.

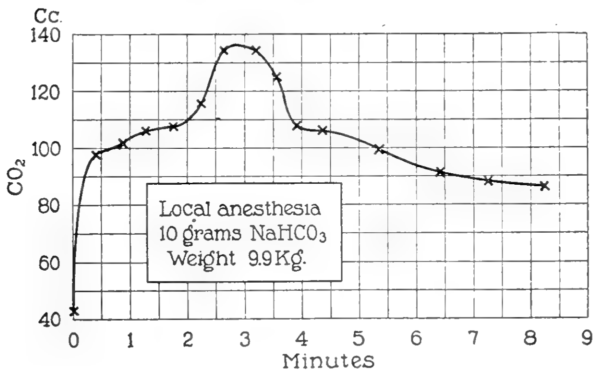

Fig. 3.

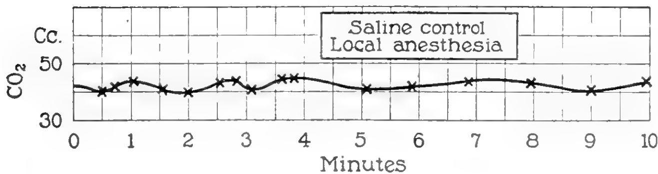

Fig. 4.

was administered. It is not a straight line, but is pulled downward and to the right, that is, some of the bicarbonate as it enters the vein must escape from the blood. Possible modes of escape are: First, the passage of bicarbonate into lymph and tissue spaces; second, amounts neutralized by metabolic acids during that space of time, probably exceedingly small; third, withdrawal of the samples; hemorrhage is known to reduce blood bicarbonate. In this case hemorrhage was probably too small to be a large factor. Fourth is the dilution with the incoming fluid; and fifth, the escape of carbon dioxide from the sodium bicarbonate in excess of ordinary metabolic carbon dioxide through the lungs. Examination of the point of inflection of the curve and its downward leg shows how abruptly the concentration in the blood falls. It also shows the general way in which the bicarbonate level of the blood returns to near the starting point.

SUMMARY.

1. Palmer and Van Slyke's formula for the estimation of the rise in blood bicarbonate following the administration of sodium bicarbonate has been investigated. Given by mouth, it is surprisingly accurate in normal individuals, but less so in various diseases.

2. The form of the curve showing the rise in blood bicarbonate after intravenous administration of sodium bicarbonate has been investigated. This shows the general way in which the blood bicarbonate rises and falls after such procedure.

COMPARISON OF BLOOD AND LYMPH BICARBONATE AFTER INTRAVENOUS INJECTION OF SODIUM BICARBONATE.

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(Received for publication, March 18, 1921.)

In the preceding paper it is shown that the curve of blood bicarbonate during the intravenous administration of sodium bicarbonate is not a straight line. One of the factors pulling it down and to the right was thought to be diffusion into the lymph. This is capable of experimental investigation. Dogs were anesthetized, and a cannula was tied into the left jugular vein after all its branches were ligated.[1] Fluid from the thoracic duct then flowed through the cannula. The dogs were fasted for about 18 hours to avoid excessive digestive products. Since the fluid coagulates very quickly, the cannulæ were paraffined and the fluid led into potassium oxalate in centrifuge tubes. Some contact with the air took place; some of the samples clotted; none contained much fat. All collections, however, were centrifuged. The supernatant fluid was removed, equilibrated with the analyst's alveolar air, and the carbon dioxide capacity determined in the usual way in the Van Slyke burette. With the errors mentioned, absolute quantities of carbon dioxide are perhaps not as accurate as desirable, but the comparative amounts are sufficient to indicate the tendency. According to Starling,[2] osmotic equilibrium between blood and lymph is established within half a minute when large amounts of dextrose are injected into the circulation.

[1] We are indebted to Professor Sweet of the University of Pennsylvania for these operations.

[2] Starling, E. H., Principles of human physiology, Philadelphia, 3rd edition, 1920, 1064.

The same phenomenon occurs in probably as rapid a time with sodium bicarbonate. The rate of the passage of fluid containing various substances is discussed, and experiments on this subject related by Smith and Mendel.[3] Sodium bicarbonate quickly leaves the blood and distributes itself in the tissue fluids. That it is equally distributed is fairly certain, since the formula for calculating the dosage gives such close agreement between the theoretical and

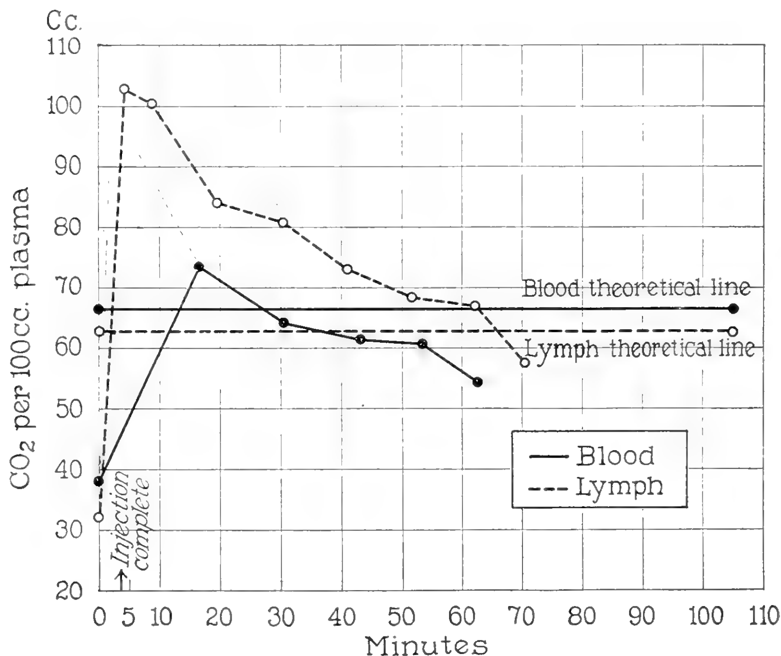

Fig. 1.

the observed results, as pointed out by Palmer and Van Slyke.[4] This quick diffusion as shown in these experiments lends another argument in its favor.

Blood samples were removed as often as possible, and at as close intervals as possible. The experimental results are shown

[3] Smith, A. H., and Mendel, L. B., *Am. J. Physiol.*, 1920, liii, 323.

[4] Palmer, W. W., and Van Slyke, D. D., *J. Biol. Chem.*, 1917, xxxii, 499.

in Figs. 1 and 2. Three dogs were used and the results were all similar.

The flow from the duct before administration of fluid per vein varied from 10 to 15 minutes for 10 cc. Immediately after the sodium bicarbonate solution began to enter the vein the rate of flow increased quite rapidly. Samples of 10 cc. were removed as fast as they collected. The graph shows the rate at which the flow occurred during the experiment. Results in the preceding

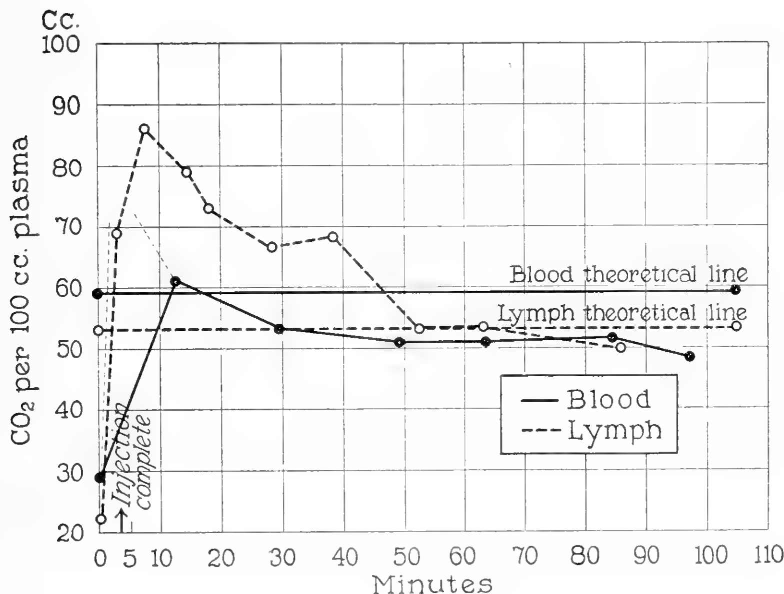

Fig. 2.

paper applied to the curve of blood carbon dioxide in these experiments, would give these blood curves a different form. This is indicated in dotted lines. The "theoretical" line is based on calculations with the Palmer-Van Slyke formula. The point of inflection of the curve of lymph carbon dioxide probably coincides with cessation of injection. "Probably" because samples could not be collected at sufficiently frequent intervals to demonstrate it. Larger samples were collected for duplicate and triplicate determinations.

SUMMARY.

Blood and lymph bicarbonate was determined after intravenous injection of 4 per cent sodium bicarbonate solution. Both increased parallel. Bicarbonate left the blood for the lymph at a remarkably rapid rate.

GROWTH AND REPRODUCTION UPON SIMPLIFIED FOOD SUPPLY. I.*

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(Received for publication, February 26, 1921.)

The purpose of this paper is to present the first results of a series of experiments in which rats have been fed upon rations consisting of only one or two articles of food. For the sake of brevity we omit the customary review of previous work in similar fields as being sufficiently familiar to readers of this Journal, but we desire to make due acknowledgment of indebtedness to all those on whose earlier researches our own are so largely based.

The rats used were of known age and relationship, having been bred by us from Osborne and Mendel stock obtained through the kindness of Professor Mendel and Professor Mary S. Rose. It is a pleasure to express our indebtedness for many helpful suggestions regarding the use and management of the rat as an experimental animal, received in the course of visits to the laboratories of Osborne, Mendel, and Ferry at New Haven, and of McCollum, Simmonds, and Parsons at Baltimore. Our technique has in general been based upon that of Osborne and Mendel. Rats fed individually have been kept in uniform round cages 9 inches in diameter. Breeding rats and other experimental groups are kept in rectangular cages, 11 by 14 inches in size and having wire cloth bottoms resting upon paper. Pure crêpe paper is used for bedding the young until they are well covered with hair; otherwise, no bedding whatever is used. Elimination of bedding not only avoids the possibility of its being eaten, but also greatly facilitates the accurate recovery of any scattered food. Quantitative determination of the food intake has received close atten-

* Published as Contribution No. 355 from the Department of Chemistry, Columbia University.

tion in all of our feeding experiments. The discussion of the quantities of food consumed is, however, deferred until the data of larger numbers of experiments become available.

Preliminary Test.—In a preliminary experiment, young rats of the same litter were fed from the time of weaning upon rations consisting of bread alone, or bread and one other article of food. The bread was made in the laboratory by one of the investigators and in its preparation only patent flour, water, and minimum amounts of yeast, sugar, salt, and lard were used. The feeding of such bread as sole food from the time of weaning (4 weeks) resulted in immediate cessation of growth followed by death after

Fig. 1. Contrasting effects of equally simplified food supplies. These two rats were twin sisters and at weaning time were of equal size and equally healthy and vigorous. One was then fed with bread and apple, the other with bread and milk. The latter had grown to five times the weight of the former, by the time this photograph was taken.

about 6 weeks. A diet of bread and meat begun at the same age resulted in slow growth for about 5 weeks followed by decline and death, the length of life being only slightly greater than when bread alone was fed. On a ration of bread and apple there was practically no growth but the animals lived about 50 per cent longer than those receiving bread alone or bread and meat. The rats of the same litter receiving bread and milk grew at a fully normal rate. Fig. 1 shows twin sisters photographed together when 12 weeks old. When placed on experimental diet at 4 weeks they were of exactly the same weight and appeared in all

respects equally healthy and vigorous. One of them (No. 53) was then fed upon bread and apple and the other (No. 54) upon bread and milk, with the result that during the next 8 weeks No. 54 grew to over five times the weight of No. 53. It will be noted that the diet was equally restricted in each case, consisting simply of bread and one other article of food. The food of No. 53 was also as pure and wholesome of its kind as that of No. 54, the bread fed the two rats being identical and the apple of as high quality as the milk. It cannot be thought that the rat receiving the apple had been injured by it, since this rat had lived longer than those of the same litter who received the same bread without the apple; *i.e.*, bread as sole food, or bread and meat. Evidently the cause of the striking difference in growth and development of the twin sisters shown in Fig. 1 is the greater efficiency of milk than of apple in making good the dietary deficiencies of the bread. Thus a one-sided diet of staple articles of food may result in such striking stunting as has been made familiar by Osborne and Mendel in their work with mixtures of purified foodstuffs.

The growth curves of typical rats on each of the four rations thus far mentioned and that of another of the same litter receiving a ration of bread and white turnip are shown in Chart 1. In each case in which the ration consisted of bread and one other food it was offered in the proportion of 4 calories of bread to 1 calorie of meat, milk, turnip, or apple. In the case of the bread and milk ration this meant equal weights of fresh bread and ordinary milk. The milk was evenly distributed over the bread and entirely absorbed by it so that the rat necessarily ate the bread and milk in the same quantitative proportions in which they were offered. The bread and meat were so fed that they also were eaten in the approximate proportions of 4 calories of bread to 1 of meat. The apple and the turnip, however, were not always eaten in the proportion offered so that in the bread and apple ration the apple did not always furnish as much as 20 per cent of the calories consumed, nor in the bread and turnip ration was as much as 20 per cent of the calories always consumed in the form of turnip. Omitting deductions and discussions which are sufficiently obvious to readers of this Journal, we desire to develop first in a quantitative way the supplementary relationships of bread or wheat and milk, postponing until later the

further investigation of meat, fruit, and vegetables in this connection.

White Bread with Very Limited Milk Supply.—It will be noted (Chart 1) that Rat 54, fed exclusively from weaning upon a ration of equal weights of white bread and milk, or 4 calories of bread to 1 calorie of milk, produced a litter of young at an early

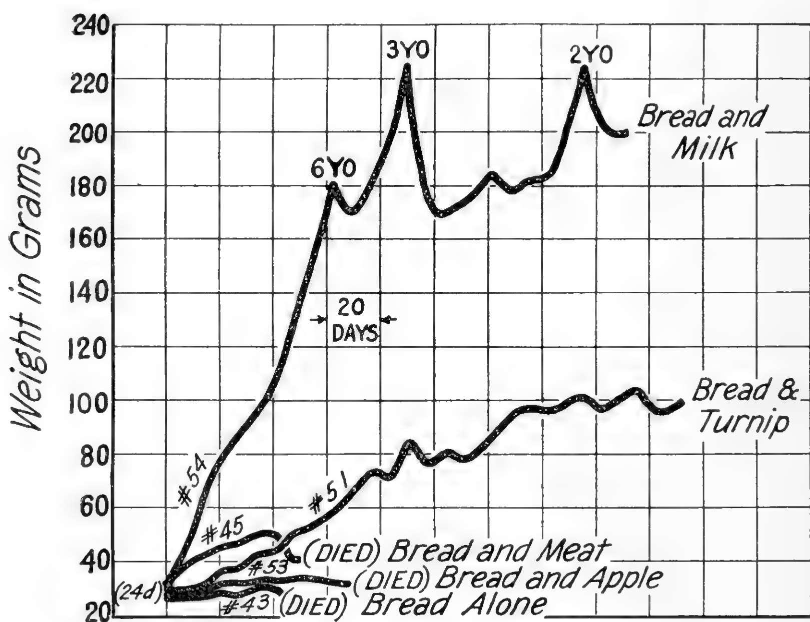

CHART 1. Showing results of feeding bread alone, bread and meat, bread and apple, bread and turnip, or bread and milk to young rats of the same litter.

age (87 days); but none of these survived. She was immediately remated and a second litter of young was born within a month but these also died, as did those of a third litter produced on the same diet after a longer interval. It will thus be seen that, while the ration of equal weights of bread and milk sufficed for normal

growth and early reproduction, it apparently did not suffice for the rearing of young. Later experiments upon equally simple and monotonous mixtures containing a larger proportion of milk have proved such mixtures to be fully adequate, particularly when the white bread was replaced by ground whole wheat.

Growth curves of a number of male rats fed on the diet of equal weights of fresh white bread and milk, or on equivalent mixtures of dry ground bread and whole milk powder, are shown

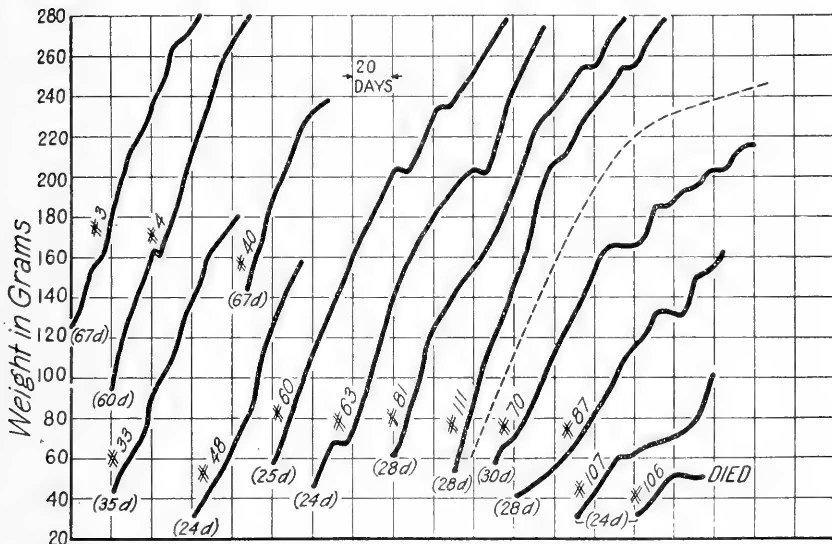

CHART 2. Growth of male rats on bread with limited proportion of milk; five-sixths bread solids to one-sixth milk solids. (See text.)

in Chart 2, the age of each animal when placed on experimental diet being indicated at the foot of the curve. Those to the left of the broken line (which indicates the normal average according to Donaldson[1]) were born of mothers on mixed food and were placed upon the bread and milk diet in most cases at the time of weaning (24 to 35 days), or in a few cases (Nos. 3, 4, and 40) at an age of about 2 months, having eaten more or less of the same

[1] Donaldson, H. H., The rat, Philadelphia, 1915, 69.

mixed food as the parents up to this time. Those to the right of the curve representing the normal average are of males kept and fed in the same way, but born of mothers which were at the time upon the same experimental ration of white bread and milk, in which the proportion of milk was restricted to about one-fifth of the total calories. It will be noted that the young males from litters produced on mixed food, even when placed upon the experimental ration at the early age of 24 to 30 days, grew at a rate

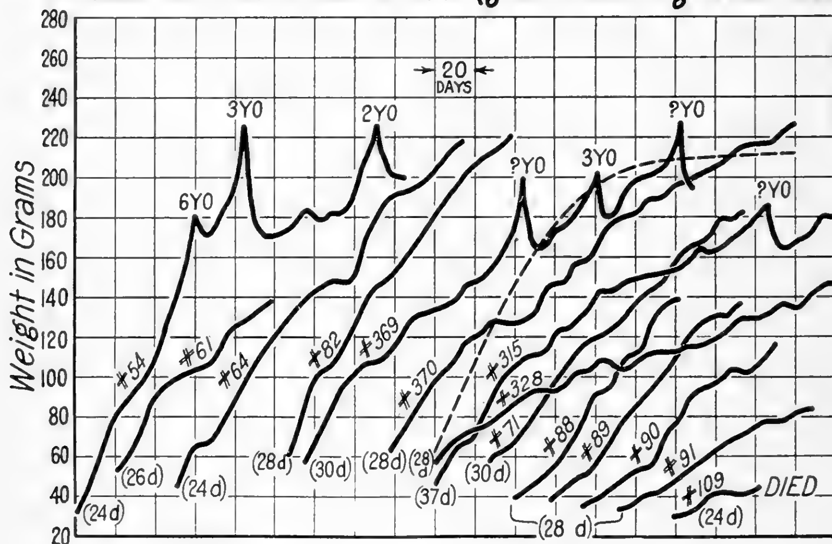

Chart 3. Growth of female rats on bread with limited proportion of milk; five-sixths bread solids to one-sixth milk solids. (See text.)

fully equal to the normal average, while the rate of growth of those from litters produced by mothers on the experimental ration was not quite so good. Evidently the proportion of milk here used was not sufficient to support reproduction without some deterioration of the stock, even when the mothers had grown to maturity on mixed food.

Growth curves of female rats on the same diet are shown in a similar manner in Chart 3. The letter Y on a curve indicates

birth of young, the numeral before the letter showing the number born, while that following shows the number successfully suckled. (It is, of course, possible that some young may be eaten without being seen, and therefore that the actual number born may be greater than the number recorded. By frequent observations we aim to reduce this possibility to a minimum.)

No. 54 (Chart 3) is the rat already discussed in comparison with the rats fed on rations of bread and meat, bread and apple, bread and turnip, or bread alone. No. 61 (Chart 3) is a twin sister of No. 60 (Chart 2); No. 64 (Chart 3) of No. 63 (Chart 2); and No. 82 (Chart 3) of No. 81 (Chart 2). Nos. 369 and 370 (Chart 3) were the offspring of vigorous mothers kept on mixed diet and themselves had eaten more or less of the mixed food before separation at the ages of 30 and 28 days.

It will be seen that, while all these rats made an almost normal rate of growth, yet they seemed to respond somewhat less favorably to this experimental ration than did the male rats of the same litters, and though kept on the ration well beyond the usual breeding age, none of them reared any young, though later they were shown to be capable of breeding when placed upon diets with larger proportions of milk.

To the right of the broken line are shown the growth curves of young females born of mothers kept on this experimental diet and it will be seen here, as in the case of the males represented on Chart 2, that the growth upon this diet is somewhat less favorable in the case of the young whose mothers were on the same experimental ration than in the case of those whose mothers received mixed food. Rats 315 and 328 (Chart 3) were of mothers on experimental diets containing similarly restricted proportions of milk, but one of these mothers received string beans in addition, while the other was fed whole wheat instead of white bread. Only one of the many females which we have raised from weaning upon the ration of white bread and milk in the proportion of 4 calories of the former to 1 of the latter has succeeded in rearing any young.

Effect of Whole Wheat in Place of White Bread, and of Increasing the Proportion of Milk.—In Chart 4 are shown the weight curves of two females which were originally fed on the bread and milk diet discussed in the previous paragraph, but later had their rations altered. In the case of No. 64 the proportion of milk in

the diet was continued at approximately one-fifth of the total calories, but at the age of about 5 months, having failed to breed on this diet, the white bread of the ration was replaced by whole wheat. Here as in all cases in which bread was replaced by flour or wheat, sodium chloride was fed in the proportion of 2 per cent of the weight of the flour or wheat, this being the proportion which had been used in making the bread. 3 weeks later she gave birth to eight young, all of which were successfully reared.

Effect Upon Mother & Young of Increasing Proportion of Milk in Food

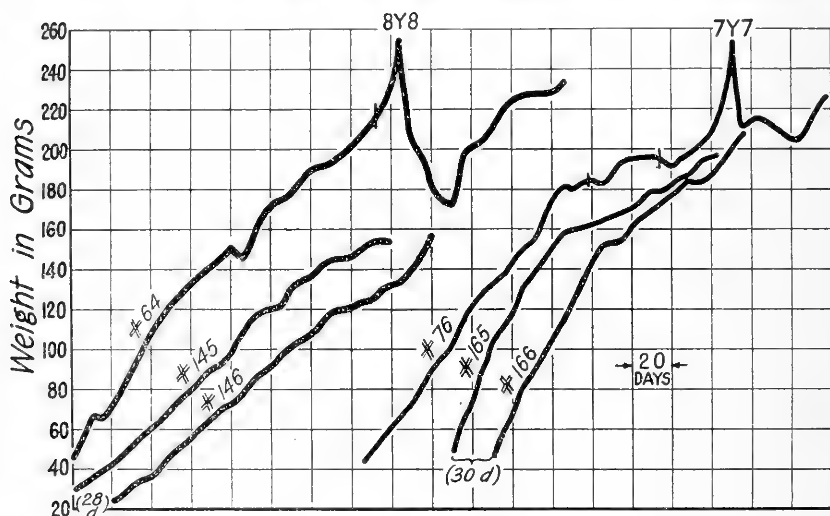

CHART 4. Showing better protection of nursing mother and better growth of young both before and after weaning when the proportion of milk solids in the diet was increased from one-sixth to one-third. (See text.)

The mother lost an unusual proportion of her body weight during the suckling period and in spite of this the young grew at somewhat less than the average rate.

No. 76, from the same stock as No. 64, having the same early history, and having grown at practically the same rate, had her diet changed at 5 months by doubling the proportion of milk in her ration, so that about two-fifths of the calories were now derived from milk and about three-fifths from bread. 3 weeks

later the white bread was replaced by whole wheat and 6 weeks after this change she gave birth to seven young, all of which were suckled successfully and grew at a fully normal rate, much more rapidly than the young of No. 64, and this with very much less loss of body weight on the part of the mother during the suckling period. Nos. 145 and 146 (Chart 4) are young of No. 64; Nos. 165 and 166 are young of No. 76. Apparently the better growth of the young and the better maintenance of the mother

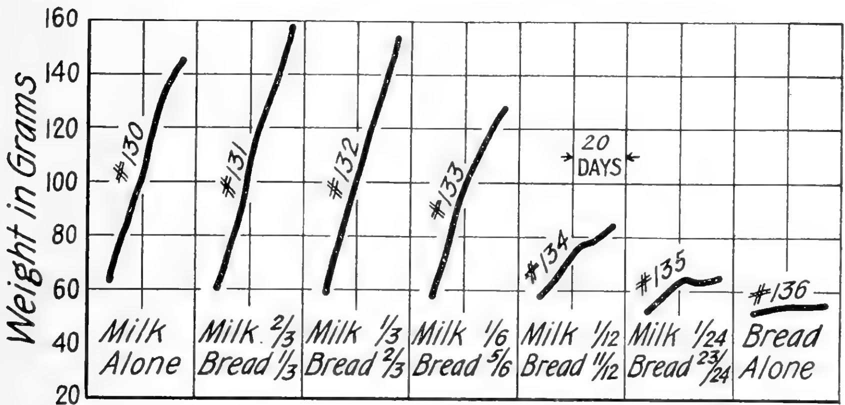

Chart 5. Showing the influence of the proportion of milk in the diet upon the rate of growth of young rats of the same litter.

while nourishing the young was directly due to the larger proportion of milk in her ration.

Different Proportions of Bread and Milk Fed to Rats of the Same Litter.—In Chart 5 are shown the results obtained by feeding different proportions of milk and bread to seven young rats of the same litter placed upon the experimental rations at 26 days of age. The proportions are given in terms of the relative weights of whole milk powder and of dry bread, the latter having been made in the laboratory as previously described, dried at a temperature of 55°C. and ground to a fine powder. It will be seen

that feeding milk in the proportion of one-third of the dry weight of the mixture resulted in more rapid growth than when the milk constituted one-sixth of the dry solids, or about one-fifth of the total calories, as in the experiments previously described. A higher proportion of milk than one-third of the weight of the dry solids, or about two-fifths of the total calories, may or may not induce a further increase in the rate of growth of the rat at this age. The effect of higher proportions of milk upon both growth and reproduction are now being studied upon a larger scale.[2]

Feeding Experiments with Dried Milk.—In describing some of the foregoing experiments we have referred to the diet as consisting of equal weights of bread and milk, or an equivalent mixture of dry bread and whole milk powder. Before assuming that milk powder could be substituted for market milk without affecting the results in such growth experiments, the question was tested upon six young female rats of the same litter, which were divided into three groups. One group was fed fresh bread and market milk; another, fresh bread and "reconstructed" milk made by mixing the milk powder with water immediately before use, the reconstructed milk being then poured over the bread so that the food should have the same texture as in the case of the ordinary fresh bread and milk ration; and the third group was fed a dry mixture of whole milk powder and dried ground bread.

The second group was included as a safeguard against a possible vitiation of the comparison of the fluid and solid milks in case the appetite for, or digestion of, the ration should be influenced by the difference in texture between the moist and dry food mixtures.

[2] Since the above was written Mattill and Conklin (Mattill, H. A., and Conklin, R. E., *J. Biol. Chem.*, 1920, xliv, 137) have published results indicating retardation of growth in its later stages and failure to secure normal reproduction when rats were fed upon cows milk exclusively. The experiments which we have described above (Chart 5) were not continued throughout the age period discussed by Mattill and Conklin and therefore are not directly comparable with theirs. From a considerable number of experiments upon mixtures of whole milk powder and ground whole wheat now in progress in our laboratory we judge that one-third to one-half is probably the optimum proportion of milk in such a diet. Discussion of the interpretation of results obtained upon diets containing more than the optimum proportion of milk is deferred until the completion of these later experiments.

The proportion of milk solids to bread solids was the same in all three cases and it will be seen from Chart 6 that the rate of growth of the three lots was so nearly identical as to be hardly distinguishable.

Until we have more quantitative knowledge regarding the limiting factors in these foods, we cannot state just what degree of precision can be expected of such a feeding experiment as a means of detecting any possible effect of heat treatment upon the dried food. It can readily be seen, however, that any marked

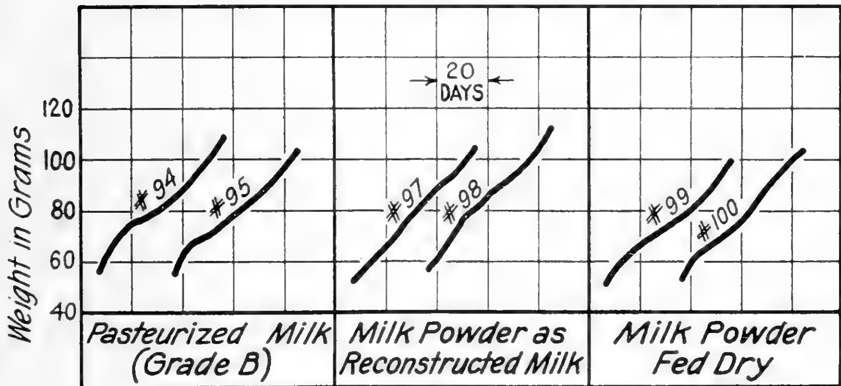

CHART 6. Showing equality of growth upon diets containing corresponding proportions of fluid and solid milks.

difference in growth-promoting value between the fluid and solid milks could hardly have escaped detection in this experiment.

Since milk furnished only 11 per cent of the solids in each of these cases, and the other 89 per cent of the solid matter of the food was that of white bread made from patent flour, it is plain that the growth-promoting substances of the milk could not have been present in the food mixture in any great excess above the quantities actually required for the support of normal growth. In fact both the rate of growth in these experiments and the results of a considerable number of other experiments with the

same two foods mixed in varying proportions, give evidence that the diet here fed was quite closely adjusted to the minimum limit of adequacy for normal growth.

That under these circumstances the animals of the three groups should all grow at the same rate throughout a period in which they more than doubled the body weights with which they entered the experiment, would seem to constitute sufficient evidence that the growth-promoting power of the milk as revealed in experiments of this type, was in no respect seriously injured by drying and that corresponding quantities of fluid and solid milk may be used interchangeably in all such cases as we are here considering. Certainly, since the proportion of milk fed in the cases represented in Chart 6 was lower than in the experiments shown in Charts 1, 2, 3, and 4 and in the first four cases on Chart 5, we are fully justified in the belief that in these cases the rations consisting of bread and market milk and those made by mixing the corresponding proportions of dry bread and whole milk powder may be treated as interchangeable.

This conclusion is substantiated by the large number of cases in which we have changed from the fluid milk to the dry milk ration in the course of a growth experiment without any material effect upon the rate of growth. Usually such a change results in the rat eating less for a few days and, therefore, losing some weight or failing to make a normal gain during the first week on the new ration, which slight loss, however, is almost invariably regained within the next week or two or as soon as the animal has become accustomed to the new food.

Bread Made with and without Milk.—Just as we find evidence of no material loss of fat-soluble or water-soluble vitamine in commercial drying as compared with the ordinary marketing of milk, so it would also appear that the value of milk in supporting growth is still fully manifest after it has been subjected to such heating as is involved in a typical cooking process such as the making of bread.

Chart 7 shows the relative rates of growth of three male rats (Nos. 5, 6, and 7) of approximately the same age and equally vigorous at the beginning of the experiment. These rats were fed as follows: No. 5 upon bread made with water and containing no milk or milk product; No. 6 upon bread made with equal parts

of milk and water as liquid and which contained in the finished product about 5 per cent of the total calories in the form of milk; and No. 7 fed bread made with milk instead of water and which contained about 10 per cent of its calories in the form of milk. It will be seen that there was no growth on the water bread except during the first 3 weeks, during which time previously stored

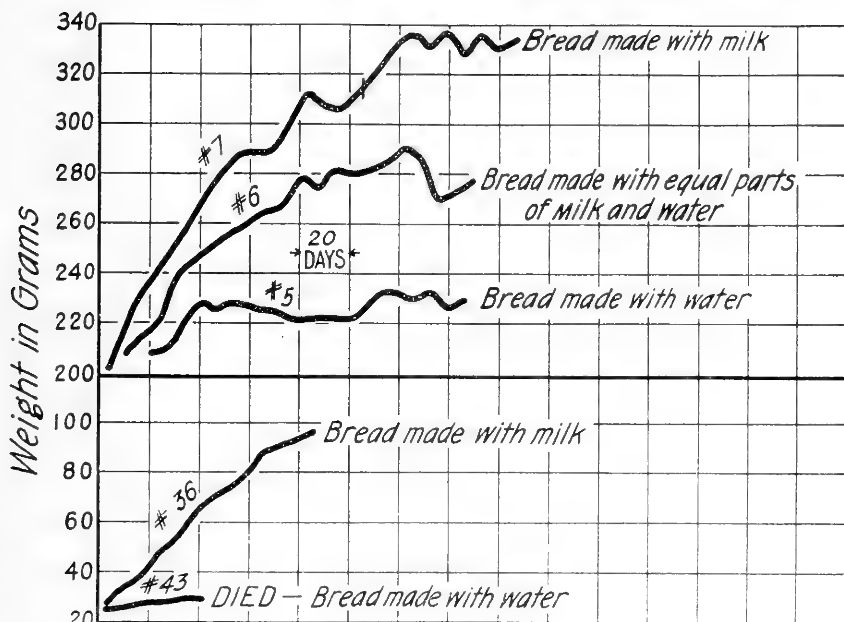

CHART 7. Illustrating the growth-promoting properties of bread made with milk. (See text.)

material was probably being used. Quite appreciable growth occurred on the ration containing only 5 per cent of its calories in the form of cooked milk, and very marked growth upon the ration containing 10 per cent of milk calories, notwithstanding the fact that this milk had been subjected to the heating involved in the baking of the bread. The lower section of Chart 7 shows the effects of bread made with or without milk when fed as a sole

food to rats from the time of weaning. With milk bread growth was nearly normal, while with water bread no growth occurred. The marked improvement in the nutritive value of bread thus shown to result from the use of milk in its preparation is, of course, attributed only in part to the vitamines and in large measure also to the proteins and mineral elements of the milk.

Growth and Reproduction after Stunting by a Diet of Bread Alone.—Osborne and Mendel have repeatedly demonstrated that an animal may be stunted by a specific deficiency in its food, such as a lack of lysine, and subsequently recover when the diet is made adequate. We have thought it of interest to determine whether the same is true when the stunting results from the use of an unbalanced dietary of ordinary food, simultaneously deficient in more than one factor, and particularly when the deficiencies are such as may frequently occur in human experience. Chart 8 shows the case of a female which was first seriously stunted by the feeding of water bread only at an early age. This rat (No. 34) was put at weaning time upon an exclusive diet of white bread (Diet 5) for between 5 and 6 weeks, at the end of which time she appeared likely to die within a few days. She was then given bread soaked with an equal weight of milk (Diet 3) on which she immediately began to grow at a fully normal rate and about 3 months later gave birth to four young but failed to rear any of them. The proportion of milk in her diet was then increased so as to yield about 30 per cent of the total calories of her food, the other 70 per cent being in the form of white bread (Diet 10). Two litters of young produced on this diet were also lost. A temporary diet of white flour, milk powder, and fresh vegetables (Diet 20A) was then fed for 4 weeks during which time seven young were born, but none successfully suckled, the failure here evidently being due to the mother not yet having recovered from the effects of her previous insufficient diet. Finally, she was placed permanently upon a mixture of one part by weight of whole milk powder to two parts of ground whole wheat (Diet 13) on which she successfully reared twelve young, some of which were continued on the same diet and produced and reared young in their turn. The growth curves of the second and third generation (Chart 8) are fully equal to the normal average. Thus the mixture of one-third whole milk

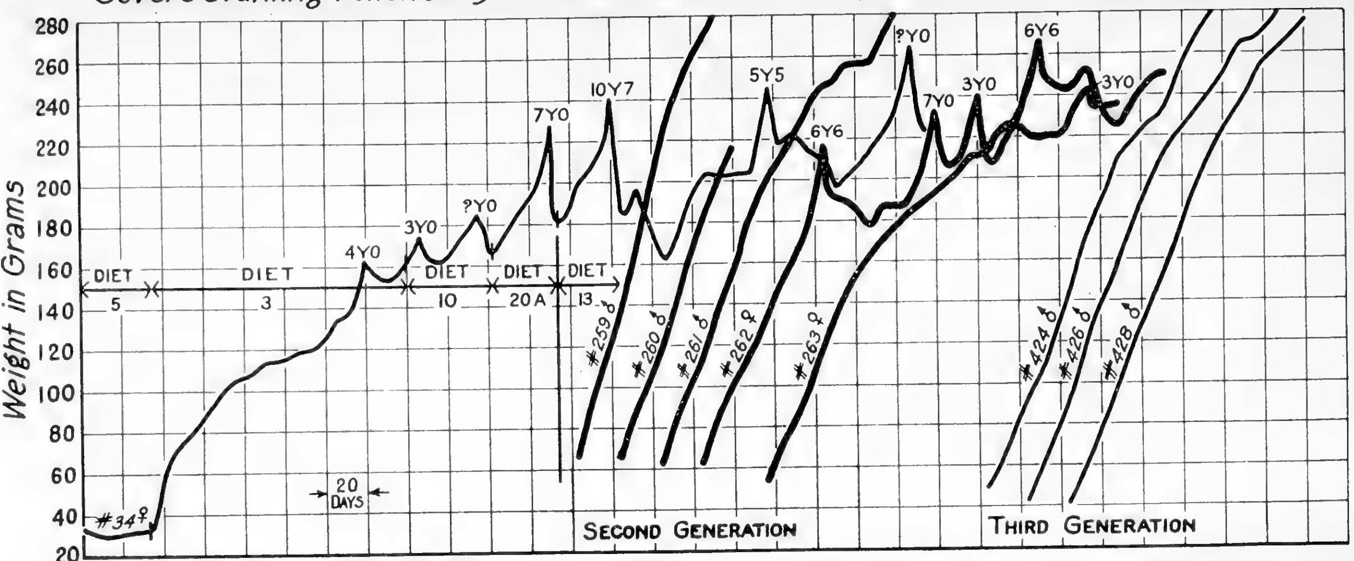

Chart 8. Showing growth curves of three generations. The original female was first stunted and brought nearly to death by feeding on water bread only for 5 weeks after weaning, during which time she should, on a good diet, have more than doubled her weight. On adding a limited proportion of milk to the diet, recovery and rapid growth ensued immediately. Later when the diet was further improved by increasing the proportion of milk and by substituting whole wheat for white bread, young were reared and these grew at a fully normal rate as did their young in turn.

powder and two-thirds ground whole wheat (Diet 13) not only restored the depleted organism of the mother and enabled her to raise young after repeated failures on less adequate food, but was also shown to meet all the needs of normal nutrition through at least three generations.

SUMMARY.

In preliminary trials with animals (rats) placed upon experimental diet at the time of weaning, bread as a sole food resulted in cessation of growth at once and death after about 6 weeks; with bread and meat there was some growth at first, but the survival period was only slightly longer than with bread alone; with bread and apple there was no growth but the survival period was considerably longer; with bread and white turnip there was long continued slow growth; with bread and milk there was continued growth at a normal rate to full adult size.

A mixture of equal weights of bread and milk in which white bread furnished four-fifths and milk only one-fifth of the total calories (or a corresponding mixture of dry bread or flour and whole milk powder), when fed from weaning time to young of both sexes from well nourished mothers, sufficed for growth at practically the normal rate but not for normal reproduction.

On a ration containing the same proportion of milk (about one-fifth of the total calories) but with ground whole wheat instead of white bread or patent flour, young were successfully suckled (though at the cost of considerable loss of weight on the part of the mother), grew to maturity at somewhat less than the average rate, and in several cases have produced and successfully suckled young of the third generation.

When the proportion of milk in the diet was made larger, so as to constitute one-third of the total solids, or about two-fifths of the total calories of the food mixture, the rest of which was ground whole wheat, the mother has suckled the young without undue loss of weight, and the young have made a fully normal rate of growth, as have also the young of the third generation.

When ordinary milk has been replaced by dried milk, or when it has been used in bread-making and, therefore, subjected to the heating involved in the baking of bread, there has been no evi-

dence of any appreciable effect of such heating upon the growth-promoting property of milk as demonstrated in experiments upon rats.

When even one-half of the water used in bread-making was replaced by milk, and still more when the bread was made entirely with milk instead of water, the improvement in the food value of the resulting breads (containing 5 and 10 per cent of their calories respectively in the form of milk solids) was strikingly apparent in feeding experiments upon growing animals.

Increased rate of growth regularly followed increases in the proportion of milk in the diet from 5 per cent up to at least 38 per cent of the total calories of the food.

A female, which had been seriously stunted in early life by feeding upon bread alone, resumed growth at a normal rate when fed equal weights of fresh bread and market milk and later, when fed with a mixture of one part whole milk powder to two parts of ground whole wheat, was able to produce healthy young and suckle them successfully so that they grew at a fully normal rate and one of them at an early age produced vigorous young of the third generation.

Several of the experiments briefly noted above are being continued, and other studies of the effects of heating under different conditions upon the vitamine values of food, of the adequacy and relative values of different simple food mixtures, and of the nutritional rehabilitation of individuals or families whose vitality has been reduced by living upon unsuitable proportions of staple articles of food, are now in progress.

THE "BIOS" OF WILDIERS AND THE CULTIVATION OF YEAST.

By M. IDE.

(*From the Laboratory of Biological Chemistry, Louvain, Belgium.*)

(Received for publication, March 1, 1921.)

Wildiers, Amand, and Devloo, who studied the "bios" from 1900–06, were my pupils in the Laboratory of Biological Chemistry at Louvain, and I am therefore intervening on their behalf in the controversy regarding vitamines in the cultivation of yeast. MacDonald and McCollum[1] repeated the slow cultivation of yeast in mineral nutrients and concluded that "either yeast must grow without 'bios,' or it must synthesize the substance to meet its own needs."

This conclusion may be correct, but it may also be misleading. There are two kinds of proliferation of yeast; one very slow without "bios," and one fast with "bios." Between the two, there is such an obvious difference, that the distinction could not be overlooked by anyone who has seen it. Of course, the difference can be appreciated only if the same method is used in both cases.

I had occasion to follow and to measure the slow proliferation without "bios" during my experiments[2] in connection with the criticism of Pringsheim in 1907 in which I used the method of Wildiers, Amand, and Devloo.

We prepared each culture of 125 cc. with 10 gm. of sugar in an Erlenmeyer flask, closed by a Chrispo or a Meissl valve, charged with H_2SO_4. Each flask shows by the loss of weight the amount of sugar consumed.

For the accurate estimation of weight in the slow proliferation, we included in the calculation the slight gain of weight, which results from the absorption of water by the H_2SO_4 of the Chrispo. This gain in the conditions of our experiments represents nearly

[1] MacDonald, M. B., and McCollum, E. V., *J. Biol. Chem.*, 1921, xlv, 307.

[2] Ide, M., *Centr. Bakt., 2te Abt.*, 1907, xviii, 193.

1 gm. in 40 days, which is equal to 0.025 gm. per day. The loss of weight in the fast proliferation is taken from Amand.[3] We were obliged to neglect the centigrams on our balance.

It can be seen from Table I that the culture without "bios" shows in the second week a daily loss of weight, which is nearly 0.05 gm., a 30th part of the loss given by a "bios" culture on the third day.

In connection with the above fact three questions arise.

1. Without "bios," there is some slow proliferation; after several weeks, weighable quantities of yeast are obtained. Laurent, Henry, Kossowicz, Pringsheim, Wildiers himself, and many others demonstrated this. MacDonald and McCollum seem to be more conservative in regard to the objection of "bios" introduced with

TABLE I.

Loss of Weight for Each Culture of 125 Cc.

Without "bios."		With "bios"	
Age of culture.	Loss of weight.	Age of culture.	Loss of weight.
days		*days*	
5	0.05 gm. in 5 days.	2	0.50 gm. in 2 days.
11	0.1 " " 6 "	3	1.60 " " 1 day.
16	0.2 " " 5 "	4	1.20 " " 1 "
21	0.2 " " 5 "	5	0.50 " " 1 "
24	0.2 " " 3 "	6	0.20 " " 1 "
25	0.05 " " 1 day.		
28	0.15 " " 3 days.		

the seedings. Before I could accept as definite the conclusion of these authors, I should have to be convinced that the purity of their seedings was controlled, not by microscopic examination after a week, but by another examination after a month, as so many cultures which seem pure the first week, later show an evident impurity of very small cocci. 20 years experience will perhaps make this remark pardonable. But we should accept provisionally the possibility of slow proliferation of pure yeast without "bios."

2. Another question is the existence of a special "biosine," which allows a proliferation thirty times more rapid. MacDonald and McCollum believe that "many other substances" have a favorable influence on the growth of yeast. This is an error.

[3] Amand, A., *La Cellule*, 1904, xxi, 333.

None of the known organic substances described up to the present has any comparable influence on yeast. We tried urea and the amino-acids including tryptophane, the nuclein bases, choline and derivatives, the four ethyl and methyl amines, many alkaloids and glucosides, but none was effective. Devloo found biosine in a fat-soluble form in lecithin, but it is neither choline nor glycol amine.[4] Now we know that it is rather soluble in methyl alcohol, and quite insoluble in ethyl alcohol.

For rapid growth, the yeast needs a large portion of its nitrogenous food in the form of biosine; half of it at least (see Devloo). "Small quantities of impurities" is an incorrect expression for the biosine food. No doubt biosine is a new substance in biology, and its influence on yeast is really a specific one.

3. Are water-soluble B vitamine and "bios" the same substance? It is too soon to answer this question. Until now, we observed no difference in the chemical properties of the water-soluble B of Myers and Voegtlin and our biosine. The similarity is remarkable, and our pupils now work with the American methods combined with our former ones. If the yeast test leads to the identification of biosine, it will become easy to show its influence on growing rats against beri-beri.

Other methods of demonstrating the presence of the substance are uncertain, until it is possible to control them with the isolated substance.

[4] Devloo, R., *La Cellule*, 1906, xxiii, 361.

THE "BIOS" OF WILDIERS AND THE CULTIVATION OF YEAST.

By MARGARET B. MacDONALD and E. V. McCOLLUM.

(From the Laboratory of Chemical Hygiene, School of Hygiene and Public Health, the Johns Hopkins University, Baltimore.)

(Received for publication, March 30, 1921.)

Through the courtesy of the Editors of the Journal, Prof. Ide's paper was submitted to us before publication. We are taking this opportunity to comment on the several points which he has raised.

The object of our study was to convince ourselves whether yeast could be utilized as a test organism for the approximate estimation of the content of the antineuritic substance (water-soluble B) in foodstuffs or preparations made from them. The investigation had its beginning in the efforts of Souza[1] to apply the methods described by Williams[2] and by Bachmann.[3] Souza was unable to obtain sufficiently uniform results to inspire confidence that the method had any value for the specific purpose for which it was designed.

Following this experience an investigation was made of the effects of extracts of wheat germ, beefsteak, and rolled oats, respectively, prepared after these materials had been heated with alkali sufficiently long to destroy all the antineuritic substance which they contained, or at least a sufficient amount to make negative efforts to detect it by administering the preparations to polyneuritic rats. Extracts of these alkalinized foods were found to stimulate the growth of yeast when added to our standard nutrient solution of inorganic substances and sugar, in a manner entirely comparable with similar amounts of extracts prepared from untreated wheat germ, muscle, or oats. Since these extracts

[1] Souza, G. deP., and McCollum, E. V., *J. Biol. Chem.*, 1920, xliv, 113.
[2] Williams, R. J., *J. Biol. Chem.*, 1919, xxxviii, 465.
[3] Bachmann, F. M., *J. Biol. Chem.*, 1919, xxxix, 235.

were entirely without curative properties when administered to polyneuritic rats, there was no parallelism between the antineuritic factor and the development of yeast, and we were forced to the conclusion that some factor other than the antineuritic substance was responsible for the stimulation of the yeast growth.

To supplement Souza's investigation, we undertook to grow pure cultures of three races of yeast in the nutrient medium containing only ammonium sulfate as a source of nitrogen, the other essential inorganic nutrients and sugar being purified by repeated precipitation from concentrated aqueous solution by means of alcohol. This nutrient solution was, we believed, far more carefully prepared so as to insure freedom from even traces of the antineuritic substance or from other unknown complexes, such as the hypothetical "bios," than any medium employed by previous investigators. Since these pure races of yeast grew just as well at the end of a series of 15 consecutive seedings in this nutrient solution as they did earlier, we were forced to the conclusion that yeast can grow without either the antineuritic substance or the hypothetical "bios."

We are fully in accord with Prof. Ide in his statement that there is a most striking difference in the rate of proliferation of yeast in the purified nutrient solution, as contrasted with the same after the addition of extracts of natural foods. The explanation for this may probably be considered of twofold character. In an attempt to explain this, Souza and McCollum inclined to the view that the added extracts were potent because of improvement in the nutrient substances. The recent publication of Fulmer, Nelson, and Sherwood[4] would seem to prove that the raising of the viscosity of the solution by the added extracts may be of equal importance.

Prof. Ide has evidently misunderstood the description of our technique when he gained the impression that the purity of our seedings was controlled by microscopic examination only after the first week. In our paper,[5] we state: "This successive seeding was continued to the fifteenth seeding with no apparent change

[4] Fulmer, E. I., Nelson, V. E., and Sherwood, F. F., *J. Am. Chem. Soc.*, 1921, xliii, 186.

[5] MacDonald, M. B., and McCollum, E. V., *J. Biol. Chem.*, 1921, xlv, 309.

in the rate of growth or diminution in the fermentative activity of the yeast. Every precaution was taken to insure pure seedings, a microscopic examination of a sample from each flask being made before seeding the next flask, because of the known influence of other fungi." By this we intended to convey the information that every one of the 15 consecutive seedings was tested microscopically in order to definitely establish the purity of the culture before seeding the next flask. The experiment extended over 3 months and a microscopic examination when the flasks were emptied showed no contamination.

In the studies reported by Souza and McCollum, and MacDonald and McCollum, it was not shown definitely what factor operated in the improvement in the growth of yeast through the addition of extracts of natural foods. In the recent publication by Fulmer, Nelson, and Sherwood, important observations are recorded bearing on this point. Their studies have shown that the concentration of ammonium salt which is optimum for the growth of yeast is identical with the concentration of the salt producing the least swelling of wheat gluten. They further observed that the addition of colloidal substances, such as starch or dextrin, so prepared as to leave no room for doubt that they were free from contaminating substances, exercised a markedly favorable influence on the growth of yeast. This affords convincing evidence that the optimum nutrient conditions for the development of yeast in media containing only known substances have not been appreciated or achieved by any foreign investigator up to the present time. The experience of Fulmer, Nelson, and Sherwood, correlates so perfectly and supplements so satisfactorily our own studies that we feel safe in asserting that it is fully established that neither the hypothetical "bios" nor the antineuritic or other uncharacterized dietary factor essential in the nutrition of mammals need be supplied in order to enable yeast to develop.

THE INVERSION AND DETERMINATION OF CANE-SUGAR.*

By A. R. ROSE.

(*From the Laboratory of Pathological Chemistry, New York Post-Graduate Medical School and Hospital, New York.*)

(Received for publication, March 24, 1921.)

In the methods for the determination of cane-sugar the crucial step is always the inversion of the saccharose into glucose and fructose. Glucose is unaltered under the conditions usually prevailing in sugar analyses, but fructose is readily converted into products with different powers of reduction and rotation of polarized light. Hence analysts have proposed numerous exact procedures for hydrolysis whereby the alteration in the fructose may be either reduced to a minimum or at least remain constant for the conditions prescribed. We prefer the method developed and used in this laboratory as being simple and safe. The cane-sugar is inverted by adding picric acid and heating in boiling water for 10 minutes, then determining the increase in the power of this solution to reduce the picric acid. The fructose thus formed is not altered and its specific reduction of the picric acid is identical with that of the glucose. The picric acid method[1]

* A preliminary report of this work was read before the Society for Experimental Biology and Medicine (Rose, A. R., *Proc. Soc. Exp. Biol. and Med.*, 1917–18, xv, 16).

[1] Lewis and Benedict first used the picric acid method for the determination of glucose in blood in 1912–13 and described the procedure in a paper read before the Society for Experimental Biology and Medicine (Lewis, R. C., and Benedict, S. R., *Proc. Soc. Exp. Biol. and Med.*, 1913–14, xi, 57); and published the details later (*J. Biol. Chem.*, 1915, xx, 61). A method for the determination of the glucose based on the reduction of picric acid was also proposed by Dehn and Hartman (Dehn, W. M., and Hartman, F. A., *J. Am. Chem. Soc.*, 1914, xxxvi, 403). These two papers give ample résumé of the literature pertaining to the determination of glucose by means of its reducing properties and the chemistry involved in the reaction between this sugar and picric acid. The Lewis and Benedict method was

usually used for samples containing minute quantities of sugar can be employed satisfactorily for larger samples when modified in some of its details as follows:

From 1 to 10 gm. of the food sample are triturated in a mortar with 100 cc. of water, including the moisture of the sample. 0.5 gm. of picric acid is added before the trituration, if the sugar content is known to be very low; it is also added when it may serve as a clarifying agent or to remove other reducing substances than the sugars. The trituration mixture is centrifuged (or filtered) and 1 cc. of the clear extract is transferred to a long narrow test-tube[2] containing 2 cc. of a saturated solution of picric acid and 1 cc. of a 20 per cent solution of sodium carbonate. The tube is then immersed in a bath of boiling water and kept there for not less than 30 minutes, nor more than 1 hour.

When cane-sugar is also to be determined, the reducing sugars are estimated as indicated above in the briefest time consistent with reasonable care, and the picric acid solution to be used is chilled (unless kept in a cool place), before it is mixed with the extract containing the cane-sugar. 1 cc. of the extract is then mixed with 2 cc. of saturated picric acid solution in a sugar tube and heated in boiling water for 10 minutes. This completely inverts the cane-sugar. On addition of 1 cc. of 20 per cent sodium carbonate solution with subsequent heating for half an hour a color develops which represents the total amount of sucrose, glucose, and fructose (dextrin and maltose must be absent or accounted for). The difference in readings before and after the heating with the picric acid gives the equivalent amount of cane-sugar in terms of glucose.

In the determination of the reducing sugars in the presence of cane-sugar it is well to work at temperatures not exceeding 20°C. and with picric acid concentrations in the sugar tube of one-half to two-thirds saturation. The error due to the inversion of the cane-sugar at room temperature (25°C.) with picric acid at three-fourths saturation is very small. A 1 per cent cane-sugar solution

later improved by Myers and Bailey (Myers, V. C., and Bailey, C. V., *J. Biol. Chem.*, 1916, xxiv, 147). While the method was primarily intended for sugar estimations in the blood, it is so simple in manipulation and requires so short a time in execution that it ought to be seriously considered in other lines of investigation, especially where a saccharimeter or polariscope are not constantly ready for use. Such application of the method was first suggested by Bernhard while in this laboratory (Bernhard, A., *Sugar*, 1915, xvii, 41).

[2] The test-tubes used in this reduction were especially designed for this work by Myers. They are made of resistance glass 12 mm. inner diameter and graduated at 3, 4, 10, 15, and 20 cc. These tubes have come to be called "sugar tubes."

under these conditions gave a reduction of 0.112 per cent of the total sugar present after standing for 1 hour.

TABLE I.

Cane-sugar solution used.	Sugar found.		Difference.
	By the picric acid method.	By direct polarization.	
	per cent	*per cent*	*per cent*
An aqueous solution	5.23	5.24	0.01
1 gm. dissolved in 10 cc. of water and diluted to 50 cc. with a saturated solution of picric acid	1.99	1.99	0.00
2 gm. dissolved in 10 cc. of water and diluted to 50 cc. with a saturated solution of picric acid	4.00	3.99	0.01
3 gm. dissolved in 10 cc. of water and diluted to 50 cc. with a saturated solution of picric acid	5.99	5.99	0.00
10 gm. dissolved in 100 cc. of water	9.98	10.01	0.03
Above solution diluted with an equal volume of saturated solution of picric acid	5.00	5.05	0.05
10 gm. dissolved in 50 cc. of water	20.02	20.04	0.02
Above solution diluted with an equal volume of saturated solution of picric acid	9.97	9.71	0.11
10 gm. dissolved in 50 cc. of water and part diluted with an equal volume of water	9.83	9.93	0.10
Another part of the same diluted with a like volume of saturated solution of picric acid		9.91	0.08
Average on the cane-sugar used, from the above	99.66	99.55	0.11

In using this method of inversion of cane-sugar it is well to adjust the solutions so that the cane-sugar does not exceed 0.1 per cent. Such a solution is completely inverted in 3 minutes at 95°C. When the concentration of the cane-sugar is as much as

10 per cent the heating must be prolonged to 15 minutes in order to insure complete inversion. 10 minutes is sufficient time for complete inversion of cane-sugar solutions under 5 per cent.

Picric acid may also be used in connection with the polariscope, either by direct readings or the method of double polarization. The presence of the picric acid does not interfere with the rotation of the carbohydrate solutions but rather seems to render the observations easier. In many instances the picric acid acts as a clarifier and also as a remover of soluble proteins. The picric acid reduction method has been compared with direct polarization both with and without the presence of picric acid. Some of these comparisons are given in Table I. The possibility of using the picric acid method of inversion in the estimation of the cane-sugar by double polarization was suggested by the following experiment.

5 gm. of glucose crystals were dissolved in 20 cc. of water and diluted to 100 cc. with a saturated solution of picric acid. The rotation of this solution in a 200 mm. tube at 26°C. averaged 5.23° for three determinations. In a 100 mm. tube at 20° the rotation came to 2.63°; 2.62° was expected from the previous determination in the longer tube. 5 gm. fructose made up in the same manner and polarized in a 100 mm. tube at 20° gave a reading of −3.74°. These two solutions, when mixed in equal parts and rotated, gave a reading of −0.57°, the algebraic sum of the two solutions taken separately is −0.58°. The two solutions were then heated over a free flame under a reflux condenser for 1 hour and finally cooled to room temperature. The rotation of each and a mixture of equal parts of the two were then determined in a 100 mm. tube at 20°. The readings were 2.64°, −3.66°, and −0.54°, respectively. The algebraic sum in this case is −0.51°. These figures show that the glucose is stable under the conditions prevailing during the procedure. The fructose is sufficiently stable for the purpose in view. In the above, four-fifths saturation of the picric acid was used, which is a considerably stronger acid solution than actually employed in the method. The time of boiling was four times longer than required in the complete inversion of a 10 per cent solution of sugar. This excess treatment caused a reduction of 2.2 per cent in the total amount of fructose present. For a similar cane-sugar solution this would amount to only 1.1 per cent.

TABLE II.

Cane-sugar solution used.	Polariscope tube.	Angular rotation.	Calculation.*		
			I	II	III
	mm.	*degrees*	*per cent*	*per cent*	*per cent*
10 gm. dissolved in 50 cc. of water	200	26.55	99.6		
	100	13.33	99.9		
A part of this solution diluted with an equal part of a saturated solution of picric acid	220	14.39	98.0		
The diluted solution heated at 98°C. for 15 min	100	−2.11		99.8	99.7
15 gm. dissolved in 100 cc. of water	220	22.22	101.0		
A part of this solution diluted with an equal part of a saturated solution of picric acid and heated at 98°C. for 15 min	100	−1.44		91.0	99.9
30 min	100	−1.44		91.0	99.9
60 min	100	−1.38		88.1	98.9
Adding an excess of picric acid crystals to portions of the diluted solution and heating at 98°C. for 15 min	100	−1.42		90.9	99.5
30 min	100	−1.41		90.3	99.4
60 min	100	−1.35		86.6	98.3
10 gm. dissolved in 50 cc. of water	200	26.61	99.8		
	100	13.29	99.7		
After 24 hrs	200	26.61	99.8		
A part of this solution diluted with an equal volume of water	220	14.54	99.2		
Another part diluted with an equal volume of a saturated	220	14.54	99.2		
solution of picric acid	200	13.21	99.1		
After 24 hrs	220	14.34	98.7		
	200	13.11	98.3		
The diluted solution again diluted with picric acid solution	220	7.18	97.8		

TABLE II—*Concluded.*

Cane-sugar solution used.	Polariscope tube.	Angular rotation.	Calculation.*		
			I	II	III
	mm.	*degrees*	*per cent*	*per cent*	*per cent*
The first diluted solution heated at 98°C. for 10 min....	100	−1.99		94.1	99.5
15 min.....................	100	−2.13		100.8	100.6
25 min.....................	100	−2.11		99.8	100.4
Another part of the same solution heated at 98°C. for 30 min.........................	100	−2.09		99.0	100.2
60 min.....................	100	−1.83		91.1	97.3
The rediluted picric acid solution heated at 97°C. for 13 min.........................	100	−1.05		100.2	100.3

* Calculation I is per cent of cane-sugar from Tollens' formula; Calculation II, per cent of cane-sugar calculated from invert sugar using the formulas of Tollens and Ost; Calculation III, cane-sugar calculated from the difference in rotation before and after heating with picric acid using the formula $\frac{100 \times A}{B - (C + D)}$ in which A represents the difference in the readings, B the $[\alpha]_D$ for sucrose, C one-half the $[\alpha]_D$ for glucose, and D one-half the $[\alpha]_D^{20}$ for fructose. With the 100 mm. tube and 20°C. the factor for a 10 per cent solution is 1.1522 and for a 7.5 per cent solution, 1.15404.

The polariscope observations were made at 20°C. with a Schmid and Haensch triple shadow instrument (Landolt type); the dilutions, with picric acid solutions at room temperature.

That the picric acid inversion of cane-sugar may also be used in connection with the polariscope was further tested by applying it to solutions of "granulated" cane-sugar and determining the rotation of these solutions before and after this method of inversion. The results from this test are arranged in Table II. It is quite evident that this method of inversion may also be used in polarization. In practice it would be advisable to add the picric acid in the form of a saturated solution, either one or two parts to each part of the sugar solution, so that the picric acid concentration is from one-half to two-thirds saturated and to heat not more than 20 minutes in the boiling water bath.

CONCLUSIONS.

The determination of glucose and fructose, either separately or as invert sugar, may be conveniently, accurately, and very rapidly accomplished by a colorimetric method as briefly described. The cane-sugar may be simultaneously determined on a duplicate sample in almost identical manner, using the picric acid as the inverting agent. The picric acid may also be employed to advantage for the inversion of the cane-sugar by those who are equipped with and prefer the polariscope.

THE DETERMINATION OF CARBOHYDRATES IN VEGETABLE FOODS.

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Our present knowledge of the carbohydrate content of vegetables is based largely upon data collected by Atwater and Bryant.[1] Their figures for total carbohydrate include "fiber," which is not digested by the human body. In some instances they record the percentage of fiber alone. The total carbohydrate has been determined generally by difference, thus causing the combined errors of all other analyses to fall upon this constituent. Olmsted[2] has determined the total available carbohydrate of certain raw and thrice boiled vegetables by two methods: first, starch hydrolysis by diastase and dilute hydrochloric acid, with estimation of reducing sugar by Bertrand's titration method; second, the determination of the sugar excreted by phlorhizinized dogs which were fed certain of the vegetables. His results agree rather closely with earlier recorded figures.

The form in which the available carbohydrate exists in foods is of significance in any attempt to develop satisfactory methods of cooking, aiming either to conserve as much as possible of the food value, or to remove most of the carbohydrate, as is desirable in the feeding of diabetic patients. Little is recorded in the literature concerning the form of available carbohydrate in vegetables. Atwater and Bryant[1] have recorded analyses of a few vegetables and fruits in which the percentage of sugar is given, in addition to the total carbohydrate. They report that one sample of carrots contained 3.6 per cent cane-sugar and 3 per cent fruit-sugar.

[1] Atwater, W. O., and Bryant, A. P., Chemical composition of American food materials, *U. S. Dept. Agric., Bull. 28*, revised 1906.

[2] Olmsted, W. H., *J. Biol. Chem.*, 1920, xli, 45.

One sample of boiled white potatoes contained 0.2 per cent cane-sugar, 0.2 per cent glucose, and 17.4 per cent starch. The edible portion of twenty-six samples of raw sweet potatoes contained an average of 2.5 per cent cane-sugar and 3.4 per cent invert sugar. One sample of turnips contained 4.4 per cent sugar. Of the fruits of which the percentages of sugar are recorded, sugars form a large proportion of the total carbohydrate. Thus the edible portion of one sample of apple contained 6.4 per cent glucose, 6 per cent cane-sugar, and 1.2 per cent starch, acids, etc. The edible portion of one sample of apricot contained 11.9 per cent sugar, as against 13.4 per cent total carbohydrate on an average of eleven samples. Eight samples of oranges contained an average of 9 per cent sugar. Lemon juice contained 2.3 per cent sugar and 7.5 per cent citric acid, making up 9.8 per cent of total carbohydrate. Wardall[3] states that among the vegetables which she studied, artichokes, celery, spinach, rhubarb, kale, carrots, asparagus, endive, cauliflower, mushrooms, beets, and parsnips, only cauliflower, parsnips, cabbage, and carrots showed the presence of even a very small amount of starch, the others apparently containing the carbohydrate in the form of soluble sugars. Most of the carbohydrate of the carrot was found to be in the form of soluble reducing substance, while sucrose appeared to be the chief form of carbohydrate in beets. Wardall emphasizes the fact that "information concerning the nature and physiologic significance of the carbohydrate will lead to a rational method of procedure in the preparation of the foods." Street and Bailey[4] in a study of the carbohydrates of the soy bean used the method of analysis by which the soluble reducing sugars and sucrose were extracted with boiling 95 per cent alcohol, the dextrins and soluble starch then extracted with water, and the insoluble starch hydrolyzed with malt extract. The final determination of the sugars was made by reduction of Fehling's solution before and after acid hydrolysis of these extracts.

The present study was undertaken to work out a relatively simple method for the determination of available carbohydrate in vegetable foods; by the use of this method to determine the amount and form of such carbohydrate in those vegetables and

[3] Wardall, R. A., *J. Am. Med. Assn.*, 1917, lxix, 1859.

[4] Street, J. P., and Bailey, E. M., *J. Ind. and Eng. Chem.*, 1915, vii, 853.

fruits most used in feeding diabetic patients; and to observe the effect of different factors in cooking on the removal of carbohydrate from vegetables.

Studies concerning the carbohydrate content of various foods have been in progress in this laboratory for several years. At the suggestion of one of us, Bernhard[5] in 1915 employed a colorimetric procedure for the determination of free reducing sugars and total carbohydrate in miscellaneous food materials. This method depends on the extraction with water of the reducing sugars and their determination by the well known color reaction with sodium picrate.[6,7] The total carbohydrates were determined by direct hydrolysis after heating the food with approximately normal hydrochloric acid on a reflux condenser for $2\frac{1}{2}$ to 3 hours, and subsequent colorimetric determination of the reducing sugar formed. Bernhard published the results of the analysis of various fruits and vegetables for free reducing sugars, and of various other food materials for free reducing sugars and total carbohydrates. Myers and Rose[8] employed a similar colorimetric method for a study of the carbohydrate changes in the ripening of bananas. In the preceding paper, Rose[9] has described a very simple method for the inversion and determination of cane-sugar, which depends upon the hydrolysis of the sucrose by boiling for 10 minutes with picric acid solution and the subsequent determination of the reducing sugar by reduction of sodium picrate.

Method.

The method here described makes use of the following principles. The free soluble sugars are extracted with water from the ground vegetable and are determined by reduction of sodium picrate to sodium picramate, both before and after hydrolysis of the non-reducing sugar with picric acid.[9] In the determination of total available carbohydrate, however, the method of direct acid hydrolysis is open to question, since some of the indigestible

[5] Bernhard, A., *Sugar*, 1915, xvii, 41.
[6] Lewis, R. C., and Benedict, S. R., *J. Biol. Chem.*, 1915, xx, 61.
[7] Myers, V. C., and Bailey, C. V., *J. Biol. Chem.*, 1916, xxiv, 147.
[8] Myers, V. C., and Rose, A. R., *J. Am. Med. Assn.*, 1917, lxviii, 1022.
[9] Rose, A. R., *J. Biol. Chem.*, 1921, xlvi, 529.

"fiber" and hemicelluloses may be hydrolyzed, and fructose and maltose partly decomposed. Accordingly, an attempt was made to hydrolyze this starch with "taka-diastase,"[10] and it was found that when the preliminary hydrolysis is completed by gentle acid hydrolysis of the dextrins and maltose with approximately 0.6 N HCl for 1 to 2 hours in a water bath, satisfactory results are obtained. The reducing sugar is then determined by the reduction of sodium picrate.

The determination of free soluble sugars is carried out in the following manner: The edible portion of the vegetable or fruit is ground up finely in a food chopper and the material is thoroughly mixed. Two or more samples[11] are accurately weighed into beakers, transferred to a mortar and thoroughly ground with sand, and finally with a measured volume of water, until all of the soluble carbohydrate has been extracted. A portion of this (cool) mixture is then saturated with dry picric acid, purified according to Halverson and Bergeim,[12] and filtered. The estimation is now carried out without delay essentially as described by Myers and Bailey[7] for blood sugar. 3 cc. of the clear filtrate are accurately measured into each of two tall glass test-tubes graduated to 3, 4, 10, 15, and 20 cc. (sugar tubes). To the first tube is added 1 cc. of 22 per cent sodium carbonate solution, both tubes are immersed in a boiling water bath and after 10 minutes 1 cc. of 22 per cent sodium carbonate is added to the second tube. Both tubes are now heated for 20 minutes, cooled, and diluted to 10, 15, or 20 cc. (or more) to match approximately the standard and compared in the Bock-Benedict colorimeter with the standard. A 0.02 per cent solution of pure glucose in saturated picric acid solution is used as a standard. 3 cc. of this mixture are heated in a sugar tube with 1 cc. of 22 per cent sodium carbonate for 20 minutes in a boiling water bath. This is cooled, diluted to 10 cc., and set at 15 mm. in the colorimeter. If there is an increase in the percentage of reducing sugar after hydrolysis of the filtrate with picric acid, this represents the non-reducing soluble sugar (sucrose) present.

Calculation:

$$\frac{D_x}{D_a} \times \frac{R_a}{R_x} \times \frac{0.0006}{3} \times \frac{V}{W} = \text{per cent of sugar in vegetable.}$$

D_x = Dilution of unknown tube.

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[10] The "taka-diastase," manufactured by Parke, Davis and Co., Detroit, was employed.

[11] The following proportions of vegetable and water were found convenient according to the amount of carbohydrate recorded as present. 1 to 5 per cent of carbohydrate; 6 to 3 gm. samples of food; 100 cc. of water. 6 to 10 per cent of carbohydrate; 3 to 2 gm. samples of food; 200 cc. of water. 15 or more per cent carbohydrate; 3 gm. samples of food; 100 cc. of water, with dilution of filtrate 1 to 5, or 10 if necessary.

[12] Halverson, J. O., and Bergeim, O., *J. Biol. Chem.*, 1917, xxxii, 164.

R_x = Reading in colorimeter of unknown tube.
R_a = Reading in colorimeter of standard.
D_a = Dilution of standard.
0.0006 = gm. of glucose in 3 cc. standard solution.
3 cc. = volume of unknown filtrate used.
V = Volume of water added to vegetable sample plus volume of water contained in the sample itself. In some cases moisture determinations were made on the vegetable; in others the average recorded values for moisture content were used.
W = Weight of the sample of vegetable.

The determination of the total available carbohydrate is carried out according to the following procedure. Samples[13] of the ground vegetable are weighed accurately into a small necked Florence flask of 250 to 300 cc. capacity, calibrated to a mark on the neck. About 100 cc. of water are added, and the material is boiled with a reflux condenser for about 1½ hours. The vegetable is then ground to a paste in a mortar and rinsed back quantitatively into the flask with the mother liquor and with water until the volume is about 150 to 200 cc. The flask is stoppered with cotton and the contents are heated to boiling, then cooled to about 40°C.; 5 cc. of a 1 per cent solution of "taka-diastase" and a few cubic centimeters of toluene are added. The contents are shaken and the flask is again stoppered with cotton and allowed to stand for 18 to 20 hours in the incubator at 35–37°C. The liquid is then allowed to cool and the volume is made up with cold water to a mark on the neck of the flask. The flask is thoroughly shaken and a portion of the liquid is filtered off. 20 cc. of this filtrate (or 10 cc. of filtrate plus 10 cc. of water, if the percentage of carbohydrate is above 10 per cent) are pipetted into a large test-tube calibrated to a 25 cc. mark, 1.4 cc. of concentrated hydrochloric acid (34 per cent) are added to make the acid concentration about 0.6 N; a small funnel is inserted into the top of the test-tube as condenser, and the tube is heated in a boiling water bath for 1 to 2 hours. The dextrin and maltose formed from the starch by the diastase are thus changed to simple reducing sugar. The cooled liquid is then almost neutralized with strong sodium hydroxide, made up with water to the 25 cc. mark, and saturated with dry picric acid. 3 cc. portions are measured into sugar tubes and the color is developed on heating with 1 cc. of 22 per cent sodium carbonate solution before and after hydrolysis by heating for 10 minutes with picric acid. Comparison is made in the colorimeter with the standard glucose solution, and the percentage of reducing sugar found represents the total available carbohydrate in the vegetable. In the calculation, allowance must be made for the reducing sugar in the

[13] For total carbohydrate the following proportions of vegetable and water were found suitable, according to the amount of carbohydrate recorded as present. 1 to 5 per cent carbohydrate; 15 to 5 gm. samples of food; about 300 cc. of water (up to graduation mark). 6 to 10 per cent carbohydrate; 5 to 2 gm. samples of food; about 300 cc. of water. 15 or more per cent carbohydrate; 5 to 4 gm. samples of food; about 300 cc. of water.

"taka-diastase," which is determined separately in the above manner for each 1 per cent solution prepared. After acid hydrolysis for 1 hour this has been found to be about 0.5 to 0.7 per cent reducing sugar in the 1 per cent diastase solution. A solution may be kept active for at least a week, if preserved with toluene in the refrigerator.

This method has been tested and found to give accurate check determinations on the same vegetable. Glucose, sucrose, and starch added to the vegetable have been recovered quantitatively. The method for total carbohydrate has been tried on starch alone, and 100 per cent recovery has been obtained when the final hydrolysis with 0.6 N hydrochloric acid has been carried on for 2 hours. When the total carbohydrate was determined on vegetables low in starch content, higher results were obtained at the end of 1 hour acid hydrolysis than at the end of 2 hours, suggesting the loss of a small percentage of the sugar by prolonged heating with hydrochloric acid. We are aware of certain criticisms which have been directed against the determination of sugar by the colorimetric picrate method, but we believe that the results are quite as accurate as those obtained with some of the more standard methods, and may be secured in a small fraction of the time.

DISCUSSION.

Determinations of free soluble sugars were made on a variety of raw and cooked vegetables and fruits and in many cases on the water drained from the vegetables after boiling; determinations of total available carbohydrate were made on certain of these vegetables. The samples of fruits and vegetables were obtained through the cooperation of the dietary department of the hospital. The vegetables which were boiled once were prepared in the manner generally employed in the household, the vegetables being cut into medium-sized pieces and boiled until the vegetables were soft in a small amount of water, generally sufficient to cover the vegetable. The "boiling water," remaining after the boiling process, was drained off at once, and the volume was measured. In the thrice boiling process the vegetables were cut into smaller pieces and the time of boiling and volume of "boiling water" were greater than for the other process. The results are tabulated in Tables I, II, and III. When classified

TABLE I.

Free Sugars in Vegetables Expressed in Terms of Glucose.

Vegetable.	Form.	Reducing sugar.	Non-reducing sugar (sucrose).	Total sugar.	Recorded total CHO minus fiber.*	Water content found.	Water content recorded.*	Time of boiling.	Ratio of amount of vegetable to boiling water.	Percentage of CHO of raw remaining in cooked vegetable.	Remarks
		per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*min.*		*per cent*	
Asparagus.	Raw.	1.20	0.01	1.21	2.5		94.0				
	1 × boiled.	0.57	0.24	0.81	1.4		91.6	35	1:0.6	67	
Lima beans.	Canned.	0.17	1.38	1.55	13.4		79.5				
Celery.											
Outside portion.	Raw.	0.54	0.02	0.56	3.3 (Includes		94.5				
	1 × boiled.	0.28	0.15	0.43	fiber).			25	1:1	77	
Hearts.	Raw.	1.70	0.24	1.94	3.3						
Lettuce.											
Green leaves.	Raw.	0.82	0.12	0.94							
White leaves.	"	1.42	0.25	1.67							
Mixed head.	"	2.01	0.20	2.21	2.2	95.3	94.7				
Cauliflower.	"	1.84	0.20	2.04	Range 2.4 to	93.2	92.3				
	1 × boiled.	1.07	0.13	1.20	5.0 (raw).	95.0		60	1:2	59	
	Raw.	1.74	0.42	2.16		91.9	92.3				
	1 × boiled.	0.90	0.15	1.05		95.05		75	1:3.4	49	

TABLE I—*Concluded.*

Vegetable.	Form.	Reducing sugar.	Non-reducing sugar (sucrose).	Total sugar.	Recorded total CHO minus fiber.*	Water content found.	Water content recorded.*	Time of boiling.	Ratio of amount of vegetable to boiling water.	Percentage of CHO of raw remaining in cooked vegetable.	Remarks.
		per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*min.*		*per cent*	
Cauliflower.	Raw.	2.05	0.29	2.34		93.3	92.3				
	1 × boiled	1.50	0.23	1.73		94.5		30	1:1	74	
	Raw.	2.02	0.50	2.52		92.5	92.3				
	1 × boiled.	1.39	0.27	1.66		93.9		50	1:1.3	66	
	Raw.	2.60	0.30	2.90		91.7	92.3				
	1 × boiled.	1.45	0.24	1.69	Average 3.7.	94.0		30	1:1.9	58	
Cucumber.											
Minimum.	Raw.	1.59	0.14	1.73	1.7						
Maximum.	"	3.40	0.00	3.40	3.1						
Average.	"	2.50	0.07	2.57	2.4		95.4				
Radishes.											
Minimum.	Raw.	3.22	0.13	3.35	2.7						
Maximum.	"	3.02	0.40	3.42	7.6						
Average.	"	3.12	0.27	3.39	5.1		91.8				
String beans.											
Green.	Raw.	2.66	0.77	3.43	5.5	91.4	89.2				
	1 × boiled.	1.17	0.34	1.51	1.6†	94.6	95.3	90	1:1.7	44	
Tomato.	Raw.	3.39	0.08	3.47	3.3		94.3				
	1 × boiled.	2.21	0.33	2.54				25	1:0.7	73	
	Canned.	2.91	0.34	3.25	3.5		94.0				

Cabbage.	Raw.	3.08	0.14	3.22	Range 2.9 to 6.4 (raw).	93.8	91.5				
	1 × boiled.	0.89	0.19	1.08	2.6†	96.6		35	1:3	33.8	
	Raw.	3.31	0.00	3.31		93.3	91.5				
	1 × boiled.	0.68	0.06	0.74	2.6†	96.8		60	1:4.1	22.4	
	Raw.	3.10	0.50	3.60			91.5				
	"	3.50	0.66	4.16		93.5	91.5				
	3 × boiled.	0.77	0.06	0.83	Average 4.5.			35	1:8.1	19.9	Boiled 15, 10, and 10 minutes.
Squash.	Raw.	4.84	0.17	5.01	9.8	91.1	87.6				
	1 × boiled.	2.48	0.43	2.91		93.7		55	1:1.3	58	
	3 × boiled.	2.26	0.40	2.66		94.0		40	1:2.6	53	Boiled 10, 15, and 15 minutes.
Green peas.	Raw.	0.39	5.10	5.49	15.2		74.6				
	1 × boiled.	0.30	2.50	2.80	8.1		73.8	50	1:1.2	51	
Carrots.	Raw.	6.20	1.30	7.50	8.2		88.2				
	1 × boiled.	3.60	0.11	3.71	5.7†			45	1:1.4	49.5	
Onions.	Raw.	5.40	2.90	8.30	9.1		87.6				
	1 × boiled.	4.40	1.70	6.10	4.1		91.2	40	1:0.5	73.5	

* Unmarked recorded values are from Atwater and Bryant.[1]

† Values from Joslin (Joslin, E. P., The treatment of diabetes mellitus, Philadelphia and New York, 1916).

according to the carbohydrate found, the vegetables and fruits in the tables generally fall into the proper groups in the classification by Joslin[14] of foods with 5, 10, 15, and 20 per cent of carbohydrate. It is seen that in those vegetables studied which belong to the 5 and 10 per cent groups of Joslin and in all the fruits studied, most of the carbohydrate is in the form of soluble sugars and often chiefly free reducing sugars. Small percentages of starch are present in most of those tested for total carbohydrate. The amount of soluble sugars for most of the fruits and vegetables compares well with the amount of total carbohydrate minus fiber in the recorded figures, and this leads to the inference that the percentage of starch is small in fruits and in many of those vegetables not tested for total carbohydrate. On the other hand, potatoes, peas, and lima beans apparently contain a large proportion of starch in comparison with soluble sugars. These findings are similar to those of Falk,[15] who studied the form of the carbohydrates in fresh and dehydrated vegetables using the method of Street and Bailey.[4] Table IV shows a comparison of the distribution of the available carbohydrate in carrots and in potatoes, as determined by Falk and by the picric acid method described here. Falk reports a distribution of the carbohydrates in cabbage and in turnips similar to that in carrots. Myers and Rose[8] found a great variation in the relative amount of sugar and starch in the banana, according to the degree of ripeness. Thus a green banana with greenish yellow peel was found to have 1.17 per cent reducing sugar, 4.6 per cent sucrose, and 15.62 per cent starch, all expressed in terms of glucose. A very ripe banana with light brown peel had 7.08 per cent reducing sugar, 11.10 per cent sucrose, and 0.71 per cent starch. A very green banana for which the starch is not recorded, was shown to have 0.55 per cent reducing sugar and 1.20 per cent sucrose.

Since most of the carbohydrate in many of the vegetables and fruits studied appears to be in a soluble form, then theoretically it should be possible to remove practically all of the sugars by extraction with water. The ordinary method of boiling vegetables for a minimum time and with a small amount of water was found (Tables I and III) to remove 23 to 66 per cent

[14] Joslin, E. P., *Am. J. Med. Sc.*, 1915, cl, 485.

[15] Falk, K. G., *J. Ind. and Eng. Chem.*, 1919, xi, 1133.

TABLE II.

Free Sugars in Fruits Expressed in Terms of Glucose.

Fruit.	Reducing sugar.	Non-reducing sugar (sucrose).	Total sugar.	Recorded total CHO minus fiber.[1]
	per cent	*per cent*	*per cent*	*per cent*
Lemon juice.	0.95	0.46	1.41	(Sugar) 2.3.
Lemon.	1.41	0.56	1.97	
Strawberry (medium ripe).	3.77	0.33	4.10	Range 3.7 to 10.0. (Glucose) 5.5.
Grapefruit.	7.28	1.35	8.63	
Cuban grapefruit.	5.23	3.72	8.95	
Orange juice.	2.70	4.08	6.78	Range
Florida orange.	5.01	4.57	9.58	11.6 to 18.5. (Sugar) 9.0.
Orange.	4.10	5.70	9.80	
California orange.	6.08	8.10	14.18	
Winter pear.	10.20	1.70	11.90	11.4
Apple.	11.00	0.10	11.10	Range
Baked apple.	11.20	0.80	12.00	7.9 to 19.9.
George Washington apple.	9.25	3.45	12.70	Average 13.0. (Sugars) 12.4.
Northern Spy apple.	11.45	2.55	14.00	
Tangerine.	4.75	8.90	13.65	
Banana.*				
Very green.	0.55	1.20	1.75	Range
Medium ripe.	2.88	12.52	15.40	15.3 to 28.8.
Very ripe.	7.08	11.10	18.18	Average 21.0.
Malaga grapes.	22.25	0.25	22.50	Average 15.0.

* The data on banana are taken from Myers and Rose.[8]

TABLE III.

Free Sugars and Total Carbohydrate in Vegetables Expressed in Terms of Glucose.

Vegetable.	Form.	Reducing sugar.	Non-reducing sugar (sucrose).	Total sugar.	Total available CHO.	Recorded total CHO minus fiber.*	Water content found.	Water content recorded.*	Time of boiling.	Ratio of amount of vegetable to boiling water.	Percentage of CHO of raw remaining in cooked vegetable.	Remarks.
		per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*min.*		*per cent*	
Celery. Outside portion.	Raw.	0.48	0.04	0.52	0.57	3.3 (Includes fiber).	95.2	91.5				
	1 × boiled.	0.21	0.06	0.27	0.36		96.7		70	1:3.1	63	
	Raw.	1.02	0.22	1.24	1.70	3.3		91.5				
	3 × boiled.	0.28	0.16	0.44	0.65				45	1:14.9	38	Boiled 15, 15, and 15 minutes.
Cauliflower.	Raw.	1.69	0.13	1.82	2.48	Range 2.4 to 5.0 (raw).		92.3				
	1 × boiled.	0.97	0.08	1.05	1.09				35	1:2.1	44	
	3 × boiled.	0.52	0.04	0.56	0.56				35	1:8.3	22.6	Boiled 10, 15, and 15 minutes.
	Raw.	1.65	0.28	1.93	2.89		92.6	92.3				
	3 × boiled.	0.62	0.10	0.72	0.79		95.0		45	1:5.4	27	Boiled 15, 15, and 15 minutes.
	Raw.	4.35	0.57	4.92	5.86	Average 4.4.						

Cabbage.	Raw	4.18	0.67	4.85	5.78	4.4		91.5				
Carrots.	"	5.23	1.02	6.25	6.20	Range 5.9 to 11.5 (raw).		88.2				
	3 × boiled.	0.63	0.17	0.80	0.79				75	1:5	11.6	Boiled 25, 25, and 25 minutes.
	Raw.	5.81	2.89	8.70	9.00	Average 8.2.		88.2				
Potatoes.	"	1.16	0.30	1.46	24.4	18.0		78.3				

* Unmarked recorded values are from Atwater and Bryant.[1]

of the carbohydrate, leaving 34 to 77 per cent of the carbohydrate of the raw vegetable in the cooked sample. The use of thrice boiled vegetables in the dietary of diabetic patients advocated by Allen,[16] has been developed on the theory that more carbohydrate is lost if the water is changed several times during the boiling process. Examination of the tables shows that 62 to 88 per cent of the carbohydrate is lost, or 11.6 to 38 per cent of the carbohydrate of the raw vegetable is retained in the thrice cooked vegetable, where the proportion of "boiling water" to vegetable is relatively large. In the thrice boiling of squash the proportion of water was low and the percentage of carbohydrate retained is 53 per cent, almost as great as the percentage retained (58 per

TABLE IV.

Comparison of the Distribution of Available Carbohydrate in Carrots and Potatoes.

Per cent of total reducing power due to.	Raw carrots.		Raw potatoes.	
	Falk.	Picric acid method.	Falk.	Picric acid method.
	per cent	*per cent*	*per cent*	*per cent*
Simple reducing sugars before acid hydrolysis	57.1	64.5	2.5	4.75
Simple reducing sugars after acid hydrolysis	92.2	96.5	4.9	6.0
Dextrins and soluble starch	2.7	3.5	0.5	94.0
Insoluble starch	5.1		94.6	

cent) in the vegetable boiled one time. Wardall[3] shows that in 3, 4, and 5 extractions, respectively, by boiling celery, fresh spinach, and rhubarb, all the reducing substance is removed. 6 extractions are required for carrots, 9 for asparagus, and 16 for cauliflower. Cammidge[17] reports similar results in finding that celery, spinach, turnips, and carrots are more easily freed from carbohydrate by extraction than are other vegetables. O'Reilly and McCabe,[18] using a technique similar to that of Olmsted,[2] found that if 20 parts of water are used as compared with 10, for each of the three boilings of vegetables, the carbohydrate extraction is more complete. In this way they were able to remove

[16] Allen, F. M., *Boston Med. and Surg. J.*, 1915, clxxii, 241.

[17] Cammidge, P. J., *Lancet*, 1919, ii, 1192.

[18] O'Reilly, L., and McCabe, E. H., *J. Biol. Chem.*, 1921, xlvi, 83.

completely the available carbohydrate from vegetable marrow, lettuce, and celery, and to remove nearly all of the carbohydrate from canned spinach, canned asparagus, turnips, beets, and onions. They found that carrots, cauliflower, canned string beans, pumpkin, and cabbage still retain about 0.5 per cent of available carbohydrate. The use of 0.05 per cent and 0.1 per cent concentrations of sodium bicarbonate in these extractions was found to favor the removal of the carbohydrate from the vegetable. Masters and Garbutt[19] and Denton[20] show that greater loss of fuel value occurs in vegetables when they are cut into small pieces and when the time of boiling and the volume of the boiling water are increased. The fact is shown clearly in Tables I and III that the greater the proportion of water used in boiling the vegetable and the longer the time of boiling, the greater is the loss of carbohydrate in the boiling process. It is shown also that the greatest loss of carbohydrate occurs in those vegetables in which most of the carbohydrate occurs in a soluble form.

SUMMARY.

1. A relatively simple colorimetric method is described for the determination of the amount and form of the available carbohydrate in vegetable foods.

2. Results are given for analyses of vegetables and fruits such as are used in feeding diabetic patients. It is shown that a large proportion of the available carbohydrate occurs in a soluble form.

3. The effect of different factors in cooking on the removal of carbohydrate from vegetables is discussed. The conclusion is reached that the soluble form of the carbohydrate and the increase in time of boiling and in volume of boiling water, favor the removal of carbohydrate from vegetables.

[19] Masters, H., and Garbutt, P., *Biochem. J.*, 1920, xiv, 75.

[20] Denton, M., *J. Home Econ.*, 1919, xi, 143, 200.

ANIMAL UTILIZATION OF XYLOSE.

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More definite knowledge of the place of the pentoses in animal metabolism is desirable, not only as to their formation, transformations, and significance, when they appear in the urine, but also knowledge of their digestibility, since their abundant occurrence in plant tissues suggests a possible potential food value.

In the literature experimental results with xylose are often indefinite, incomplete, or conflicting. Thus a number of experimenters, including Cremer[1] and Voit,[2] reported that animals can utilize a limited amount of this pentose, converting it into glycogen; Slowtzoff[3] stated that 33 to 82 per cent of xylan was absorbed from the intestine of rabbits and that 90 per cent does not reappear in the urine; Ebstein[4] believed that man can use very little; Frentzel[5] proved that xylose is not a glycogen-former in rabbits; and Brasch[6] found that results with rabbits were inconclusive and, with dogs, negative in this respect.

The plan used by us was to determine the difference in the amount of xylose ingested by mouth and that passed in the urine and feces. Tests were made upon rabbits as representatives of herbivorous animals, upon cats and dogs as carnivorous animals, and upon man. The food constituents given with the xylose were selected so as to furnish varying quantities of other carbohydrates and of protein. The results show the extent to which the pentose is taken up by the organism; the path after absorption has not been followed.

[1] Cremer, M., *Z. Biol.*, 1892, xxix, 484.

[2] Voit, F., *Deutsch. Arch. klin. Med.*, 1896–97, lviii, 523.

[3] Slowtzoff, B., *Z. physiol. Chem.*, 1901–02, xxxiv, 181.

[4] Ebstein, W., *Arch. path. Anat. u. Physiol.*, 1892, cxxix, 401.

[5] Frentzel, J., *Arch. ges. Physiol.*, 1894, lvi, 273.

[6] Brasch, W., *Z. Biol.*, 1908, l, 113.

The xylose was prepared from chopped wheat straw or from corn-cobs. The method was essentially that of Monroe;[7] extracting first with sodium hydroxide then, after washing out the alkali, hydrolyzing the xylan by long boiling with dilute sulfuric acid, neutralizing the acid with barium carbonate, and removing the excess of barium by phosphoric acid. After decolorizing with blood charcoal the xylose was dissolved out by alcohol and the solution evaporated. Usually the pentose was not crystallized but the thick syrup used for feeding. The hexoses were removed by fermenting 24 hours with yeast and the concentration of the xylose was determined by titrating with Fehling's reagent and checking these results with the polariscope.

Before making the quantitative feeding experiments the approximate tolerance of the animals was learned by feeding the syrup until its presence could be detected in the urine. The urine and feces of the experimental animals were repeatedly tested for pentoses when they were on their usual diet but none was present. The usual qualitative tests were employed, that of Bial (orcinol-ferric chloride-hydrochloric acid) being the most satisfactory. To avoid confusing with the hexoses the urine was previously fermented with yeast.

For the quantitative determination of the xylose of the urine, Fehling's reagent was employed. This was checked by converting the xylose into furfural by distilling with hydrochloric acid, adding aniline acetate to the distillate, and comparing the color with that from a known furfural solution. The color fades, but by treating under the same conditions, a fair comparison could be obtained.

Much modification of the diet for rabbits was not possible. They were given oatmeal for a high protein food, bread and grass for a moderate, and melon with sugar for a diet low in protein and relatively high in carbohydrate (Table I).

The results indicate that of the ingested xylose from 1.5 to 1.75 gm. per kilo of body weight do not reappear in the urine. Where the food contained less protein and more carbohydrates somewhat less of the xylose disappeared.

With dogs the high protein, low carbohydrate diet was meat. The high carbohydrate, low protein diet was bread with the

[7] Monroe, K. P., *J. Am. Chem. Soc.*, 1919, xli, 1002.

addition of starch and sugar, and the intermediate diet was bread with milk and starch (Table II).

The tolerance for xylose was found to be decidedly lower in dogs than in rabbits. The maximum which was not recovered

TABLE I.

Utilization of Xylose by Herbivora (Rabbit).

Food.	Body weight.	Xylose.		Elimination.			Xylose utilized.		
		Total given.	Per kg. of body weight.	First day.	Second day.	Third day.	Total.		Per kg. of body weight.
	gm.	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*per cent*	*gm.*
Oatmeal	2,570	2.58	1.0				2.58	100.0	1.0
"	1,500	2.63	1.75	0.07			2.56	97.4	1.70
"	1,500	3.00	2.00	0.62			2.38	79.3	1.59
"	2,670	4.50	1.68	0.26			4.24	94.2	1.60
"	2,670	5.00	1.87	1.11			3.89	77.8	1.46
Bread and grass	2,670	5.00	1.87	0.54	0.62		3.84	76.8	1.44
Melon and sugar	2,670	3.50	1.31				3.50	100.0	1.31

TABLE II.

Utilization of Xylose by Carnivora (Dog).

Food.	Body weight.	Xylose.		Elimination.			Xylose utilized.		
		Total given.	Per kg. of body weight.	First day.	Second day.	Third day.	Total.		Per kg. of body weight.
	gm.	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*per cent*	*gm.*
Meat	7,200	4.50	0.63		0.95		3.55	78.9	0.49
"	7,200	6.75	0.94		2.72	0.47	3.56	52.8	0.50
"	7,200	4.75	0.66	0.93	0.15		3.67	77.3	0.51
"	6,900	3.00	0.44	0.85	0.03		2.12	70.7	0.31
Bread, milk, and starch	6,900	3.00	0.44		0.71	0.18	2.11	70.3	0.31
Bread, milk, and starch	6,900	3.50	0.51	1.54		0.42	1.54	44.0	0.22
Bread, sugar, and starch	9,000	4.20	0.46	2.00			2.20	52.4	0.24

was about 0.5 gm. per kilo of body weight. With an increased content of carbohydrates in the food there was a decrease in the amount of xylose which disappeared. The same observation was made in the case of rabbits.

TABLE III.

Utilization of Xylose by Carnivora (Cat).

Food.	Body weight.	Xylose.		Elimination.			Xylose utilized.		
		Total given.	Per kg. of body weight.	First day.	Second day	Third day.	Total.		Per kg. of body weight.
	gm.	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*per cent*	*gm.*
Meat	1,700	3.55	2.16	0.50	0.35	0.04	2.66	74.9	1.57
Bread, milk, and starch	1,040	1.50	1.43	0.47	0.15		0.81	54.0	0.78
Bread, milk, and starch	1,040	1.75	1.68	0.67	0.16		0.92	52.6	0.88
Bread, sugar, and starch	1,040	1.75	1.68	0.59	0.41		0.75	42.8	0.71

TABLE IV.

Utilization of Xylose by Man.

Food.	Body weight	Xylose.		Elimination.			Xylose utilized.		
		Total given	Per kg. of body weight.	First day.	Second day.	Third day.	Total.		Per kg. of body weight.
	kg.	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*per cent*	*gm.*
Meat and eggs	60	15.75	0.26	5.67	0.83	*	8.70	55.3	0.145
Meat, sugar, and melon	60	6.00	0.10	1.80			4.20	70.0	0.070
Sugar and other carbohydrates	60	7.65	0.13	2.03	1.53		4.09	53.5	0.068

* 0.55 gm. was recovered from the feces, none was found on the other days.

The variations made in the food of cats were similar to those for dogs. With these animals, again, less of the xylose reappeared

in the urine when the food contained no other carbohydrate (Table III). When the food carbohydrates were abundant the amount of xylose which appeared in the urine was greater. On the basis of body weight more of the xylose disappeared than with dogs but less than with rabbits. It was about 1.5 gm. per kilo with a meat diet and less than 1 gm. when other carbohydrates were eaten.

For a man the diet was meat and eggs for high protein; less meat with melon and sugar for the intermediate; and practically a protein-free diet for the third (Table IV).

TABLE V.

Utilization of Xylose.

Food.	Rabbit.	Cat.	Dog.	Man.
	gm. per kg.	*gm. per kg.*	*gm. per kg.*	*gm. per kg.*
Low carbohydrate.	1.70	1.57	0.49	0.145
	1.59		0.50	
	1.60		0.51	
	1.46		0.31	
Mean.	1.57	1.57	0.45	0.145
Medium carbohydrate.	1.44	0.78	0.22	0.070
		0.88		
Mean.		0.83		
High carbohydrate.	1.31	0.71	0.24	0.068

The quantity of xylose which did not appear in the urine or feces was very small in proportion to the body weight. It was largest when the food was meat and eggs, and smaller when sugar and melon were substituted for a part of these food materials.

Table V summarizes the relation of xylose utilized to body weight. Only those experiments are included where the limit of tolerance was reached.

CONCLUSIONS.

From these experiments it is evident that there is a limit to the amount of xylose which can be utilized by animals and that this limit is low. The term, utilization, is used here to signify the

disappearance of the xylose with no theory as to whether it is oxidized, stored, or converted into some other compound. In view of this slight extent to which the animal organism can dispose of xylose there is no encouragement for our expecting a valuable food material from this source.

Less of the xylose is used, that is, more appears in the urine or feces, when the diet contains an abundance of other sugars and starch. The latter, through their greater instability appear to protect the xylose. When these other carbohydrates are absent the organism disposes of more of the xylose.

The disappearance, or utilization, of the xylose is greatest with the rabbit, an animal with a long intestine. It is much less with the carnivorous cat and dog. Although the results with man are few they indicate that he has an intermediate position in the series.

THE RELATION OF PLANT CAROTINOIDS TO GROWTH AND REPRODUCTION OF ALBINO RATS.*

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Considerable interest has been shown recently in the possible relation of the fat-soluble vitamine to the carotinoid pigments of the plant plastids. After one of us[1,2] had shown that the plant carotinoids are the cause of the so called lipochromes of the higher animals by reason of a direct transference of the pigments from the diet, the rapid development in vitamine research seemed to indicate that a certain analogy existed between the simultaneous presence of carotinoid and fat-soluble vitamine in certain foods and their absence from others. This idea has been considerably strengthened by the experiments of several investigators, which will not be cited at this time, but which support the belief that all of the known vitamines have their origin in vegetable matter.

Drummond[3] seems to have been the first to test the possibility of fat-soluble vitamine being a carotinoid by feeding both impure and pure crystalline preparations of carotin to rats suffering from fat-soluble vitamine deficiency. The carotin was fed at the rate of 0.003 per cent of the ration. The results were negative, especially with the pure crystals of pigment. Steenbock[4] and coworkers, a few weeks later called attention to the relation of yellow pigmentation and vitamine content of vegetable foods and suggested that the two were at least associated in some way. The statement was made, however, that fat-soluble vitamine "is not carotin." This statement was later[5] retracted and the provisional assumption advanced

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[1] Palmer, L. S., and Eckles, C. H., *J. Biol. Chem.*, 1914, xvii, 191. Palmer, L. S., *J. Biol. Chem.*, 1915, xxiii, 261.

[2] Palmer, L. S., *J. Biol. Chem.*, 1916, xxvii, 27.

[3] Drummond, J. C., *Biochem. J.*, 1919, xiii, 81.

[4] Steenbock, H., Boutwell, P. W., and Kent, H. E., *J. Biol. Chem.*, 1920, xli, p. xii.

[5] Steenbock, H., *Science*, 1919, l, 352.

that the fat-soluble vitamine is one of the yellow plant pigments. Several papers[6] have since appeared in support of this idea, in which fat-soluble vitamine has been shown to occur more or less abundantly only in the plant organs which are relatively richly pigmented with yellow (presumably carotinoid) colors. It is interesing also that Osborne and Mendel[7] have shown that dried tomato, which is rich in lycopin, an isomer of carotin, is also rich in fat-soluble vitamine. Steenbock's most recent paper[6] shows that the unsaponifiable matter (containing carotinoids) obtained from alfalfa hay can be fractionated into a carotin and fat-soluble vitamine-rich fraction and a xanthophyll and fat-soluble vitamine-poor portion by means of the usual relative solubility method of separating carotin and xanthophylls. It appears also that Steenbock[8] has made a crystalline preparation of carotin containing the vitamine.

Rosenheim and Drummond[9] were also much attracted to the idea of an intimate relationship between carotinoids and fat-soluble vitamine notwithstanding the earlier negative tests secured by Drummond. They were, however, unable to establish an identity of the vitamine with either carotin or xanthophyll, but were nevertheless very reluctant to abandon the idea that the two classes of substances are in some manner associated.

Drummond and Coward[10] later submitted 24 different fats and oils (both animal and vegetable), variously pigmented with carotinoids, to examination for fat-soluble vitamine by feeding them to rats suffering from deficiency. No correlation could be discovered between vitamine efficiency and pigment content. Most significant were the results showing that carotinoid-free dog body fat and perinephritic pig fat, also devoid of pigment, are relatively rich in fat-soluble vitamine. The failure of these investigators to find carotinoids in cottonseed oil does not coincide with Palmer's observations.[11] The discrepancy can be explained at present only on the ground that the natural yellow pigment either had been destroyed in the oil examined by Drummond and Coward, or had been replaced by other pigments. The natural coloring matters of cottonseed oil are frequently removed before the oil is placed on the market.

The demonstration of fat-soluble vitamine in lard or in the adipose tissue of swine is an important point in connection with the relation of pigment and vitamine. Daniels and Loughlin[12] have presented data which they interpret as indicating that lard (and also commercial cottonseed oil) is not always as devoid of vitamine as we have been led to believe. While

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[6] Steenbock, H., and Boutwell, P. W., *J. Biol. Chem.*, 1920, xli, 81. Steenbock, H., and Gross, E. G., *J. Biol. Chem.*, 1920, xli, 149. Steenbock, H., and Boutwell, P. W.. *J. Biol. Chem.*, 1920, xli, 163; xlii, 131.

[7] Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1920, xli, 549.

[8] *Wisconsin Agric. Exp. Station, Bull. 323*,1920, 19.

[9] Rosenheim, O., and Drummond, J. C., *Lancet*, 1920, cxcviii, 862.

[10] Drummond, J. C., and Coward, K. H., *Biochem. J.*, 1920, xiv, 668.

[11] Palmer, L. S., and Eckles, C. H., *Missouri Agric. Exp. Station, Bull. 10*, 1914.

[12] Daniels, A. L., and Loughlin, R., *J. Biol. Chem.*, 1920, xlii, 359.

Osborne and Mendel[13] have not been able to substantiate this finding for lard using a basal ration which had been especially purified from the standpoint of fat-soluble vitamine, Drummond[14] and his coworkers have shown that diets containing 15 per cent pig fat can in some measure promote recovery and renew the growth of rats suffering from a deficiency of this vitamine. It appears, however, that the fat, to be efficient, must be deposited in the pig's body on rations rich in fat-soluble vitamine. Drummond and his coworkers believe also that the vitamine deficiency of lard is to be explained on the grounds that the usual fattening ration of pigs is deficient in fat-soluble vitamine and also because the methods usually employed in the manufacture of lard destroy the vitamine. The significant point for the present paper is that fats completely devoid of pigment are not necessarily devoid of vitamine.

The most recent evidence on the alleged identity of fat-soluble vitamine and carotinoid pigments has been presented by Miss Stephenson.[15] This investigator finds that a crude preparation of carotin from carrots when dissolved in vitamine deficient palm-kernel oil may be fairly rich in vitamine, but that purified carotin fed in the same way is devoid of the fat-soluble accessory. The carotin was fed to rats in each case at the rate of 0.0006[16] per cent of the ration. Especially significant was the observation that butter fat can be decolorized of its carotin by adsorbing the pigment with charcoal (a well known property of carotinoids) without, however, impairing in any way the vitamine content of the fat.

Of the two possible view-points on the question of the relation of fat-soluble vitamine to carotinoids the writers have been most strongly inclined to the view that the substances cannot be identical and that their relations are fortuitous. This conclusion has been based on the success attained in a previous experiment[17] in raising a large number of White Leghorn fowls from hatching to maturity on rations which contained at the most mere traces of carotinoids. An especially significant feature of that work was the fact that the mature hens of the flock could be made to produce *carotinoid-free* eggs on a special ration prepared from corn-starch, skim milk powder, and limestone, and that a number of these eggs proved to be fertile and normal chicks were hatched from them. Other evidence against the alleged identity of vitamine and pigment was later presented by one of us[18] based partly on theoretical grounds and partly on the experiments of others.

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[13] Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1921, xlv, 277.

[14] Drummond, J. C., Golding, J., Zilva, S. S., and Coward, K. H., *Biochem. J.*, 1920, xiv, 742.

[15] Stephenson, M., *Biochem. J.*, 1920, xiv, 715.

[16] The oil content of the rations is unfortunately, not stated in Miss Stephenson's paper, but we have assumed it to be 15 per cent inasmuch as this is the usual fat content of the ration fed to rats in Dr. Hopkins' laboratory where the investigation was carried out.

[17] Palmer, L. S., and Kempster, H. L., *J. Biol. Chem.*, 1919, xxxix, 299.

[18] Palmer, L. S., *Science*, 1919, l, 501.

Several writers have criticized certain features of the experiments with fowls. Miss Stephenson[15] has suggested that the bone meal which was fed in those experiments may have contained carotinoids inasmuch as freshly ground bones will yield yellow fat on extraction with ether. This criticism would be valid if the bone meal which was used had not been the steamed bone meal of commerce, which yields no color to fat solvents.

Rosenheim and Drummond[9] and van den Bergh and Muller[19] have objected to the fact that pork liver was used to bolster up the fat-soluble vitamine of the rations during the growing period, on the grounds that pork liver contains "lipochromes," the inference being that the rations were therefore not as devoid of carotinoids as was stated. This point is of very great importance inasmuch as the evidence showed conclusively that the successful raising of the fowls depended upon the use of the pork liver. The writers of both papers also seem to see in the statement that the carotinoid-free egg yolks contained residual pigment, an admission that the egg yolks were not entirely devoid of carotinoid. This is also a critical point because carotinoid-free egg yolks containing this residual pigment were used as the source of fat-soluble vitamine in one of the experiments reported below.

The question of carotinoids in pork liver will be considered first. Before reviewing the evidence upon which the investigators mentioned based their conclusions it may be remarked in passing that it is very difficult to reconcile the presence of plant carotinoids in the liver of swine with the fact that the blood of this species is *completely* devoid of these pigments even when pigs are on rations rich in carotinoids.[2] Van den Bergh and Muller have confirmed the absence of carotinoids from the blood of swine, the rations of the animals, however, not being stated. The corpus luteum of swine is also devoid of carotinoids, according to these investigators, a fact which coincides with unpublished observations of one of us (Palmer). It may be remarked also that further evidence of the absence of carotinoids from pork liver has been obtained in experiments carried out in the Department of Agricultural Chemistry in the University of Wisconsin.[20]

Considering now the evidence which has been presented in favor of the presence of carotinoids in pork liver, Rosenheim and Drummond state that "substances resembling the lipochromes do occur in pig's liver," and again that, "we have found in liver tissue, besides carrotene and xanthophyll, a substance which gives certain reactions similar to those given by the lipochromes, and which is not identical with any known member of that class." These statements are not open to discussion because the evidence has not yet been presented upon which they are based, the authors stating merely that they "tested for the presence of lipochromes by the usual chemical tests."

In the investigation by van den Bergh and Muller, which dealt largely with the distribution of carotinoids in the human body in both normal and

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[19] Van den Bergh, H., and Muller, P., *Biochem. Z.*, 1920, cviii, 279.

[20] *Wisconsin Agric. Exp. Station, Bull. 323*, 1920, 20.

pathological states, the statement is made that the liver of swine contains equal amounts of carotin and xanthophyll, but that the total amount of carotinoid in this organ is small. No details are given of the examination of the liver except that the statement is made in the early part of the paper that carotinoids were demonstrated solely by means of the phase method of separating carotin and xanthophylls, using light petroleum and 80 per cent alcohol, and by the blue color reaction with concentrated sulfuric acid. Apparently these investigators have made the mistake of regarding the relative solubility of carotin and xanthophylls in the solvents mentioned as a specific *test* for carotinoids. This is not a test for carotinoids but merely a means of effecting the separation of two distinct classes of carotinoid pigments. Many substances with a carotinoid color respond to this separation between light petroleum and 80 per cent alcohol. For example, the reddish yellow aldehyde resin produced in impure alcohol by KOH responds readily to the phase separation and thus indicates the presence of both carotin and xanthophyll. It was partly for this reason that one of us[1] cautioned against the formation of aldehyde resins in the saponification of fatty material with alcoholic potash previous to the isolation of carotinoids from fats or oils. Bixin, the pigment of annatto seed, when examined by the phase method, also indicates the presence of both classes of carotinoids, but no true pigments of this character are present.

Criticism can also be directed at the use of the blue color reaction with concentrated sulfuric acid as a specific test for carotinoids. It is true that these pigments when in the crystalline or fresh, amorphous condition, and even when precipitated as microchemical crystals in plant tissues, dissolve in strong sulfuric acid with a blue color. It does not seem to be generally recognized, however, that a number of substances which are not carotinoids, *e.g.* bixin, likewise respond to this test. Again, there are substances of unknown composition whose *solutions* turn blue when strong sulfuric acid is added. The liver, especially, may contain such substances. For example, a carbon disulfide or carbon tetrachloride solution of cod liver oil gives a beautiful blue color reaction.[21] This has been ascribed to the "lipochrome" in the oil, but it cannot be due to carotin because *solutions* of carotin, freshly extracted from carrots, alone or when dissolved in colorless oil, do not give the reaction. Solutions of butter fat, whose "lipochrome" is known to be carotin, also fail to give the reaction. According to Rosenheim and Drummond, a substance which is neither carotin nor xanthophyll but which gives the cod liver oil "lipochrome" reaction, is present in the liver of all animals. As noted later, we have found such a substance to be present in the liver of the adult rat.

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[21] This test, as applied to cod liver oil, and which fails when applied in the same manner to butter fat and CS_2 solutions of carotin from carrots is performed by dissolving 1 drop of oil in 20 drops of carbon disulfide or carbon tetrachloride and adding 1 drop of concentrated sulfuric acid.

The facts just cited show clearly that there is danger of overlooking several important points in attempting to establish a relation between carotinoids and vitamine. In the first place it is not possible to identify animal "lipochromes" with plant carotinoids merely on the basis of color or solubility or any other one of a number of single tests which are also given by other substances. The complete identity can be established only when the pigment in question corresponds in *all* its properties (color, relative solubility, adsorption, spectroscopic, etc.) with one of the plant carotinoids, and particularly when it can be shown by physiological experiment that the coloring matter thus identified is *derived from the food*. Although it seems likely that many of the yellow pigments formerly regarded as of animal origin are in reality plant carotinoids, definite proof of this has been furnished only for certain pigments of the higher animals.

Another important point to be emphasized is that if fat-soluble vitamine is a plant carotinoid it must be present *as such* in the animal tissues containing fat-soluble vitamine, and not as some vague substance which may respond to a color test which may or may not be specific for carotinoids. The existence of a leuco form of the vitamine postulated by Steenbock to cover the exceptions to the association of pigment and vitamine also seems rather fanciful in the light of what is known of the chemistry of the carotinoids. The only leuco forms of these substances so far produced are oxidation products. Especially significant is the fact that oxidation not only bleaches the carotinoids but also destroys the efficacy of fat-soluble vitamine.[22]

A word may be said with reference to the possibility of the residual pigment in the carotinoid-free egg yolks being due to traces of carotin or xanthophyll. It is possible that the special ration of corn-starch and skim milk powder may have contained traces of carotin, inasmuch as the skim milk powder contained 0.5 per cent fat, which may have been more or less pigmented with carotin. However, it is possible to detect carotin in the egg yolk of fowls only when highly pigmented eggs are examined. This makes it extremely doubtful on theoretical ground alone that carotinoids could have been present in the eggs in question.

[22] Hopkins, F. G., *Biochem. J.*, 1920, xiv, 725. Drummond, J. C., and Coward, K. H., *Biochem. J.*, 1920, xiv, 734.

It is certain that the ration contained no detectable amount of the xanthophylls which normally pigment the egg yolk. Moreover, the pigment itself failed to show the characteristic properties of carotinoid, although it was slightly soluble in ether and more so in acetone. Especially, it failed to give the characteristic ferric chloride reduction test. Van den Bergh and Muller[19] have criticized the use of this test apparently because it is not one of the usual "lipochrome" tests, overlooking the fact that it was not the only criterion by which it was judged that carotinoids were absent. However, it may be noted by way of parenthesis that the reduction of ferric to ferrous chloride by carotinoids is very characteristic and never fails as a test for these pigments[23] when applied correctly (*i.e.*, to carotinoid dissolved in oil or fat or to freshly extracted carotinoid free from moisture and carotinoid solvents) even when less pigment is present than is the case with the egg yolks in question. For example, the fat from ewe's milk, used in Experiment 2, responded readily to the test although the actual amount of carotinoid present, estimated colorimetrically, was almost infinitesimal.

The great practical importance of determining the identity of fat-soluble vitamine demands that the possibility of the vitamine being identical with carotinoid pigment be settled conclusively. In view of the fact that the functions of the carotinoids in plant life have not been determined and the reasons for their distribution in various plant organs therefore unknown, it has appeared to us to be more logical to attack this question from the animal side. This point of view is supported strongly by the wide variation in the amount of carotinoids appearing in different species of higher animals. This suggests that if fat-soluble vitamine is carotinoid pigment there is either a wide variation in the vitamine requirements of different species, or, if the substances are not identical, certain species are able to effect a sepa-

[23] It should be stated in this connection that substances other than carotinoids can act as strong reductases towards ferric chloride. The alcohol extract of dried yeast (both baker's and brewer's) leaves a brown oily residue on evaporation, which reduces ferric chloride very strongly. Yeast has been known for some time to be rich in reductase, and this enzyme apparently appears in the alcohol extract. The question might be raised whether this is of significance in connection with the potency of the alcoholic extract of dried yeast as a source of water-soluble vitamine.

ration of the two substances. As to the first possibility practically nothing is known as yet as to the vitamine requirements of various species, the assumption apparently being that the requirements of the rat are an index of the requirements of all species of animals, including man. As to the second possibility it may be that some species require both vitamine and pigment, and others only vitamine. As far as fowls are concerned we are convinced that fat-soluble vitamine is required but not carotinoids. This is also true of swine, at least during the period of most rapid growth, namely the suckling period, for we have observed that the milk of swine (even the colostrum milk) is absolutely devoid of carotinoid pigments. As to a possible pigment requirement of animals, a high pigmentation in the body might indicate a low pigment requirement, the pigment being superfluous, or *vice versa*.

One of us[18] has already raised the question of the validity of testing the pigment-vitamine hypothesis on species of animals which contain no carotinoid pigments. The statement was made at that time that the albino rat is devoid of carotinoids. This statement was based on somewhat superficial evidence. Inasmuch as the rat was adopted for the present experiments it seemed advisable, in view of the argument advanced, to examine the albino rat critically from the point of view of carotinoid distribution in the body. This examination forms the basis of Experiment 1.

According to our view, the conclusive proof that pigment and vitamine are neither identical nor necessarily associated, which is the thesis we have adopted, must be furnished in either of two ways: (1) by showing the failure of growth and other functions when supplying the pure pigments in rations otherwise *absolutely devoid* of both pigment and fat-soluble vitamine, or (2) by showing normal growth and reproduction on rations rich in fat-soluble vitamine but devoid of carotinoid pigment. The first method is subject to the difficulty and expense of preparing perfectly pure pigments and also to their very labile nature when in the pure state. We have adopted the second method as being less subject to experimental error. Ordinarily we believe that conclusive experiments on points of this character should not be terminated until the animals have been carried through at least two life cycles in order to avoid the possibility of storage in the body of

the young rats with which such experiments are usually begun. This does not seem necessary in the present case, however, inasmuch as we have found no storage of carotinoids in the albino rat. Nevertheless, we regret that the exhaustion of the supply of special fats used in our experiments necessitated their termination shortly after the birth of young.

It occurred to us that another method of throwing light on this question would be a quantitative comparison of the carotin content of rations containing the minimum quantities of various carotinoid-containing foods required to furnish the fat-soluble vitamine. Data are available in the literature upon which approximately quantitative calculations of this character can be based. Drummond and Coward[10] have approached the problem from this point of view but their data for the carotin content of the rations are only relative. The xanthophyll content of the rations was ignored in our calculations in view of the result secured by several investigators that the fat-soluble vitamine has followed the carotin in the attempts which have been made to isolate the vitamine on the basis of its possible carotinoid nature. A word may be said here in explanation of this result. The final separation of carotin from xanthophylls is effected because of the fact that carotin is quantitatively extracted from 80 per cent alcohol by light petroleum, especially that boiling between 40 and 60°C. When one bears in mind that the solvents employed in this separation are respectively exceedingly poor and very excellent fat solvents, it is not surprising that the fat-soluble vitamine will follow the substance which goes into the better fat solvent.

The data reported in this paper include, (1) a critical study of the presence of carotinoids in the albino rat, (2) a study of the effect on growth and reproduction of albino rats of feeding practically colorless ewe milk fat as the sole source of fat-soluble vitamine, (3) a similar study using *carotinoid-free* egg yolks as source of the vitamine, and (4) a quantitative comparison of the carotin content of various rations containing minimum quantities of carotin-containing food required to supply the requisite amount of fat-soluble vitamine.

EXPERIMENTAL.

Experiment 1.—Examination of Albino Rats for Carotinoids.—Two adult rats and nine rats 1 to 2 days old were examined critically for the presence of carotinoids. One of the adult rats was a male weighing 285 gm. It had been on a ration of purified foods containing butter until 2 weeks before the examination for pigments. During the interval it had received the regular ration of the breeding colony, which included alfalfa hay. The other adult rat was a female, weighing 246 gm. It had been on a diet containing an abundance of yellow corn and alfalfa hay since weaning. This animal was pregnant. The young rats were animals which had been discarded from unusually large litters in the breeding colony.

All the rats were chloroformed and the adult rats bled from the jugular. 10 to 12 cc. of blood were obtained from each animal. The oxalated blood from each rat was desiccated with plaster of Paris, moistened to a paste with either absolute ethyl or methyl alcohol, and the mixture shaken vigorously with 10 to 15 cc. of petroleum ether (boiling point 40–60°C.). Both the supernatant and lower layers of solvent were examined carefully for pigment. In addition to the blood, the liver, spleen, suprarenals, adipose tissue, and, in the case of the pregnant female, the ovaries were examined for carotinoids. The adipose tissue was merely ground with sand and allowed to render at 40°C. The various visceral organs were ground with sand and plaster of Paris and the desiccated tissue boiled for a few seconds with methyl alcohol. The mixture, after cooling, was shaken vigorously with petroleum ether. The liver was treated slightly differently, it being ground up with a little milk of lime in addition to the sand and plaster of Paris, in order to bind any biliary pigment which might be present. This desiccated material was then extracted with light petroleum after moistening with alcohol.

The examination of the young rats consisted in removal of the liver only, the material from the nine animals being combined and treated as noted for the liver of the adult rats. When the young rats were dissected it was noticed that the stomach of each

was distended with freshly curdled milk. This was removed, about 3 cc. in all being obtained. The fat was extracted by adding an equal volume of alcohol and shaking with two volumes of ether. The ether extract was allowed to evaporate spontaneously and the fatty residue examined for pigment. A large drop of oily fat was secured in this way.

The results of the examination of the various organs were as follows:

Milk Fat.—Absolutely water-white oil.

Adipose Tissue.—Absolutely colorless fat.

Blood.—Petroleum ether and alcohol layers water-white. Petroleum ether layer left no color on evaporation.

Suprarenals.—Entirely devoid of any pigment soluble in alcohol or petroleum ether.

Spleen.—Extracts entirely free from pigment.

Ovaries.—No evidence of any yellow pigments. Alcohol and petroleum ether extracts absolutely colorless.

Liver.—The liver tissue of the new born rats was entirely devoid of any alcohol- or ether-soluble pigment. The extracts were colorless. The liver of the adult rats, however, yielded a yellowish petroleum-ether extract by the method described above. The alcoholic layer of the extract was colorless.

The petroleum-ether solution of pigment thus obtained was washed gently with water and evaporated to dryness at low temperature. The residue consisted of a few drops of yellow oil. When taken up in carbon disulfide there was no great intensification of color. The carbon disulfide solution in a volume of 3 to 4 cc. was examined spectroscopically using a Hilger wave-length spectroscope. At a depth of 1.5 cm. all the green and blue and a great deal of the violet were transmitted. There were no absorp-

CORRECTION.

On page 569, Vol. XLVI, No. 3, May, 1921, 8 lines from the bottom, for 485μ read $485\mu\mu$.

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The examination of the blood, milk, and various organs of the rat confirms completely our previous conclusion that this species of animal is devoid of carotinoids. The yellow coloring matter present in the liver of the adult animal is a lipochrome in the wide, ambiguous sense in which this term may be applied. It is not a carotinoid, however, in any sense of the word. The pigment is probably identical with the substance referred to by Rosenheim and Drummond[9] as being present in the liver of all animals, and as being the probable cause of the characteristic color reaction given by cod liver and other liver oils.

The complete absence of color from the milk fat of albino rats, as indicated by our observation, shows rather strikingly that young rats manage to survive the suckling period very satisfactorily with no pigment whatever in their food.

Experiment 2.—Fat from Ewe's Milk as Source of Fat-Soluble Vitamine.—The almost complete absence of pigment from the fat of ewe's milk has been previously noted by one of us.[2] That the faint yellow tinge of the melted fat is probably due to traces of carotinoids is shown by the fact that this practically colorless fat responds positively to the ferric chloride test. This result furnishes striking proof of the delicacy of this test for the qualitative detection of carotinoid pigments. In the case of the ewe milk fat the green color did not develop at once on addition of a small crystal of ferric chloride to the warm melted fat, but only after the fat had been left in a warm place for several minutes.

We did not feel justified in sparing enough of the ewe milk fat for the determination of the character of the carotinoid present. We have assumed it to be carotin and estimated the amount present by applying the colorimetric method of Willstätter and Stoll[24] directly to the melted fat, using 0.2 per cent $K_2Cr_2O_7$ as standard. The Kober colorimeter was used. By constructing both ordinary and logarithmic curves based on the relations between this standard and 0.00268 per cent carotin solution as given by Willstätter and Stoll and referring the readings obtained for the ewe fat to these curves the amount of carotin present could be determined with some degree of accuracy. The colorimetric ratios between the standard bichromate and the ewe fat

[24] Willstätter, R., and Stoll, A., Untersuchungen über Chlorophyll Methoden und Ergebnisse, Berlin, 1913, 107.

and the carotin content of the fat based on these ratios were as follows:

Ewe fat.	0.2 per cent $K_2Cr_2O_7$.	Carotin content of ewe fat.
mm.	*mm.*	*per cent*
100	3.47	0.00017
50	1.70	0.00011
25	0.98	0.00015

These data show that the carotin content of the ewe fat was, on the average, 0.00014 per cent.

The ewe milk was obtained from several ewes on pasture. The cream was separated by centrifugal force, churned into butter, and the fresh butter rendered at 40–50°C. About 400 gm. of fat were secured. It was stored at ice box temperature.

The effect of feeding this fat as the sole source of fat-soluble vitamine is shown in Chart 1.

The basal ration used in our experiments was shown by special examination to be devoid of carotinoids. This was ascertained by extracting suitable portions for several hours with boiling absolute alcohol and following this with a similar extraction with ether. The combined extracts were evaporated to dryness and the residue tested for carotinoids with ferric chloride. As already noted, extracts obtained from dried yeast in this way yield residues which reduce ferric chloride very strongly, but this cannot be due to carotinoids.

The casein used was made by us from separator skim milk by the grain-curd method recently published by Clark[25] and coworkers. The fresh curd, after thorough washing with distilled water acidified to a pH of 4.8, was purified further as follows. The curd was drained and then extracted twice with cold 95 per cent alcohol for periods of 24 hours each. The alcohol was drained off and the curd submitted to two similar washings with ether. The second ether extract was always colorless. We believe that a much purer product (from the standpoint of vitamines) is secured

[25] Clark, W. M., Zoller, H. F., Dahlberg, A. O., and Weimar, A. C., *J. Ind. and Eng. Chem.*, 1920, xii, 1163. Through the courtesy of Dr. Zoller we were able to put the details of this method into practice some months before the publication of the method.

by this method than by attempting to purify the dried commercial casein, as is done by many investigators using purified rations.

The salt mixture used in this and the following experiment was that given by McCollum.[26]

The experiment was begun with all the rats receiving 5 per cent ewe fat. When the rats failed to gain materially the fat was increased to 9 per cent. The following week dried baker's yeast was added to furnish the rats 0.2 gm. of dried product per day. The immediate effect of this addition on the growth of the rats showed that the wheat embryo extract in the ration was, for some reason, inadequate as a source of water-soluble vitamine. Three of the rats were now returned to the former 5 per cent fat level, and the other three continued at the 9 per cent level throughout the experiment. Both the wheat embryo extract and the dried yeast were continued, however, the latter being incorporated at the rate of 2 per cent of the ration. Records of food intake throughout the experiment showed an average of 10.8 gm. per day per rat.

As will be seen by referring to Chart 1, three of the rats had large litters. Two of these individuals were practically normal in weight but Rat 161 was somewhat light and less sturdy in appearance. The litters were reduced to six in each case. The young of Rats 157 and 161 were normal and did well up to the time the exhaustion of the food forced the termination of the experiment. The young of Rat 159 did not do so well. At the end of 27 days they weighed only 20 gm. each. 10 days later, after the mother had been placed on a mixed diet, they had increased to 45 gm. each.

It is regrettable that the supply of ewe fat was not ample to carry the experiment further into the lactation period of the mother rats. Nevertheless, the chart shows clearly that normal growth and reproduction of rats can be secured on rations containing 9 per cent ewe milk fat as the sole source of fat-soluble vitamine, in spite of the fact that the ration contained only 0.0000127 per cent carotin or 0.127 parts per million. It is possible that even a lower level of ewe fat would be adequate, as

[26] McCollum, E. V., Simmonds, N., and Pitz, W., *J. Biol. Chem.*, 1916, xxv, 123.

one of the three rats on the 5 per cent level of fat showed normal growth and produced a litter of eleven.

Incidentally the experiment is the first to show the relative amount of fat-soluble vitamine in milk fat from animals other than the cow. The abundant evidence of the adequacy of goat's milk in infant nutrition indicates that liberal quantities of fat-

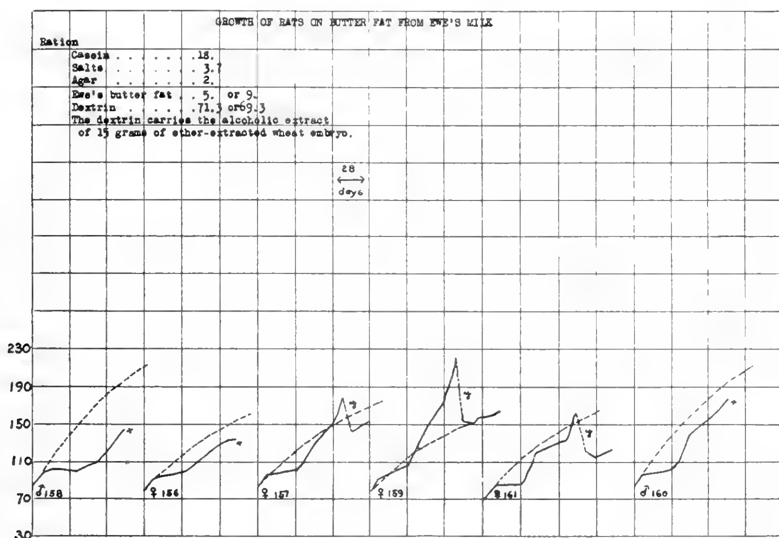

CHART 1.—Rats 156 to 160. Curves for Rats 156 to 158 illustrate the growth of rats on 5 per cent butter fat from ewe's milk. For 3 weeks of the experiment the ration of Rats 159 to 161 carried the same amount of ewe's fat. It was then increased to 9 per cent. As none of the curves approached the normal at the end of 4 weeks, 2 per cent of dried baker's yeast was added to the ration. The rats with one exception responded at once with excellent growth which would indicate that the alcoholic extract of the embryo was for some reason deficient. Nos. 156, 158, and 160 were taken off the ration to conserve the food. Nos. 157, 159, and 161 produced litters of eleven normal young.

soluble vitamine are present in the milk fat from this species of animal. The fat of goat's milk, however, is characteristically almost devoid of pigment. Traces of carotinoid (presumably carotin) are present, as in the case of ewe milk fat. We determined the percentage of carotin present in one sample of goat's

milk fat to be 0.00019 per cent, which is slightly higher than for the fat from ewe's milk.

Experiment 3.—Carotinoid-Free Egg Yolks as Source of Fat-Soluble Vitamine.—In the spring of 1920, ten hens remained of the original carotinoid-free flock of White Leghorn fowls hatched in April, 1918, at the University of Missouri. These fowls were raised from hatching to maturity on a ration consisting almost wholly of selected white corn, skim milk, and an occasional feeding of fresh pork liver, with bone meal or oyster shells *ad libitum.* The hens of the flock which remained had never been off this diet. Inasmuch as they were laying fairly well during their second season of fecundity the opportunity presented itself of testing the effect of the carotinoid-free eggs on the growth of rats. Eggs were accordingly collected during a period of several weeks. The weekly ration of the ten fowls during this period consisted of about 14 pounds of white corn, a like amount of skim milk, and 2 pounds of fresh pork liver, fed at two different times, with the usual mineral matter *ad libitum.* The total ration on the dry matter basis contained about 4 per cent liver tissue.

The eggs were received from the University of Missouri in one shipment, about 8 dozen in all. The eggs were placed in water at 75°C. for about 5 minutes, which was sufficient to curdle the white so that it could be more easily separated from the yolk. The slightly cooked yolks were dried before an electric fan, for the most part in a room at a temperature of 30–35°C. The resulting product was ground to a fine powder. It was soft and oily and had a grayish white color. It was stored in the ice box and underwent no decomposition during the course of the experiment.

The egg yolk powder, which was entirely lacking in yellow color, readily gave up a pale yellow fat to a mixture of acetone and ether, but the pigment, as previously noted, failed to respond to the ferric chloride test for carotinoids.

The effect on growth and reproduction of rats when this powder formed the sole source of fat-soluble vitamine is shown in Chart 2. The ration contained 15 per cent of the powder or about 9 per cent egg oil. The food consumption records of the rats showed an average intake of 8.5 gm. of ration per rat per day for the entire experiment. The basal ration was the same as in

Experiment 1, except that it contained wheat embryo extract only as source of water-soluble vitamine.

As indicated in the chart all the rats made better than normal growth. Only three of the six were carried to maturity, however, because of the limited supply of egg yolk powder. The mature rats were especially vigorous and healthy. Both females produced strong vigorous litters before the supply of egg powder was

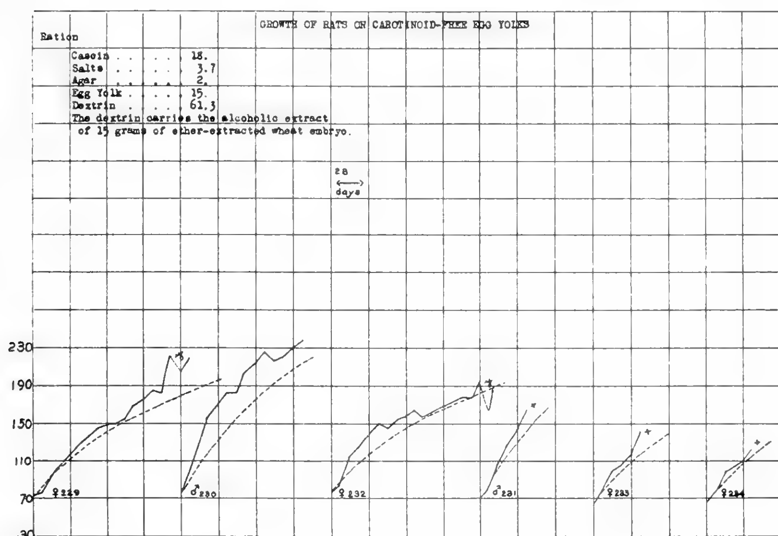

CHART 2.—Rats 229 to 234. These curves illustrate the excellent growth of rats on a ration in which carotinoid-free egg yolks were the sole source of the fat-soluble vitamine. Nos. 231, 233, and 234 were taken off the ration to conserve food. The two remaining females produced seven normal young each. The mothers were in an exceptionally good condition after the birth of the young, coming back to their normal weight very quickly.

exhausted although in order to secure this result it was necessary to replace the male rat No. 230 by one from the breeding colony.[27]

The results seem to us to show conclusively that carotinoid pigment is not necessary for normal growth and reproduction of albino rats.

[27] The male rat carried to adult size on the egg yolk ration proved to be sterile, or at least failed to breed, even after several weeks on a mixed diet, although he appeared to be normal in every way.

The Quantitative Relation of Carotin to Growth of Rats.

Steenbock and Gross[6] and Osborne and Mendel[7] have presented data showing approximately the quantitative amounts of certain carotin-containing foods required to support normal growth of rats. According to the former authors this requirement is met with rations containing 15 per cent dried carrots, or 85 per cent yellow maize, or 5 per cent spinach, or 5 per cent dried lettuce. The latter investigators state that 0.1 gm. daily of dried spinach, grass, carrots, or alfalfa permit growth to adult size. Other data of a similar character are presented by both investigators but the foods mentioned are those whose carotin content has either been determined or can be calculated readily from available data.

The carotin content of dried carrots is at least 0.026 per cent, inasmuch as Willstätter and Stoll[24] state that Escher isolated 125 gm. of pure carotin from 472 kilos of dried carrots. The figure of 0.026 per cent will be recognized, therefore, as very conservative.

The carotin content of dry yellow maize is at least 0.0166 per cent according to the statement of van den Bergh and Muller[19] that the ratio of carotin in dried maize to that in dried carrots is 1.6 : 2.5. From this ratio and the carotin content of dried carrots given above the carotin content of maize is calculated to be that mentioned. This figure is also very conservative.

Arnaud[28] has determined the carotin content of dried spinach to be 0.16 per cent and that of dried grass to be 0.106 per cent.

Using this figure for spinach the carotin content of dried lettuce is at least 0.028 per cent as calculated from the ratio of the carotin in lettuce to that in spinach (0.76 to 4.4) as given by van den Bergh and Muller.

The carotin content of dried alfalfa is approximately 0.093 per cent based on Jacobson's[29] finding that the total carotinoids comprise 0.28 per cent and assuming that the normal chloroplastid ratio of xanthophylls to carotin of 2:1 holds true for alfalfa leaves.

Using the above figures as a basis the carotin content can be calculated for various rations containing minimum quantities of the selected foods, as found by the authors mentioned. An average food intake of 10 gm. a day per rat is assumed where such an assumption is necessary. The results of such a calcu-

[28] Arnaud, A., *Compt. rend. Acad.*, 1889, cix, 911.

[29] Jacobson, C. A., *J. Am. Chem. Soc.*, 1912, xxxiv, 1263.

lation in comparison with the carotin content of the rations used in our experiments are presented in Table I. The results are self-evident in showing wide and incongruous relations between the carotin content and fat-soluble vitamine efficiency of the various rations.

TABLE I.

Quantitative Comparison of Carotin Content and Fat-Soluble Vitamine Efficiency of Various Rations.

Food supplying fat-soluble vitamine.	Amount of food in ration.	Carotin content of ration.	Ratio of carotin content to 9 per cent ewe milk fat ration.
	per cent	*per million*	
Carotinoid-free egg yolk..........	15	0.0	
Ewe milk fat....................	5	0.0733	
" " "	9	0.1263	
Carrots (dry)....................	15	390.0000	3,080:1
Yellow maize (dry)...............	85	1,400.0000	11,070:1
Spinach (dry)....................	5	800.0000	6,324:1
Lettuce (dry)....................	5	140.0000	1,107:1
Spinach (dry)....................	1	160.0000	1,265:1
Grass (dry)......................	1	106.0000	838:1
Carrots (dry)....................	1	2.6000	20:1
Alfalfa (dry)....................	1	9.3000	74:1

SUMMARY.

The status of the alleged relation of carotinoids to fat-soluble vitamine is reviewed and discussed.

A critical study is reported of the possible presence of carotinoid pigments in the albino rat, showing the complete absence of these coloring matters in this species of animal.

Growth and reproduction of rats is reported with ewe milk fat containing only 0.00014 per cent carotin as the sole source of fat-soluble vitamine, the ration showing the best results containing only 0.0000126 per cent carotin.

Growth and reproduction of rats are reported using *carotinoid-free* egg yolk as the sole source of fat-soluble vitamine.

A quantitative comparison of the carotin content and fat-soluble vitamine efficiency of various rations is given showing irreconcilable divergencies from the view that vitamine and carotinoid are identical. The data show that the substances are not even quantitatively associated in the plant tissues in which both are presumably synthesized.

ANAEROBIC RESPIRATION IN SOME PELECYPOD MOLLUSKS.

THE RELATION OF ANAEROBIC RESPIRATION TO GLYCOGEN.

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It has been shown by Collip (1) that the pelecypod mollusk, *Mya arenaria*, can remain alive and continue to produce carbon dioxide for considerable periods under conditions in which the free oxygen present is reduced to a minimum and the conclusion is tentatively advanced that this animal is capable of anaerobic respiration.

The present work was undertaken to endeavor to explain the process whereby this respiration is accomplished in *Mya arenaria*. Most of the experiments recorded in this paper have, however, been carried out on two other species of pelecypod mollusk, *Paphia staminea* and *Saxidomus gigantea*, since *Mya arenaria* was not readily obtainable during the winter months.

The mechanism of anaerobic respiration in animals does not appear to have been very fully investigated though, as far back as 1776, Spallanzani (2) showed that certain "animalculae" could live in the absence of air. More recently Bunge (3), Packard (4), and others have shown that certain animals can live under anaerobic conditions for varying periods of time. The faculty is a well known characteristic of certain moulds, yeast, and bacteria and the subject has been very fully studied in the case of the last named group of organisms since the time of Pasteur owing to the great technical importance of some of the anaerobic bacteria.

In the case of the vegetable organisms, hydrogen, carbon dioxide, methane, and other gaseous compounds have been recorded among the products of anaerobic respiration. The observation by Collip (1) of the production of carbon dioxide by *Mya arenaria*

appears to be the first definite record of the nature of a respiratory product of an animal living under anaerobic conditions. Any attempt to find an explanation of the formation of the various products of anaerobic respiration in the former case is complicated by the presence of food materials in the media on which the organisms are grown, but in the latter case the situation is simplified by the absence of food material and the problem, then, resolves itself into finding a means whereby the oxygen necessary for the formation of carbon dioxide is obtained; the carbon must be derived from the animal itself.

There seem to be only two possible alternatives. Either the oxygen is also derived from the body substance of the animal and carbon dioxide results from a molecular rearrangement, or it is obtained by the dissociation of water. This latter assumption is the basis of the theory of respiration advanced by Mathews (5). According to Mathews respiration is due to dissociation of water with the liberation of hydrogen. In normal (aerobic) respiration this hydrogen is recombined with atmospheric oxygen to form water. In anaerobic respiration it is disposed of by combination with some readily reduced substance present in the organism or set free in gaseous form. The maintenance of the anaerobic respiratory process would, in the former case, depend upon the presence of the appropriate readily reducible substance and the theory seems to have received experimental support by demonstrations that the provision of such a substance does indeed enable certain organisms to live for increased periods under anaerobic conditions. This has been done by Packard (6) by supplying sugars to *Fundulus heteroclitus* and by many workers in the case of bacteria. The necessity for provision of a sufficiency of carbohydrate material in culture media on which most anaerobic bacteria are to be grown is well known.

It seems possible, however, that in these cases the carbohydrates supplied serve either as a direct source of oxygen or as a source of energy by reason of their ready oxidizability by means of oxygen derived from the breakdown of some other compound present in the organism, rather than as polarizers enabling the organism to dispose of hydrogen set free by the dissociation of water.

Whatever explanation of their action is accepted carbohydrates appear to be the only class of substances which has hitherto been

shown to possess this power of stimulating vital activity under anaerobic conditions and they are amongst the most readily oxidizable, as well as the most readily reducible, of the substances known to occur in the animal body. It seemed advisable, therefore, to attempt in the first place to correlate the property of anaerobic respiration in the animals investigated with their carbohydrate constituents.

Lang and Macleod (7) have shown in the case of *Schizothoerus nuttalli* that only extremely small quantities of reducing substances are to be found in the circulating fluid and it is doubtful how many of these are carbohydrate in nature. This is probably true of other mollusks of the same class.[1] Glycogen, on the other hand, has been shown by Kilborn and Macleod (8) to occur in considerable quantities in all the main tissues of *Schizothoerus nuttalli* and experiment has shown the same thing to be true of the mollusks dealt with in this paper.

Although it would not be anticipated that glycogen, as such, would undergo either reduction or oxidation and so play a part in anaerobic respiration, dextrose derived from its hydrolysis would readily do so. The glycogen would therefore seem to be the most likely source of any sugar participating in the process, and, in this case, there should be an appreciable decrease in the glycogen content of the organisms after anaerobic respiration had been maintained for some time. Attempts to trace such a connection are recorded in this paper.

EXPERIMENTAL.

Methods.

The animals used for the experiments to be described were taken from clam-beds in the neighborhood of the Marine Biological Station, Nanaimo. In the case of *Mya arenaria* the bed consists of fine muddy sand and the animals are usually found buried some 6 to 9 inches below the surface. *Paphia staminea* and *Saxi-*

[1] In a paper which has appeared since this was written, Albrecht (*J. Biol. Chem.*, 1921, xlv, 395) records finding material in the alcoholic extract of *Tivella stultorum* which yielded a large quantity of reducing sugar on hydrolysis. The clams I have used have not been examined for such a substance. If present its relation to anaerobiosis will be worth following.

domus gigantea are found nearer the surface and in rather coarser muddy sand.

In most cases the animals were placed under experimental conditions within a few hours of digging. The glycogen content was usually determined immediately in individuals of as nearly as possible the same size and weight as those submitted to experiment and used for experimental controls.

The weight of the animal in its shell was found to be only a rough guide to that of the animal itself, mainly owing to the variation in the quantity of water held. They were kept exposed to the air and allowed to discharge all the water they would before weighing and, in this way, fairly uniform specimens could be selected.

In all cases the entire animal was taken for analysis since glycogen occurs in all the tissues examined. The animal was removed from the shell, cut up into small pieces which were freed from adherent moisture by pressing between filter paper, and weighed. These operations were performed as quickly as possible. Results were calculated as percentages based on the weight of this roughly dried material. Pflüger's method was used for the determination of glycogen with the following modifications suggested by the work of Starkenstein and Henze (9).

1. Only one volume of alcohol was used to precipitate the glycogen from the alkaline digest. Starkenstein and Henze contend that certain glucosamine derivatives and pentosans which occur in the livers of the mollusks examined by them are liable to escape complete destruction during the digestion with alkali and to be precipitated together with the glycogen if the usual two volumes of alcohol are used and lead to too high a result in the determination. This, they say, is avoided if only one volume is used.

Although no pentosan could be detected by means of Bial's orcinol reagent in the precipitates obtained during the work to be described even if two volumes of alcohol were used, it was found that the whole of the glycogen was carried down by one volume, so that there was no object in using two. It seemed advisable, moreover, to avoid the possibility of the error from glucosamine derivatives indicated by Starkenstein and Henze.

2. The whole precipitate thrown down by alcohol was transferred to boiling water, boiled, cooled, made up to volume with any

undissolved material in suspension, and the turbid liquid, or an aliquot part of it, taken for hydrolysis; instead of dissolving on the filter paper and hydrolyzing the filtrate in the usual manner.

Starkenstein and Henze state that, in determining glycogen in the tissues of marine animals, the results are liable to be too low due to adsorption of a portion of the glycogen by metallic hydroxides formed by the action of the potassium hydroxide on compounds present in the animals and in the adherent sea water. This adsorbed glycogen is said to be left undissolved during the ordinary process of treating the precipitate with hot water on the filter and is thus lost to the analysis. Kilborn and Macleod (8) have recently confirmed this statement.

The method described eliminates this risk. Very little material remained undissolved after boiling the precipitates in water and the greater part of such material as did remain consisted of fragments of organized tissue which consisted mainly of chitin.[2]

Natural Variation in Glycogen Content of Mya arenaria.

Before proceeding with further experiments it was necessary to know to what extent the glycogen content varied amongst individual clams of the same species which had been selected of as nearly the same size and weight as possible. Some specimens of *Mya arenaria* which had been kept in a cage floating in the sea for some time were used for this purpose (Table I).

Thea greement between the results obtained from the clams of like size seemed as good as could be expected when the inevitable approximation introduced by the method of drying is regarded.

[2] This was particularly marked in the case of *Mya arenaria*. The form and the color of the fragments showed that they were derived from the tips of the siphons. That they consisted largely of chitin was indicated by their insolubility in boiling 60 per cent potassium hydroxide and by the following properties: (*a*) They contained nitrogen. (*b*) They gave the red-violet coloration characteristic of chitosan on treatment with dilute sulfuric acid and iodine. (*c*) They dissolved readily on boiling in concentrated hydrochloric acid. (*d*) The solution in hydrochloric acid gave off ammonia on making alkaline and boiling. (*e*) The solution in hydrochloric acid had a strong reducing action. (*f*) The solution in hydrochloric acid gave a phenylosazone having the characteristic appearance of glucose phenylosazone.

From these results, coupled with those subsequently obtained with freshly dug specimens of all the three species used for experiment, which are recorded in later tables, it was concluded that

TABLE I.

Mya arenaria.

Weight of animal in shell.	Weight of animal.	Total glycogen.	Glycogen.	Remarks.
gm.	*gm.*	*gm.*	*per cent*	
87.0	23.1	0.97	4.19	In cage for 1 week.
85.3	23.3	0.99	4.25	" " " 1 "
50.5	8.8	0.35	3.97	" " " 3 to 4 weeks.
46.5	8.6	0.29	3.37	" " " 3 " 4 "

the natural variation in the percentage of glycogen in clams of the same species, weight, and size, which had been living under like conditions, was not great.

Effect of Submersion in Oxygen-Free Sea Water in Sealed Vessels.

A series of experiments was carried out with each of the three species of clams to determine the effect on the glycogen content of submersion in boiled out sea water in sealed vessels.

The water was boiled briskly for about half an hour, making up losses by evaporation with boiled tap water, and cooled out of contact with air. Water so treated was found to contain only traces of dissolved oxygen, or in some cases to be quite free from it, as far as could be detected by the method of Winkler (10). It was not, however, essential that the water should be absolutely free from dissolved oxygen when the animals were first put into it since it was found that they rapidly used up the oxygen present even if put into unboiled water.[3]

The vessels used were glass cylinders, holding 1,330 cc., sealed with tightly fitting rubber bungs through which a stop-cock tube was inserted so that the bung could be pushed in tight and any bubbles of air set free by the clams after the vessel was closed could be displaced. They were kept in the open. The air temperature over the period of the experiments was rarely over 10°C.

[3] This was true of each of the three species experimented with. They are thus shown to be facultative rather than obligatory anaerobes.

or below the freezing point. The controls were kept in a similar vessel under identical conditions except that a current of air was aspirated through the vessel by means of a suction pump.

It was found that all three species of clams remained alive for several days in the sealed vessels. In the case of *Mya arenaria* and *Saxidomus gigantea* it was easy to determine whether the animals were alive without removing them from the apparatus since they usually lie with their siphons extended, particularly in the absence of oxygen, and withdraw them if the container is given a sudden jar. Moreover, the valves are only slightly relaxed as long as the animal remains alive, but begin to gape as soon as they die. In the case of *Paphia staminea* it was more difficult since they often extend their siphons and relax their valves very little or not at all. The experiments were never carried to the death-point of the animals, since, before this was reached, the water in the sealed vessels became turbid and evil-smelling, presumably due to excretory products, and it was thought this might lead to faulty estimates of carbon dioxide production. The experiments were concluded before, or as soon as, turbidity of the water in the sealed vessels began to set in. The extreme limits of endurance of anaerobiosis have thus not been determined. The water was invariably found quite free from dissolved oxygen when the vessels were opened. The water in the control vessel was usually clear and free from odor.

The animals always showed every sign of life and seemed to be perfectly normal in every respect when removed from the apparatus. So far as could be judged by comparison on the basis of the weights in the shell no significant decrease in body weight took place either as the result of anaerobiosis or starvation which both the experimental animals and the controls underwent during the period of the experiments; but, as is shown in Tables II to VI, there was usually some decrease in the glycogen content of the tissues in both cases.

The bicarbonate present in the water in which the animals had been kept was determined by titration with 0.1 N hydrochloric acid, using methyl orange as indicator, immediately on opening the vessels. After allowing for the small amount present at the start this gave a measure of the carbon dioxide produced in the sealed vessel. The amount was calculated on the full 1,300 cc.

of water held by the vessels. Actually a part of this was displaced by the clams, but this was a relatively small volume and, as is indicated by the work of Collip (1, 11), it is to be anticipated that the bicarbonate concentration of the body fluids of the animal would be at least as high as that of the surrounding water. The only significant source of error would be in respect to the water displaced by the shells. As Collip (1) points out only half the carbon dioxide found as bicarbonate is to be directly attributed to respiration, the other half being due to the solution of the carbonate of the shells according to the equation

$$CaCO_3 + CO_2 + H_2O = Ca(HCO_3)_2$$

Any free carbon dioxide present in the water was neglected in this series of experiments, but the amount which failed to react with the carbonate of the shells in accordance with the above equation can hardly have been a significant quantity.

TABLE II.

Mya arenaria.

	Weight of tissue.	Total glycogen.	Glycogen.	Average glycogen.
	gm.	*gm.*	*per cent*	*per cent*
As taken from natural habitat....................	29.3	1.9	6.5	
" " " " "	20.6	1.2	5.8	6.15
After 7 days in boiled water in sealed vessel.......	22.6	0.85	3.8	
" 7 " " " " " " "	28.5	0.85	3.0	3.57
" 7 " " " " " " "	23.9	0.95	3.9	
After 7 days in boiled water continuously aerated.	19.5	0.45	2.3	
" 7 " " " " " "	22.6	0.52	2.3	3.03
" 7 " " " " " "	23.5	1.14	4.5	

In the case of the controls the carbon dioxide produced could not be measured by determining the increase of bicarbonate in the water because, as is shown in later experiments, a large proportion of it was carried away by the air current. The increase of bicarbonate in the control was in every case relatively small (Table II).

The results for *Mya arenaria* indicate that in this species no loss of glycogen accompanies anaerobiosis. A comparison of the

average of the results obtained from the experimental animals and from the controls seems, in fact, to point in the opposite direction, but the difference is not sufficiently large to be outside the limits of natural variation.

The bicarbonate content of the water from the sealed vessel corresponded to a total production of 0.87 gm. of carbon dioxide, or 0.435 gm. due to respiration (Table III).

In the experiments recorded in Table III two sets of controls were kept, the first set in boiled water with air passing through

TABLE III

Saxidomus gigantea.

	Weight of tissue.	Total glycogen	Glycogen	Average glycogen.
	gm.	*gm.*	*per cent*	*per cent*
As taken from natural habitat	36.9	1.49	4.0	
" " " " "	36.2	1.59	4.4	4.2
After 10 days in boiled water in sealed vessel	33.9	0.95	2.8	
" 10 " " " " " " "	39.0	1.19	3.0	2.7
" 10 " " " " " " "	34.3	0.78	2.3	
After 14 days in boiled water continuously aerated.	33.8	1.04	3.1	
" 14 " " " " " "	21.4	0.37	1.7*	3.5
" 14 " " " " " "	36.7	1.46	3.9	
After 14 days in unboiled water open to the atmosphere	39.5	1.41	3.6	
" " "	32.0	0.87	2.7	3.0
" " "	34.9	0.90	2.6	

* This seemed to be an exceptional individual. The gonad was quite undeveloped. The result is therefore not included in the average.

constantly, the second in a similar vessel containing ordinary sea water and left open to the air.

The results indicate only a small difference in respect of glycogen consumption between the two controls or between the controls taken as a whole and the experimental animals, but, if the average result obtained from the latter be compared with that of the controls with air passing continuously, the difference is sufficiently large to indicate some destruction of glycogen accompanying anaerobiosis. There would seem to be some justification

in excluding the controls in still water from the comparison since in this case the conditions were not strictly aerobic. The water in the neighborhood of the clams at the bottom of the cylinder cannot have been kept fully oxygenated by diffusion from the small surface exposed to the atmosphere.

TABLE IV.

Paphia staminea.

	Weight of tissue.	Total glycogen.	Glycogen.	Average glycogen.
Experiment 1.				
	gm.	*gm.*	*per cent*	*per cent*
As taken from natural habitat	4.0	0.16	4.0	
" " " " "	4.3	0.19	4.4	3.97
" " " " "	5.1	0.18	3.5	
After 8 days in boiled water in sealed vessel	5.0	0.11	2.2	
" 8 " " " " " " "	4.7	0.14	2.9	2.67
" 8 " " " " " " "	5.7	0.17	2.9	
After 8 days in boiled water continuously aerated.	5.0	0.24	4.8	
" 8 " " " " " "	5.0	0.11	2.2	3.50
After 8 days in filtered water open to atmosphere	4.8	0.18	3.7	
" 8 " " " " " " "	3.9	0.13	3.3	3.47
" 8 " " " " " " "	5.5	0.19	3.4	
Experiment 2.				
As taken from natural habitat	7.6	0.18	2.4	
" " " " "	8.4	0.24	2.8	2.5
" " " " "	8.7	0.19	2.2	
After 21 days in boiled water in sealed vessel	8.2	0.35	4.3	
" 21 " " " " " " "	7.4	0.23	3.1	3.7
" 21 " " " " " " "	6.7	0.25	3.7	
After 21 days in boiled water continuously aerated.	8.7	0.27	3.1	
" 21 " " " " " "	7.8	0.24	3.1	2.8
" 21 " " " " " "	3.9	0.09	2.3	

The bicarbonate content of the water in which the experimental animals had been kept indicated a total production of 0.79 gm. of carbon dioxide, or 0.395 gm. due to respiration.

In the first experiment with *Paphia staminea* recorded in Table IV two sets of controls were again kept, but, in the case of the

vessel open to the atmosphere, the water was filtered in order to remove any food material which would have been destroyed by boiling in the other cases.

The analysis of one of the three animals kept in continually aerated water was lost and the remaining two gave such widely divergent results that little value can be attached to their average. A comparison of the average of the results obtained from the animals kept under anaerobic conditions and that from those kept in still water open to the atmosphere indicates a destruction of glycogen accompanying anaerobiosis, but there is only just a large enough difference between the averages to fall outside the limits of natural variation. Moreover, it is probable that the animals in still water were subjected to some measure of shortage of oxygen for the reason advanced in the foregoing section.

No increase occurred in the bicarbonate content of the water in the sealed vessel.

In order to render the results obtained from this animal more definite a second experiment was carried out. The control in still water was eliminated. Six animals were taken for preliminary analysis and the same number kept under anaerobic and fully aerated conditions respectively. They were analyzed in pairs in each case so as to reduce the risk of error involved in handling the rather small amount of material of which one animal consists.

This experiment was carried out during a spell of colder weather than the first and, probably for this reason, it was found possible to carry it on for 3 weeks. It was in fact only stopped then to make way for other tests. No turbidity had set in in the water in either vessel and the animals were perfectly normal when taken out.

The results show definitely that there has been no destruction of glycogen accompanying anaerobiosis. It is not likely that the apparent gain has any significance.

The production of carbon dioxide by respiration indicated by the bicarbonate determination in the water of the anaerobic vessel amounted to 0.036 gm.

Experiments in a Current of Hydrogen.

It has been mentioned above that a limit to the periods for which the experiments in sealed vessels could be conducted was set by the production of turbidity and an unpleasant odor in the water. It seemed probable that, if the volatile products of vital activity could be removed by means of a current of gas, these periods might be prolonged and thus any consumption of glycogen involved become more marked.

With this object in view a few experiments were carried out in which anaerobic conditions were maintained by passing a current of pure hydrogen through the previously boiled out water in the vessel. Advantage was taken of the fact that, in these circumstances, any free carbon dioxide would be washed out of the water by the gas currents to obtain a complete measure of the amount produced in each case.

In the preliminary experiment and the one with *Paphia stáminea,* the modification of the Kipp apparatus described by Dolch (12) was used to generate the hydrogen. The gas produced, as long as the generation was unbroken, was found so nearly free from oxygen that the small residue could easily be absorbed by alkaline pyrogallate, but the apparatus does not lend itself to continuous working and it was found extremely difficult to avoid slight leakages of air into the system during the resting periods. For this reason electrolytic hydrogen from a cylinder of the compressed gas was used in the later experiments with *Saxidomus gigantea.* With this continuous working was possible and a constant positive pressure could be maintained in the system so that no leakage of air could take place.

The hydrogen was first passed through a large wash bottle of alkaline pyrogallate and then through a smaller one. The alkali was not added to the pyrogallic acid until all the air had been displaced from the bottles by hydrogen. In this way the liquid was obtained practically colorless and the maintenance of this condition in the second bottle served as a check on the efficient absorption of any oxygen present in the hydrogen by the first. The alkaline pyrogallate would have served also to absorb any carbon dioxide had any been present in the hydrogen. The air aspirated through the control vessel was freed from carbon diox-

ide by bubbling it through a large volume of strong sodium hydroxide solution. In both cases the carbon dioxide in the emerging gases was absorbed by passing them through standard barium hydroxide solution. The sum of the carbon dioxide thus measured and half that found as bicarbonate in the water, as in the previous experiments, gave a complete measure of the carbon dioxide produced by respiration.

A preliminary test was carried out with *Saxidomus gigantea* to find out how the animals behaved in hydrogen and to get an approximate idea of the amount of free carbon dioxide produced. It was found possible to keep the test running for 2 weeks, whereas 10 days was the longest period attained in a sealed vessel. The increase of the period over which the test could be maintained did not, however, hold in subsequent experiments and was probably due to the hydrogen experiment having been carried out during a period of exceptionally low air temperature.

The difference between the average glycogen content of the animals kept in hydrogen and when first dug was slightly greater than when they were kept in a sealed vessel, but no control was kept in an air current in this experiment so that no estimate of how far this difference is to be attributed to anaerobiosis was formed. Also the amount of free carbon dioxide to be anticipated was underestimated and the whole of the standard baryta taken for absorption was neutralized. The test is therefore not reported in detail.

Paphia staminea.

Nine individuals were taken for experiment and control, respectively. They were so selected that the combined weight of the animals in their shells was the same in both cases. No analysis was made of the animals when dug. The clams kept their shells tightly shut and did not extrude their siphons in the anaerobic vessel and, as a very slight turbidity had set in, it was decided to conclude the experiment after running a week. The condition of the clams when opened suggested that it might have been run longer with safety. All the nine clams were taken together for glycogen determination in both experiment and control. The results obtained are summarized in Table V.

The figures agree in the two cases remarkably closely. There has been no loss of glycogen accompanying anaerobiosis and practically the same amount of carbon dioxide is produced by unit weight of tissue under anaerobic as under aerobic conditions.

Considerably more free carbon dioxide is produced in both cases than is fixed as bicarbonate, but the ratio of free to fixed carbon dioxide is notably greater under aerobic than anaerobic conditions; that is to say, there is relatively more carbon dioxide fixed as bicarbonate in the latter case.

TABLE V.

Paphia staminea.

	Weight of tissue.	Total glycogen.	Glycogen.	Free CO_2.	CO_2 as bicarbonate.	Total CO_2 respired.	Respired CO_2.
	gm.	*gm.*	*per cent*	*gm.*	*gm.*	*gm.*	*per cent*
Experiment.........	49.9	1.83	3.6	0.236	0.091	0.282	0.54
Control............	50.2	1.86	3.7	0.240	0.081	0.281	0.56

Saxidomus gigantea.

Two experiments were carried out in a continuous hydrogen current using three individuals for experiment and control, respectively. Individuals selected from the same lot of clams were used for both experiments, but those used in the second case had been in a floating cage in the sea for 16 days. In each instance glycogen determinations were made in each of three animals before starting the experiment and individual determinations were also made at the end (Table VI).

In both cases there is evidence of loss of glycogen accompanying anaerobiosis confirming the indication obtained in the experiment recorded in Table III.

In the first experiment the amount of free carbon dioxide produced by anaerobic respiration is about the same as that fixed as bicarbonate. In the second, the production of free carbon dioxide under these conditions seems to have been accelerated, 0.0023 gm. of carbon dioxide per unit weight of tissue having been produced in 10 days in the first case, 0.0039 gm. per unit weight of tissue in 7 days in the second. The determination of bicarbonate under anaerobic conditions was lost in the second experiment.

There is considerably less total carbon dioxide produced per unit weight of tissue under aerobic than under anaerobic conditions

TABLE VI.

Saxidomus gigantea.

	Weight of tissue.	Total glycogen.	Glycogen.	Average glycogen.	Free CO_2.	CO_2 as bicarbonate.	Total CO_2 respired.	Respired CO_2.
Experiment 1.								
	gm.	*gm.*	*per cent*	*per cent*	*gm.*	*gm.*	*gm.*	*per cent*
As taken from natural habitat	34.2	0.84	2.46					
" " "	32.4	0.80	2.47	2.39				
" " "	25.6	0.57	2.23					
After 10 days in boiled water with hydrogen passing continuously	34.6	0.54	1.56					
" " "	33.9	0.53	1.56	1.77	0.243	0.564	0.525	0.510
" " "	34.4	0.75	2.18					
After 10 days in boiled water continuously aerated.	33.8	0.93	2.75					
" " "	29.5	0.72	2.44	2.46	0.256	0.203	0.358	0.376
" " "	32.0	0.70	2.19					
Experiment 2.								
After 10 days in floating cage in sea	30.6	0.72	2.35					
" " "	25.0	0.51	2.04	2.30				
" " "	24.4	0.61	2.50					
After further 7 days in boiled water with hydrogen passing continuously.	24.4	0.23	0.94					
" " "	24.2	0.50	2.07	1.45	0.275			
" " "	20.9	0.28	1.34					
After further 7 days in boiled water continuously aerated	25.6	0.57	2.23					
" " "	29.7	0.54	1.82	2.09	0.399	0.133	0.466	0.612
" " "	20.8	0.46	2.21					

in the first experiment, but the difference is almost entirely in the bicarbonate. Very much less carbon dioxide is fixed as bicar-

bonate under aerobic than under anaerobic conditions. The corresponding comparison cannot be made in the second experiment owing to the absence of the bicarbonate determination under anaerobic conditions, but there is in this case a markedly larger production of free carbon dioxide under aerobic than under anaerobic conditions.

The production of free carbon dioxide per unit weight of tissue under aerobic conditions is nearly twice as great in the second experiment as in the first although the latter was carried on for 10 days and the former only 7. It would seem therefore that, while the power of carbon dioxide production under anaerobic conditions has increased to some extent as a result of keeping the clams in the floating cage for 16 days, the power of doing so under conditions of strong aeration has increased still more. The chief difference between the conditions in the cage and those of their natural environment was probably in respect to aeration. The surface temperature of the water varied between 5.5 and 7.5°C. during the period, probably not very different from that in the clam-beds.

DISCUSSION.

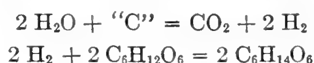
It is clear from the foregoing that the two species of mollusk which have been sufficiently closely investigated differ in that in the case of *Paphia staminea* the loss of glycogen under anaerobic conditions does not occur, and in the case of *Saxidomus gigantea* it does. As far as the evidence for *Mya arenaria* goes it seems to behave like *Paphia staminea*. In these latter cases, therefore, some explanation of anaerobiosis other than that involving the destruction of glycogen must be looked for.

The glycogen lost during anaerobiosis by *Saxidomus gigantea* is not nearly sufficient to account for the carbon dioxide produced on the assumption that the oxygen of the carbon dioxide is obtained by the dissociation of water and that all the hydrogen freed thereby goes to reduce the glucose resulting from the hydrolysis of the glycogen.

It would be anticipated that sorbitol would result from this reduction since this compound is produced by the action of nascent hydrogen on glucose under laboratory conditions and is also formed from it under the action of anaerobic bacteria. Sor-

bitol, or an isomeric alcohol, is in fact the only compound which can be formed by addition of hydrogen without disruption of the glucose molecule.

For this reaction two molecules of glucose would disappear for each molecule of carbon dioxide produced.



$$2\,H_2O + \text{"C"} = CO_2 + 2\,H_2$$
$$2\,H_2 + 2\,C_6H_{12}O_6 = 2\,C_6H_{14}O_6$$

In the case recorded in Experiment 1, Table VI, we find, however, that a production of 0.525 gm. of carbon dioxide is accompanied by a disappearance of 0.69 per cent of glycogen in 102.9 gm. of tissue; that is 0.71 gm. of glycogen or 0.76 gm. of glucose; whereas 4.30 gm. of glucose would be reduced in order to produce 0.525 gm. of carbon dioxide according to theory. The amount of glycogen necessary for the purpose on this assumption is, in fact, considerably more than the whole amount contained in the clams.

By a similar line of reasoning it can be shown that there is insufficient loss of glycogen to account for the carbon dioxide produced if the glucose is acting as a direct oxidizing agent, giving up its oxygen to unite with carbon, derived either from its own disruption or from some other compound present in the tissues, unless the whole of the oxygen present is so utilized. In this case a molecule of glucose could account for three molecules of carbon dioxide. This is very nearly the relation found in Experiment 1, Table VI.

It is conceivable that anaerobic respiration in *Saxidomus gigantea* means nothing else but disruption of the glycogen molecule in accordance with the equation

$$C_6H_{10}O_5 + H_2O = 3\,CO_2 + 3\,CH_4$$

and some probability is lent to this conception by the close agreement of the relation between glycogen and carbon dioxide indicated and that actually found, and by the fact that cellulose, an isomer of glycogen, is known to decompose in this way under the action of anaerobic bacteria.

The absence of any trace of gas formation in the experiments conducted in sealed vessels seems to argue against the formation of methane in accordance with the above equation. It also argues

against the possibility that the oxygen of the carbon dioxide was derived by dissociation of water and hydrogen set free.[4] It is possible, however, that either of these gases was formed in the experiments in which a current of hydrogen was running through the apparatus. There is reason, based on the comparative carbon dioxide production, to suppose that the behavior in sealed vessels and in open ones with hydrogen passing is not identical. The emergent gases will be examined from this standpoint in future work, but it will be necessary to make use of some indifferent gas other than hydrogen for the purpose.

That there is a difference in carbon dioxide production in sealed and unsealed vessels under anaerobic conditions is concluded from a comparison of the experiment quoted in Table III with that quoted in Table VI (Experiment 1). Both these experiments ran for 10 days under conditions which are strictly comparable. The amount of carbon dioxide found as bicarbonate is 0.0037 gm. per unit weight of tissue in the first case and 0.0027 gm. in the second; but, in addition to the bicarbonate, 0.0024 gm. of free carbon dioxide per unit weight of tissue is found in the latter case, so that the total carbon dioxide production per unit weight of tissue is much the greater.[5]

A similar relationship is found in the case of *Paphia staminea* by a comparison of Experiment 2, Table IV, and that quoted in Table V. The difference is even more marked in this case since the experiment in the sealed vessel was running for 3 weeks and that in the hydrogen current for only 1 week. It is apparent, therefore, that more carbon dioxide per unit weight of tissue is formed in the current of hydrogen than in a sealed vessel.

A comparison of the carbon dioxide production under anaerobic conditions of the three species of mollusk experimented with goes to show that in sealed vessels *Mya arenaria* is the most active, *Paphia staminea*, the least; though *Paphia staminea* seems to be more active than *Saxidomus gigantea* in a hydrogen current. Table VII illustrates the relationship.

[4] This argument from the absence of any trace of hydrogen in the sealed vessels applies also to the cases of *Paphia staminea* recorded in Table IV.

[5] Free carbon dioxide was not determined in the sealed vessels owing to the difficulty introduced by the presence of magnesium and lack of gasometric apparatus. It is unlikely that any significant quantity would remain uncombined with the carbonate of the shells.

The experiments should, however, be carried out under constant temperature conditions to be strictly comparable, but this was not the case. Temperature would doubtless be an important factor in determining the metabolic activity in question.

Finally it is perhaps worthy of note that, in the experiments in which carbon dioxide production in a current of hydrogen was compared with that in an air current, the gas invariably showed itself, by formation of a precipitate in the baryta, much sooner in the air than in the hydrogen. This may be connected with the larger quantity of carbon dioxide fixed as bicarbonate in hydrogen than in air and suggests the necessity for a period of readjustment in the physiological processes of the animal to meet the anaerobic conditions.

TABLE VII.

Specimen.	Carbon dioxide per 100 gm. of tissue per day.	
	In sealed vessel.	In hydrogen current.
	gm.	*gm.*
Mya arenaria	0.083	
Saxidomus gigantea	0.037	0.051
Paphia staminea	0.008	0.082

SUMMARY.

1. Collip's observation that *Mya arenaria* can produce carbon dioxide under anaerobic conditions is confirmed.

2. The same thing is found true of *Paphia staminea* and *Saxidomus gigantea.*

3. No disappearance of glycogen accompanies anaerobiosis in the cases of *Mya arenaria* and *Paphia staminea.*

4. A disappearance of glycogen invariably accompanies anaerobiosis in *Saxidomus gigantea.*

5. In this case there is not sufficient glycogen consumed to account for the carbon dioxide produced on the basis of Mathews' theory of anaerobic respiration and the assumption that glucose derived from glycogen is reduced by the hydrogen set free by dissociation of water.

6. The relation between glycogen consumption and carbon dioxide production corresponds to a complete breakdown of the glycogen into carbon dioxide and methane.

7. More carbon dioxide is produced under anaerobic conditions in a current of hydrogen than in a sealed vessel by both *Paphia staminea* and *Saxidomus gigantea.*

8. More carbon dioxide is produced in a current of hydrogen than under aerobic conditions by *Saxidomus gigantea;* practically identical amounts by *Paphia staminea.*

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THE PREPARATION AND PROPERTIES OF SOME SALTS OF URIC ACID.

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INTRODUCTION.

In some of the recent methods for the determination of uric acid in blood, use has been made of the fact that uric acid may be precipitated by certain of the heavy metals. In the method of Morris (1) the uric acid is precipitated by means of a zinc salt. Curtman and Lehrman (2), after making a comparative study of various metallic precipitants, decided on the use of a nickel salt for the preliminary precipitation of uric acid. In both of these methods a large excess of the metallic salt is added to the solution containing a relatively small amount of uric acid. On adding an excess of sodium carbonate, the metal is completely precipitated together with the uric acid. The precipitate, therefore, consists of a large amount of basic carbonate of the metal mixed with the uric acid which is either carried down by adsorption or is coprecipitated as a metallic salt. Since a search of the literature failed to reveal any further data concerning the existence of a zinc or nickel salt of uric acid, it was considered worth while to attempt the preparation of these compounds and to determine their properties. Furthermore, since there was some doubt as to the composition of the copper salt of uric acid, Bensch (3) giving one formula and Balke (4) another, we decided to include in this investigation the preparation of the copper salt and to determine its formula and properties.

Preparation of Monopotassium Urate, $KHC_5H_2N_4O_3$.

A consideration of the properties of uric acid and its salts as reported in the literature, led to the choice of the acid potassium salt as the most suitable compound for the preparation of the zinc, nickel, and copper salts. Uric acid in aqueous solution could not be used because of its insoluble nature. The phosphate standard solution of uric acid devised by Benedict and Hitchcock (5) was objectionable because of the presence of phosphate which would yield insoluble phosphates with the heavy metals. A lithium carbonate solution of uric acid was also rejected because of the disturbing influence of the carbonate radical. The so called neutral sodium or potassium urates, though more soluble than the acid salts, were found inapplicable as precipitants because of their instability. On exposure to air, these salts absorb carbon dioxide; moreover, these salts yield aqueous solutions which are strongly alkaline and as a consequence when treated with solutions of the salts of the heavy metals, would cause the precipitation of the usual hydroxides or oxides. A large amount of the monopotassium salt was prepared for this investigation by the method of Behrend and Roosen (6) with slight modifications.

Procedure.

In a stoppered liter flask, 800 cc. of a 5 per cent KOH solution were shaken thoroughly with 50 gm. of uric acid until the former was saturated. The mixture was then filtered to remove the excess of uric acid. From a Kipp generator, carbon dioxide, washed with H_2SO_4 (1:1), was passed into the clear solution until precipitation was complete. This operation required about 1 hour. The white granular precipitate which formed was filtered by suction on a Buchner funnel and washed with cold water until the washings failed to react alkaline towards litmus. The precipitate was washed twice with 50 per cent ethyl alcohol, then with 95 per cent alcohol, and finally dried in a water-jacketed oven for about 36 hours. The yield was 47 gm. Several samples were made by the above method and analyzed for nitrogen by the Kjeldahl method.[1] The potassium was determined as

[1] Throughout this work the nitrogen was determined by the Kjeldahl method as given on page 5 of Bulletin 107 (revised 1907) of the Bureau of Chemistry.

potassium sulfate by the following procedure: A weighed amount of the salt was treated in a Kjeldahl flask with a mixture of concentrated H_2SO_4 and concentrated HNO_3 and boiled (with addition of more HNO_3) until the solution became colorless. It was then diluted with water, transferred to a platinum dish, evaporated to dryness, ignited, and weighed.[2] The results of these analyses are recorded in Table I.

From the results in Table I it is evident that the percentage of potassium is too low while that of nitrogen is somewhat high. Behrend and Roosen (6) recrystallized their preparations

TABLE I.

Analysis of Various Samples of Monopotassium Urate.

Preparation No.	Potassium.		Nitrogen.	
	per cent	*per cent*	*per cent*	*per cent*
1*	18.41	18.35	28.52	28.36
2	18.72	18.68	28.57	28.50
3	18.47	18.60	28.35	28.29
4	18.30	18.42	28.40	28.51
5†	18.61	18.69	28.24	28.13

	Calculated for $KHC_5H_2N_4O_3$.
N	27.19
K	18.97

* This was a commercial preparation which was purchased.

† This preparation was made from Kahlbaum's uric acid.

from hot water and obtained results for potassium much lower than our figures. In one sample which they did not recrystallize they found 18.63 per cent potassium which is in close agreement with our results. It is of interest to note that in their preparations of the sodium salt of uric acid, Behrend and Roosen found the percentage of sodium also low. They make no mention of the percentage of nitrogen in any of their preparations.

Effect of Recrystallizing the Potassium Salt.—5 gm. of Preparation 3 (Table I) were dissolved in 400 cc. of hot water, the resulting solution filtered and allowed to cool. The precipitate which separated out of the cold solution, was filtered by suction, dried in the water oven, and analyzed. The results are given in Table II.

[2] Potassium was also determined by precipitating with H_2PtCl_6. The results checked with those obtained by the K_2SO_4 method.

The results in Table II show that the recrystallized salt is less pure than that originally obtained and confirm the findings of Behrend and Roosen. The higher percentage of nitrogen and the lower value for potassium in the recrystallized product may be explained on the assumption that hydrolysis takes place when the salt is dissolved in hot water.

Properties of Monopotassium Urate.—Monopotassium urate was found to be slightly soluble in cold water but much more soluble in hot water. It is insoluble in ethyl alcohol and ether.[3] Further experiments showed it to be insoluble in methyl alcohol, acetone,

TABLE II.

The Effect of Recrystallizing the Monopotassium Salt.

Preparation No.		Potassium.		Nitrogen.	
		per cent	per cent	per cent	per cent
3	Before recrystallization.	18.47	18.60	28.35	28.29
3	After "	18.09	18.13	28.51	28.58

acetic acid, hydrochloric acid, tartaric acid, boric acid, sodium hydroxide, ammonium hydroxide, sodium potassium tartrate solution, borax solution, and in Benedict's phosphate mixture (5). Experiments also showed that while monopotassium urate is insoluble in dilute H_2SO_4, it dissolves completely in the hot concentrated acid forming a yellow solution. We also found that it is soluble in nitric acid with evolution of NO_2 fumes which were slight with the dilute but very copious when the concentrated acid was used.

Zinc Salt of Uric Acid.

Except for the record of the unsuccessful attempts of Morris (1) to isolate a zinc salt of uric acid, no mention of this compound could be found in the literature. Morris' failure to obtain this salt was due to the fact that he uniformly worked in alkaline solutions and as a consequence invariably obtained a precipitate of zinc hydroxide, carbonate, or phosphate, according to the medium which was used to dissolve the uric acid. Some preliminary work on the part of the authors showed, however, that when

[3] These observations are in agreement with those found in the literature.

an aqueous solution of monopotassium urate which is neutral to litmus, is added to a water solution of zinc acetate, a white flocculent precipitate is formed. This observation suggested a method for the preparation of the salt.

1. Preparation of the Zinc Salt, Using an Excess of Zinc Acetate.—An aqueous solution of monopotassium urate was made by dissolving 8 gm. of the salt in 8 liters of boiling water. The solution after cooling to room temperature was treated with 500 cc. of a 10 per cent solution of zinc acetate. This amount provided a large excess of zinc. A white flocculent precipitate was immediately formed which settled rapidly. The mixture was allowed to stand over night. The next morning, the supernatant liquid was poured off and the precipitate filtered by suction on a Buchner

TABLE III.

Analysis of Zinc Urate Formed by Adding an Excess of Zinc Acetate.

Preparation No.	Zinc.		Nitrogen.	
	per cent	*per cent*	*per cent*	*per cent*
1	24.30	24.59	20.87	20.93
2	24.14	24.09	20.92	21.03
3	24.46	24.38	20.89	20.93
4	24.22	24.30	20.80	20.92

	Calculated for $ZnC_5H_2N_4O_3.2H_2O$.
N..	20.97
Zn..	24.34

funnel. The precipitate was washed several times with water, then with 95 per cent ethyl alcohol, and finally dried in a water-jacketed oven. The yield was 8 gm.

Method of Analysis.—Several preparations made by the above method were analyzed for nitrogen and zinc. The results are recorded in Table III. Inasmuch as the precipitate contained no other metal but zinc the following method was used for its determination. About 0.5 gm. of the sample was carefully ignited in a porcelain crucible to destroy the organic matter. After cooling, a few drops of concentrated nitric acid were added, the resulting mixture evaporated to dryness, ignited, and finally weighed as ZnO. This method was found to give results which agreed with those obtained by weighing the zinc as pyrophos-

phate; and since the former method is considerably more rapid than the latter, it was used in all the determinations of zinc recorded in this paper.

2. Preparation of Zinc Salt, Using an Excess of Monopotassium Urate.—The same procedure as that given above was employed except that only 70 cc. of 10 per cent zinc acetate solution were used, thus supplying an insufficient amount of zinc to completely precipitate the uric acid. A similar white flocculent precipitate was obtained. The yield was only 3.5 gm. The analyses of these preparations made in this manner are given in Table IV. The results of Table IV show that the same compound is formed regardless of the quantity of zinc acetate which is added.

TABLE IV.

Analysis of Zinc Urate Formed by Adding an Insufficient Amount of Zinc Acetate.

Preparation No.	Zinc.		Nitrogen.	
	per cent	*per cent*	*per cent*	*per cent*
1	24.47	24.29	20.92	20.80
2	24.28	24.16	20.85	20.93
3	24.40	24.45	20.87	20.75

	Calculated for $ZnC_5H_2N_4O_3.2H_2O$.
N...	20.97
Zn...	24.34

3. Experiments with a Pyridine Solution of Uric Acid.—To separate 50 cc. portions of a pyridine standard solution of uric acid prepared in accordance with the directions supplied by Benedict and Hitchcock (5), varying amounts of a 10 per cent solution of zinc acetate were added. A white precipitate was obtained in each case. These experiments were repeated using a pyridine solution of the same strength containing no uric acid. No precipitate was obtained in any case even on standing for 24 hours. It appears from these experiments that a salt of uric acid might be made by adding zinc acetate to a pyridine solution of uric acid.

4. Experiments with a Glycerine Solution of Uric Acid.—Colasanti (7) gives the solubility of uric acid in glycerine as 0.74 part in 100. This fact suggested the possibility of forming a zinc

salt by the use of a glycerine solution of uric acid. Accordingly, 1 gm. of uric acid was dissolved, with the aid of heat, in 200 cc. of glycerine. A clear yellow solution was obtained. To 10 cc. of the solution 10 cc. of 10 per cent zinc acetate were added and the mixture was shaken vigorously. Upon standing for a few minutes, a white gelatinous precipitate separated out which was similar in appearance to that obtained with an aqueous solution of monopotassium urate. No precipitate formed when zinc acetate solution and glycerine were mixed. On adding 50 cc. of water to an equal volume of the glycerine solution of uric acid and thoroughly mixing, a precipitate of uric acid was formed on standing. This observation is in harmony with the statement made by Colasanti that uric acid separates from its glycerine solution on standing. Because of the instability of the glycerine solution of uric acid, no attempt was made to use it for the preparation of a zinc salt.

Conclusions.—From the results in Tables II and III it is evident that a zinc salt of uric acid with a definite formula exists and that it may conveniently be prepared from monopotassium urate and zinc acetate. Experiments with pyridine and glycerine solutions of uric acid also point to the existence of a zinc salt as well as the possibility of preparing the salt by these methods.

Properties of Zinc Urate.—Experiments showed that zinc urate is insoluble in water, ethyl alchohol, methyl alcohol, ether, acetone, and acetic acid. The method used in ascertaining the above data was as follows: A small amount of zinc urate was shaken in a test-tube with a little of one of the above solvents. The mixture was boiled for a few moments and then filtered. The filtrate was made alkaline with NaOH and an excess added to form Na_2ZnO_2. The solution was then treated with H_2S. In no case was a precipitate of ZnS obtained, showing that zinc urate is insoluble in each of the solvents mentioned above. Following this procedure we found that zinc urate was somewhat soluble in HCl, dilute HNO_3, NaOH, and NH_4OH. Further experiments showed that it is slightly soluble in an aqueous solution of NH_4Cl, but much more soluble in an aqueous solution of NH_4Cl and NH_4OH than in either of these substances. Zinc urate dissolves completely in concentrated H_2SO_4. It also dissolves readily in concentrated HNO_3 with a copious evolution of NO_2 fumes.

Nickel Salts of Uric Acid.

Except for a statement by Allan and Bensch (8) that they were unable to prepare a nickel salt of uric acid and the new volumetric method of Curtman and Lehrman (2) in which nickel acetate is used as a precipitant for uric acid in alkaline solution, there is no mention of this salt in the literature.

1. Preparation of a Nickel Salt, Using Insufficient Nickel Acetate.—It was expected that the method which was successfully used in the preparation of the zinc salt would also be suitable for the preparation of a nickel compound of uric acid. Accordingly, 18 liters of an aqueous solution of monopotassium urate containing 18 gm. of the salt were treated with 70 cc. of 10 per cent nickel acetate. No precipitate formed even after standing for several days. A 20 per cent solution of potassium carbonate was then added, drop by drop, until the solution was just alkaline to litmus. A green precipitate then formed which did not settle and could not be separated by filtering or centrifuging.[4] The addition, however, of 450 cc. of a potassium acetate solution containing 500 gm. of the salt in 500 cc. of solution, caused the precipitate to agglutinate and settle rapidly. The mixture was allowed to stand over night. The next morning, the supernatant liquid was poured off and the precipitate centrifuged at 3,000 revolutions per minute. It was then dried in the water oven. The yield was 4.5 gm.

Analysis.—Several preparations were made by the above procedure and analyzed for nitrogen and nickel. The results are recorded in Table V. The nickel was determined as NiO by a method similar to that used for the determination of the zinc.

[4] To coagulate the precipitate the following methods were tried: (*a*) 50 cc. of the mixture were evaporated to 25 cc. with no apparent change. (*b*) Separate 50 cc. portions were respectively treated with varying amounts of ethyl alcohol, methyl alcohol, and acetone. The result was the same as in (*a*). (*c*) To separate 50 cc. portions of the mixture varying amounts of potassium acetate solution (100 gm. of salt in 100 cc. of water) were added. It was found that 2.5 cc. of this solution readily coagulated the green precipitate and caused it to settle in a few minutes. A control was run by adding 2.5 cc. of the acetate solution to 50 cc. of monopotassium urate solution; but no precipitate of uric acid or of the potassium salt separated even on standing for 2 days.

The results obtained by this method agreed with determinations made by precipitating the nickel with dimethylglyoxime and drying and weighing the product.

2. Preparation of a Nickel Salt with an Excess of Nickel Acetate.—To 8 liters of an aqueous solution of monopotassium urate containing 8 gm. of the salt, 500 cc. of 10 per cent nickel acetate were added. A very light green precipitate slowly formed but settled rapidly. The precipitate was filtered by suction on a Buchner funnel, washed several times with water, and finally with 95 per cent ethyl alcohol. It was then dried in the water oven. The yield was 8 gm. Several preparations made by this

TABLE V.

Analysis of a Nickel Salt of Uric Acid Formed by Using Insufficient Nickel Acetate.

Preparation No.	Nickel.		Nitrogen.	
	per cent	*per cent*	*per cent*	*per cent*
1	30.11	30.21	15.87	15.75
2	30.95	30.83	15.78	15.77
3	30.30	30.42	15.69	15.80

	Calculated for $NiC_5H_2N_4O_3.NiCO_3.2H_2O$.
N..	14.79
Ni..	30.98

method were analyzed for nitrogen and nickel. The results are given in Table VI.

Conclusions.—When an insufficient amount of nickel acetate is added to a solution of monopotassium urate and the solution rendered just alkaline by the addition of potassium carbonate, a product of variable composition is obtained which approximates the formula $NiC_5H_2N_4O_3.NiCO_3.2H_2O$. When, however, an excess of nickel acetate is added, compounds are obtained having the formula $Ni(C_5H_3N_4O_3)_2.xH_2O$. The amount of water probably depends upon the extent to which the precipitate is dried. The analytical results in Table VI show that Preparations 1 and 2 conform to the formula with $4H_2O$; while Preparations 3, 4, and 5 have $6H_2O$.

3. Action of Nickel Acetate on a Pyridine Solution of Uric Acid.—To separate 50 cc. portions of the standard pyridine solution

of uric acid prepared as described by Benedict and Hitchcock (5) varying amounts of 10 per cent nickel acetate solution were added. In no case was a precipitate obtained.

4. Action of Nickel Acetate on a Glycerine Solution of Uric Acid.—To 10 cc. of a solution containing 1 gm. of uric acid in 200 cc. of glycerine, 10 cc. of nickel acetate solution were added and the mixture was shaken vigorously. No precipitate was obtained on standing for 24 hours. On longer standing, the solution clouded

TABLE VI.

Analysis of a Nickel Salt of Uric Acid Formed by Using an Excess of Nickel Acetate.

Preparation No.	Nickel.		Nitrogen.	
	per cent	*per cent*	*per cent*	*per cent*
1	12.19	12.15	23.79	23.83
2	12.25	12.11	24.24	24.28
3	11.63	11.79	22.78	22.81
4	11.66	11.64	22.34	22.26
5	11.70	11.75	22.41	22.61

	Calculated for $Ni(C_5H_3N_4O_3)_2.4H_2O$.
N..	24.10
Ni..	12.63

	Calculated for $Ni(C_5H_3N_4O_3)_2.5H_2O$.
N..	23.20
Ni..	12.16

	Calculated for $Ni(C_5H_3N_4O_3)_2.6H_2O$.
N..	22.37
Ni..	11.72

slightly due to the separation of uric acid. This behavior on long standing is characteristic of a glycerine solution of uric acid.

Properties of the Nickel Salts of Uric Acid.—The nickel salts were found to be insoluble in water, ethyl alcohol, methyl alcohol, ether, acetone, and acetic acid. When treated with dilute or concentrated hydrochloric acid or sulfuric acid, a green solution and a white precipitate are obtained. The latter is probably uric acid resulting from the decomposition of the salt. In concentrated sulfuric acid, nickel urate dissolves with the formation of a greenish brown

solution. When treated with concentrated nitric acid, the salt dissolves with the formation of a green solution and the copious evolution of NO_2 fumes.

Copper Salts of Uric Acid.

In 1845 Bensch (3) made several metallic salts of uric acid including a copper salt. The latter was prepared by adding copper sulfate solution to a solution of monopotassium urate. The precipitate after filtration was boiled with water and finally dried. To his preparation, Bensch assigns the formula $3CuO + 2(C_5N_2HO_2) + 2HO$ which when rearranged to conform with modern methods of formulation becomes $2CuC_5N_4H_2O_3.CuO.2H_2O$. Bensch's analytical data for his salts agree very well with the calculated values except in the case of the copper salt. Here, a divergence of 1.5 per cent from the theoretical value for copper may be noted. Balke (4) found that by the action of hydroxylamine hydrochloride and Fehling's solution on an alkaline solution of uric acid, a precipitate was formed which on analysis approximated the formula $C_5N_4H_4O_3.Cu_2O$. No further data concerning the existence of a copper salt of uric acid could be found in the literature.

1. Preparation of a Copper Salt, Using an Excess of Copper Acetate.—To 8 liters of a solution of monopotassium urate containing 8 gm. of the salt, 400 cc. of a saturated solution of copper acetate were added. A greenish brown precipitate formed immediately which was filtered by suction on a Buchner funnel. The precipitate was washed several times with water and finally with 95 per cent ethyl alcohol. It was then dried in the water-jacketed oven. The yield was 9.5 gm. Several preparations were made by this method and analyzed for nitrogen and copper. The latter was determined volumetrically. The results of these analyses are given in Table VII.

From the results in Table VII, it is evident that the compound formed under the conditions described, possesses the formula $7CuC_5H_2N_4O_3.3CuO.4H_2O$ and not the formula given by Bensch.

2. Preparation of a Copper Salt, Using an Excess of Copper Sulfate.—To 8 liters of an aqueous solution of monopotassium urate containing 8 gm. of the salt, 250 cc. of 10 per cent copper sulfate were added. A greenish brown precipitate was obtained.

The precipitate was filtered off, washed with water, then with 95 per cent alcohol, and finally dried in the water-jacketed oven. The yield was 8 gm. Three preparations were made by this method and analyzed for copper and nitrogen. The results are given in Table VIII.

TABLE VII.

Analysis of a Copper Salt of Uric Acid Formed with an Excess of Copper Acetate.

Preparation No.	Copper.		Nitrogen.	
	per cent	*per cent*	*per cent*	*per cent*
1	32.38	32.40	19.14	19.62
2	32.40	32.45	19.56	19.63
3	32.16	32.25	19.32	19.44

	Calculated for $7\ CuC_5H_2N_4O_3.3CuO.4H_2O$.
N..	19.78
Cu..	32.09

	Calculated for $2CuC_5H_2N_4O_3.CuO.2H_2O$. (Bensch's formula).
N..	19.48
Cu..	33.22

TABLE VIII.

Analysis of a Copper Salt of Uric Acid Formed with an Excess of Copper Sulfate.

Preparation No.	Copper.		Nitrogen.	
	per cent	*per cent*	*per cent*	*per cent*
1	17.61	17.74	22.63	22.26
2	17.68	17.77	22.16	22.21
3	17.71	17.65	22.29	22.20

	Calculated for $5Cu(C_5H_3N_4O_3)_2.2CuO.20H_2O$.
N..	22.34
Cu..	17.75

Two more preparations of the copper salt were made by a procedure similar to that given above, except that an insufficient amount of copper sulfate (100 cc., 10 per cent of $CuSO_4.5H_2O$) was used. The yield was 7.5 gm. for 8 gm. of monopotassium urate. The results of the analyses of these preparations were in close agreement with those given in Table VIII.

Conclusion.—It appears from the results in Table VIII that the compound formed by adding an excess of copper sulfate to a solution of monopotassium urate possesses the formula $5Cu(C_5H_3N_4O_2)_2.2CuO.20H_2O$ and that the same compound is formed when an insufficient amount of copper sulfate is used. It is noteworthy that the compound formed with copper sulfate is different in composition from that which is formed when copper acetate is used.

3. The Action of a Pyridine Solution of Uric Acid on Copper Sulfate Solution.—Two pyridine solutions were prepared according to the directions supplied by Benedict and Hitchcock (5).

TABLE IX.

Action of Pyridine Solution of Uric Acid on Copper Sulfate Solution.

No.	10 per cent $CuSO_4$.	Result with pyridine solution containing uric acid.	Control with pyridine solution containing no uric acid.
	cc.		
1	0.25	Dark green precipitate, turning brown.	Clear deep blue solution remaining clear after 24 hrs.
2	0.50	" " "	" " "
3	1.00	" " "	Clear deep blue solution becoming cloudy after 8 hrs.
4	1.50	" " "	Clear deep blue solution becoming cloudy in 0.5 hr.
5	2.00	" " "	Cloudy blue solution, forming a heavy light blue precipitate in 0.5 hr.

One of these solutions contained no uric acid and was used for purposes of control in the following experiments. To separate 10 cc. portions of each of the pyridine solutions varying amounts of 10 per cent copper sulfate solution were added. The results obtained are recorded in Table IX.

Pyridine acts somewhat like ammonia on copper sulfate, forming first $Cu(OH)_2$ which dissolves in an excess of pyridine to a clear deep blue solution. Hence the object of the above experiment was to determine how much $CuSO_4$ could be added to a pyridine solution of uric acid without causing the simultaneous precipitation of $Cu(OH)_2$. The results in Table IX show that for every 100 cc. of pyridine solution, 5 cc. of 10 per cent $CuSO_4$ are a safe amount. Based on the above considerations the following

procedure was carried out in the expectation of forming a copper salt of uric acid. To 2 liters of the usual pyridine solution of uric acid, 50 cc. of 10 per cent copper sulfate were added. The precipitate formed was filtered by suction and washed and dried in the usual way. The yield was 1.5 gm. Analysis of the product showed that it contained 32.54 per cent copper and 19.69 per cent nitrogen. The substance, therefore, possesses the formula $7CuC_5H_2N_4O_3.3CuO.4H_2O$, and is identical with that formed when an excess of copper acetate is added to an aqueous solution of monopotassium urate (Table VIII).

4. Action of a Glycerine Solution of Uric Acid on Copper Sulfate.—To 10 cc. of a solution of 1 gm. of uric acid in 200 cc. of glycerine, 10 cc. of 10 per cent copper sulfate were added and the mixture was shaken vigorously. In a few minutes a green gelatinous precipitate was obtained which slowly turned brown while settling. No precipitate was obtained on mixing the copper sulfate solution with pure glycerine, thus indicating that a copper salt of uric acid was probably formed in the first place.

Properties of the Copper Salts of Uric Acid.—There is no mention made in the literature of the properties of the copper salts of uric acid. We therefore undertook to supply this information. The copper salts were found to be insoluble in water, ethyl alcohol, methyl alcohol, ether, acetone, acetic acid, and ammonia. With hydrochloric acid, a green solution is obtained together with a white precipitate which is probably uric acid. Dilute sulfuric acid yields a similar white precipitate and a light blue solution. When treated with a solution of sodium hydroxide, the salt undergoes a change in color from greenish brown to white, while the solution above the precipitate remains colorless. Concentrated sulfuric acid dissolves the copper salts completely as does also concentrated nitric acid. The latter yields a blue solution and copious fumes of NO_2.

SUMMARY.

1. Monopotassium urate was prepared by the method of Behrend and Roosen with slight modifications. Experiment showed that the recrystallized salt was not as pure as the uncrystallized product due to the tendency of the salt to undergo hydrolysis in the hot water solution from which it was recrystallized. For this reason the uncrystallized salt was used as the

basic material for the preparation of the zinc, nickel, and copper salts of uric acid.

2. A zinc salt of uric acid having the formula $ZnC_5H_2N_4O_3.2H_2O$ was prepared by adding an aqueous solution of zinc acetate to a water solution of monopotassium urate. Precipitates were also obtained when a solution of zinc acetate was added either to a pyridine or a glycerine solution of uric acid, thus indicating other methods for the formation of the zinc salt.

3. When to an aqueous solution of monopotassium urate, an insufficient quantity of nickel acetate was added, no precipitate formed. When, however, the mixed solution was rendered just alkaline with potassium carbonate, a green precipitate was obtained which could not be filtered. The addition of a definite quantity of potassium acetate caused the precipitate to agglutinate and to settle rapidly. Analysis showed that the precipitate approximated the formula $NiC_5H_2N_4O_3.NiCO_3.2H_2O$.

An excess of nickel acetate added to an aqueous solution of monopotassium urate yields products of the formula $Ni(C_5H_3N_4O_3)_2 \cdot x\, H_2O$, the amount of water depending upon the extent to which the preparations are dried.

4. A copper salt of the formula $7CuC_5H_2N_4O_3.3CuO.4H_2O$ was formed on mixing an aqueous solution of monopotassium urate with copper acetate solution. A pyridine solution of uric acid when treated with an aqueous solution of copper acetate gave a product of the same composition.

With solutions of copper sulfate and monopotassium urate, precipitates were obtained having the formula $5Cu(C_5H_3N_4O_3)_2 2CuO. 20H_2O$. The same compound was formed when either the copper sulfate or the potassium urate was present in excess.

5. The properties of the potassium, zinc, nickel, and copper salts of uric acid were determined.

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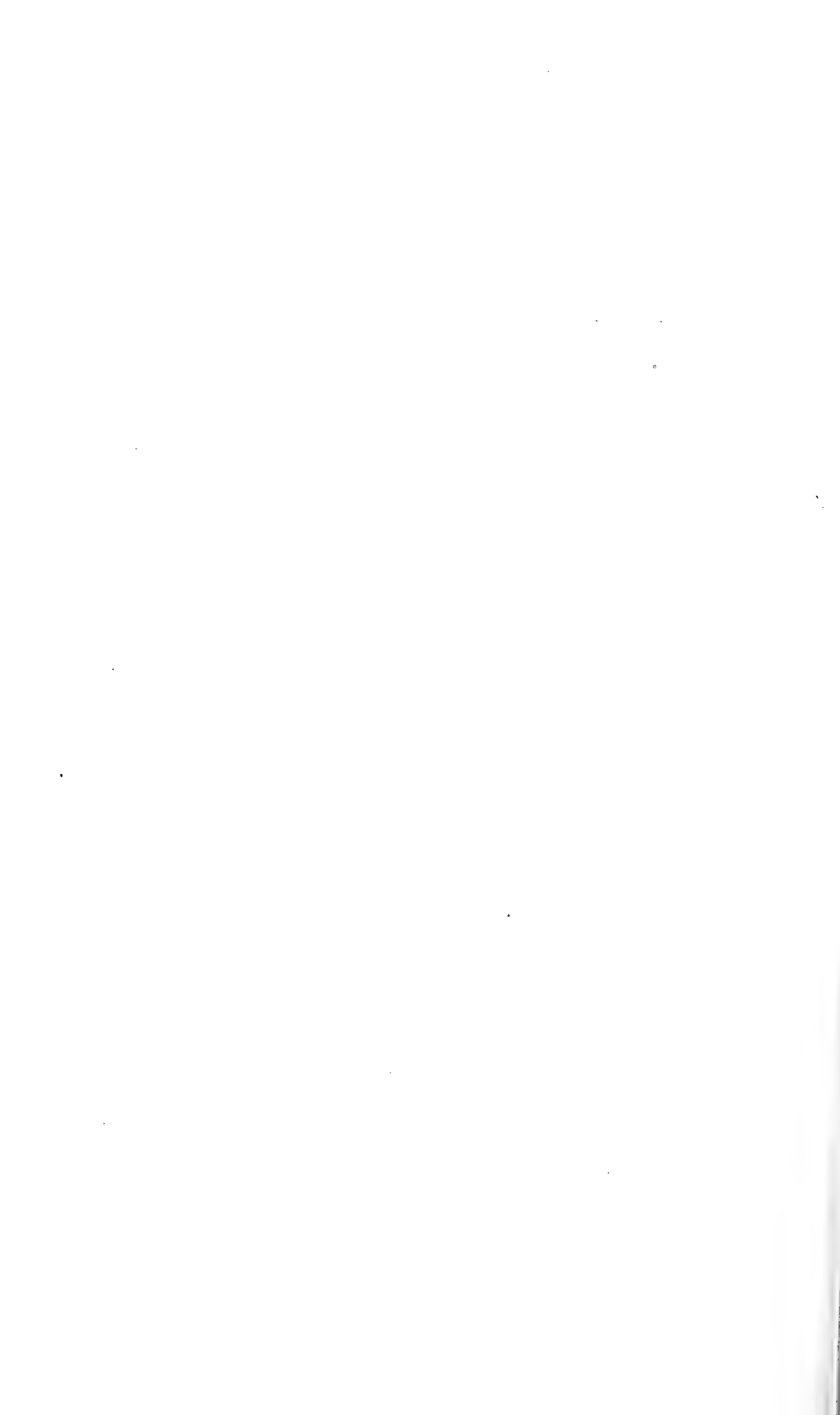

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PROCEEDINGS OF THE AMERICAN SOCIETY OF BIOLOGICAL CHEMISTS.

FIFTEENTH ANNUAL MEETING.

Chicago, Ill., December 28–30, 1920.

A PHYSICOCHEMICAL METHOD OF CHARACTERIZING PROTEINS. II.

By EDWIN J. COHN.*

(From the Laboratories of the Harvard Medical School, Boston.)

At a previous meeting of this Society (1919) Henderson and Cohn described a physicochemical method of characterizing proteins in terms of their amphoteric constants. We pointed out that although the large number of acidic and basic valences involved and the uncertain molecular weights of the proteins rendered it impracticable to evaluate these constants it was possible to represent their strength graphically by plotting the ratio of acid to protein—and of protein to base—as ordinate, and the hydrogen ion concentration as abscissa. If this ratio is expressed as mols of HCl or NaOH added to 1 gm. of anhydrous isoelectric protein the hydrogen ion concentration corresponding to the ordinate *O* becomes the isoelectric point of the protein.

The titration curve of gluten was then presented, with an estimate of the number of acidic and basic valences required in the construction of a curve to approximate the experimental one at all hydrogen ion concentrations. Since then the writer has determined the titration curve of tuberin and of very pure serum globulin, and Loeb has reported the titration curves of pure gelatin and of recrystallized egg albumin. The titration curves of these characteristic proteins reveal important differences.

Each of the proteins has a different isoelectric point. The hydrogen ion concentration corresponding to the isoelectric point determines the ratio of the acid to the basic dissociation constants of the proteins in the equation: $(H^+)^2/Kw = Ka/Kb$, since, at the isoelectric point, we can express the sum of the active valences by one constant, Ka, and of the active basic valences by Kb.

The slope of a tangent drawn to the titration curve at the isoelectric point graphically represents the apparent strength of

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the multivalent protein as acid and as base, for the greater the slope of the titration curve of equimolal equivalent protein solutions, the greater the amphoteric constants. However, the strength of the amphoteric constants of a protein appear greater, the smaller its molecular weight. The following are the approximate slopes of tangents to the titration curves of different proteins at their isoelectric points.

	(H^+) at isoelectric point.	Ka/Kb at isoelectric point.	Approximate slope of titration curve at isoelectric point.
Gluten	About 1×10^{-7}	1	2
Serum globulin	40×10^{-7}	1,600	10
Potato " (tuberin)	500×10^{-7}	250,000	13
Gelatin	200×10^{-7}	40,000	18
Egg albumin	160×10^{-7}	25,600	19

The slope is smallest for proteins which are insoluble in water at their isoelectric points (gluten, glutenin); steeper for proteins which are slightly soluble (serum globulin, tuberin); steepest for egg albumin which is relatively soluble at its isoelectric point.

Since experiments of another kind suggest that glutenin and serum globulin are precipitated as undissociated molecules at their isoelectric point, and since the degree of dissociation of a protein is a function of the strength of its amphoteric constants the differences manifested by the slopes of the tangents to these protein titration curves at their isoelectric points suggest a physicochemical basis for the present classification of simple proteins.

THE SYNTHESIS AND ELIMINATION OF HIPPURIC ACID IN NEPHRITIS: A NEW RENAL FUNCTION TEST.

By F. B. KINGSBURY and W. W. SWANSON.

(From the Biochemical Laboratory, University of Minnesota Medical School, Minneapolis.)

The rate of hippuric acid synthesis and elimination in normal individuals and in patients suffering from various renal disturbances has been studied, the dosage of sodium benzoate being 2.4 gm. or its equivalent of hippuric acid neutralized with sodium bicarbonate.

After ingestion of the above dosage of sodium benzoate the normal individual will eliminate 90 to 100 per cent of it in the form of hippuric acid within 3 hours, in most of the cases between 95 and 100 per cent.

In cases of chronic nephritis the rate of elimination is much slower, but is almost if not quite the same after benzoate ingestion as after hippurate ingestion. More evidence is brought out that the kidney has little to do with the synthesis of hippuric acid. No unconjugated or free benzoic acid is excreted in any case, normal or otherwise. These results are confirmatory of the work of Kingsbury and Bell with nephrectomized dogs.

Our findings confirm those of Lewis and Karr that hippuric acid is excreted more slowly after hippurate ingestion than after the equivalent benzoate ingestion in normal cases.

In nephritis and other renal disturbances the rate of hippuric acid excretion after benzoate ingestion is much slower than with the normal subject. This *rate of elimination* is used as the renal function test, not the *synthetic ability of the kidney* which was the criterion referred to by earlier investigators, since the kidney apparently is not concerned in the actual process of synthesis.

Our results apparently show that the above renal function test varies in the same general direction as the phenolsulfonephthalein test, being low when the latter is low, etc., but seems to be capable of showing kidney conditions to a much finer degree than the phenolsulfonephthalein test.

A CRYSTALLINE URIC ACID COMPOUND IN BEEF BLOOD.

By ALICE ROHDE DAVIS and STANLEY R. BENEDICT.

(From the Department of Chemistry, Cornell University Medical College, New York.)

One of us has reported the presence of a non-protein uric acid combination in beef blood. The compound has been isolated from whole blood which has been freed from protein by heat and acetic acid followed by colloidal iron. The uric acid was quantitatively removed with mercury and the compound precipitated from the filtrate by mercury in the presence of sodium acetate. The mercury was removed with hydrogen sulfide and, after con-

centrating the mercury-free filtrate, the compound crystallized in colorless square plates. The crystals are soluble in hot water and in a concentration of 10 mg. to 1 cc. or greater form on cooling a stiff, water-clear gel which when seeded crystallizes rapidly. The compound is not precipitated by silver-magnesia mixture but on hydrolysis shows the reactions of uric acid and of a pentose. A quantitative estimation of the uric acid in the compound gives the theoretical figure for a combination of uric acid and a pentose in molecular proportions.

Studies are being continued to include ultimate analysis and identification of the carbohydrate radical.

FURTHER OBSERVATIONS ON THE MECHANISM OF THE KETOLYTIC (ANTIKETOGENIC) ACTION OF GLUCOSE.

By P. A. SHAFFER.

(From the Department of Biological Chemistry, Washington University Medical School, St. Louis.)

The following evidence was presented in support of the hypothesis that the effect of glucose in inhibiting the appearance of the acetone bodies is due to a chemical reaction between some unknown derivative or oxidation product of glucose and acetoacetic acid; the product of the reaction being readily susceptible of further oxidation in the body, whereas acetoacetic acid (formed in the main from fatty acid) is, in the absence of its reaction with the glucose derivative, slowly if at all oxidized in the body. For the complete oxidation of fatty acid the simultaneous oxidation of a definite minimum molecular equivalent, probably 1 molecule, of glucose is necessary.

1. Diets, which in different human subjects barely cause the appearance of traces of acetone in breath and of the three acetone bodies in urine, supply 1 molecule of fatty acid to 1 molecule of glucose from all sources (carbohydrate, protein, and glycerol of fat). Such diets are approximately: 10 per cent protein calories, 10 per cent carbohydrate calories, and 80 per cent fat calories, of the total energy requirement of the subject. Diets containing relatively more carbohydrate do not give rise to acetone bodies in the non-diabetic.

These facts enable a rational calculation of low carbohydrate diets which should avoid acetone body production.

2. The respiratory quotient of fasting subjects, or those receiving such diets as will produce traces of acetone bodies, is usually, *when acetone bodies first appear*, 0.74 to 0.76, which values indicate the oxidation at such times of fatty acid and glucose in approximately equimolecular quantities.

3. *In vitro* experiments show that acetoacetic acid in alkaline solution is very slowly oxidized by hydrogen peroxide, while if glucose, fructose, or glycerol is present, the acetoacetic acid rapidly disappears even at room temperature. 1 molecule (or possibly ½ molecule) of the "ketolytic" substance appears to react with, and accomplish the oxidation (or decomposition) of 1 molecule of acetoacetic acid. This phenomenon is believed to be analogous to the "antiketogenic" action of glucose in the body. Butyric acid, hydroxybutyric acid, and acetone do not appear to give similar reactions with glucose; and lactic acid has *in vitro* no ketolytic action.

THE LIPOID BALANCE IN THE BLOOD.

By W. R. BLOOR.

(From the Laboratories of Biochemistry and Pharmacology, University of California, Berkeley.)

In previous communications to the Society it has been pointed out that there is a relationship between fat, lecithin, and cholesterol and also between cholesterol and its esters, in normal blood, and that this relationship is constant within rather narrow limits for the individual and within wider limits for the species. Various factors, such as the absorption of fat, may disturb the balance but in most cases it is soon restored either by elimination of the disturbing constituent or in case that is not immediately possible (as in lipemia) by raising the level of all the lipoid constituents until the equilibrium is established at a higher level. The present communication is a report of a continuation of the study consisting of examination of the lipoid balance under conditions such as fasting, hemorrhage of various kinds, injection of strong salt solutions, etc. In general it appears that if the experimental procedure is severe enough in its effects to produce a marked

disturbance, the balance tends to preserve its constancy although at a higher or lower level than normal while if the disturbance is not severe, one or another of the lipoid constituents may change independently. Lecithin and cholesterol especially tend to preserve a constant relationship to each other while fat is much more variable. There appears to be a threshold both in amount and time within which changes may occur in one of the lipoid constituents without disturbing the others. Thus there may be a moderate change of fat alone for a short time without any change in lecithin or cholesterol. A greater change or longer lasting change in fat will result in changes in the lecithin while cholesterol appears to be least readily affected by changes in fat.

THE NATURE OF BLOOD CLOTTING, AS VIEWED FROM THE ACTION OF TISSUE EXTRACTS.

By C. A. MILLS and G. M. GUEST.

(From the Biochemical Laboratory, University of Cincinnati, Cincinnati.)

The addition of lung extract, well sedimented in a centrifuge, and calcium to a citrated horse plasma in various proportions gives fibrin yields varying from 1 to 156 per cent greater than that obtained merely by recalcifying such plasma without tissue extract addition. This added material seems to be chemically bound, since inert suspended material, such as starch paste or diluted boiled egg white, is removed in the washing of the fibrin to a very much greater degree.

The phospholipin of the active tissue coagulant is essential to such fibrin increase, since, if it is removed by benzene at room temperature, no such increases take place and the clotting is greatly inhibited.

Regarding 41.6 per cent of the added active material as phospholipin, it was found that thorough phospholipin extraction of the fibrins never yielded as much phospholipin as it was calculated had entered into the fibrin. That the missing phospholipin is fixed in the fibrin is indicated by the presence of much larger amounts of phosphorus in the phospholipin-extracted fibrin than in the normal fibrin.

Since the protein-phospholipin compound of tissue extracts not only accelerates blood clotting, but also seems to enter directly

into the composition of the fibrin, and since the phospholipin is essential to such action and is in part bound in the fibrin in a non-extractable form, it is considered that the union of the tissue substance to the blood fibrinogen takes place through the phospholipin molecules. Calcium also being necessary for clotting and being found in the washed fibrin in definite amounts, it is thought that the union of the phospholipin to blood fibrinogen may be through the calcium atom, both the tissue and blood fibrinogen being negatively charged so as to combine with the doubly positive calcium atom.

Thrombin is considered as an end-product of clotting (Wooldridge, Nolf) and not as a factor in bringing about the normal clotting of blood.

Antithrombin, made artificially by removing the phospholipin from the active tissue coagulant, is similar to the antithrombin of peptone plasma in that it is coagulated by heat and may readily be neutralized by cephalin or tissue extract. When neutralized by cephalin, not only does the antithrombic action disappear, but the action becomes strongly thromboplastic. Thus this antithrombin differs from the active tissue coagulant only in its lack of cephalin.

THE ANTISCORBUTIC PROPERTIES OF COMMERCIALLY DRIED ORANGE JUICE.

By J. F. McCLENDON, W. S. BOWERS, and J. P. SEDGWICK.

(From the Department of Physiology, University of Minnesota Medical School, Minneapolis.)

(The method of drying is reported under separate title.) The dried juice was fed to guinea pigs on a basal diet of equal parts of alfalfa meal and white flour. The quantities are expressed in cc. of original juice in order to compare them with fresh orange juice. Thirteen guinea pigs of small size were used. Three on the basal diet declined rapidly in weight and died. One fed 0.25 cc. per day lost 5 per cent in weight in 5 weeks and died of scurvy. One fed 0.5 and another 0.75 cc. maintained weight but died of scurvy. All the others gained in weight and the gain was more or less proportional to the quantity of juice. Those getting 1.25 cc. or more juice never developed scurvy. Since

this is about as favorable an outcome as with fresh juice, we conclude that the drying did not significantly impair the antiscorbutic properties of the juice. We fed it to infants.

Juice freed from water-soluble B, colloids (pectin), citric acid, and calcium retained antiscorbutic properties.

THE COMMERCIAL DRYING OF ORANGE JUICE RETAINING ITS ANTISCORBUTIC PROPERTIES.

By J. F. McCLENDON and S. M. DICK.

(From the Department of Physiology, University of Minnesota Medical School, Minneapolis.)

A unit or dehydrating cell consists of an octagonal chamber 20 feet in diameter and 20 feet high with 12 hot air ports in three tiers. The upper tier admits air at a velocity of 300 feet per minute, the middle at 600 feet, and the lower 150 feet. The centrifugal spray is in the center and just below the level of the upper tier of ports. It consists of a hollow shaft (admitting the juice) and a head made of a series of concave disks serrated at their margins (12 inches in diameter). Between the disks is a series of grooved collars distributing the juice from the hollow shaft to the concave sides of the disks. The shaft and head rotate on ball bearings 5,000 revolutions per minute and transform the juice into a fine spray or fog. The air currents cause eddies and retard the falling of the spray until it is dried. The exhaust ports are at the bottom of the cell and are so large as not to retard the falling of the dried juice in the dead air space. The incoming air is heated to 55–70°, but the evaporation on the surface of the droplets prevents their reaching this temperature before they fall into the dead air space where the temperature is lower. Since the droplet is exposed but 1 minute to the hot air, any temperature effect on the vitamine is minimized. Orange juice is one-eighth solids. If it is evaporated to one-fifth of the original weight the pectin forms a clot from which a syrup oozes. This separation is not hastened by the centrifuge but is by a filter press. If the juice is evaporated to one-sixth or less, no clot separates. The high content in monosaccharides makes it hygroscopic when dried. The taste is practically unaffected and the dried juice, reconstituted with water, makes a pleasant drink. Cane-sugar may be added before drying.

Milk is dried in the same type of cell and the orange preparation may be added to milk powder to restore the antiscorbutic lost in pasteurization. (The milk is first pasteurized since wild yeasts and some bacteria are not killed by drying.) The orange juice imparts a lactic acid flavor to the reconstituted milk in proportion to the amount added, but the milk does not curdle unless enough citric acid (juice) is added to bring it to the isoelectric point of casein.

THE ANTISCORBUTIC PROPERTY OF SOME DESICCATED FRUIT JUICES.

By MAURICE H. GIVENS AND ICIE G. MACY.

(From the Research Laboratories, Western Pennsylvania Hospital, Pittsburgh.)

If the proper nutrition of man is to be maintained it is necessary that he receive an adequate intake of the antiscorbutic vitamine as well as the other known dietary essentials. The influence of temperature on this vitamine is marked, especially if the heat is excessive. Therefore it is important to determine whether this essential substance is present in foods after *any* heat treatment.

Desiccated foods are now offered for human consumption. Such dried products have been subjected to more or less heat in their preparation. By feeding them daily to guinea pigs on a diet of oats, bran, and hay we have investigated the antiscorbutic potency of the following substances dehydrated by a known commercial process:[1] lemon juice, raw and raw neutralized; grape juice; tomato juice; raspberry juice; grapefruit juice; and orange juice. All these substances except grape juice and raspberry juice contained a significant amount of the antiscorbutic vitamine.

Whether or not dried foods will retain their antiscorbutic potency after long ageing is important. We have reported previously that dried orange juice was a satisfactory antiscorbutic agent.[2] It can now be added that this material is still effective

[1] Our best thanks are due the Merrell-Soule Company, Syracuse, New York, for kindly preparing for us the dried products.

[2] Givens, M. H., and McClugage, H. B., *Am. J. Dis. Child.*, 1919, xviii, 30.

in this respect 2 years after dehydration. All the above substances with the exception of dried raw lemon juice were from 14 to 20 months old. Details of this work as well as a study of dried strawberry and pineapple juices will be published in the near future.

HYDROLYSIS OF LACTALBUMIN.

By CARL O. JOHNS AND D. BREESE JONES.

(From the Protein Investigation Laboratory, Bureau of Chemistry, United States Department of Agriculture, Washington.)

Lactalbumin, or the total coagulable protein of milk after removal of the casein, was hydrolyzed by boiling for 4 hours with 20 per cent hydrochloric acid. The following percentages of amino-acids were determined: proline 2.25, aspartic acid 9.37, glutaminic acid 13.00. Evidence has been obtained showing the presence of considerable hydroxyglutaminic acid. One of the most striking results of this hydrolysis is the high yield obtained of aspartic acid, which is over nine times the amount found by Abderhalden and Pribram.[3]

GLYCINE AND GLYCYL-GLYCINE SYNTHESIS.

By J. LUCIEN MORRIS AND FLOYD DeEDS.

(From the Biochemistry Laboratory, Western Reserve University School of Medicine, Cleveland.)

The progress of amino-acid formation at successive stages of the reaction of ammonia with monochloroacetic acid was studied. Quantitative determinations of amino nitrogen and total nitrogen were made after driving off the ammonia. When aeration was used for the removal of ammonia the subsequent analytical data paralleled Kraut's observations by means of chloride determinations. When ammonia was removed by evaporation with calcium hydroxide, both amino nitrogen and total nitrogen values were irregular. Upon investigation of the irregularities, the conclusion was reached that glycyl-glycine is formed when glycine, monochloroacetic acid, and ammonia are treated with calcium hydroxide at water bath temperature. The formation of glycyl-

[3] Abderhalden, E., and Pribram, H., *Z. physiol. Chem.*, 1907, **li**, 409.

glycine under these conditions indicates a new method of peptide synthesis which differs from both the diketopiperazine and acid chloride methods. The extent of its application to the experimental preparation of peptides and possible relationships with amino-acid and peptide synthesis in metabolism are being further investigated in this laboratory.

EFFECT OF THE INJECTION OF SMALL AMOUNTS OF ADRENALIN CHLORIDE ON SOME OF THE CHEMICAL CONSTITUENTS OF THE BLOOD.

By ROGER S. HUBBARD and FLOYD R. WRIGHT.

(*From the Clifton Springs Sanitarium, Clifton Springs.*)

The effect of doses of 0.5 and 1 cc. of a 1 to 1,000 solution of adrenalin chloride on the blood sugar, carbon dioxide-combining capacity of the plasma, and acetone from acetone plus aceto-acetic acid, and from β-hydroxybutyric acid in the blood was studied in five normal subjects. It was found that, accompanying the decrease in carbon dioxide-combining capacity of the plasma, there was an increase in both the acetone bodies. This increase lasted for an hour and normal values were reached again after 2 hours, while the lowered carbon dioxide-combining capacity of the plasma persisted for at least 2 hours. No relationship could be demonstrated between increased blood sugar concentration and these changes in acetone and β-hydroxybutyric acid.

THE CONCENTRATION OF AMINO-ACIDS IN THE BLOOD IN RELATION TO FEED AND MILK SECRETION.

By C. A. CARY.

(*From the Research Laboratories of the Dairy Division of the United States Department of Agriculture.*)

Since it has been shown[4] that the proteins of milk are formed in the mammary gland from the amino-acids of the plasma of the blood one might expect to find some relation between the rate of formation of these proteins and the level of amino-acid N of the plasma. Experiments were tried with milking cows in which

[4] Cary, C. A., *J. Biol. Chem.*, 1920, xliii, 477.

the total nutriment and protein of their diet were changed and in which the protein alone or the total nutriment alone was altered.

As has frequently been found the protein of the diet, the milk yield, the percentage of protein in the milk, and total protein secreted daily varied together directly. In some instances and under some conditions the level of amino-acid N of the plasma varied with these factors. Under other conditions these factors could not be directly correlated with the amino-acid level in either the plasma or the whole blood. This lack of correlation was probably due to variations in the quality of the plasma mixture of amino-acids.

THE ANIMAL UTILIZATION OF PENTOSE.

By ELBERT W. ROCKWOOD and KRIKOR G. KHOROZIAN.

(*From the Department of Chemistry, the State University of Iowa, Iowa City.*)

Feeding experiments with cats, dogs, rabbits, and man showed that xylose is utilized by carnivorous, herbivorous, and omnivorous animals. The degree of utilization is markedly modified by variations in the rest of the diet, being greatest on a low carbohydrate diet and least when the diet contains much sugar and starch.

THE PHOSPHORIC ACID COMPOUNDS IN THE BLOOD OF NORMAL INFANTS.

By GRACE M. McKELLIPS and ISABEL M. De YOUNG.

(*From the Departments of Biochemistry and Pediatrics, University of California, Berkeley.*)

The work was undertaken preliminary to the study of these compounds in the blood of infants and young children in certain pathological conditions, especially anemia. The amounts and distribution of the various compounds in the corpuscles were found to be practically the same as in the corpuscles of adults. In the plasma the total phosphate is about 10 per cent lower, inorganic phosphate practically the same, while the lipoid phosphoric acid is 25 per cent lower and the organic considerably higher than in the plasma of adults.

CAN AN INDICATOR METHOD BE USED FOR MEASUREMENT OF CO_2 PRODUCTION FROM ISOLATED NERVE?

By SHIRO TASHIRO and E. M. HENDRICKS.

(From the Laboratory of Biochemistry, University of Cincinnati, Cincinnati.)

By immersing nerves in Ringer solution colored with an indicator and by determining extent of the change of the color, it has been claimed by some that the activated nerve does not give off more CO_2 than the resting. Tashiro has demonstrated elsewhere that the activated nerves give off a volatile base—presumably ammonia—which would tend to neutralize the CO_2 formed during excitation. The best way, therefore, to measure CO_2 from isolated nerve and other tissues as well is to estimate it as such and not as acidity produced thereby. If one prefers to use an indicator, three precautions should be taken: (1) Let it respire in CO_2-free air, not in Ringer solution; (2) let it respire in a chamber over non-volatile acid to remove a volatile base formed in the tissue; and (3) unbuffered alkaline solution must be used with the indicator. The new apparatus is presented to show these devices. Although less accurate and less delicate than the original $Ba(OH)_2$ method (biometer), with these devices an indicator method can be used for demonstration that during passage of the nerve impulse, the activated nerve does give off more CO_2 than the resting.

ISOLATION OF CITRIC ACID FROM NORMAL URINE.

By S. AMBERG and MARY E. MAVER.

(From the Laboratory of the Children's Memorial Hospital, Otho S. A. Sprague Memorial Institute, Chicago.)

Urine is treated with a slight excess of lead acetate, then ammonia is added to a rather strongly alkaline reaction. The filtrate is evaporated over a small flame to a small volume, whereby it becomes acid. It is treated with H_2S. The excess of H_2S is removed from the filtrate by boiling. On renewed evaporation some crystalline masses separate which are filtered off. To the filtrate Ba acetate is added and just enough $Ba(OH)_2$ to make it partly alkaline. Then about two to three times the volume of 95 per cent alcohol is added. The precipitate is filtered off and

washed well. It is extracted with hot water, acidified with H_3PO_4, and the Ba removed with H_2SO_4. The solution is made rather strongly acid with H_2SO_4 and evaporated on the water bath under addition of plaster of Paris to dryness. The dry residue is extracted in the Soxhlet with ether. The ether is evaporated and the residue taken up in water. Lead carbonate is added in slight excess under heating. Later NH_3 is added to strongly alkaline solution and filtered. The filtrate is evaporated and treated with H_2S. After removal of the H_2S from the filtrate, mercuric sulfate is added, the precipitate is filtered off, and the Hg removed by H_2S. The solution is again treated with lead carbonate, etc. To the final solution Ba acetate is added. A precipitate is formed which gives the Sabanin-Laskowski reaction and liberates CO when heated on the water bath with H_2SO_4 in a current of CO_2, whereby the solution does not blacken, but remains light yellow. From some of the Ba precipitate the Ba was removed with H_2SO_4 and the solution heated with Denigès' reagent ($HgSO_4$ in H_2SO_4), whereupon $KMnO_4$ solution was added. The precipitate was decomposed with H_2S and the filtrate extracted with ether and evaporated to dryness. The residue gives a strong purple color on addition of $FeCl_3$. It melts at about 117°C. Pure acetone dicarbonic acid melts at 134°C. The Hg was not determined for several reasons, chiefly because the precipitate obtained according to Denigès from citric acid is said not to be of uniform composition. Residue obtained in the manner described by Denigès for the preparation of acetone dicarbonic acid from citric acid gave a melting point of about 117°C. While Denigès gives the Hg content of the Hg compound obtained with acetone dicarbonic acid prepared according to Pechman, he fails to give the melting point either of the acetone dicarbonic acid prepared according to Pechman or of the one prepared in his way. The ether residue obtained from the substance isolated from urine as well as that obtained from citric acid gave the Legal test and the $FeCl_3$ test.

The barium precipitate yields pentabromoacetone. The barium precipitate was not pure. It did not have the water of crystallization or the proper Ba content after drying at 162°. The amount of CO obtained from this Ba precipitate corresponded to about the amount of pentabromoacetone derived from it.

THE ADAPTATION OF THE PENTABROMOACETONE METHOD TO THE QUANTITATIVE DETERMINATION OF CITRIC ACID IN URINE.

By W. D. McCLURE.

In Collaboration with H. McDonald.

(From the Laboratory of the Children's Memorial Hospital, Otho S. A. Sprague Memorial Institute, Chicago.)

Several difficulties arise in the quantitative determination of citric acid by the pentabromoacetone method in urine. Urine acidified with H_2SO_4 and treated with an excess of Br yields a precipitate which might interfere, although the treatment with $KMnO_4$ may minimize the possible error arising from this cause. Urine acidified and treated with animal charcoal gives very little of this Br precipitate, but urine treated this way loses citric acid. The Br precipitate is partly soluble in alcohol, ether, and acetone and the solution, on adding water, becomes very turbid. Such precipitate heated to 100–105°C. for 9 hours loses about 25 per cent of its weight, while pentabromoacetone is all lost. Mixtures of pentabromoacetone and this Br precipitate when heated lose a little more weight than corresponds to the amount of pentabromoacetone present. If, therefore, the precipitate obtained by the pentabromoacetone method from the urine is heated for several hours at 100–105°C., the possible error from the admixture of a Br precipitate other than pentabromoacetone is minimized considerably.

STUDIES ON THE INFLUENCE OF PHENYLCINCHONINIC ACID, SALICYLIC ACID, AND SOME OF THEIR DERIVATIVES ON RENAL EXCRETION.

By VICTOR C. MYERS and JOHN A. KILLIAN.

(From the Laboratory of Pathological Chemistry, New York Post-Graduate Medical School and Hospital, New York.)

It is now well known that the administration of salicylates and phenylcinchoninic acid (cinchophen) results in an increased excretion of uric acid in the urine and a marked drop in the uric acid content of the blood. An effort has been made to obtain further light on the mechanism of the action of these drugs by experiments on both man and animals. Studies on the effect of these drugs, especially cinchophen and the ethyl ester of its

methyl derivative (tolysin), on the composition of the blood have been made in a series of more than forty human cases, data on ten cases being presented.

In four cases the blood urea, as well as the uric acid, was influenced. The action on the uric acid was very pronounced in all four cases, while the urea nitrogen dropped from 25 to 29 mg. per 100 cc. to practically normal figures. It has previously been reported that of the three nitrogenous waste products, uric acid, urea, and creatinine, uric acid is the most difficultly eliminated, with urea standing in somewhat of an intermediate position. One would therefore expect that drugs, which have a general stimulating effect on the kidney, would effect the excretion of uric acid first, urea next, and creatinine last. If the observations reported for urea are correct, it would indicate that the action of these drugs on the kidney is not absolutely *specific* for uric acid, as has generally been considered.

Data were presented on three cases of arthritis, which were clinically benefited by the administration of tolysin, although in this case the influence on the uric acid was slight. In one case which received daily for 10 days 200 grains (four times the ordinary therapeutic dose), there was a tendency to return to the initial blood uric acid level. It appears that the analgesic effect of these drugs is unrelated to their influence on renal excretion.

In one case with comparatively slight renal involvement, tolysin apparently had no influence on the blood uric acid.

In no case did the administration of cinchophen or tolysin cause proteinuria, nitrogen retention, or diminished phenolsulfonephthalein output—thus differing from the data reported by others for salicylates.

Some preliminary studies have been made on the action of other phenylcinchoninic acid derivatives.

STUDIES ON LACTIC ACID.

By S. W. CLAUSEN.

(*From the Department of Pediatrics, Washington University Medical School, St. Louis.*)

A modified Ryffel method is described for 1 or 2 cc. amounts of blood, determining the α-hydroxy acids as lactic acid. Normal figures in children vary from 18 to 35 mg. per 100 cc. and average

25 mg. In cases of alimentary intoxication in infants, the lactic acid is increased; and the alkali reserve decreased. One severe case showed lactic acid of 180 and a carbon dioxide-combining power of plasma of 21 volumes per cent. This acidosis may yield to measures which supply water to the tissues and restore the blood volume and blood flow to normal. Alkaline therapy unless very guarded may result in tetany. A case of severe cardiac decompensation with lactic acid of 160 and CO_2 of 20, recovered symptomatically after bleeding, and without the use of alkalies, the lactic acid falling to 35 and the CO_2 rising to 50.

OSTEOPHAGIA IN CATTLE.

By H. H. GREEN.

(From the Veterinary Research Division, Pretoria, South Africa.)

Investigations, under the direction of Sir Arnold Theiler, on lamziekte,[5] a fatal bovine disease in South Africa, have concentrated attention upon a form of bone eating (osteophagia). Affected cattle search for bone debris and eagerly devour their finds though these may still be putrid. Acute osteophagia is displayed by almost all the cattle in particular regions for 10 months of the year, only abating while the grass is still very young. The abnormal craving is shown to be due to phosphorus deficiency in soil and vegetation, can be produced experimentally upon phosphorus-low rations, and removed by administration of phosphorus compounds or by phosphatic manuring. In itself osteophagia is relatively harmless and has no independent mortality incidence, although it may affect the growth of young stock and the milk yield of cows. Its great economic importance arises from its position in the etiological chain of lamziekte, summarized as follows: (1) A toxin which poisons the cattle, (2) produced by toxicogenic saprophytic anaerobes, (3) in carcass debris, (4) which is only eaten by cattle suffering from osteophagia, (5) due to phosphorus deficiency in soil and vegetation.

[5] A preliminary report by Theiler, in collaboration with Viljoen, Green, du Toit, and Meier, may be obtained from the Director of Veterinary Research, Box 593, Pretoria, Union of South Africa.

THE DETERMINATION OF IODINE IN THE BLOOD AND TISSUES.

By E. C. KENDALL.

(From the Division of Chemistry, Mayo Foundation, Rochester.)

Thyroxin contains 65 per cent of iodine. The maximum concentration of thyroxin in the blood and tissues may be established by determining the amount of iodine in the blood and tissues. Whether 100 per cent of the iodine present is in the form of thyroxin cannot be known, but the iodine content establishes the maximum content of thyroxin. Recent improvements in the determination of iodine have permitted the study of the iodine content of the blood, tissues, and food. The normal content of iodine in the blood and tissues and in certain pathological conditions will be outlined.

RELATION OF THE NERVOUS SYSTEM TO THE PARATHYROIDS.

By FREDERICK S. HAMMETT.

(From the Wistar Institute of Anatomy and Biology, Philadelphia.)

Two series of observations were made of the effect of thyreoparathyroidectomy and parathyroidectomy on the albino rat. The animals of one series were progeny of rats which had for several generations been subjected to constant contact with men so that their natural defensive instincts of fear and rage had been submerged, the result of which was to increase the stability of their emotional neural activities and consequently their metabolism. The other series was made up of rats from the standard colony in which no such regulation had been established, and in which there was evident a condition of excitability expressed as timidity and characteristic irritability with consequent metabolic instability. The first series is designated as "gentled" the second as "untamed." All rats were originally from the same ancestral group. The removal of the parathyroids or of the complete thyroid appàratus from the gentled rats gave but a 16 per cent mortality. Similar operative procedure on the untamed group gave a mortality of 79 per cent, all rats dying in evident parathyroid tetany. No sex variations were obtained. These results are interpreted as being primarily due to differences

in stability of the nervous system with consequent differences in metabolic stability and show the great dependency of emotional and metabolically unstable animals on the secretion of the parathyroid glands.

OBSERVATIONS ON THE OCCURRENCE OF CREATININE AND CREATINE IN BLOOD.

By H. JEANETTE ALLEN and STANLEY R. BENEDICT.

(From the Department of Chemistry, Cornell University Medical College, New York.)

Repeated extractions of the protein residue of blood (whether obtained by picric acid precipitation or by heat coagulation) show a continuous yield of creatinine by the colorimetric reaction. A practically minimum yield corresponding to about 0.3 mg. of creatinine per 100 cc. of blood is finally obtained. From certain considerations we may tentatively conclude that about 0.5 mg. of the creatinine found per 100 cc. of blood (beef, dog, or human) is certainly not creatinine.

In connection with creatine determinations in blood we have found that heating with weak acids (picric, acetic) gives a much higher yield of creatine than when hydrochloric acid is employed. This is contrary to the behavior of creatine in pure solution or when added to blood. Details of the work will be published in the near future.

A NEW REAGENT FOR THE ESTIMATION OF SUGAR IN NORMAL AND DIABETIC URINE.

By JAMES B. SUMNER.

With the assistance of Vi A. Graham.

(From the Department of Physiology and Biochemistry, Medical College, Cornell University, Ithaca.)

A new reagent, 4-6-dinitroguaiacol, is reduced to the intensely colored 4-nitro-6-aminoguaiacol when heated with solutions of glucose in the presence of sodium carbonate. The reaction is quantitative and the amount of glucose can be determined by colorimetric comparison of the unknown solution with a standard.

Since dinitroguaiacol itself has nearly the same quality of color as nitroaminoguaiacol, the coloration due to the excess of dinitroguaiacol is subtracted in calculating the results.

The advantages of dinitroguaiacol as a reagent for sugar in urine are due to the fact that it is reduced by no other of the urinary constituents than the reducing sugars. Uric acid and possibly polyphenols cause some increase in color when glucose is present. These substances are removed by using norite.

Parallel determinations by the new method and by the method of Benedict and Osterberg[6] for the estimation of sugar in normal urine have been made upon samples of diabetic, sugar-free diabetic, nephritic, and normal urine.

SOME OBSERVATIONS ON LOSS OF NITROGEN IN MUSCULAR ATROPHY IN MAN AND ANIMALS.

By AMOS W. PETERS and A. E. GUENTHER.

(From the College of Medicine, University of Nebraska, Omaha.)

These experiments were made as a beginning on the determination of the nature of the loss of weight incurred by muscular atrophy consequent on nerve section. They resulted from an endeavor to correlate or explain observations we had made on the chemical pathology of human cases of muscular atrophy.

The continuity of the sciatic nerve in one leg of each rabbit was interrupted by the removal of a section of nerve about 2 cm. long. After 3 weeks the rabbits were killed and examined to determine the success of the nerve section and comparison of the extensor and soleus muscles of the two legs showed a very marked diminution in volume of these two muscles in the leg the sciatic nerve of which had been sectioned. Various physical and chemical comparisons were made of the normal and atrophied muscles of eleven rabbits taken individually and subsequently of a group of four rabbits of which the muscles of the same kind were compounded for analysis. The following is a summary of the *averages* of the results obtained.

[6] Benedict, S. R., and Osterberg, E., *J. Biol. Chem.*, 1918, xxxiv, 195.

	Eleven rabbits, individual determinations.				
	Average loss of weight. Atrophied.	Average loss of water.		Average total N content.	
		Normal.	Atrophied.	Normal.	Atrophied.
	per cent	*per cent*	*per cent*	*per cent*	*per cent*
Soleus muscle........	From 23.9 to 45.0.	79.0	79.1	3.08	2.66
Extensor, 1st digit...	" 5.81 " 56.7.	78.2	78.8	3.36	3.07
	Four rabbits, group determination.				
Soleus muscle........		78.5	79.1	3.08	2.66
Extensor, 1st digit...		76.4	79.5	3.00	2.20

The total loss of weight in the atrophied muscle is physiological, *i.e.* occurred in the living animal and is quite variable, though by mere inspection even, the loss in size is very marked. The practical equality of the loss of water on desiccation at about 60–70°C. for both normal and atrophied muscles shows that the atrophy is not dependent on loss of water. The results on the total nitrogen content show an unmistakable loss of nitrogen by the atrophied muscle. Our experiments have not as yet been carried far enough to determine what form of combined nitrogen was lost. The loss of nitrogen observed should be compared with similar results obtained by Greene[7] on the muscle of king salmon during the fast of spawning migration. The rabbit muscles show a marked maintenance of physiological capacity although atrophied, as shown by experimental tests (unpublished).

A 4 HOUR METABOLISM PERIOD.

I. THE ELIMINATION OF URINARY NITROGEN AND CHLORINE IN NORMAL MEN AND WOMEN.

By C. FERDINAND NELSON and MAY KINNEY.

(*From the Laboratory of Biochemistry, University of Kansas, Lawrence.*)

The elimination of total nitrogen, urea, uric acid, creatinine, and sodium chloride in nine healthy individuals was measured over 4 hour periods at irregular intervals varying from 3 weeks

[7] Greene, C. W., *J. Biol. Chem.*, 1919, xxxix, 435.

to 9 months. On a standard meal consisting of three shredded wheat biscuits, 1 pint of whole milk, 50 gm. of cane-sugar, and two glasses of water (preceded by an ordinary breakfast of fruit, toast, and coffee, with or without an egg) average variations in urinary outputs were obtained as follows:

	gm.	per cent of total nitrogen
Total nitrogen	0.06	
Urea	0.07	1.6
Ammonia	0.014	0.77
Uric acid	0.007	0.2
Creatinine	0.004	0.2
Sodium chloride	0.73	

The practicability of using the above test meal and 4 hour metabolism period in determining kidney functioning is being investigated.

RATE OF NITROGEN ELIMINATION.

By H. O. POLLOCK and W. S. McELLROY.

(From the Department of Physiological Chemistry, School of Medicine, University of Pittsburgh, Pittsburgh.)

The results of experiments on the rate of nitrogen elimination in dogs following the ingestion of protein fed in the form of lean beef are reported. Immediately after the ingestion of the meat the urine was collected at 2 hour intervals by catheterization and blood drawn from a vein at 1 hour intervals. The total nitrogen and urea nitrogen output in the 2 hour urines and the total non-protein nitrogen and urea nitrogen in the 1 hour blood samples were determined by analysis.

A parallelism was found between the curves for the total nitrogen and urea nitrogen output in the urine and the non-protein nitrogen and urea nitrogen in the blood.

The variations in the curves of nitrogen excretion we attribute to variations in the rate of alimentation and absorption, as indicated by the curves for the non-protein nitrogen and urea nitrogen in the blood.

THE OXIDATION OF CYSTINE IN THE ANIMAL BODY.

By HOWARD B. LEWIS and LUCIE E. ROOT.

(From the Laboratory of Physiological Chemistry, University of Illinois, Urbana.)

A study of the conditions which determine the oxidation of the sulfur fraction of the cystine molecule has been made. The amino group of the cystine has been protected from deamination by conjugation with phenylisocyanate to form phenyluraminocystine and the behavior of this compound in the organism of the rabbit studied. The phenyluraminocystine was administered *per os* or subcutaneously, in solution in NaOH or Na_2CO_3. After parenteral administration there was a slight increase in the elimination of SO_4 sulfur but the greater portion of the ingested sulfur was eliminated as unoxidized sulfur. After subcutaneous injection no oxidation of any kind was observed, the sulfur of the molecule being recovered in the urine as unoxidized sulfur. Control experiments on the same animals in which cystine was fed or injected showed an almost quantitative oxidation of the sulfur to sulfates. These experiments indicate that the oxidation of the sulfur fraction of the cystine molecule is probably connected with the process of deamination or with the oxy-acids, products of deamination.

THE NUTRITIVE VALUE OF THE PROTEINS OF THE DRIED YELLOW PEA, PISUM SATIVUM.

By CARL O. JOHNS and A. J. FINKS.

(From the Protein Investigation Laboratory, Bureau of Chemistry, United States Department of Agriculture, Washington.)

Nutrition experiments with the dried yellow pea, *Pisum sativum*, show that when cooked, and when the other necessary non-protein dietary ingredients are added, it furnishes adequate protein and water-soluble vitamine for the normal growth of albino rats. Cystine is not required, as in the case of the seeds of the genus *Phaseolus*.

THE PROTEINS OF THE TOMATO SEED, SOLANUM ESCULENTUM.

By CARL O. JOHNS and CHARLES E. F. GERSDORFF.

(From the Protein Investigation Laboratory, Bureau of Chemistry, United States Department of Agriculture, Washington.)

Experimental studies on the proteins of tomato seed have shown that a 4 per cent aqueous solution of sodium chloride extracts the maximum amount of protein; namely, 60 per cent of the total protein (calculated from N × 6.25). Fractional precipitation with ammonium sulfate disclosed the presence of two globulins, both high in sulfur, containing 1.07 and 1.80 per cent respectively. No indication of the presence of albumin or glutelin has been found.

FATE OF PHENYLACETYL DERIVATIVES OF THE AMINO-ACIDS IN THE ANIMAL ORGANISM.

By CARL P. SHERWIN and GEORGE J. SHIPLE.

(From the Chemical Research Laboratory, Fordham University, New York.)

Phenylacetyl ornithine was isolated from the excreta after feeding phenylacetic acid to hens. Phenylacetyl glutamine was isolated from urine after the ingestion of phenylacetic acid by human beings. The following compounds were prepared synthetically:

Phenylacetyl glycocoll (phenaceturic acid).
" alanine.
" glutamic acid.
" asparaginic acid.
" asparagine.
" tyrosine.
" leucine.

The tyrosine, alanine, and leucine compounds which had not previously been prepared were studied. All these phenylacetyl derivatives were fed or injected, using dogs, rabbits, chickens, and human beings as subjects. The glutamine, glycocoll, and ornithine compounds proved non-toxic when introduced into the organisms where they do not occur as detoxication products of phenylacetic acid. Phenylacetyl glutamine undergoes partial hydrolysis into phenylacetyl glutamic acid and partial cleavage

into phenaceturic acid when fed to the dog. Phenylacetyl ornithine undergoes destruction when fed to the dog or human being, but apparently not when injected. Phenylacetyl alanine, phenylacetyl leucine, and phenylacetyl tyrosine undergo little or no alteration when fed.

A METHOD FOR THE UTILIZATION OF THE NUTRITIVE SALTS OF SEA WATER.

By J. F. McCLENDON.

(From the Department of Physiology, University of Minnesota Medical School, Minneapolis.)

Little is known of the quantities of the minor inorganic constituents of foods or of our requirements of them. There seems likely to be a shortage of some of them unless we use products from seaweed or sea salts. The evaporation of sea water produces an unstable, hygroscopic, bitter, and laxative mass. On heating the residue to complete dryness, HCl is evolved and the formation of insoluble carbonates and double salts abolishes the bitter and hygroscopic properties. My method is to add 6 gm. of H_3PO_4 per liter to ocean water and evaporate by heat to complete dryness. The resulting mass is non-hygroscopic and of pleasant taste and contains possibly every metallic element. It may be used in cooking or as shaker salt, and insures iodine for the thyroid and fluorine for bones and teeth as well as more common elements. It may be added to common salt to prevent caking of the latter and improve its nutritive qualities.

POWDERED SPINACH AND SEA SALTS IN THE DIET OF ADULTS AND INFANTS.

By J. F. McCLENDON and J. P. SEDGWICK.

(From the Department of Physiology, University of Minnesota Medical School, Minneapolis.)

Spinach is difficult to obtain fresh in winter and yet seems indispensable. Carrots are substituted for spinach in winter as an addition to a milk diet. Milk is deficient in iron and pasturized milk in vitamines. On the basis of calorific units, spinach has ten times as much iron as is contained in carrots. Spinach

has twice as much bone-forming mineral and fat-soluble A as is contained in carrots. By drying and reducing to a powder, the digestibility of spinach is increased and its preparation for consumption facilitated. Powdered spinach mixed with 6 per cent of sea salts will keep indefinitely and may be prepared for an infant by boiling in water 2 minutes or more.

Adults may not like the taste of dried spinach and are especially liable to avoid the pot-liquor that contains about half of some of the valuable constituents (vitamines, salts).

Bread was made of yeast and powdered spinach 1 per cent, soy bean meal 10, sea salts 5, and wheat flour 84 per cent. Rats grew at two-thirds the normal rate on this diet alone.

The mixture was standardized on rats. The addition of 5 per cent of powdered spinach doubled the increase in weight of weaned rats on a whole wheat-casein-salt-mixture diet. These rats weighed 30 gm. As the weight increased the percentage of spinach for normal growth decreased so that 1 per cent of spinach was sufficient for rats of 100 gm. With 2.5 per cent of spinach in above bread and substitution of part white with Graham flour the gain was three-fourths the normal rate and was not affected by substitution of corn for wheat.

	Average gain per wk.
	ounces
10 babies	2.3
With spinach	3.2
" " and $\frac{1}{12}$ oz. dried ($\frac{1}{2}$ oz. fresh) orange juice	5.7
6 babies	2.2
With spinach	4.4
" " and $1\frac{1}{2}$ oz. fresh orange juice	11.2
8 babies	2.4
With spinach	10.9

INFLUENCE OF PHYSICAL AND CHEMICAL TREATMENT ON THE ADSORPTIVE CAPACITY OF FULLERS' EARTHS.

By G. H. A. CLOWES, A. L. WALTERS, and G. B. WALDEN.

(*From the Biochemical Research Laboratory, Eli Lilly and Company, Indianapolis.*)

Following the experiments reported last year on the adsorptive capacity of fullers' earths for alkaloids and their release in physical and biological systems, experiments have been conducted to

determine the influence of chemical and physical treatments on the adsorptive capacity and other properties of Lloyd's reagent. The iron and alumina present in Lloyd's reagent may be removed by treatment with sulfuric acid. The adsorptive capacity for alkaloids appears to be a direct function of the amount of iron and aluminum so contained in the Lloyd's, and to be adversely affected by silica.

Water loss on heating causes a similar effect, the adsorptive curve for alkaloids falling off with loss of water.

The toxicity of Lloyd's reagent when introduced into the peritoneal cavity of rats or guinea pigs appears to follow a similar curve showing a rapid falling off in toxicity with loss of iron and aluminum and also with water, but in the case of iron and aluminum a minimum toxicity point is reached when about 60 per cent of the iron and aluminum has been removed.

THE CHEMICAL NATURE OF THE ACTIVE BLOOD COAGULANT OF TISSUE EXTRACTS.

By C. A. Mills.

(*From the Biochemical Laboratory, University of Cincinnati, Cincinnati.*)

The active blood coagulant of tissue extracts is not cephalin alone as claimed by Howell, since a milky cephalin emulsion will not quicken the clotting of blood any more than will a saturated lung extract diluted 500 to 1,000 times. Furthermore, such a cephalin emulsion injected intravenously into rabbits in doses up to 5 cc. fails to produce any symptoms, while 0.05 cc. of the saturated lung extract kills the rabbit within a minute by intravascular clotting.

The active coagulant is a protein phospholipin compound, having the solubility characteristics of a globulin, and an isoelectric point at a H^+ concentration of $N \times 10^{-5}$ to 10^{-6}. For a moderately strong extract the addition of acid sufficient to give a calculated acidity of 0.002 N produces complete precipitation of the active substance, the supernatant fluid having the above mentioned H^+ concentration. Properly precipitated and redissolved, the entire coagulative activity of tissue extracts can be recovered through this one substance, while the remaining proteins present in the extract are without action.

This purified compound consists of 41.6 per cent of phospholipin and 58.4 per cent of protein. Accepting the usual molecular weight figures for these substances as cephalin 800 and protein 16,000, a molecule of the active compound would consist of 1 protein molecule to which are attached about 14 phospholipin molecules. Both protein and phospholipin fractions are essential to the activity on coagulation for the following reasons:

1. Addition of acid or alkali sufficient to form *m*-proteins while apparently improving the state of solution of the dissolved material, destroys the coagulative activity almost entirely. This is probably only through change in the condition of the protein fraction.

2. Digestion with a lipase-free trypsin solution very rapidly inactivates the material. Digestion with a strong lipase solution almost trypsin-free also produces complete inactivation but at a much slower rate.

3. Extraction of the phospholipin with fat solvents that leave the protein unaltered in its solubility, gives a protein practically phospholipin-free, which acts to inhibit strongly the normal clotting of blood *in vivo* or *in vitro*. Addition of the phospholipin back to this antithrombic protein restores the positive coagulation activity more than 100 times as much as the activity of the cephalin alone could account for.

4. The addition of an excess of fresh lung phospholipin to a lung extract of any strength will increase its coagulative activity about fourfold. This is an increase many thousand times that to be accounted for by such an amount of cephalin acting alone.

THE HYDROLYSIS OF SUCROSE IN THE STOMACH OF MAN.

By HOWARD B. LEWIS and ROBERT M. HILL.

(*From the Laboratory of Physiological Chemistry, University of Illinois, Urbana.*)

The hydrolysis of sucrose *in vivo* in the human stomach has been studied by the fractional method. Following ingestion of protein (egg white, cottage cheese washed as free as possible from reducing sugars), sucrose has been administered and samples of the gastric contents were removed at 20 minute intervals. These were analyzed for reducing sugar, and for total carbohydrate

present after hydrolysis with HCl. Portions were also incubated for 2 hours and reducing sugar was determined after incubation. The results of the experiments showed a slight hydrolysis of sucrose in the first period, with slightly greater hydrolysis in succeeding periods. The velocity with which the sucrose left the stomach, however, made these effects almost negligible in relation to the total amount of sucrose ingested. Although intestinal regurgitation has occurred in almost every case, no evidence was obtained of the presence of an active intestinal sucrase in the gastric juice. The degree of hydrolysis observed seemed to be due entirely to the acidity of the gastric juice and to be negligible for practical considerations.

A VOLATILE SPERM-STIMULATING SUBSTANCE DERIVED FROM MARINE EGGS.

By G. H. A. CLOWES and E. BACHMAN.

(From the Biochemical Research Laboratory, Eli Lilly and Company, Indianapolis.)

The eggs of starfish, sea urchins, and sand-dollars give off a volatile substance which exerts, under extraordinary dilutions, a non-specific stimulating influence on sperm and promotes the fertilization of eggs by sperm so stimulated.

The quantity of this substance produced is directly proportional to the number of eggs employed and its production is maintained for a period of several days from mature sea urchins' eggs. It may be distilled from the extract and the distillate exerts an effect almost equal to that of the original extract. It is not destroyed by heating in a sealed tube in an autoclave for several hours in a neutral or alkaline solution, but is weakened by heating with acid.

It is extraordinarily susceptible to the action of oxidizing agents like iodine, 10 minutes contact with an 0.002 N iodine solution prior to removal of excess iodine with thiosulfate lowering the activity of the solution to one-hundredth of its original strength.

Similar sperm-stimulating and fertilization-promoting effects are exerted by upwards of 50 simple organo substances of the type of propyl, allyl, and cinnamyl alcohol, propylene, etc., which function at a dilution of 1:100,000,000 or more. The

stimulating substance derived from the eggs is in all probability a mixture of volatile autolytic products. It probably contains unsaturated groups and has a boiling point not far removed from that of water, but on account of the difficulty in procuring sufficient material at the end of the season, no individual volatile substance has thus far been identified.

THE FAT-SOLUBLE VITAMINE.

By H. STEENBOCK, MARIANA T. SELL, E. M. NELSON, and MARY V. BUELL.

(*From the Laboratory of Agricultural Chemistry, University of Wisconsin, Madison.*)

In harmony with our observations that yellow maize contains an abundance of the fat-soluble vitamine while white maize contains little or none of it, it has now been found that similar relations obtain with carrots, sweet potatoes, and squash. In peas the vitamine content runs parallel to the yellow pigment content of different varieties. Furthermore in the yellow corn kernel the vitamine is localized in the endosperm which in distinction from the embryo contains most of the pigment.

Carotin of constant melting point through a number of crystallizations was always found to induce growth in rats after growth had been suspended by a lack of the fat-soluble vitamine in the diet. In spite of this it is not meant to infer that the vitamine is necessarily a pigment as is evident from the fact that in butters the vitamine content is not of the same order of magnitude as the pigment content. Furthermore in other materials such as cod liver oil an abundance of the vitamine may occur in the presence of little or no pigment.

So far studies of its distribution do not allow conclusions to be drawn as to its nature but physiologically it is suggestive that in the plant kingdom it has now often been found closely associated with yellow pigments. Chemically it is suggestive that light which destroys these pigments also rapidly destroys the vitamine.

Studies of the stability of the fat-soluble vitamine as extracted from alfalfa hay have indicated that it is not destroyed by saponification in the hot as carried out in the determination of the Koettstorfer number, nor is it destroyed by oxidation with

hydrogen peroxide or reduction with nascent hydrogen. Crystalline acetyl derivatives of constituents in the non-saponifiable vitamine fraction have been prepared without resultant destruction of the vitamine which apparently suggests means which can be adopted in the further fractionation of the crude vitamine preparation.

AMINO-ACIDS IN NUTRITION.

III. IS PROLINE A GROWTH-LIMITING FACTOR IN THE PROTEINS OF PEAS?

By BARNETT SURE.

(From the Department of Agricultural Chemistry, University of Arkansas, Fayetteville.)

McCollum, Simmonds, and Parsons[8] have recently demonstrated that zein supplements the proteins of peas. Zein is entirely lacking in tryptophane and lysine and probably has very little, if any, cystine, and, since that protein belongs to the class called prolamines, it was thought that it might possess its supplementing value by virtue of its high proline content. It is true, zein is also high in leucine and is quite satisfactory for its tyrosine content. If proline should not be the nucleus of the supplementary character, then either tyrosine or leucine or a combination of these amino-acids might be, if implicit faith is to be placed in chemical methods of analysis. Although McCollum, Simmonds, and Parsons found that gelatin does not supplement peas, gelatin being high in proline, it was thought that since it is so indigestible the fragment containing the proline nucleus may escape digestion and hence fail to bring about response; therefore, in view of that possibility, peas were chosen as a further basis for exploring the capacity of the animal body to synthesize the pyrrolidine nucleus of the protein molecule.[10]

No response was obtained to individual additions of proline, leucine, the leucine fraction, composed of alanine, leucine, and valine, cystine, or tyrosine; or to the addition of proline, in the

[8] McCollum, E. V., Simmonds, N., and Parsons, H. T., *J. Biol. Chem.*, 1919, xxxvii, 287.

[9] Totani, G., *Biochem. J.*, 1916, x, 382.

[10] Sure, B., *J. Biol. Chem.*, 1920, xliii, 443, 457.

presence of cystine, leucine, and tyrosine. This eliminates, then, alanine, leucine, valine, cystine, tyrosine, proline, lysine, and tryptophane as limiting amino-acids that may be responsible for supplementing the proteins of peas; and, since zein is very low in the diamino-acids of arginine and histidine, it is suggested, particularly in view of the latest findings of Dakin[11] using his refined methods of protein analysis, that zein may contain one or more amino-acids as yet not isolated that are necessary for growth.

THE EFFECT OF SUBCUTANEOUS INJECTIONS OF SOLUTIONS OF POTASSIUM CYANIDE ON THE CATALASE CONTENT OF THE BLOOD.

By WILLIAM H. WELKER and J. L. BOLLMAN.

(From the Laboratory of Physiological Chemistry, College of Medicine, University of Illinois, Chicago.)

Cyanide is generally supposed to act by paralyzing the oxidases of the cells. It has been shown experimentally to have a marked effect on the catalase of the blood *in vitro*. If the view concerning the nature of the action of cyanide on the living organism is correct, it should have a marked effect on the catalase content of the blood if the latter enzyme is related to oxidase as has been assumed by some investigations.

This work was undertaken to study this point. Our series of eight experiments shows that cyanide, injected subcutaneously, has little or no effect on the catalase activity of the blood.

THE PROPERTIES OF RENAL LIPASE.

By VICTOR E. LEVINE and SALVER A. GIANELLI.

(From the Biochemical Laboratory, School of Medicine, Creighton University, Omaha.)

The lipolytic activity of the kidney was studied with the rabbit, dog, sheep, pig, and cow, using ethyl acetate, ethyl butyrate, methyl salicylate, olive oil, and castor oil as substrates or zymolytes. Chloroform-water extracts of the cortex, upper medulla, and lower medulla (papillary region) served as the source of enzyme.

[11] Dakin, H. D., *J. Biol. Chem.*, 1920, xliv, 499.

Renal lipase differs from pancreatic lipase in its behavior towards bile. Even in small concentrations bile inhibits markedly the lipolytic activity of the kidney. That the inhibition is due to the bile salts is shown by the findings with the salts of glycocholic and taurocholic acids, which also depress the lipolytic power of the kidney. Renal lipase behaves like hepatic lipase, for the observations of Loevenhart, confirmed by Quinan, Simmonds, and more recently by Wishart,[12] indicate the existence in the bile of an inhibitor for the latter.

The extent of lipolysis depends upon the order in which enzyme, zymolyte, and inhibitor are mixed. Less zymolyte is hydrolyzed when lipase and bile or bile salts are brought together before the addition of zymolyte than is hydrolyzed when bile or bile salt is added to a mixture of lipase and zymolyte. This behavior argues for a protective action exerted by the zymolyte on the enzyme and is an indirect confirmation of the view that a combation of enzyme with its zymolyte precedes enzyme action and that the inhibitor directly affects the enzyme.

Renal lipase resembles pancreatic lipase in the influence exercised by blood serum, which, according to Pottevin, Rosenheim and Shaw-Mackenzie, and Tsuji,[13] contains a substance that accelerates the activity of steapsin. The serum accelerator is, like the bile accelerator or inhibitor, organic in nature and thermostabile; but, unlike the bile substance, it is non-dialyzable and insoluble in alcohol. The order of mixing enzyme, zymolyte, and accelerator is also very important. No increase in lipolysis or a very slight increase is observed when serum is added to a mixture of enzyme and zymolyte. The acceleration, however, is most striking when the following order is adhered to: enzyme, serum, zymolyte. This fact tends to prove that the accelerator acts directly on the enzyme.

The similarity in the action of serum upon the lipase of kidney and of pancreas leads to the belief that the two enzymes contain

[12] Loevenhart, A. S., and Pierce, G., *J. Biol. Chem.*, 1906–07, ii, 397. Loevenhart, A. S., *J. Biol. Chem.*, 1906–07, ii, 427. Quinan, C., *J. Med. Research*, 1915, xxxii, 73. Simmonds, J. P., *J. Exp. Med.*, 1918, xxviii, 663. Wishart, G. M., *Biochem. J.*, 1920, xiv, 406.

[13] Pottevin, H., *Compt. rend. Acad.*, 1903, cxxxvi, 767. Rosenheim, O., and Shaw-Mackenzie, J. A., *J. Physiol.*, 1910, xl, p. xii. Tsuji, K., *Biochem. J.*, 1915, ix, 53.

the same chemical groupings or nuclei in their molecular structure. The dissimilarity in the action of bile salts, however, indicates a difference in the stereoisomeric configuration of the two lipases or a variation in the side chains or substituents in the major groupings of the enzyme molecule. The results of preliminary experiments differentiate sharply the exolipase of the pancreas from the endolipase of other tissues by the contrasting behavior of the bile salts. Serum, however, seems to accelerate all tissue lipases.

Renal lipase is a reversible enzyme. It not only possesses the power to hydrolyze esters like ethyl acetate, ethyl butyrate, methyl salicylate, and glycerides in olive and castor oils, but it is also capable of synthesizing an ester like ethyl butyrate from ethyl alcohol and butyric acid or a glyceride from glycerol and oleic acid. Fatty degeneration of the kidney may be the result of a reversion of the normal lipolytic process.

ACERIN: THE GLOBULIN OF MAPLE SEED (ACER SACCHARINUM).

By R. J. ANDERSON.

With the Collaboration of W. L. Kulp.

(From the Biochemical Laboratory, New York Agricultural Experiment Station, Geneva.)

The principal protein of the seed of the Silver Maple (*Acer saccharinum*) has been isolated and purified. This protein for which we propose the name acerin is a globulin. It could not be obtained in crystalline form but separated on dialysis in small globular particles. The purified acerin is a nearly white, heavy powder which on combustion leaves no weighable ash. A number of analyses were made on different preparations all of which showed close agreement. The average composition of acerin is as follows: C = 51.44; H = 6.80; S = 0.55; N = 18.34; O = 22.87 per cent.

The nitrogen distribution in acerin is shown in the table below:

	per cent
Amide nitrogen	2.53
Humin "	0.15
Basic "	4.86
Non-basic "	10.63

When analyzed by the Van Slyke method it was found that a considerable percentage of the basic nitrogen was present as lysine.

CREATINURIA.

By E. G. GROSS and H. STEENBOCK.

(From the Laboratory of Agricultural Chemistry, University of Wisconsin, Madison.)

Taking advantage of the technique for the study of creatinuria which had previously been developed in this laboratory when it was demonstrated that creatinuria can always be induced in the pig when given a sufficient amount of protein we have now found it possible to demonstrate that arginine administration also causes an increase in the creatine excretion. Cystine likewise was observed to increase the creatinuria but this increase was apparently due to the resultant acidosis as it was never observed when sodium acetate was administered simultaneously. Under similar conditions the casein or arginine creatinuria was not prevented.

A great variation in the ease with which creatinuria was induced in different animals was observed. That this may have been due, in part at least, to differences in the functioning of the thyroid mechanism, as is well known to occur in pigs, was supported by observations indicating the effect of thyroid administration on creatinuria. Thyroid creatinuria was not prevented by sodium acetate administration.

BRAIN AUTOLYSIS AND MEMORY.

By H. C. BRADLEY.

(From the Laboratory of Physiological Chemistry, University of Wisconsin, Madison.)

Examination of a large number and variety of brains shows that autolysis proceeds in the same way and is subject to the same conditions as in all other tissues thus far examined. Increased acidity leads to increased speed and extent of autolysis. Titration figures are about one-third those obtained on an equal amount of liver or thymus tissue. But the total protein of the brain is only about one-half that of liver or other gland tissues, and the neurokeratin

and other non-available structural proteins make up a considerable fraction of this. The amount of protein that can act as substratum in autolysis is therefore very much less than in other glands.

The outstanding feature of normal brain tissue is the permanence of its impressions, memory and habit. This permanence is achieved by its remarkably perfect mechanism for preventing conditions leading to brain autolysis. The large blood supply insures against asphyxial conditions and abnormal hydrogen ion concentration. The respiratory center makes available every resource of the organism to combat an abnormal rise in the hydrogen ion level in the brain. With a normal hydrogen ion level maintained, autolysis cannot go on, and the protein framework is not altered.

THE TITRATION OF ORGANIC ACIDS IN MILK.

By BENJAMIN KRAMER and CARL H. GREENE.

(*From the Department of Pediatrics, the Johns Hopkins University, Baltimore.*)

The Van Slyke and Palmer method for the determination of the titratable organic acids in urine may be applied to milk if protein, fat, part of the milk sugar, phosphates, and carbonates are removed by means of copper sulfate and a suspension of calcium hydroxide. Determinations were made on cow's milk, human milk, protein milk, and buttermilk. 98 per cent of acetic acid added to cow's milk and 90 per cent of added lactic acid were recovered.

The Method.

10 cc. of milk are placed in a 100 cc. volumetric flask and diluted to 50 cc. with distilled water. 2 to 5 cc. of 20 per cent copper sulfate are then added and 10 cc. of a 10 per cent suspension of calcium hydroxide. The mixture should be shaken at intervals of a few minutes. At the end of 15 minutes the volume is made up to 100 cc. with distilled water and the material mixed and filtered with suction through hardened filter paper on a Buchner funnel.

A 50 cc. aliquot representing the organic acid content of 5 cc. of milk is titrated according to the method of Van Slyke and

Palmer.[14] 0.5 cc. of a 1 per cent solution of phenolphthalein is added to the filtrate which has been transferred to a large test-tube (30 mm. in diameter, 200 mm. in length) and 0.1 N hydrochloric acid added until the pink color just disappears (pH = approximately 8). 5 cc. of 0.02 per cent tropeolin OO are then added and the addition of 0.1 N hydrochloric acid is continued until the color matches that of a standard solution in a test-tube of the same dimensions containing 0.6 cc. of 0.2 N HCl, 5 cc. of tropeolin OO solution, and water to a total volume of 60 cc. Sufficient water should be added to the unknown, when the titration approaches the end-point, to make the volume equal that of the standard solution. A correction must be made for the 0.1 N hydrochloric acid used in the blank titration of distilled water from pH 8.0 to 2.7.

CLINICAL METHODS FOR THE QUANTITATIVE DETERMINATION OF CALCIUM AND MAGNESIUM IN SMALL AMOUNTS OF SERUM OR PLASMA.

By BENJAMIN KRAMER and FREDERICK F. TISDALL.

(From the Department of Pediatrics, the Johns Hopkins University, Baltimore.)

A modified McCrudden technique for the quantitative determination of calcium and magnesium has been applied directly to small amounts of blood serum or plasma. A complete report will appear shortly in the *Johns Hopkins Hospital Bulletin.* The procedure is as follows.

The Calcium Method.

1 or 2 cc. of serum or citrated plasma are measured into an ordinary 15 cc. graduated centrifuge tube containing 2 to 3 cc. of distilled water. The tube should be gently agitated after the addition of each drop of serum. 2 drops of 0.01 per cent phenolsulfonephthalein are added followed by 1 drop of N sulfuric acid. 0.1 cc. of 30 per cent ammonium chloride is then added followed by 1 cc. of approximately N oxalic acid. The sample should be shaken after each addition. 0.5 cc. of a saturated solution of sodium acetate is added and the tube allowed to stand for at

[14] Van Slyke, D. D., and Palmer, W. W., *J. Biol. Chem.*, 1920, xli, 567.

least 1 hour. The pH of the sample at this point is about 5.4 (dibrom-cresol). The sample is then made up to a definite volume, preferably 6 cc., and centrifuged for at least 20 minutes at high speed. The supernatant fluid is blown off by means of a glass tube, the lower end of which is drawn out to a bore of 1 mm. and curved upward. The lower opening in this tube should be at least 3 or 4 mm. above the precipitate. The precipitate is suspended in the residual liquid by stirring with a glass rod. Enough 2 per cent NH_4OH (2 cc. of concentrated NH_4OH diluted to 100 cc.) is then added to bring the volume to 3 cc., care being taken to wash the rod and the sides of the centrifuge tube free of adherent oxalic acid. The tube is then centrifuged for 10 minutes. This procedure is repeated twice, thus making three washings in all. After the third washing the supernatant fluid is blown off, the tube is shaken to suspend the precipitate, and 2 cc. of approximately N sulfuric acid are added. The tube is then warmed in the boiling water bath for a few minutes and titrated with 0.01 N potassium permanganate until a definite pink color persists for at least 1 minute when viewed under a good light against a white background. The strength of the permanganate solution is determined by titrating against an 0.01 N sodium oxalate (Sörensen).

Calculation.—The number of cc. of 0.01 N potassium permanganate used diminished by 0.02 cc. (the blank) multiplied by 0.2 equals the mg. of calcium in the sample.

The Magnesium Method.

5 cc. of the supernatant fluid from the calcium determination corresponding to 1.66 cc. of serum are measured into a 30 cc. beaker, 1 cc. of 2 per cent $(NH_4)_2HPO_4$ solution is added and then 2 cc. of concentrated ammonia. Next day the sample is filtered through a well packed Gooch crucible, washed ten times with 5 cc. of 1:10 NH_4OH (10 cc. of concentrated ammonia to 100 cc. of water), and then twice with 95 per cent alcohol made alkaline with NH_4OH. The crucible is returned to the beaker and dried for a few minutes at 80°C. in the oven.

10 cc. of 0.01 N HCl are added to the crucible and after a few hours the entire material is transferred to a test-tube, centrifuged,

and 5 cc. of the supernatant fluid are measured into a flat bottomed colorimeter tube graduated for 10 cc., which contains 2 cc. of the 0.3 per cent iron thiocyanate solution. The volume is then made up to 10 cc. with 0.01 N HCl, a rubber stopper inserted, and the fluid mixed. A series of standards is prepared by adding varying amounts of a known NH_4MgPO_4 solution to the thiocyanate solution and bringing the volume up to 10 cc. as in the unknown samples. The color is compared by looking through the entire length of the liquid column against a white background.

Calculation.—The calculation is the same as in the method of Marriott and Howland. Reading (cc. of standard solution) $\times 0.01 \times 2 \times \frac{6}{5} \times 50 =$ mg. Mg in 100 cc. serum, when 2 cc. of serum are used. The standard NH_4MgPO_4 solution should contain 0.01 mg. of magnesium per cc.

STUDIES ON THE DIGESTIBILITY OF PROTEINS IN VITRO.

I. THE EFFECT OF COOKING ON THE DIGESTIBILITY OF PHASEOLIN.

By HENRY C. WATERMAN and CARL O. JOHNS.

(*From the Protein Investigation Laboratory, Bureau of Chemistry, United States Department of Agriculture, Washington.*)

Preliminary work on a method for the comparison of protein digestibilities is described, the possibility of developing an approximately quantitative method is discussed, and further work outlined.

The experiments were made in approximate imitation of conditions of natural digestion. The samples were digested successively with pepsin in 0.1 N acid and trypsin in N/120 alkali and the extent to which proteolysis had proceeded was ascertained by determining amino nitrogen after the digestion. The experiments indicate that phaseolin is rendered more readily digestible by boiling with distilled water. Cooking for 5 minutes gave a detectible increase in digestibility, while cooking for $\frac{3}{4}$ hour was apparently sufficient to produce the maximum effect.

The finding of Johns and Finks that cooked phaseolin gave better results in feeding experiments than did the raw is apparently to be explained as due to an increase in digestibility.

THE SULFOCYANATE CONTENT OF THE SALIVA AND URINE IN PELLAGRA.

By M. X. SULLIVAN and PAUL R. DAWSON.

(*From the Pellagra Hospital, United States Public Health Service, Spartanburg.*)

The sulfocyanate content of the saliva and the urine of patients with active pellagra is, as a rule, less than that of the saliva and the urine of the same patients about to be discharged from the hospital as free from the signs and symptoms characteristic of pellagra.

The increase of the sulfocyanate of the saliva and urine seems to be associated with the betterment of the general condition of the patient, with better assimilation, a higher protein metabolism, and presumably a greater detoxifying power of the system as a whole.

The increase of sulfocyanate of the urine at discharge, over that at entrance, however, is not proportional to the increase of the total nitrogen of the urine.

DIALYSIS INDICATOR METHOD FOR THE DETERMINATION OF THE HYDROGEN ION CONCENTRATION OF MILK.

By BENJAMIN KRAMER and CARL H. GREENE.

(*From the Department of Pediatrics, the Johns Hopkins University, Baltimore.*)

The hydrogen ion concentration of milk may rapidly and accurately be determined by the dialysis indicator method of Levy, Rowntree, and Marriott. 5 cc. of milk are placed in a collodion sac and dialyzed against an equal volume of 0.9 per cent NaCl solution. Tubes are used of such size that the level of the salt solution is above that of the contents of the sac. Dialysis is allowed to proceed for at least 5 minutes. The collodion sac is then removed and a suitable indicator added to the dialysate. The color developed is compared against a series of standard solutions of known pH. The Clark and Lubs series of indicators were used in conjunction with the standard phosphate solutions of Sörensen and the acid phthalate solutions of Clark and Lubs as well as the Gillespie color standards. Identical results were obtained in each case. The indicator solutions used

were brom-phenol blue pH 3.0 to 4.6, methyl red pH 4.4 to 6.0, brom-cresol purple pH 5.2 to 6.8, and phenol red pH 6.2 to 7.0, made up according to the directions of Clark and Lubs.

A series of parallel comparative determinations by the colorimetric and gas-chain methods was made on cow's milk, human milk, protein milk, and buttermilk. The agreement between the two methods was excellent.

The pH of the following samples of milk was determined.

Breast milk, 3 samples	6.8, 6.9, 6.9
Cow's " 6 "	6.7, 6.7, 6.7, 6.7, 6.6, 6.5
Protein " 5 "	5.5, 5.9, 5.0, 5.1, 4.8
Buttermilk, 4 "	5.2, 4.4, 4.7, 4.9

Comparison between the Colorimetric and Electrometric Methods for Determination of pH.

Sample.	Electrometric.	Colorimetric.
	pH	*pH*
Breast milk	6.83	6.8
	6.82	
Cow's "	6.45	6.4
	6.45	
Protein "	4.75	4.8
	4.8	
Buttermilk	4.35	4.4
	4.40	

A STUDY OF A CASE OF RENAL GLYCOSURIA.

By ALFRED P. LOTHROP.

(*From the Laboratory of Biological Chemistry, Queens University, Kingston, Ontario.*)

The subject was a medical student who discovered the presence of sugar during a routine examination of his urine in the laboratory. The blood sugar concentration was 0.078 per cent and showed a normal response to the ingestion of 100 gm. of glucose without any increase in the amount of glucose in the urine. The quantity of urinary sugar never exceeded 12 gm. in a 24 hour sample and was usually less than that. The sugar promptly disappeared from the urine on a restricted diet and could not be

increased above 12 gm. by a mixed diet rich in carbohydrate to which 125 gm. of extra sucrose were added. The results indicate that the case is one of so called "renal glycosuria." A more detailed study of the case is contemplated.

THE CHEMISTRY OF NEOARSPHENAMINE.

By GEORGE W. RAIZISS AND M. FALKOV.

(From the Dermatological Research Laboratories, Philadelphia.)

Neoarsphenamine is one of the most important remedies in the treatment of syphilis. Very little is known of its chemical composition. It is supposed to be a condensation product of arsphenamine with 1 or 2 molecules of sodium formaldehyde sulfoxylate. In the opinion of the authors, it is important to ascertain whether one or both amino groups of arsphenamine are combined with sulfoxylate. Neoarsphenamine has been analyzed by the authors for arsenic, nitrogen, and sulfur. The distribution of the sulfur was studied in particular. The results show that neoarsphenamine consists of from 58.5 to 69.5 per cent of arsenical condensation products. The balance is accounted for by uncombined sodium formaldehyde sulfoxylate, sodium chloride, and methyl alcohol of crystallization.

Some samples of neoarsphenamine contain only the monosubstituted product, while others consist of a mixture of both the mono- and di-substituted products.

CONPHASEOLIN: A NEW GLOBULIN FROM THE NAVY BEAN.

By CARL O. JOHNS AND H. C. WATERMAN.

(From the Protein Investigation Laboratory, Bureau of Chemistry, United States Department of Agriculture, Washington.)

It has been shown that the navy bean contains besides phaseolin (S, 0.30 per cent) a small amount of another globulin, the sulfur content of which is over 1 per cent.

RAPID DETERMINATION OF PROTEIN IN URINE.

By H. C. BRADLEY and WILLIAM ELLIOTT.

(From the Laboratory of Physiological Chemistry, University of Wisconsin, Madison.)

The sulfur of proteins appears to be a fairly characteristic quantity, and can be used as the basis for quantitative determinations except where the highest accuracy is required. Protein is digested in boiling NaOH, decomposing very quickly, and the sulfide titrated in acid solution by standard iodine. Figures obtained check well with total nitrogen. The method is particularly applicable to the coagulable protein of nephritic urine, which can be estimated by this method in 15 to 20 minutes.

The possibilities of more rapid, though less accurate, colorimetric reactions for the sulfide are being studied with a view to finding a clinical method of greater accuracy than Esbach's test.

STUDIES ON ORGANIC REGULATION.

I. THE URINARY EXCRETION AND BLOOD PICTURE OF THE HIBERNATING FROG.

By H. C. van der HEYDE.

(From the Department of Physiology and Physiological Chemistry, School of Medicine, Morgantown, West Virginia.)

It is remarkable that of the urinary excretion of the frog, which has been the animal of experimentation *par excellence* for centuries, nothing is known, as far as I could detect. The only material of comparison is given in two studies of Denis on fish urine.

One of the causes of this is the lack of method. The methods of Folin and Denis, of Folin and Wu, of Van Slyke, and of others open a wide field of investigation. Another difficulty is the gathering of urine in sufficient quantities. The method used in this study will be given, along with quantitative results, in the full paper to be published later.

It may be sufficient to say that the urinary figures show great irregularity, though they agree amongst the others of the series. That is, the total nitrogen, urea nitrogen, uric acid, creatinine, etc. figures parallel one another throughout. The influence of

temperature seems to be very considerable. Moreover, the humidity of the surroundings is a factor of great importance. The almost complete absence of chlorides and of phosphates in connection with the neutral reaction of the urine is one of the questions raised by this work. The figures of the influence of temperature on excretion are compared with those of Krogh and Snyder in analogous cases. The skin excretion was studied in its relation to urinary excretion.

Regarding the blood, some constituents, such as creatinine and creatine, are too small in amount to be quantitatively determined. Jaffe's test is negative on blood filtrates. Satisfactory determinations of the quantities of total non-protein nitrogen, urea, and of uric acid of the blood were made by the methods of Folin and Wu.

The study is being made as a groundwork for future investigation of the problem of regulation in the process of metamorphosis and for the factors involved in hibernation.

AUTOLYSIS OF ERYTHROCYTES.

By WITHROW MORSE.

(*From the Department of Physiology and Physiological Chemistry, School of Medicine, Morgantown, West Virginia.*)

The following study aims to meet the objections which have been raised against the conclusions of investigators of the enzyme content of erythrocytes; namely, to preclude clearly the participation of other form constituents, the leucocytes and platelets; to insure that no possibility of inhibition by serum occurs; to use an adequate quantitative method rather than the qualitative scheme used by the most convincing of the papers thus far reported; and finally to control the reaction of medium.

Method.

Potassium oxalate sufficient to make a 20 mg. per cent solution with blood was placed in paraffined 50 cc. centrifuge tubes and blood was received in these tubes immediately from a carotid cannula of an etherized dog. The tubes were centrifuged so that no laking occurred, after which the leucocyte and platelet zones, along with sufficient of the erythrocyte layer to insure that all

the other form elements were removed, were decanted and discarded. The original volume was restored by the addition of 0.9 per cent saline solution and the centrifuging was repeated three times, making four in all. The washings at this time were colorless.

The blood was divided into two portions, the first having such an amount of concentrated HCl added (1:50 blood) as leaves a slight free acidity after the adsorption of the acid by the blood protein. Xylene was added to inhibit bacterial development. In the second case, the blood was left intact, nothing being added. After sampling for N non-precipitable by tannic acid the flasks were stoppered with cotton and placed in a thermostat and additional sampling was made at 42 hour and at 9 day intervals. A small amount of filterable nitrogen appears in the initial samples, as shown below. The figures are in milligrams of nitrogen per 100 cc. of whole blood.

Sample 1.	*mg.*	Sample 2.	*mg.*
Initial	39	Initial	47
42 hrs	34	42 hrs	34
9 days	50	9 days	42

The exceeding difficulty in obtaining consistent results owing to the viscous mass one has to deal with in filtering introduces the errors which explain the anomalous fact that less nitrogen appears in the 42 hour sample of Sample 1 and throughout Sample 2. The amounts dealt with here are almost insignificant, for the difference in Sample 1 involves 11 mg. of nitrogen, corresponding to 68.75 mg. per 100 cc. of blood protein digested. At the end of the 9 day period, the flasks are without odor and the viscosity does not seem to have been altered, pointing to no alteration in the colloidal dispersion, as we should expect if digestion of the proteins took place. Comparison of these figures with those obtained by Bradley[15] for slowly autolyzing tissues such as muscle will exhibit the insignificance of the 11 mg. of nitrogen increase in the above experiment, remembering that the tannic acid filtrate involves peptide as well as amino nitrogen.

CONCLUSION.

Autolysis of erythrocytes is not demonstrated.

[15] Bradley, H. C., *J. Biol. Chem.*, 1915, xxii, 113.

THE ENZYMES OF THE ABDOMINAL ADIPOSE TISSUE OF THE COMMON TURKEY, MELEAGRIS GALLOPAVO.

By JOSEPH S. HEPBURN.

(From the Constantine Hering Laboratory, Hahnemann Medical College, Philadelphia.)

Study was made of the abdominal adipose tissue (crude gizzard fat) of six fresh, dry picked, air-chilled, dry packed, well bled, uneviscerated hen turkeys. Catalase, lipase (acting on tributyrin), and esterase (acting on ethyl butyrate) were always found. Simple reductase and oxidase, acting on phenolphthalein, were usually present. Tests for oxidases, which act on α-naphthol and on tricresol, and for protease gave negative results. Aldehyde reductase and peroxidases were found in several of the samples.

DEXTROSE CONTENT OF THE HEN'S EGG.

By JOSEPH S. HEPBURN and E. QUINTARD ST. JOHN.

(From the Constantine Hering Laboratory, Hahnemann Medical College, Philadelphia, and the Philadelphia Clinical Laboratory, Philadelphia.)

The procedure devised by Folin and Wu for blood analysis may be applied to eggs (white, yolk, or whole egg) for the preparation of a clear aqueous solution, and for the colorimetric determination of the dextrose content of that solution. A 0.02 per cent solution of dextrose is used in the colorimetric standard. In individual *fresh hennery eggs*, not over 36 hours old, the following maximum, minimum, and average percentages of dextrose were obtained; each average is based on 6 samples: *Whole egg* 0.36 to 0.49, average 0.45; *white free from yolk* 0.29 to 0.57, average 0.47; *yolk free from white* 0.11 to 0.15, average 0.14; *yolk separated from white in the commercial manner* 0.16 to 0.35, average 0.25. In *eggs preserved in sodium silicate solution* for 9 months, the percentage of dextrose in individual whole eggs (0.50), whites free from yolk (0.47 to 0.54), and commercially separated yolks (0.29 to 0.32) apparently lay within the values found for fresh eggs. *Commercial frozen whites* contained 0.41 to 0.43 per cent of dextrose, average of three samples 0.42 per cent; a *commerical frozen whole egg* contained 0.28 per cent of dextrose; the period of cold storage of these samples was unknown. Dextrose was not present in a sample of *putrid white.*

HEAT ELIMINATION AND GASEOUS EXCHANGE IN CELERY AND EGGS DURING STORAGE.

By C. F. LANGWORTHY and H. G. BAROTT.

(From the Office of Home Economics, States Relations Service, United States Department of Agriculture, Washington.)

Some years ago the respiration calorimeter was used for studies of the energy elimination and gaseous exchanges of ripening fruit[16] but the investigations were interrupted by the pressure of other work during the war. Recently similar experiments have been resumed in cooperation with the Bureau of Markets, this time with special reference to providing data fundamental to the handling and storage of food products. The specific heat and heat elimination of vegetables, fruits, and other commodities are factors which are little understood, but which are of great importance because they affect the optimum conditions and the cost of storage. These factors were determined by use of the respiration calorimeter in the case of freshly dug prime celery from the Department's Experimental Farm boxed (crated) for market. The mean specific heat was determined over two temperature ranges, namely 12–16°, and 17–21°C., and amounted to 0.83 and 0.93 compared to water as 1.00. Heat elimination was determined at three temperatures, namely 12.0°, 16.5°, and 21.0°C., and was found to be respectively 0.19, 0.21, and 0.24 calories per kilo per hour. The celery, which was in good condition, was then placed in cold storage for approximately 1 month, when its heat of elimination was again determined at 13.5–17.5°C. and was found to be respectively 0.20 and 0.33 calories per kilo per hour; its mean specific heat over this average temperature was 1.00 compared to water as 1.00. During a continuation of the experimental period for 8 days longer, the celery deteriorated rapidly and at the end was in an advanced stage of decay. The determination of gaseous exchange was difficult, owing to the large elimination of water. The results obtained in these experiments probably are in error in the second decimal place by an unknown amount because of corrections necessarily made for adhering dirt.

[16] Langworthy, C. F., Milner, R. D., and Barott, H. G., *J. Biol. Chem.*, 1920, xli, p. xlix.

Another series of experiments was made with hens' eggs, the mean specific heat being determined over two temperature ranges, 12.0–15.0°, and 15.0–19.0°C., and found to be 0.64 and 0.71 as compared with water at 1.00. The heat elimination at three temperatures, 12.0°, 15.0°, and 19.0°C., was found to be 0.01, 0.02, and 0.06 calories per kilo per hour. The data obtained were obviously of an entirely different magnitude from those with celery, the eggs showing a small moisture elimination (due to evaporation) and almost no heat output, while the celery gave out very much moisture and much heat.

The results of these experiments, like the earlier ones with apples, provide definite data regarding matters which must be taken into account in storage; the best results in storage will be possible when such definite information supersedes empirical data, a fact appreciated by the storage industry in this country and elsewhere.

THE QUANTITATIVE RELATION BETWEEN THE VITAMINE CONTENT OF FEED EATEN AND MILK PRODUCED.

By J. S. HUGHES, J. B. FITCH, and H. W. CAVE.

(*From the Kansas State Agricultural College, Manhattan.*)

During the past year two cows have been fed a diet, consisting of pearl hominy, tankage, cottonseed meal, and beet pulp, which by experiment has been shown to be very low in each of the three classes of vitamines. During this time each of these cows has gained in weight, improved in appearance, and has produced an apparently normal calf.

The vitamine content of the milk produced by these cows as well as that produced by cows receiving a feed rich in vitamines has been determined by the usual feeding experiments. The results show no difference in the antiscorbutic property of the milk produced by the cows receiving the two kinds of feeds. The water-soluble and the fat-soluble vitamine content of the milk produced by the cows receiving the feed rich in vitamine was decidedly greater than that of the milk produced by the cows receiving the feed low in vitamines.

Butter made from the milk having a high fat-soluble vitamine content contained much more of this vitamine than the butter made from the milk deficient in this vitamine.

BIOLOGICAL ANALYSIS OF THE GEORGIA VELVET BEAN.

By BARNETT SURE and J. W. READ.

(From the Department of Agricultural Chemistry of the University of Arkansas, Fayetteville.)

Unlike most seeds so far studied, the Georgia velvet bean was found to be very abundant in the fat-soluble vitamine. It proved to be stable in this seed after autoclaving for 1 hour at 15 pounds pressure. Best response was obtained on low levels of seed intake. The proteins of the seed, recently isolated by Johns and Jones, and chemically found by them to be quite satisfactory for the amino-acid content, biologically were found by us to be very deficient in character. Response to additions of casein was found to be greater at a lower level of velvet bean intake. It is suggested, therefore, that like cottonseed, for growth, and wheat, for reproduction, the Georgia velvet bean is toxic in high concentrations for growth. The seed was also found to be poor in salts and low in the water-soluble concentration. The hulls have no supplementary value in the seed. The leaf was found to be the most nutritious part of the whole plant. It furnishes the necessary salts and is fairly abundant in the water-soluble vitamine. The biological value of the whole plant is being studied at the present time.

IODOMETRIC METHODS FOR THE DETERMINATION OF REDUCING SUGARS IN URINE, BLOOD, MILK, AND OTHER SOLUTIONS.[17]

By A. F. HARTMANN and P. A. SHAFFER.

ON THE INFLUENCE OF MENSTRUATION ON THE FOOD TOLERANCE IN DIABETES MELLITUS.

By JACOB ROSENBLOOM.

[17] Shaffer, P. A., and Hartmann, A. F., *J. Biol. Chem.*, 1920–21, xlv, 365.

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