THE JOURNAL

OF

BIOLOGICAL CHEMISTRY

FOUNDED BY CHRISTIAN A. HERTER AND SUSTAINED IN PART BY THE CHRISTIAN A. HERTER MEMORIAL FUND

OFFICIAL ORGAN OF THE AMERICAN SOCIETY OF BIOLOGICAL CHEMISTS

VOLUME XLV
BALTIMORE
1920–21

PUBLISHED BY THE ROCKEFELLER INSTITUTE FOR MEDICAL RESEARCH FOR THE JOURNAL OF BIOLOGICAL CHEMISTRY, INC.

WAVERLY PRESS
THE WILLIAMS & WILKINS COMPANY
BALTIMORE, U. S. A.

CONTENTS OF VOLUME XLV.

No. 1. December, 1920.

No. 2. January, 1921.

No. 3. February, 1921.

DISODIUM PHOSPHATE AS A CATALYST FOR THE QUANTITATIVE OXIDATION OF GLUCOSE TO CARBON DIOXIDE WITH HYDROGEN PEROXIDE.

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(Received for publication, July 13, 1920.)

The experiments described in this paper represent a confirmation and extension of part of Löb's observations on the influence of phosphates on oxidative glycolysis. By the experiments herein described it is proved that disodium phosphate catalyzes the quantitative oxidation of glucose to carbon dioxide by hydrogen peroxide. Additional experiments on the influence of the carbonates of sodium and other compounds are included and a partial interpretation of the results is offered.

Previous experiments on the influence of phosphates on the oxidation of butyric acid[1] with hydrogen peroxide were being extended by further experiments when it was realized that if the results of Löb and his coworkers,[2, 3] on the influence of phosphates on glucose oxidation with peroxide, could be demonstrated by an adequate method the results would help clarify the influence of phosphates on butyric acid oxidation and have considerable interest in other ways. The results of some earlier work on the oxidation of glucose[4] indicated that probably the amount of oxidation observed by Löb could be exactly determined by the method suggested by those data. This was confirmed.

[1] Witzemann, E. J., *J. Biol. Chem.*, 1918, xxxv, 83.

[2] Löb, W., and Pulvermacher, G., *Biochem. Z.*, 1910, xxix, 316. Löb, W., and Gutmann, S., *Biochem Z.*, 1912, xlvi, 288. Beysel, W., and Löb, W., *Biochem. Z.*, 1915, lxviii, 368.

[3] Löb, W., *Biochem. Z.*, 1911, xxxii, 43.

[4] Witzemann, E. J., *J. Am. Chem. Soc.*, 1916, xxxviii, 150.

The statements under consideration as given in Löb's summary[3] are quite definite and are in part as follows:

"(1) In salt-free sugar solutions hydrogen peroxide produces only a vanishingly small amount of oxidative glycolysis.

(2) The glycolysis is markedly increased by raising the hydroxyl ion concentration.

(3) With the small OH ion concentrations in solutions having the alkalinity of blood, which is only slightly different from that of water, glycolysis is very slight if it is not accelerated by phosphates.

(4) The phosphate ions accelerate the glycolysis by the OH ions; the most favorable OH ion concentration within the limits tested lies at pH 8.302 to 7.070. At pH $\lesseqgtr$ about 5.600 there is no longer a perceptible OH ion effect exceeding that of pure water, even in the presence of phosphate ions.

(5) The acceleration of the glycolysis increases with constant OH ion concentration with increase in the absolute amount of phosphate added."

An examination of the experimental data, however, leaves one in doubt as to whether Löb really measured the oxidative glycolysis. In fact Michaelis and Rona[5] were not convinced by Löb's data and interpreted his observations differently. Obviously his determination of optical rotation and the reduction of Fehling solution by the glucose solutions before and after oxidation was not a determination of the absolute amount of oxidation. Consequently the term "oxidative glycolysis," which he uses to describe these phenomena, might include two processes.

(*a*) Destruction of glucose by oxidation at the expense of oxygen from the hydrogen peroxide used. This is what Löb meant.

(*b*) Destruction of glucose by intramolecular rearrangement under the influence of alkali. This kind of chemical change is what Michaelis and Rona appear to think Löb really saw at least in part.

If a neutral phosphate system such as Löb used, which is known to be a constituent of many living organisms, has any considerable effect upon oxidation the scope and nature of the effects should be known. The possible importance of such facts biologically for instance, when considered in relation to the well known indispensible relationships between phosphates and much normal cellular oxidation, is too obvious to require further comment.

[5] Michaelis, L., and Rona, P., *Biochem. Z.*, 1912, xlvii, 447.

The data described in this paper are sufficiently definite to give a new interest to the many facts already in hand in this field and to serve as a definite point of reference in the further study of these questions.

The results described here have a general interest in another way also. In the interpretation of the action of alkaline substances on sugars two points of view are recognized. According to the one the known effects of alkaline substances on sugars are due essentially to the hydroxyl ions. The other older view recognizes that the undissociated molecules and other ions may also aid or produce other effects than those of the so called hydroxyl ion effects. Without reviewing this problem any further in this paper it may simply be stated that the data herein presented offer varied and interesting support for the latter view.

EXPERIMENTAL.

Considering the importance of Löb's claims from several points of view it seemed highly important to determine accurately how much oxidation actually took place in his experiments with phosphates. This, it was thought, could be done, by applying the results of the author's previous study of the complete oxidation of glucose with potassium permanganate[4] to the analysis of the results obtained by Löb's experiments.

1. Methods of Analysis.—Previous experiments on the oxidation of glucose showed that in alkaline solution it is quantitatively oxidized to carbon dioxide and oxalic acid with potassium permanganate. The oxalic acid in turn is quantitatively oxidized to carbon dioxide by permanganate in sulfuric acid solutions. The plan was therefore as follows:

1. Oxidize glucose with hydrogen peroxide in the presence of phosphates just as Löb did.
2. After the expiration of the proper time interval add excess manganese dioxide to decompose unchanged hydrogen peroxide.
3. After decomposition is complete filter off the manganese dioxide, washing the filter and the original flask thoroughly.
4. Add excess sodium hydroxide solution.
5. Add an accurately known amount but excess of a strong accurately standardized solution of potassium permanganate

(about 3 gm. per 100 cc.). Heat this mixture to boiling and set aside over night after covering the top of the hot flask.

6. Add excess concentrated sulfuric acid.

7. Add an accurately known amount but excess of an accurately standardized solution (about 6 gm. per 100 cc.) of oxalic acid.

8. Dilute the clear colorless solution to a convenient definite volume and using an aliquot portion titrate back the excess oxalic acid with dilute potassium permanganate solution (0.1 N).

9. Calculate the total permanganate required for complete oxidation of the solution in No. 8, add the permanganate added in the beginning, and substract the permanganate equivalent of the oxalic acid used. The result is the amount of permanganate utilized by the glucose or other incompletely oxidized compounds present and may easily be calculated to its glucose equivalent.

In order to test the accuracy of the above method a solution of pure glucose containing 10 gm. per liter was prepared. 20 cc. of this solution, containing 0.200 gm. of glucose, 15 cc. of 35 per cent sodium hydroxide solution, and 75 cc. (= 2.028 gm.) of potassium permanganate solution were heated to boiling. After standing over night excess concentrated sulfuric acid was added and then 50 cc. (≡ 1.519 gm. of $KMnO_4$) of an oxalic acid solution. This colorless solution was diluted to 500 cc. in a graduated flask. 25 cc. portions were titrated back with 0.0996 N potassium permanganate. 5.70 cc. were required. $5.70 \times 20 \times 0.003146 = 0.359$ gm. of $KMnO_4$ required for the excess oxalic acid that was added.

2.028	gm.	$KMnO_4$ originally added.
0.359	"	$KMnO_4$ required for excess oxalic acid.
2.387	"	$KMnO_4$ used (total).
1.519	"	$KMnO_4$ equivalent of oxalic acid added.
0.868	"	$KMnO_4$ reduced by the glucose.

Since 2.40 molecules of $KMnO_4$ are required to oxidize 1 molecule of glucose to carbon dioxide the equation

$$758.4 : 180 = 0.868 : x$$
$$x = 0.206 \text{ gm. glucose}$$

gives the amount of glucose originally present.

Another oxidation made at the same time gave 0.198 gm. of glucose.

These were the results obtained with the first pair of oxidations tried and give fairly the maximum analytical error as demonstrated by subsequent experience. The results show that the method will be satisfactory provided the amount of oxidation observed exceeds the experimental error of 2 or 3 per cent.[6]

Experiments with the Phosphates of Sodium.

2. Repetition of Löb's Experiments.—Having established the fact that it is possible to determine glucose quantitatively in the proposed way the author repeated and analyzed by the method described above a number of the experiments carried out by Löb.

In Table I the results obtained in five oxidations carried out at room temperature for just 1 week are given. The results are calculated as though the oxygen required by the unoxidized compounds in the solution was all consumed by unchanged glucose. This is almost certainly not entirely true but since the incompletely oxidized compounds are possibly a complex mixture, difficult to analyze,[7] it seemed permissible and correct for the purposes of comparison to calculate the permanganate consumed to glucose. The results show that the influence of the phosphates

[6] A similar method was developed by Greifenhagen and coworkers (Greifenhagen, W., König, J., and Scholl, A., *Biochem. Z.*, 1911, xxxv, 169), and was found sufficiently accurate in use by Levene and Meyer (Levene, P. A., and Meyer, G. M., *J. Biol. Chem.*, 1912, xii, 265). These results were discovered after the completion of my own work.

[7] On the basis of Löb's earlier work (Löb, W., *Biochem. Z.*, 1908, xii, 78, 466; 1909, xvii, 132. Löb, W., and Pulvermacher, G., *Biochem. Z.*, 1909, xvii, 343. Löb, W., *Biochem. Z.*, 1909, xx, 516; xxii, 103; 1910, xxiii, 10; xxvi, 231), but specifically on the basis of a later statement (Löb, W., *Biochem. Z.*, 1915, lxviii, 368) it might be concluded that the incompletely oxidized compounds are formic and polyhydroxy acids arising from formaldehyde and pentoses. Tests on solutions from complete oxidations known to reduce permanganate equivalent to 0.02 to 0.04 gm. glucose in 75 cc. gave distinct tests for sugar with Haines' or Fehling's solution. Since this is near the limits of sensitiveness of these reagents, it appears that no large proportion of intermediate oxidation products (between hexose and CO_2) can be present. This appears to conform with the observations of Smolka (Smolka, A., *Sitzungsb. Math. Natur. Akad. Wiss.*, 1887, xcv, pt. ii, 5) on the oxidation of glucose with insufficient neutral permanganate, who recovered only final oxidation products (HCO_2H, $H_2C_2O_4$, and CO_2) and the calculated amount of unchanged glucose.

is progressively greater with increasing concentration, but that it is not a linear function of the concentration since the relative acceleration diminishes with increasing phosphate concentration.

The results in Table II were obtained under exactly the same conditions as those in Table I except that the solutions were kept 98 hours (4 days, 2 hours) in an incubator at 37°C.

TABLE I.

Glucose + H_2O_2 + Phosphates at Room Temperature.

20 cc. glucose solution (0.200 gm.) + 20 cc. 3 per cent H_2O_2 in total volume of 75 cc.

No.	0.33 M Na_2HPO_4.	0.33 M NaH_2PO_4.	H_2O	Reaction.	Glucose recovered.	Glucose oxidized.
	cc.	*cc.*	*cc.*	*pH*	*gm.*	*per cent*
1	0.0	0.0	35	7.07	0.2006	0.00
2	1.6	0.4	33	7.347	0.1863	6.85
3	6.4	1.6	27	7.347	0.1210	39.50
4	16.0	4.0	15	7.347	0.0658	67.10
5	25.6	6.4	3	7.347	0.0442	77.90

TABLE II.

Glucose + H_2O_2 + Phosphates at 37°.

20 cc. glucose solution (0.200 gm.) + 20 cc. 3 per cent H_2O_2 in total volume of 75 cc.

No.	0.33 M Na_2HPO_4.	0.33 M NaH_2PO_4.	H_2O	Reaction.	Glucose recovered.	Glucose oxidized.
	cc.	*cc.*	*cc.*	*pH*	*gm.*	*per cent*
1	0.0	0.0	35	7.07	0.186	6.50
2	1.6	0.4	33	7.347	0.1163	41.85
3	6.4	1.6	27	7.347	0.019	90.50*
4	16.0	4.0	15	7.347	0.034	83.00
5	25.6	6.4	3	7.347	0.035	82.50

* In the experiments at 37° it was generally observed that oxidation was less complete in No. 5 than in Nos. 3 or 4. Special experiments to interpret this apparent anomaly have not been done but the effect appears to be due to the fact that the velocity of oxygen activation by the Na_2HPO_4 is greater than the velocity of oxygen consumption and consequently the excess active oxygen is lost as such from the reaction mixture. This interpretation is so far supported by facts given in this paper and by others not mentioned.

3. Fate of the Glucose.—The results given above prove quite conclusively that the glucose is oxidized. Of the large number of compounds into which it could conceivably be converted without oxidation only a small number are not completely oxidizable to carbon dioxide by permanganate in acid or alkaline solution.[8] Nevertheless it seemed necessary to demonstrate actually that carbon dioxide was an important product of this oxidation.

All the experiments on carbon dioxide recovery were done with mixtures corresponding to No. 5 in Tables I and II. In determining the CO_2 the oxidation mixture was placed in a round bottom flask attached to a reflux condenser and arranged so that CO_2-free air could be bubbled through the mixture and then passed through wash bottles containing clear barium hydroxide solution.[9] On warming the flask nearly all the CO_2 was driven over. Excess dilute sulfuric acid was finally added and the mixture heated to boiling.

A. 75 cc. of such a solution, which had been kept in the incubator until all peroxide was gone and in which oxidation was nearly complete, gave in the CO_2 apparatus 0.13 gm. of barium carbonate or about 10 per cent of the calculated CO_2 yield. The rest of the CO_2 had been lost into the air.

B. 75 cc. of such a solution after 10 days at room temperature gave 0.69 gm. of $BaCO_3$, equivalent to 0.154 gm. of CO_2 or a 52.6 per cent yield of CO_2. The solution, to which excess sulfuric acid had been added while in the CO_2 apparatus, was alkalinized with sodium hydroxide, treated with MnO_2 to remove unchanged peroxide, filtered, and treated as usual with permanganate. The permanganate consumed was equivalent to 0.0749 gm. of glucose or 37.5 per cent recovered. 52.6 + 37.5 = 90.1 per cent of the 0.200 gm. of glucose used recovered in this way.

Results similar to this were obtained under the same conditions a number of times.

[8] It was not until the experiments described above had been completed that it was suspected that oxidation to CO_2 was nearly quantitative. Löb expressed the opinion that formic and polyhydroxy acids are the main products and there was no reason to doubt this until the small amount of permanganate required to complete the oxidation suggested that the oxidation might already be largely completed to CO_2.

[9] Evans, W. L., and Witzemann, E. J., *J. Am. Chem. Soc.*, 1912, xxxiv, 1086.

C. In order to obtain a more complete conversion into CO_2 and a good recovery the oxidation was set up in the incubator. Two strong round bottom 300 cc. flasks, one of which contained barium hydroxide solution and the other the glucose oxidation mixture, were connected by a glass tube having two right angle bends in rubber stoppers. The stoppers were wired in and then covered over with molten paraffin. The whole was placed in the incubator at 37°C. and agitated a few moments every day for a week. It was then taken out and allowed to stand at room temperature several days with occasional agitation. The oxidation mixture gave 0.28 gm. of $BaCO_3$ in the CO_2 apparatus. The attached $Ba(OH)_2$ flask gave 0.83 gm. of $BaCO_3$. This corresponds to 0.2474 gm. of CO_2 altogether or an 84.4 per cent yield of CO_2. The oxidation mixture treated as in (B) reduced permanganate equivalent to 0.0195 gm. of glucose or 9.8 per cent of the glucose used. $84.4 + 9.8 = 94.2$ per cent of the glucose recovered in this way.

On the basis of these results there can be no doubt that the glucose unaccounted for by the permanganate consumed is really oxidized to CO_2.

In developing the above proof that practically quantitative oxidation to CO_2 is obtained several other facts were observed.

1. The carbon dioxide formed is freely and easily lost from the solution during the oxidation even at the room temperature. In this the oxidation resembles vital oxidation in which the carbon dioxide is spontaneously lost during respiration. As much as two-thirds or more of the carbon dioxide obtained is evolved and absorbed by barium hydroxide in a closed apparatus at 37°C.

Ordinary alkaline oxidation systems, although they undergo changes in many ways similar to those occurring in living organisms, differ in that the CO_2 formed is bound and held in the system as carbonate or bicarbonate. This easy formation and loss of CO_2 is probably the most important physical characteristic of a vital oxidation system. It is not yet certain to what extent the phosphate systems can carry out the other functions belonging to alkaline systems, that are so important in the non-oxidative transformations of sugar in organisms, but indications are not lacking that they can also aid in some of these changes under suitable conditions.

2. The solutions in which all hydrogen peroxide had disappeared and which contained material oxidizable by permanganate equivalent to only 0.02 to 0.04 gm. of glucose in 75 cc. (*i.e.*, 0.027 to 0.053 per cent) reduced Fehling solution distinctly. Since this is close to the limits of sensitiveness of this test with pure glucose it is clear that most of the glucose attacked had been completely burned to carbon dioxide, and that no appreciable quantity of intermediate products such as polyhydroxy acids could be present.

4. Influence of Additional Glucose and Peroxide.—On the basis of the results in the preceding section it was of considerable interest to know whether the same phosphate mixture would repeatedly catalyze the oxidation of glucose. In other words whether the products of the reaction in any way "poison" the catalyst. If CO_2 is the sole final product and if it is evolved as was shown above, the phosphate mixture should serve repeatedly in this oxidation just as it is known to do in the fermentation of glucose.[10]

Experiment 5, Table II, was set up in the incubator. After 3 days it was free from peroxide. 0.20 gm. of glucose and 20 cc. of 3 per cent peroxide were again added. After 1 week in the incubator the peroxide had again disappeared. The same materials were again added. After another week this was repeated. On determining the permanganate consumed in the usual way it was found to correspond to 0.0831 gm. of glucose. Since 0.80 gm. of glucose had been used this corresponds to 10.4 per cent of the glucose used, which is about what is recovered from a single experiment of this kind.

These results show that the functional activity of the disodium phosphate is not impaired in the catalysis. Since this does not occur it is clear that the disodium salt is not changed into monosodium phosphate by the carbonic acid, nor any other acid intermediate oxidation product, to any marked extent. If sodium bicarbonate were formed in this way the oxidation would be retarded or stopped in the typical way in which this compound acts (*cf.* Section 7).

[10] Harden, A., and Young, W. J., *J. Chem. Soc.*, 1905, xxi, 189; *Proc. Roy. Soc. London, Series B*, 1906, lxxvii, 405; 1908, lxxx, 299; 1909, lxxxi, 336. Young, W. J., *Proc. Roy. Soc. London, Series B*, 1909, lxxxi, 529. Harden, A., and Young, W. J., *Biochem. Z.*, 1911, xxxii, 173. Young, W. J., *Biochem. Z.*, 1911, xxxii, 177.

5. Influence of Changing the Ratio of the Phosphates.—The results in Table III constitute a repetition of part of Löb's experiments (Table XI)[3] on the influence of a change in the ratio of the two phosphates. All the experiments were set up in 250 cc. Florence flasks and kept in the incubator for 45 hours before analyzing.

TABLE III.

25 cc. (0.25 gm.) glucose + 25 cc. H_2O_2 + 20 cc. salt solution + 5 cc. water at 37°.

No.	0.33 M Na_2HPO_4.	0.33 M NaH_2PO_4.	H_2O	Reaction.	Glucose recovered.	Glucose oxidized.
	cc.	*cc.*	*cc.*	*pH*	*gm.*	*per cent*
1	0	0	25	7.07	0.2407	3.7
2	16	4	5	7.347	0.1992	20.3
3	10	10	5	6.813	0.2068	17.3
4	4	16	5	6.239	0.2316	7.4
5	2	18	5	5.910	0.2342	6.3

TABLE IV.*

20 cc. glucose (0.200 gm.) + 20 cc. 3 per cent H_2O_2 at 37° for 10 days.

No.	0.33 M Na_2HPO_4.	0.33 M NaH_2PO_4.	H_2O	Glucose recovered.	Glucose recovered after 2 days.
	cc.	*cc.*	*cc.*	*gm.*	*gm.*
1	25.6	6.4	59	0.0242	0.0387
2	25.6	32.0	34	0.0180	0.0271
3	25.6	64.0	2	0.0166	

* All the experiments in this table have a total volume of 131 cc. The reaction of No. 1 is distinctly alkaline to litmus paper while that of No. 3 is distinctly acid. Accordingly pH passes from a point on the alkaline side (about 7.347) to a point decidedly on the opposite or acid side of neutrality.

The results show a diminishing velocity of glucose oxidation as the ratio of monosodium phosphate used increases or as the ratio of disodium phosphate decreases.

From these experiments alone it might be concluded that the OH ion is significant in this oxidation but results given in the next paragraph do not confirm this idea.

When the ratio of the two phosphates is changed by changing the amount of monosodium phosphate but keeping the disodium phosphate constant in amount different results are obtained.

The results in Table IV show that in the presence of a constant amount of disodium phosphate increasing amounts of monosodium phosphate do not retard the oxidation of glucose. In fact the presence of the monosodium phosphate seems to facilitate the completion of the glucose oxidation in spite of the fact that relatively No. 3 is comparable with No. 4 in Table III as far as the proportion of the two phosphates is concerned. Exactly the same result was obtained with Nos. 1 and 2 when they were allowed to react only 2 days. No velocity experiments have been made to determine whether the excess NaH_2PO_4 retards the oxidation as it does the evolution of O_2 from H_2O_2 but the results as given indicate that it does not.

It seems clear that if Löb had done these experiments, as well as some others described below, he would have found it impossible to ascribe so much influence to the OH ions in this catalysis, as he did.

6. Influence of Time on the Oxidation.—In order to follow the glucose oxidation from day to day a large experiment containing

TABLE V.

Glucose.

					gm.
	At beginning contained				0.200
(*a*)	After	24 hrs.	"		0.1869
(*b*)	"	48	"	"	0.1611
(*c*)	"	72	"	"	0.1551*
(*d*)	"	96	"	"	0.1212
(*e*)	"	120	"	"	0.1173*
(*f*)	"	168	"	"	0.0823

* When the results described in this table are plotted the two values marked with the asterisk lie considerably outside the curve. This is due to the fact that undecomposed hydrogen peroxide was still present when the potassium permanganate was added. Thus when two solutions containing exactly the same amount of glucose but one of which also contained 5 cc. of 3 per cent hydrogen peroxide were analyzed, without decomposing the peroxide, the former was found to contain 0.1802 gm. of glucose by the complete oxidation method. The other containing the peroxide apparently contained 0.2165 gm. of glucose when calculated on the basis of the oxygen consumed. This difference is due to the well known fact that hydrogen peroxide reduces permanganate with the evolution of oxygen.

The results are given in this form in order to illustrate this error.

six times as much material as No. 2 in Table II was set up and placed in the incubator at 37°. 75 cc. of this solution (corresponding to 0.200 gm. of glucose) were taken out for analysis at definite intervals as indicated in Table V.

Experiments with the Carbonates of Sodium.

The preceding results clearly confirm Löb's claim that in the presence of phosphate mixture glucose is oxidized by hydrogen peroxide. Since he failed to observe appreciable amounts of oxidation when he used the other common reaction regulator mixtures it seemed unnecessary to test these again for the present. It did seem advisable, however, to make some experiments with the carbonates of sodium for several obvious reasons.

7. Influence of Sodium Carbonate-Bicarbonate.—If the phosphates do not exercise a catalytic effect in this oxidation and the effect observed is due to OH ions then an equimolecular amount of sodium carbonate and bicarbonate should have fully as much effect. That this is not true was definitely established by the following experiment in which 2.43 gm. of $Na_2CO_3.10H_2O$, 0.72 gm. of $NaHCO_3$, 35 cc. of distilled water, 20 cc. (0.200 gm.) of glucose solution, and 20 cc. of 3 per cent H_2O_2 were kept 4 days at 37°. Upon analysis the peroxide was found to have been completely decomposed and equivalent of 0.1902 gm. of glucose was recovered; *i.e.*, 5 per cent was apparently oxidized as against 80 per cent oxidized with the corresponding phosphate mixture.

In the above experiment the two carbonates were used in the same molecular amounts and proportion as the two phosphates in Experiments 5 in Tables I and II. The solution therefore contained at least the same amount of available alkali but had a somewhat higher OH ion concentration than the phosphate mixture referred to. If OH ion concentration and available alkali are the controlling factors in these oxidations this experiment should have shown as much or more oxidation than was obtained with the phosphate mixture.

8. Influence of H_2CO_3 and $NaHCO_3$.—The results in Table V suggest that the velocity of decomposition of sodium bicarbonate, possibly produced in the oxidation, may be a factor in determining the velocity of oxidation. The following three experiments were

done in order to test the influence of this condition. The experiments were set up in similar 250 cc. flasks and kept in the incubator for 24 hours at 37° after which they were analyzed in the usual manner.

(1) 32.0 cc. of 0.33 M NaH_2PO_4 solution + 1.22 gm. of $Na_2CO_3.10H_2O$. This mixture effervesced in the cold. It was heated to boiling to expel CO_2, cooled, and the following were added:

20 cc. (0.200 gm.) of glucose solution, 20 cc. of 3 per cent H_2O_2, 3 cc. of distilled water. 0.0761 gm. of glucose was recovered.

(2) Components the same as in (1).

All ingredients were mixed except the peroxide before adding the $Na_2CO_3.10H_2O$ in order to prevent the loss of CO_2 as much as possible. 0.1906 gm. of glucose was recovered.

(3) 25.6 cc. of 0.33 M Na_2HPO_4 solution.
6.4 " " 0.33 " NaH_2PO_4 "
20.0 " (0.200 gm.) of glucose solution.
20.0 " of 3 per cent H_2O_2.
3.0 " " distilled water.

0.0774 gm. of glucose was recovered.

On the basis of the conditions of the experiments the results of (1) and (3) were expected to be identical because the reaction mixtures as used were identical. As a matter of fact the amount of glucose recovered was nearly the same in (1) and (3). It was expected that the oxidation in (2) would be somewhat slower. In fact only 5 per cent of the glucose was oxidized in (2) as compared with over 60 per cent in the others. This indicates that not only do H_2CO_3 and $NaHCO_3$ not catalyze the oxidation of glucose with hydrogen peroxide but that they actually retard it.

The interpretation of the influence of the sodium carbonate added in (2) has not been fully established as yet. There are several factors to be considered, three of which are as follows: (*a*) Na_2CO_3 may under the conditions in (2) not react completely to give only Na_2HPO_4 and H_2CO_3; (*b*) if so, any Na_2CO_3 or $NaHCO_3$ remaining would rapidly catalytically decompose the H_2O_2; (*c*) the presence of CO_2 to the point of supersaturation may retard the activation or dissociation of H_2O_2.

9. Influence of Sodium Carbonate.—The following experiments were done in order to determine what influence sodium carbonate exercises on the action of disodium phosphate.

(1) 32.0 cc. of 0.33 M Na_2HPO_4 solution.
3.0 " " water.
20.0 " " glucose solution (0.200 gm.).
20.0 " " 3 per cent hydrogen peroxide.
0.0311 gm. of glucose was recovered.

(2) Same as in (1) with 0.61 gm. of $Na_2CO_3.10H_2O$.
0.1741 gm. of glucose was recovered.

(3) Same as (1) with 1.22 gm. of $Na_2CO_3.10H_2O$.
0.1737 gm. of glucose was recovered.

The solutions were kept in the incubator at 37°C. for 45 hours and on analysis the amounts of unchanged glucose given were found. All hydrogen peroxide present had been decomposed.

The results show that sodium carbonate exercises a strongly negative influence on this oxidation in spite of the fact that the OH ion concentration is higher in (2) and (3) and the available alkali in (3) is twice what it was in Experiments 5, Tables I and II.

This negative influence on the final result of the oxidation may be due simply to the fact that the velocity of decomposition of peroxide by Na_2CO_3 is many times greater than that by Na_2HPO_4 and that the glucose oxidation induced by Na_2CO_3 itself is relatively small in comparison.

The above observations on the influence of the carbonates of sodium permit us to conclude that, whatever the mechanism of CO_2 formation in this oxidation may be, carbonates of sodium are not intermediate stages in the process of CO_2 liberation.

10. Influence of Sodium Hydroxide.—The following three experiments were done in order to determine the influence of sodium hydroxide on the effect of the phosphate mixture.

(1) 25.6 cc. of 0.33 M Na_2HPO_4 solution + 6.4 cc. of 0.33 M NaH_2PO_4 + 20 cc. (0.200 gm.) of glucose solution + 20 cc. of 3 per cent H_2O_2 + 3 cc. of water.

(2) The same as (1) except that one-half the water was replaced with 1.5 cc. (0.0857 gm.) of NaOH solution.

(3) The same as (1) except that all the water was replaced with 3 cc. (0.1714 gm.) of NaOH solution.

After $50\frac{1}{2}$ hours at 37°C. the solutions contained no unchanged peroxide. They were analyzed and found to reduce $KMnO_4$ corresponding to glucose as follows:

(1) 0.0088 gm. of glucose.
(2) 0.0140 " " "
(3) 0.0618 " " "

The sodium hydroxide has a perceptible but not a large retarding effect which is interpreted tentatively in the light of other experiments as due simply to its effect in increasing the decomposition of the hydrogen peroxide. In this respect its activity is not so great as that of the carbonates which coincides with its smaller retarding effect on the action of the phosphate mixture.

11. Partial Interpretation of the Influence of Disodium Phosphate.—Since there are three compounds actively concerned in this oxidation reaction and since glucose and peroxide alone do not react appreciably there remain three possible ways of interpreting the reaction on the basis of the formation of molecular complexes, which are so frequently found to underlie catalytic phenomena.

(1) Na_2HPO_4 and H_2O_2 may give an unstable complex which in turn reacts to oxidize glucose.

(2) Na_2HPO_4 and glucose may form a hexose phosphate which is more sensitive to H_2O_2 than free glucose.

(3) The three compounds may form a single complex the instability of which gives rise to the oxidation.

(1) and (2) are readily capable of being tested experimentally by known methods. (3) could conceivably take place in several ways none of which appears to be readily capable of experimental confirmation.

The Na_2HPO_4-H_2O_2 Complex.—That such a complex may be formed is suggested by the experiments of Petrenko on H_2O_2 derivatives of Na_3PO_4.[11] A perphosphate of Na_2HPO_4 is unknown. Moreover perphosphoric acid is apparently unknown.[12] However, pyrophosphoric acid gives a peracid with H_2O_2, stronger

[11] Petrenko, G., *J. russ. phys.-chem. Ges.*, 1902, xxxiv, 204, 391; *Chem. Zentr.*, 1902, i, 1263; ii, 95. *Cf.* also, Gemlin-Kraut, Handbuch der anorganische Chemie, Heidelberg, 7th edition, 1906, i, pt. 1, 146.

[12] Price, T. S., Per-acids and their salts, New York, 1912, 77.

than Caro's acid and which oxidizes Mn to $KMnO_4$ and its sodium salt $Na_4P_2O_7$ gives a stable persalt with 3 per cent H_2O_2.[13]

There is therefore some basis in fact, even in this little studied field, for the idea that Na_2HPO_4 may form an unstable perphosphate as is suggested in the succeeding paragraphs.

Decomposition of Hydrogen Peroxide by the Phosphate Mixture.—Various experiments were done on the influence of Na_2HPO_4 on hydrogen peroxide although it is definitely stated[14] that it is without influence on peroxide. My own experiments, which will not be described here, show that it does decompose hydrogen peroxide and that the presence of an equimolecular amount of NaH_2PO_4 retards but does not stop the decomposition.

TABLE VI.

0.1 N $KMnO_4$ Consumed by 5 Cc. of the Mixture.

No.	23.5 hrs.	47.2 hrs.	96.2 hrs.
	cc.	cc.	cc.
1	22.70	22.62	22.45
2	22.10	21.06	18.45
3	19.35	14.53.	2.75
4	17.06	11.65	4.31
5	15.72	9.86	4.08

The only experiments to be described here represent a repetition of the glucose oxidations at 37° in Table II in which the glucose was omitted and in which the peroxide content was determined at intervals during 96 hours. The results given in Table VI indicate the amount of peroxide remaining, at the various intervals, in terms of cc. of 0.1 N $KMnO_4$ consumed by 5 cc. of the mixture.

The phosphate mixtures and the hydrogen peroxide were warmed for 24 hours at 37°C. before being mixed in order to prevent a lag which is otherwise observed during the first 24 hours.

[13] Schenck, R., Vorländer, F., and Dux, W., *Z. angew. Chem.*, 1914, xxvii, pt. 1, 291.

[14] Gemelin-Kraut,[11] p. 137.

The data show a progressively increasing decomposing effect with increasing phosphate content although the mixture is neither appreciably acid nor alkaline.[15]

In conclusion it may be stated that there are clear indications that Na_2HPO_4-H_2O_2 may form an unstable complex, but as yet there is no satisfactory evidence.

The Na_2HPO_4-Glucose Complex.—Harden and Young, von Lebedew, and others[10,16] showed that, in the yeast fermentation of glucose, Na_2HPO_4 combines with glucose to form a hexose phosphate ester. The presence of this complex was demonstrated in part by the fact that much of the phosphate was no longer precipitated with "magnesia mixture." Other hexose phosphoric esters have been obtained by chemical methods[17] but the laboratory preparation of von Lebedew,[16] and the commercial manufacture[18] of hexose phosphate ester are carried out only in the presence of growing yeast. It therefore seemed necessary to determine experimentally whether such a complex is formed in aqueous solutions of glucose and the phosphate mixture alone.

A solution corresponding to No. 5 in Table II except that it contained 20 cc. of water instead of the peroxide solution was kept 3 weeks in the incubator at 37°C. At the end of this time the glucose content was found by reduction methods to be unchanged. After standing 3 weeks more in the laboratory the

[15] It should be noted that the interpretation of this decomposition of H_2O_2 in this case cannot be attributed to the OH ion, since the solution has about the OH ion concentration of water, which is without influence. It is interesting to note in this connection that Schenck, Vorländer, and Dux[13] found that $Na_4P_2O_7$ solutions, which are so alkaline as to feel "soapy," actually stabilize H_2O_2 by forming a stable perpyrophosphate. Moreover, it was found in experiments which will not be given here that the presence of $Na_4P_2O_7$ with Na_2HPO_4 retards or prevents the oxidation of glucose with H_2O_2, but not by decomposing the H_2O_2 as with $NaHCO_3$ or Na_2CO_3.

[16] von Lebedew, A. V., *Biochem. Z.*, 1910, xxviii, 213; 1911, xxxvi, 248. Embden, G., and Laquer, F., *Z. physiol. Chem.*, 1914–15, xciii, 94. Embden, G., Griesbach, W., and Laquer, F., *Z. physiol. Chem.*, 1914–15, xciii, 124.

[17] *Cf.* foot-note, Meyer, V., and Jacobson, P., Lehrbuch der organischen Chemie, Leipsic, 2nd edition, 1902, ii, pt. 2, 927.

[18] *Cf.* for instance Bayer and Company, German Patent 292,817, February 26, 1915; *Chem. Abstr.*, 1917, xi, 1519.

phosphate was precipitated with "magnesia mixture" and weighed as the pyrophosphate. A solution of the phosphates alone made up to the same volume was similarly precipitated at the same time. The two precipitates after ignition showed the same weight, within a small fraction of 1 per cent, which shows that hexose phosphate ester was not formed to any significant extent.

Similar solutions containing glucose and the phosphate mixture were kept under observation in the polariscope in comparison with glucose solutions without phosphates. In 4 days there was no measurable change in optical rotation in either solution.

These data, taken with the absence of positive data in the literature, seem to prove that a hexose phosphate ester such as was found by Harden and Young is not formed under these conditions and consequently has no part in bringing about this oxidation of glucose. If some other type of complex is formed its presence was not demonstrated by these methods.

Influence of Time on the Glucose-Phosphate-Peroxide Reaction.—When it was found that the rate of peroxide decomposition has a definite relation to the phosphate concentration it was of interest to learn what relation the rate of glucose oxidation bears to the rate of peroxide decomposition. The results given in Table VII are typical for the rate of glucose oxidation as obtained by compiling experimental results obtained in conditions like those used for Table II.

In order to test this more fully experiments like Nos. 1 to 5 in Table II were set up having a total volume of 150 cc. and which were placed in the incubator at 37°. The phosphate-glucose mixture and the peroxide were warmed separately for 24 hours before mixing to eliminate what appeared to be a temperature lag in the curves. 10 cc. were removed and analyzed at definite intervals and the results were calculated and recorded in Table VIII on the basis of 75 cc. and thus correspond to the results in Table VII. The materials used in Table VII were not warmed before mixing which accounts for the difference in the slope of the curves when the data are plotted.

The results in both series are substantially the same and show that the rate of glucose disappearance in the presence of the phosphate mixture is appreciably faster than the rate of H_2O_2 disappearance in the absence of glucose (Table VI).

The above constitutes a partial experimental interpretation of the catalytic influence of disodium phosphate on the oxidation of glucose with hydrogen peroxide. The results clearly suggest that the glucose is really oxidized by an unstable disodium perphosphate, formed by the action of peroxide on disodium phosphate.

TABLE VII.

Glucose Remaining from 0.200 Gm. Used.

No.	50 hrs.	97 hrs.
	gm.	*gm.*
1		0.1897
2		0.1843
3		0.1249
4	0.1037	0.0135
5	0.0088	

TABLE VIII.

Glucose Remaining from 0.1902 Gm. Used.

No.	26.5 hrs.	49.2 hrs.
	gm.	*gm.*
1	0.1702	0.1455
2	0.1362	0.1074*
3	0.0489	
4	0.0195	
5	0.0273	

* The behavior of No. 2 is quite variable. Sometimes oxidation is as slow as in No. 1 without phosphates and sometimes it is nearly as fast as in No. 3, but more frequently it is about as given in these results.

12. Is a Glucose-Phosphate Solution Oxidized by Air?—Having shown that the disodium phosphate plays a specific rôle in this catalysis the question arises as to whether the use of peroxide is necessary. A few experiments were done in order to determine whether air could be used instead of peroxide. It is well known that caustic alkalies catalyze the oxidation of sugars by air, with the formation of more or less CO_2 depending on the conditions of the experiment. In the absence of definite data it was possible that the phosphate mixture might play the rôle of caustic

alkali. A mixture like No. 5 in Table II was placed in a wash bottle. A rapid air stream was bubbled through it for 48 hours during 6 days. The permanganate required by 10 cc. was determined at the beginning and at the end of the experiment and showed that no perceptible oxidation had taken place. This shows that disodium phosphate does not act like alkali in this respect, but rather conforms to the rôle of a true peroxidase.[19]

SUMMARY.

1. The work of Löb on the accelerative effect of phosphate mixtures on the oxidation of glucose with hydrogen peroxide was repeated and confirmed.

2. The confirmation consisted in proving by an adequate method that the destruction of glucose, conceded by all in this case, is oxidation.

3. It was shown that glucose may be quantitatively oxidized to CO_2 with hydrogen peroxide in the presence of the phosphate mixture. This fact it appears was not suspected by Löb, and increases the importance of his observations considerably.

4. The results as a whole show that although optimal OH ion concentration is possibly necessary it is less important than

[19] *Cf.* Bach, A., in Oppenheimer, C., Handbuch der Biochemie des Menschen und der Tiere, Jena, 1st edition, 1913, suppl., 160.

Inorganic compounds known to play the rôle of peroxidase in *in vitro* oxidations have usually been, as Bach states, metallic salts of the heavy metals such as iron and manganese. The synthetic peroxidases of Trillat (Trillat, M. A., *Compt. rend. Acad.*, 1904, cxxxviii, 274), of Dony-Hénault (Dony-Hénault, O., *Bull. acad. roy. belg.*, 1908, 105), etc., prepared from manganese were prepared to resemble and imitate what it was thought are the essential properties of an oxidizing enzyme. The peroxidase disodium phosphate differs from these inorganic peroxidases in that the peroxidase property is dependent on the phosphate part of the molecule. Other sodium compounds do not exhibit the same effect. On the other hand dipotassium phosphate, as was shown by experiments not yet published, has the same effect. That the remaining alkali and alkaline earth dibasic phosphates may act in the same way seems likely.

From this point of view then these results are of considerable interest because we have a compound playing the rôle of peroxidase, in which the non-metallic part of the molecule carries the characteristic property. In this respect it seems likely that it resembles the biological peroxidases more closely than the heavy metal derivative peroxidases do.

Löb's experiments and interpretation would indicate, when the phosphate mixture is used, and that the optimal limits, if they exist, are wider than he states.

In fact it seems more accurate to refrain from emphasizing the segregated OH ion in interpreting the reaction and simply state that the effect is specifically related to the presence of disodium phosphate under suitable conditions.

5. The amount of disodium phosphate used is the most significant factor in determining the reaction. Little or much monosodium phosphate was used with a constant amount of disodium phosphate without producing a marked negative effect on the reaction.

6. The phosphate mixture may be used repeatedly at 37° for the oxidation of additional amounts of glucose owing to the fact that the product (CO_2) is evolved from the reaction mixture during the process of oxidation. Disodium phosphate accordingly plays the rôle of a typical catalyst.

7. Consequently disodium phosphate functioning in the manner described in this paper is the only chemical substance known to be generally necessary to the life of organisms, that is known to catalyze the quantitative oxidation of glucose to carbon dioxide.

8. That compounds like hexose phosphate ester are the intermediates involved in the acceleration of oxidation described in this paper seems almost certain at first, in the light of the results of Harden and Young,[10] of von Lebedew,[16] and of Embden, Griesbach, and Laquer.[16] The attempts so far made to establish the formation of such a compound failed to demonstrate its formation under these conditions.

9. On the other hand a close parallelism between the rate of spontaneous decomposition of peroxide and the rate of glucose oxidation in the same solutions was established. This together with other facts developed gives experimental basis for the idea that the oxidation really depends upon the intermediate formation of a highly reactive perphosphate.

10. In producing this accelerating effect upon glucose oxidation disodium phosphate does not play the rôle of both oxygenase and peroxidase, as some inorganic compounds do, but acts only as a peroxidase. It is unable to activate atmospheric oxygen to any appreciable extent.

11. That the phosphate catalysis does not depend alone on unlimited capacity to decompose peroxide is clearly shown by the fact that the hydroxide and carbonates of sodium, which are much more effective in decomposing peroxide, diminish the glucose oxidation roughly in proportion to their increased ability to decompose peroxide.

12. Glucose is not oxidized by hydrogen peroxide in solutions containing $NaHCO_3$ and Na_2CO_3 when these are used in the same molecular concentrations as the two phosphates in the phosphate mixture.

13. These results together with those with sodium hydroxide show that available alkali, contrary to what was observed in the permanganate oxidation of glucose,[4, 20] is without appreciable influence on the oxidation of glucose with hydrogen peroxide.

[20] Witzemann, E. J., *J. Am. Chem. Soc.*, 1917, xxxix, 2657.

STUDIES ON MOLLUSCAN CELOMIC FLUID.

EFFECT OF CHANGE IN ENVIRONMENT ON THE CARBON DIOXIDE CONTENT OF THE CELOMIC FLUID.

ANAEROBIC RESPIRATION IN MYA ARENARIA.

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(Received for publication, September 10, 1920.)

INTRODUCTION.

It was noted in a previous communication (1) that the content of combined carbon dioxide of molluscan celomic fluid tends to rise when the animals are removed from their natural environment whereas a fall was noticed in this factor in the case of fish removed from their natural habitat. In order to determine what was the cause of this peculiar effect in the molluscan forms a series of experiments was undertaken, the results of which are herein reported.

EXPERIMENTAL.

Effect of Exposure to Atmospheric Air on the Combined Carbon Dioxide Content of the Celomic Fluid.

The method of securing samples of celomic fluid or "clam juice" from the various specimens was the same as that detailed previously (1). Specimens of seven species of pelecypod Mollusca were exposed to atmospheric air in a closed glass container for varying periods of time. One species of the Amphineura and two species of the Gastropoda were similarly studied. Several non-molluscan forms were also exposed to atmospheric air under similar conditions. These included the calcareous shelled arthropod *Balanus aquilla,* the common brachiopod *Terebretella transversa,* various Crustacea of the decapod type, starfish, sea urchins, and certain varieties of marine fish. The container

TABLE I.

No. used.	Specimen.	Time out of water.	CO_2 content of 100 cc. celomic fluid equilibrated with atmospheric air.	Remarks.
		hrs.	*cc.*	
1	*Macoma secta*	0	11.2 } one experiment.	
1	" "	6	48.6 }	
1	*Mya arenaria*	0	11.2 } one experiment.	
1	" "	17	27.3 }	
1	" "	1	19.9	
1	" "	4	19.5	
1	" "	0	10.2 } one experiment.	
1	" "	24	32.3 }	Same specimen as above.
1	" "	24	28.2	
9	" "	0	8.0	Kept for some days in floating carriage attached to landing stage.
11	" "	28	36.0	Kept for some days in floating carriage attached to landing stage.
3	" "	25	34.8	
3	" "	24	28.2	
4	" "	24	38.0	Kept in sealed container placed in sea water off landing stage.
4	" "	120	55.0	
12	" "	90	44.0	
12	" "	0	6.5	Kept for some days in floating carriage attached to landing stage.
10	" "	48	65.0 } one experiment.	Kept in sealed container placed in sea water off landing stage.
11	" "	96	74.5 }	Kept in sealed container placed in sea water off landing stage.

6	*Mya arenaria*.....	0	6.5	Kept for some days in floating carriage attached to landing stage.
4	" "	24	38.0	Kept in sealed container placed in sea water off landing stage.
10	" "	48	69.0	Kept in sealed container placed in sea water off landing stage.
8	" "	72	78.2	Kept in sealed container placed in sea water off landing stage.
2	" "	96	105.0	Kept in sealed container placed in sea water off landing stage.
				one experiment.
11	*Paphia staminea*.....	0	12.8	
1	" "	0	27.9	
3	" "	6	39.0	
1	" "	6	34.5	
1	" "	16	40.0	
1	" "	44	59.0	
8	" "	28	39.5	
1	*Schizothoerus nuttalli*.....	0	8.3	
1	" "	12	23.2	
3	" "	32	30.7	
1	*Saxidomus gigantea*.....	0	8.1	
1	" "	12	24.2	
1	" "	6	29.0	
1	" "	24	40.0	
8	" "	30	44.0	
1	" "	30	53.0	

TABLE 1.—*Concluded.*

No. used.	Specimen.	Time out of water.	CO_2 content of 100 cc. celomic fluid equilibrated with atmospheric air.	Remarks.
		hrs.	*cc.*	
1	*Cardium corbis*.....	0	7.9	
5	" "	2	12.6	
1	" "	6	14.9	
1	" "	14	16.5	
1	" "	24	18.6	
8	" "	24	16.3	
5	" "	30	20.0	
4	" "	32	18.0	
1	*Mytilus edulis*	0	8.1	
1	" "	0	9.9	
1	" "	30	27.3	
1	*Cryptochiton*.....	0	9.0	
1	"	16	18.0	
1	*Polynices* (Lunatia) *lewisii*.....	0	12.5	
1	" " "	24	31.0	
1	" " "	30	77.4	
1	" " "	52	61.2	
1	" " "	52	63.0	
1	*Thais lamellosa*.....	0	18.5	
1	" "	10	28.2	
1	" "	24	45.8	
1	*Balanus aquilla*.....	0	11.2	
1	" "	9	17.7	Same specimen as above.

1	*Terebretella transversa*.....	0	6.8	
3	" "	30	12.7	
1	*Strongylocentrotus drobachiensis.*	0	10.2	
1	" "	4	14.8	Same specimen as above.
1	" "	0	10.2	
1	" "	8	6.6	Same specimen as above.
1	*Pisaster ochracea*.....	0	6.5	
1	" "	8	6.5	Same specimen as above.
1	" "	32	7.0	
1	" "	0	7.0	
1	" "	24	10.0	Same specimen as above.
1	" "	0	6.0	
1	" "	24	7.0	Same specimen as above.
1	*Cancer magister*.....	0	18.0	
1	" "	5	18.0	Same specimen as above.
1	" "	0	18.4	
1	" "	8	19.5	Same specimen as above.
1	*Platichtys*, plasma.....	0	10.2	
1	" whole blood.....	0	3.7	Dead some hrs.
1	*Sebastodes*.....	0	11.2	
3	"	3	7.3	
1	"	0	9.3	On line $\frac{1}{2}$ hr.
1	*Squalus sucklii*.....	0	5.6	
1	" "	4	3.7	

used was of good size and a full supply of oxygen was assured. It was kept covered to prevent loss of water by evaporation. Filter paper moistened in sea water was frequently placed in the container with the specimens. Specimens which were exposed to air were kept in certain instances at a fairly constant temperature by immersing the container in sea water while in others they were kept in the laboratory and were thus subject to the temperature changes of the latter. Table I illustrates the effects of exposure to air for various periods upon the combined carbon dioxide content of the celomic fluid of the different species investigated. The rapid increase in the carbon dioxide content of the celomic fluid of the molluscan forms and the arthropod *Balanus aquilla* is very striking. That this increase is due to bicarbonate is evident since the samples were equilibrated with atmospheric air before being submitted to analysis. The decapod crustaceans examined failed to show this reaction, while a very slight increase was in some instances manifested in the echinoderms. As the latter reaction was not uniform it is of very doubtful significance.

The survival time for specimens exposed to atmospheric air varied greatly. It was early noted that *Mya arenaria* was peculiarly resistant to long exposure to atmospheric air at the temperatures which prevailed in the surface water and the air at Departure Bay during the summer months. It was for this reason used extensively in later investigation. It is regretted that no facilities were available which would enable one to keep specimens at a low as well as constant temperature. The results obtained will therefore have to be considered in the light of this condition. The greatest increase in the carbon dioxide content of the blood was in two specimens of *Mya arenaria* which had been exposed for 96 hours. The increase here was from 6.5 volumes per cent in the controls kept in sea water to 105 volumes per cent in the specimens exposed to atmospheric air at the temperature of surface sea water. The container used was a 6 liter cylindrical glass museum jar and it was opened daily both for the purpose of removing specimens for examination and to allow a change of air. The temperature of the sea water in the vessel which was taken on the 4 consecutive days during which these specimens were exposed was 19.8°, 18.8°, 19.3°, and 18.9°C. Slightly over a

sixteenfold increase in the combined carbon dioxide in the blood was noticed in this instance.

The increase in the carbon dioxide in the small pelecypod *Macoma secta* from 11.2 to 48.6 volumes per cent in 6 hours is noteworthy, as is also that observed in the gastropod *Polynices lewisii* following 30 hours exposure. The combined carbon dioxide rose in this latter instance from 12.5 volumes per cent in the control to 77.4 volumes per cent in the exposed specimen. There were few forms which would survive an exposure to atmospheric air of more than 24 hours at the prevailing land and surface water temperatures. The amphineuran *Cryptochiton,* the cockle *Cardium corbis,* and the eagle barnacle *Balanus aquilla* were very sensitive to exposure. The horse clam *Schizothoerus nuttalli,* and the butter clam *Saxidomus gigantea* withstood an exposure of 24 to 48 hours. The little neck clam *Paphia staminea* and the edible form *Mya arenaria* were very resistant to exposure and in some instances survived as long as 5 days when placed in the air, the evaporation of water being practically excluded. The nudibranch *Anisodoris* was most sensitive of all forms, dying shortly after being brought into the laboratory. The absence of a calcareous shell is probably associated with the lack of resistance to exposure to air in this form although the temperature factor must also be of great importance.

Effect of Exposure to Atmospheric Air on the Carbon Dioxide Capacity of the Celomic Fluid.

Several samples of celomic fluid taken from specimens exposed to atmospheric air for varying periods were analyzed in the Van Slyke apparatus (2) when equilibrated with atmospheric air and also when equilibrated with alveolar air of the normal subject after the manner described by Van Slyke and Cullen (3). Table II illustrates the results obtained in this series of experiments. It will be noted that in every instance the carbon dioxide capacity of the sample was considerably in excess of the carbon dioxide content of the same when equilibrated with atmospheric air.

TABLE II.

No. used.	Specimen.	CO_2 content of 100 cc. celomic fluid.		Remarks.
		Equilibrated with atmospheric air.	Equilibrated with alveolar air.	
		cc.	cc.	
1	*Echidnocerus formatus*	22.5	28.0	3 hrs. out of water.
1	*Pisaster ochracea*	4.1	9.8	Fresh.
1	" "	7.4	11.2	9 hrs. out of water.
1	" "	7.0	12.2	32 " " " "
1	*Strongylocentrotus drobachiensis*	7.4	9.8	Fresh.
1	*Dermasterias imbricata*	5.5	8.8	"
1	*Schizothoerus nuttalli*	9.9	14.4	"
3	" "	30.7	39.8	30 hrs. out of water.
1	*Balanus aquilla*	11.2	18.6	Fresh.
3	*Mya arenaria*	34.8	39.4	25 hrs. out of water.
10	" "	42.0	50.0	60 " " " "
4	" "	17.6	23.3	24 " in boiled sea water in sealed container.
11	" "	36.0	40.0	28 hrs. out of water.
3	" "	28.2	33.0	24 " " " "
10	" "	6.5	13.0	Fresh.
2	" "	18.8	25.4	25 hrs. in desiccator over alkaline pyrogallol.
8	*Paphia staminea*	39.5	45.0	28 hrs. out of water.
1	*Cardium corbis*	12.6	18.0	2 " " " "
1	*Saxidomus*	39.6	43.2	30 " " " "
1	*Polynices lewisii*	77.4	82.8	30 " " " "
1	" " (fluid from foot)	16.5	21.5	Fresh.

Effect of Exposure to Atmospheric Air on the Total Nitrogen Content of the Celomic Fluid.

In order to determine if the increase in the carbon dioxide content of molluscan celomic fluid brought about by exposure to atmospheric air was in any way due to an increase in its protein content the total nitrogen was determined in from 25 to 100 cc. of composite samples of celomic fluid taken from several specimens. The estimation was made by the usual Kjeldahl method. The results are expressed in Table III. As there was a slight

TABLE III.

No. used.	Specimen.	Total nitrogen per 100 cc. of fresh material.	Total nitrogen per 100 cc. after exposure to atmospheric air.	Time in air.
		mg.	*mg.*	*hrs.*
1	*Schizothoerus nuttalli*.....	37.5		0
2	" "	34.0		0
3	" "		34.4	32
15	*Saxidomus gigantea*.....	50.3		0
8	" "		51.8	30
23	*Paphia staminea*.....	70.0		0
8	" "		61.6	28
6	*Cardium corbis*.....	37.5		0
6	" "		50.4	30
14	*Mya arenaria*.....	33.6		0
11	" "		40.7	28
1	*Polynices lewisii* (fluid from foot)...		9.6	52

increase in two species, *Cardium corbis* and *Mya arenaria*, practically no change in *Saxidomus gigantea*, and a slight decrease in *Schizothoerus nuttalli* and *Paphia staminea*, it is very unlikely that protein plays any appreciable part in the increase in the combined carbon dioxide or alkali reserve of the celomic fluid of the mollusk which has been exposed to atmospheric air.

Effect of Exposure to Atmospheric Air upon the Calcium and Magnesium Content of the Celomic Fluid.

Calcium and in a few instances magnesium were determined in composite samples of celomic fluid taken from several specimens which had been exposed to atmospheric air for varying

TABLE IV.

No. used.	Specimen.	CO_2 per 100 cc. of fluid equilibrated with atmospheric air.	Ca per 100 cc.	Mg per 100 cc.	Alkalinity.	Reactivity.	Remarks.
		cc.	*mg.*	*mg.*	*cc. 0.01 N NaOH*	*cc. 0.01 N H_2SO_4*	
	Experiment I.						
10	*Mya arenaria*.....	6.5	35.4	49.0	−9.0	90.0	Fresh. Kept some days in floating carriage off landing stage.
4	" "	38.0	92.0	79.0			24 hrs. in air at temperature of sea water.
10	" "	69.0	186.0	82.0	−24.0	720.0	48 " " " " " " " "
8	" "	78.2	220.0		−60.0	1,080.0	72 " " " " " " " "
2	" "	105.0	240.0	90.0	−130.0	1,140.0	96 " " " " " " " "
	Experiment II.						
10	*Mya arenaria*.....	6.5	30.0	44.5			Fresh. Kept some days in floating carriage attached to landing stage.
10	" "	65.0	135.0	48.0			48 hrs. in air at temperature of sea water.
11	" "	74.5	223.0	57.3			96 " " " " " " " "
	Experiment III.						
2	*Mya arenaria*.....	7.5	38.0		−6.0	76.0	Fresh.
3	" "	38.7	140.0		−18.0	450.0	24 hrs. in air at temperature of sea water.
4	" "	61.2	208.0		−30.0	690.0	48 " " " " " " " "
3	" "	72.5	231.0	75.0	−24.0	860.0	72 " " " " " " " "
1	" "	84.0					96 " " " " " " " "
	Experiment IV.						
5	*Cardium corbis*....	9.3	48.0	78.0	−17.6	140.0	Fresh from Sand Beach.
8	" "	16.3	84.0	78.0	−32.0	200.0	Kept 24 hrs. in air in laboratory.
	Experiment V.						
5	*Cardium corbis*....	8.4	48.0	100.8	−16.0	95.0	Fresh from Sand Beach.
7	" "	19.0	98.0	100.2	−30.0	220.0	Kept 20 hrs. in air in laboratory.

periods of time. The method of McCrudden (4) was followed, the calcium being estimated by the titration of the oxalate with 0.1 N potassium permanganate. 25 cc. of celomic fluid were used in most instances. This was evaporated to dryness on the water bath, fused, dissolved by the aid of concentrated hydrochloric acid, and the calcium finally precipitated as the oxalate. The results are shown in Table IV. It will be noted that, whereas magnesium increases only very slightly in the blood of an exposed mollusk, the calcium increases to a great extent and also the increase in this latter constituent is more or less parallel with the increase in the combined carbon dioxide. It is therefore evident that the great increase in the alkali reserve of the blood of molluscan forms when exposed to atmospheric air is due to an increase in the concentration of bicarbonate which is balanced for the most part by an increase in the concentration of the calcium ions. Myers (5) has reported finding 307 mg. of calcium calculated as oxide in the blood of *Saxidomus nuttalli* and 197 mg. in *Schizothoerus nuttalli*. He has also commented on the very high calcium content of molluscan blood. I have failed to find that the calcium content of the celomic fluid of fresh molluscan forms differs materially from that of sea water. It is only after exposure to air that the calcium content becomes high.

Effect of Exposure to Atmospheric Air upon the Total Alkalinity and the Buffer Value or Reactivity of the Celomic Fluid.

Lacking the means of determining the hydrogen ion concentration, a most important factor in these experiments, a method was employed to determine approximately the total alkalinity of the blood and also its buffer value. The method adopted was similar to that previously described (1) based on the principle made use of in the method of double titration for bicarbonates by Brown and Escombe (6). The presence of small amounts of protein would of course introduce an error but as has been shown the protein content of the celomic fluid does not vary to any appreciable extent and therefore approximately the same degree of error would exist in all the titrations. The alkalinity was determined by noting the amount of 0.01 N alkali required to produce a just noticeable pink tint when phenolphthalein had

been added to the celomic fluid. The reactivity of Moore and Wilson (7) or the buffer value of Sörensen (8) was determined by titrating from the phenolphthalein to the methyl orange point using 0.01 N sulfuric acid. 0.2 N acid was used in those instances where the reactivity was of large proportion. The results are shown in Table IV. It will be noted that the rate of increase in the reactivity of the celomic fluid is in close agreement with the rate of increase of calcium and also of the combined carbon dioxide content of the same.

Effect of Exposure to Air Followed by Submersion in Fresh Water.

Table V illustrates that, whereas exposure to air causes a rapid increase in the alkali reserve of the celomic fluid, the subsequent immersion in fresh sea water causes a return to approximately the normal value for this factor.

TABLE V.

Specimen.	CO_2 content of 100 cc. of fluid equilibrated with atmospheric air.	Remarks.
	cc.	
Mya arenaria..	8.2	Fresh.
" " ..	32.0	72 hrs. in glass container in laboratory.
" " ..	23.3	Submerged 4 hrs. in fresh sea water.
" " ..	14.9	" 16 " " " " "
" " ..	9.2	" 64 " " " " "

Effect of Submersion in Sea Water in a Sealed Container.

Several fresh specimens of *Mya arenaria* were immersed in a relatively small volume of sea water in a cylindrical glass container which was then tightly sealed. After varying periods of time the celomic fluid of the specimens was examined. An analysis of the sea water which was used in the experiment was also made. The results are expressed in Table VI. A few experiments were carried out in which boiled out sea water was used in place of fresh sea water. In one instance sea water, the buffer value of which had been greatly increased by the addition of 5 gm. of basic sodium phosphate per liter, was used. It will

be noted that the behavior of *Mya arenaria* kept in a sealed container in either boiled or fresh sea water is very similar to that observed when the specimens were kept in the air. The alkali reserve and the calcium content of the celomic fluid mount steadily until the animal dies. The increase in the carbon dioxide and calcium content of sea water is also considerable. The values for these two factors in sea water are, however, lower in the case of the celomic fluid except after long submersion of the specimens, in which instance there is a tendency for the concentrations of these latter substances in the celomic fluid and sea water to equalize. The addition of basic sodium phosphate to the boiled sea water used in one experiment did not materially alter the results. A dense precipitate was formed in the sea water in this experiment which consisted for the most part of calcium phosphate.

If one considers the increase in the combined carbon dioxide in the sea water used in an experiment one finds that there is very little difference in the rate of increase in this factor in specimens exposed to air and in specimens submerged in a relatively small volume of sea water. Thus in Experiment I, Table VI, an increase of 29.3 volumes per cent was noted in the combined carbon dioxide content of the celomic fluid, while an increase of fully 20 volumes per cent took place in the sea water. The total bulk of the eight specimens used in this experiment was 550 cc. while the volume of sea water used was 750 cc. There was therefore an increase of 150 cc. of combined carbon dioxide due to the activity of the specimens over and above the increase noted in their celomic fluid. The shells of the eight specimens displaced 85 cc. of water. Not allowing for the water entrapped in the mantle cavities, there were 465 cc. of clam tissue present in this experiment so that the increase of 150 cc. of carbon dioxide found in the sea water would mean that 32 cc. of this carbon dioxide had resulted from the activity of each 100 cc. of clam tissue. This added to the carbon dioxide content of 100 cc. of celomic fluid indicates that approximately 61.3 cc. of combined carbon dioxide resulted from the activity for 46 hours of 100 cc. of clam tissue, an amount which is in close agreement with the observed increase of the volume per cent of carbon dioxide in the celomic fluid of similar specimens exposed to atmospheric air in a closed vessel for a corresponding period of time (Table IV).

TABLE VI.

No. used.	Specimen.	CO_2 per 100 cc. equilibrated with atmospheric air.	Ca per 100 cc.	Mg per 100 cc.	P_2O_5 per 100 cc.	Alkalinity.	Reactivity.	Remarks.
		cc.	*mg.*	*mg.*	*mg.*	*cc. 0.01 N NaOH*	*cc. 0.01 N H_2SO_4*	
	Experiment I.							
10	*Mya arenaria*.....	7.5	38.0			−6.0	74.0	Fresh.
8	" " (550 cc.).....	36.8	149.0			−25.0	550.0	46 hrs. in 750 cc. sea water in sealed container at temperature of sea water.
	Sea water (750 cc.).....	23.4	102.0	95.0		−13.5	140.0	8 *Mya arenaria* in this 46 hrs.
	Experiment II.							
	Boiled sea water.....	2.2	33.0			3.0	11.3	
10	*Mya arenaria*.....	6.5	35.4	49.0		−9.0	90.0	Fresh control.
1	" "	31.5						One of fifteen in boiled sea water in sealed container 42 hrs. All alive.
12	" "	51.0	121.0					90 hrs. in boiled sea water; 3 dead, 12 living; analysis of latter only.
	Boiled sea water (700 cc.)....	51.0	121.0			−21.4	350.0	15 *Mya arenaria* (600 cc.) in this 90 hrs.
	Experiment III.							
	Boiled sea water.....	2.2			273.0	−30.0	160.0	5 gm. of Na_2HPO_4 added per liter.

11	*Mya arenaria*.....	6.5	29.0			−11.2	80.0	Fresh control.
11	" "	26.6	125.0		51.0	−70.0	480.0	All alive (600 cc.). 44 hrs. in boiled sea water as above.
	Boiled sea water (700 cc.)....	26.6	50.0	50.0	50.0	−82.0	265.0	11 *Mya arenaria* in this 44 hrs.
	Experiment IV.							
	Boiled sea water.....	3.7				4.0	16.7	
10	*Mya arenaria*.....	6.5	56.0	66.0		−9.0	90.0	Fresh control.
4	" "	17.5						24 hrs. in boiled sea water (250 cc.). All alive.
	Boiled sea water (250 cc.)....	9.4				−5.5	54.0	4 *Mya arenaria* in this 24 hrs.

TABLE VII.

No. used.	Specimen.	CO_2 per 100 cc. equilibrated with atmospheric air.	Ca per 100 cc.	Mg per 100 cc.	Cl	Alkalinity.	Reactivity.	Remarks.
		cc.	*mg.*	*mg.*	*per cent*	*cc. 0.01 N NaOH*	*cc. 0.01 N H_2SO_4*	
	Experiment I.							
12	*Mya arenaria*..	6.5	35.4	49.0		−9.0	90.0	Fresh control.
20	" " ..	25.0	84.0	28.5		−24.0	330.0	45½ hrs. in distilled water in sealed container. 10 alive, 1 dead. Bulk of clams 390 cc. Water 400 cc.
	400 cc. water...	13.0	30.0	15.0		−11.0	95.0	11 *Mya arenaria* in this 45½ hrs.
	Experiment II.							
6	*Mya arenaria*..	7.5	38.0		1.301	−9.0	90.0	Fresh. Cl in sea water 1.303 mg.
6	" " ..	19.5	77.6	24.0	0.747	−9.0	250.0	24 hrs. in fresh distilled water in sealed container. Bulk of clams 530 cc. Water 770 cc.
	770 cc. water...	4.0	15.0	10.0	0.211	−3.5	38.0	6 *Mya arenaria* in this 24 hrs.
	Experiment III.							
2	*Mya arenaria*..	20.5			0.802			20 hrs. in distilled water in sealed container. Bulk of clams 200 cc. Water 1,100 cc.
	1,100 cc. water.				0.132			2 *Mya arenaria* in this 20 hrs.

Effect of Submersion in Distilled Water.

The results of some experiments in which specimens of *Mya arenaria* were submerged in fresh distilled water in a glass container and kept at the temperature of surface sea water appear in Table VII. The combined carbon dioxide and calcium content of celomic fluid rise after much the same manner as was observed when specimens were placed in either fresh or boiled sea water. Experiments I and II, Table VII, are of interest in that they show that a fall had taken place in the concentration of magnesium in the celomic fluid of the specimens, while a rise occurred in the concentration of calcium. There is also a marked difference in the total alkalinity and in the reactivity of the water and the celomic fluid. The chlorine content of the celomic fluid and of the water in which the specimens of *Mya arenaria* were submerged was determined in Experiments II and III, Table VII. The manner in which the concentration of the chloride in the celomic fluid is kept at a relatively high level under the circumstances obtaining in these experiments is remarkable. Meigs (9) has shown that the adductor muscle of the clam *Venus mercenaria* is peculiarly resistant to hypertonic solutions of sodium chloride and to double strength sea water, the concentration of sodium chloride rising to only about one-half that of the surrounding medium. He also found that the mantle is almost impermeable to sodium chloride. The ability of *Mya arenaria* to withstand immersion in water, the osmotic pressure of which is very low, is of interest in the light of the observation of Meigs.

Effect of Exposure to a Hydrogen Atmosphere.

Several specimens of *Mya arenaria* were kept in a small volume of sea water for 2 hours in order that the oxygen content of their tissues should be reduced to a low level. One specimen was then bled as a control, the others were placed in a hydrogen atmosphere over alkaline pyrogallic acid, two separate containers being used. One group of three was exposed for 26 hours; the other for 48 hours. The results of the analyses of the celomic fluids of these specimens are shown in Table VIII. The maximum and minimum temperatures for the 2 days during which

TABLE VIII.

No. used.	Specimen.	CO_2 per 100 cc. of fluid equilibrated with atmospheric air.	Ca per 100 cc.	Mg per 100 cc.	Alkalinity.	Reactivity.	Remarks.
		cc.	*mg.*	*mg.*	*cc. 0.01 N NaOH*	*cc. 0.01 N H_2SO_4*	
1	*Mya arenaria*....	8.2					Kept in small volume of sea water 2 hrs.
3	" "	26.4	156.0	75.0	−16.0	410.0	Kept in hydrogen atmosphere over alkaline pyrogallol 26 hrs.
3	" "	45.5			−22.0	690.0	Kept in hydrogen atmosphere over alkaline pyrogallol 48 hrs.
4	" "	53.0			−45.0	950.0	Kept in hydrogen atmosphere 56 hrs.

this experiment was performed were on the 1st day 24.4° and 10.9°C., on the 2nd day 23.9° and 11.4°C. It will be noticed that the increase observed in the concentration of bicarbonate in the celomic fluid is very marked. It is not, however, so great as that which is found when the specimens are exposed to atmospheric air. The increase in the calcium and magnesium is comparable to that observed for bicarbonate.

Effect of Exposure to a Nitrogen Atmosphere.

Three specimens of *Mya arenaria* were placed in a small desiccator containing a concentrated solution of pyrogallic acid in 40 per cent sodium hydroxide. A glass tube was so attached

TABLE IX.

No. used.	Specimen.	CO_2 per 100 cc. of fluid equilibrated with atmospheric air.	Remarks.
		cc.	
3	*Mya arenaria*.	8.0	Fresh control.
3	" " .	34.8	25 hrs. in glass container in laboratory.
3	" " .	17.8	25 " " desiccator over alkaline pyrogallol.

to the exhaust cock that as oxygen was absorbed the air which entered first bubbled through the alkaline pyrogallol in the bottom of the desiccator. Three other specimens of like size were placed at the same time in a glass container which was kept at the same temperature as the desiccator for the duration of the experiment. After 25 hours the specimens were bled and an analysis was made of the composite samples of celomic fluid. The results are expressed in Table IX. It will be observed that the combined carbon dioxide of the celomic fluid did not increase to the same extent in the specimens which were kept in the desiccator over alkaline pyrogallol as it did in the controls which were kept in the air.

TABLE X.

Specimen.	Fluid examined.	CO_2 per 100 cc. equilibrated with atmospheric air.	Remarks.
		cc.	
Mya arenaria.....	Celomic fluid from sinus.	8.3	Fresh.
" "	Fluid from exhalent siphon.	4.6	" Same specimen as above.
" "	Celomic fluid from sinus.	39.6	25 hrs. out of water.
" "	Fluid from mantle cavity.	36.0	25 " " " " Same specimen as above.
Cryptochiton.....	Celomic fluid from sinus.	9.0	Fresh.
"	Fluid from foot.	3.6	" Same specimen as above.
Polynices (Lunatia) *lewisii*.	Celomic fluid from sinus.	20.7	Fresh.
" " "	Fluid from foot.	17.1	" Same specimen as above.
" " "	Celomic fluid from sinus.	21.0	"
" " "	Fluid from foot.	16.5	" Same specimen as above.
" " "	Celomic fluid from sinus.	14.9	"
" " "	Fluid from foot.	12.5	" Same specimen as above.
" " "	Celomic fluid from sinus.	31.0	24 hrs. out of water.
" " "	Fluid from foot.	31.0	24 " " " " Same specimen as above.
" " "	Celomic fluid from sinus.	12.1	Fresh.
" " "	Fluid from foot.	12.1	" Same specimen as above.
" " "	Celomic fluid from sinus.	65.7	52 hrs. out of water.
" " "	Fluid from foot.	63.0	52 " " " " Same specimen as above.
" " "	Celomic fluid from sinus.	77.4	30 " " " "
" " "	Fluid from foot.	59.4	30 " " " " Same specimen as above.
" " "	Celomic fluid from sinus.	61.2	52 " " " "
" " "	Fluid from foot.	37.8	52 " " " " Same specimen as above.

Comparison of the Carbon Dioxide Content of Celomic Fluid and Other Fluids Obtained from Mollusca.

A comparison was made between the carbon dioxide content of the celomic fluid and other fluids of different Mollusca. The results of this study are shown in Table X. It was found that the carbon dioxide content of the fluid which exuded from the exhalant siphon of fresh *Mya arenaria* was just slightly higher than that of sea water. When specimens of this species were exposed to atmospheric air for some time the bicarbonate content in the fluid of the mantle cavity closely approximated that in the celomic fluid. Somewhat similar observations were made on the amphineuran form *Cryptochiton*. The fluid in the foot of the large gastropod *Polynices lewisii* bears somewhat different relation to the blood and celomic fluid of this form as far as the bicarbonate content is concerned from that which holds in the fluid between the mantle cavity and the blood and celomic fluid of a pelecypod such as *Mya arenaria*. The combined carbon dioxide content of the fluid of the foot of *Polynices lewisii* approximates the value observed for the celomic fluid. The drawing in, therefore, of the foot in this form causes a considerable decrease in the total alkali reserve of the animal. It is of interest in this connection to note that this animal does not withdraw its foot unless subjected to rather violent irritation.

Effect of Submersion of Dead Specimens of Mya arenaria in Sea Water.

The results of two experiments are shown in Table XI. It is evident that the bicarbonate content of sea water in which dead clams are immersed rises quite rapidly once decomposition has set in. It will be noted, however, that there is little change during the first 24 hours submersion. The behavior of pelecypod mollusks exposed to air or submerged in a relatively small volume of water is therefore quite distinct from that of dead clams which are undergoing decomposition.

TABLE XI.

Specimen.	Total CO_2 per 100 cc.	Remarks.
	cc.	
Experiment I.		
4 *Mya arenaria* (175 cc.)....		Placed in 275 cc. of boiling sea water.
Sea water (boiled).....	3.3	
" " "	4.7	After 24 hrs. No decomposition of clam tissue.
" " "	42.0	After 48 hrs. Decomposition clearly manifested.
" " "	55.0	After 72 hrs.
" " "	67.0	" 96 "
" " "	72.0	" 144 "
Experiment II.		
10 *Mya arenaria* (500 cc.)....		Placed in 700 cc. of boiled sea water containing 9.5 per cent alcohol.
Sea water (boiled).....	2.8	
" " "	3.3	After 24 hrs.
" " "	11.7	" 48 " Milky, decomposition evident.
" " "	35.0	After 96 hrs.

DISCUSSION.

As has already been indicated the marked increase in the bicarbonate content of the celomic fluid, and therefore in all probability of the blood and tissues of the calcareous shelled pelecypod mollusks and the arthropod *Balanus aquilla* on exposure to air is quite opposite to the effect observed in fishes when they are removed from their natural habitat. This phenomenon is undoubtedly associated with the presence of a calcareous shell the calcium carbonate of which furnishes an alkali reserve which is added to that of the body fluids and tissues, and which it appears can be readily utilized.

As specimens of *Mya arenaria* appear to remain practically normal even after long exposure to atmospheric air there is no reason to suppose that any material change has been effected in their metabolic processes as a result of the change in environment. If one assumes, therefore, that combustion still proceeds in the

exposed specimens, then the increase in the bicarbonate content of the body fluids can be explained according to the equation

$$CO_2 + H_2O + CaCO_3 \rightleftharpoons Ca(HCO_3)_2$$

The carbon dioxide resulting from the respiratory process would, by slightly increasing the hydrogen ion concentration, dissolve calcium carbonate from the shell and the concentration of the calcium ions and of bicarbonate ions would therefore steadily rise as combustion in the tissues proceeded. The amount of carbon dioxide actually excreted from the specimens in the gaseous form was not determined. If this factor were known one could calculate the intensity of metabolism in these forms by considering the amount of carbon dioxide excreted in addition to the amount retained as bicarbonate. It would appear that 50 per cent of the increase observed in the carbon dioxide content of the celomic fluid is due to carbon dioxide formed by combustion in the tissues, while the remaining 50 per cent results from the solution of calcium carbonate of the shell.

The degree of alkalinity of the celomic fluid determined by titration is by no means an indication of the hydrogen ion concentration, but the ratios observed between the alkalinity figures and the reactivity values suggest that no marked increase in the hydrogen ion concentration takes place during the early part of the exposure at least. The reactivity or buffer value is, in nearly every instance, in close agreement with the calcium content and the carbon dioxide concentration of the celomic fluid.

The increase in the alkali reserve as indicated by an increase in bicarbonate concentration in specimens exposed to atmospheric air is due for the most part to increase in the calcium content. Magnesium, which in the normal animal in its natural habitat exceeds calcium in the degree of its concentration, and therefore balances a greater proportion of bicarbonate ions than does calcium, increases only slightly as compared with calcium when a specimen is exposed to air. It is probable that the relative increase in calcium and magnesium concentrations under these circumstances is somewhat similar to the relative amounts of these substances in the shell from which solution of bicarbonate is taking place. It is of interest to note here that no increase

was observed in the concentration of magnesium in the cockle (*Cardium corbis*) on exposure to air.

As specimens submerged in boiled sea water and kept in a sealed container continue to develop an increased carbon dioxide content, calcium concentration, and buffer value, after much the same manner as specimens exposed to atmospheric air, and since a similar effect is manifested by specimens kept in an atmosphere of hydrogen or nitrogen, one is led to ask the question "Can anaerobic respiration be manifested by these forms?"

If one considers the results of an experiment recorded in Table VIII, one finds that after 48 hours in a hydrogen atmosphere the combined carbon dioxide rose from 8.2 to 45.5 volumes per cent, or an observed increase of 37.3 volumes per cent. If one assumes that aerobic respiration was taking place and that carbohydrates were being burned, then a volume of oxygen equivalent to the volume of carbon dioxide produced would be required. If 50 per cent of the observed increase in the combined concentration of carbon dioxide is indicative of the amount of this substance produced due to combustion then an amount of oxygen equivalent to 50 per cent of 37.3 volumes per cent, or 18.65 volumes per cent, would be required. As the specimens used in this experiment were kept in a small volume of sea water for 2 hours before they were transferred to a hydrogen atmosphere, one fails to see how any appreciable amount of oxygen could be contained in the tissues of the specimens. As there is no apparent source for 18.65 volumes per cent of oxygen in these clams it is therefore evident that they must be respiring anaerobically or else the increase in the carbon dioxide, calcium, and buffer value of the celomic fluid is due to some other cause than that suggested earlier in the paper. Decomposition of the clam tissue can be excluded since the specimens were very active, responding to stimulation like normal animals, after they were removed from the hydrogen atmosphere.

There is the possibility of the solution of the calcium carbonate of the shell due simply to the solvent action of the tissue fluids containing free carbon dioxide. This would result in the formation of calcium carbonate the solubility of which is considerably increased by an excess of carbon dioxide in the water (10). In dealing with a closed system, however, such as the individual

clam in an atmosphere of hydrogen, the solution of calcium carbonate due to the solvent action of the free carbon dioxide would require a constant supply of the latter if the process is to continue; otherwise equilibrium would be established between the dissolved bicarbonate, the calcium carbonate of the shell, and the free carbon dioxide, and no further increase would be manifested unless this balance were disturbed. It will be noted that the rate of increase in the carbon dioxide content and the calcium concentration of the blood is for a considerable period practically constant. If respiratory activity accounts for the increase in bicarbonate concentration of the tissue fluids, then this uniformity in the rate observed would fit in well with the fairly constant rate of metabolism which might be expected under such circumstances, anaerobic respiration being possible.

The fact that the rate of increase in the bicarbonate concentration, the calcium content, and the buffer value is greater in air than it is in either hydrogen or nitrogen would indicate that absence of oxygen does exert an influence on the intensity of the metabolic processes but by no means causes a complete cessation in the respiratory function.

The extreme sensitivity of the cockle and the horse clam to exposure to air at the prevailing summer temperatures made experiments with these forms of the same type as were conducted with *Mya arenaria* temporarily impossible. Neither of these forms is normally submitted to the same degree of low oxygen tension as is *Mya arenaria*. It is hoped that experimental work of a similar nature to that carried out with *Mya arenaria* may be done on other forms at a more favorable time of the year.

It has long been known that animals show a very unequal resistance to lack of air. Bunge (11) in his work upon respiration of intestinal parasites and mud-dwelling organisms showed that parasites in the intestine of warm blooded animals must live practically in the absence of oxygen, while worms living in the mud were also subject to similar conditions, decomposition processes, with the formation of reducing substances, keeping the oxygen absent. Packard (12) found that worms and mud-dwelling Crustacea are resistant to the lack of oxygen for some time.

The ability of an animal to resist a lack of oxygen may or may not be connected with an anaerobic respiratory mechanism. If one finds complete evidence of metabolism in an animal exposed to anaerobic conditions, then anaerobic respiration would be indicated. Such seems to be the condition in the case of *Mya arenaria*. Crustacean types which were exposed to the air or submerged in boiled sea water died within a few hours. The carbon dioxide content of the blood was, however, practically unaltered by such procedures. These forms do not use the calcium carbonate of their carapace as a protective measure when removed from their normal habitat.

In the light of the results of the experiments which have so far been conducted upon *Mya arenaria* the writer has tentatively to conclude that individuals of this species behave as facultative anaerobic organisms. It is realized, however, that in these preliminary experiments absolutely anaerobic conditions were not secured. It is the intention to continue this work at another time when it is hoped to determine the hydrogen ion concentration of the celomic fluid and the rate of oxygen consumption under various conditions.

SUMMARY.

1. Calcareous shelled pelecypod Mollusca and the arthropod *Balanus aquilla* have in the calcium carbonate of their shells a potentially great alkali reserve.

2. Exposure of these forms to atmospheric air causes a marked increase in the combined carbon dioxide of the celomic fluid.

3. There is under these circumstances a parallel increase in the calcium concentration and the buffer value of the celomic fluid.

4. Various other marine forms studied did not so react.

5. There is no increase in the total nitrogen of the celomic fluid of the pelecypod Mollusca exposed to atmospheric air.

6. *Mya arenaria* is particularly resistant to long exposure to atmospheric air.

7. When specimens of *Mya arenaria* are placed in a relatively small volume of fresh sea water, boiled sea water, distilled water, or in a hydrogen or a nitrogen atmosphere much the same reaction is observed as when specimens are exposed to atmospheric air.

8. The rate of increase in the content of carbon dioxide, the calcium concentration, and the buffer value of the celomic fluid under all the above conditions is, during the first period of several hours, constant.

9. The rate of increase in the rate of combined carbon dioxide, the concentration of calcium, and the buffer value is not so great in a hydrogen or nitrogen atmosphere as it is in air.

10. It is suggested that *Mya arenaria* is a facultative anaerobic organism which continues to produce carbon dioxide under anaerobic conditions.

In conclusion I wish to express my thanks to Dr. C. McLean Fraser, the Curator of the Biological Station, Departure Bay, British Columbia, for his cooperation during the carrying out of the experimental work reported in this communication.

My thanks are also due to the Biological Board of Canada, by whom the expenses in connection with this investigation have been defrayed.

BIBLIOGRAPHY.

1. Collip, J. B., *J. Biol. Chem.*, 1920, xliv, 329.
2. Van Slyke, D. D., *J. Biol. Chem.*, 1917, xxx, 347.
3. Van Slyke, D. D., and Cullen, G. E., *J. Biol. Chem.*, 1917, xxx, 289.
4. McCrudden, F. H., *J. Biol. Chem.*, 1911–12, x, 187.
5. Myers, R. G., *J. Biol. Chem.*, 1920, xli, 119.
6. Brown, H. T., and Escombe, F., *Phil. Trans. Roy. Soc.*, 1900, cxciii, 289.
7. Moore, B., and Wilson, F. P., *Biochem. J.*, 1906, i, 297.
8. Sörensen, S. P. L., in Moore, B., Prideaux, E., and Herdman, G., *Trans. Biol. Soc. Liverpool*, 1915, xxix, 174.
9. Meigs, E. B., *Am. J. Physiol.*, 1914, xxxiii, p. xxii.
10. Reuter, M., *Z. anorg. Chem.*, 1898, xvii, 170.
11. Bunge, G., *Z. physiol. Chem.*, 1883–84, viii, 48; 1888, xii, 565; 1890, xiv, 318.
12. Packard, W. H., *Am. J. Physiol.*, 1905–06, xv, 30.

RELATION OF THE ALKALI RESERVE OF THE BLOOD TO GLYCOSURIA AND HYPERGLYCEMIA IN PANCREATIC DIABETES.

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(Received for publication, October 7, 1920.)

The failure of the body to utilize carbohydrate and the increased combustion of fat in diabetes are regarded by most workers as responsible for the production of an excessive amount of acids, particularly aceto-acetic and β-hydroxybutyric acid, which cause a diminution of the alkali reserve of the blood and presumably of the tissues. It has been shown, on the other hand, by Elias (1912–13), and Elias and Kolb (1913), and others,[1] that the administration of mineral acids to dogs causes glucosuria and hyperglycemia. Jensen's (1918) work, however, seems to indicate that if the acid is sufficiently dilute much larger quantities may be given to dogs without causing an excretion of glucose by the kidney. He was able to increase glucosuria in dogs by administering 600 cc. of 0.1 N hydrochloric acid solution, only after seven-eighths of the pancreas had been removed.

Murlin and Kramer (1916) were able to increase the amount of glucose in the blood and urine of depancreatized dogs by giving, by mouth, a solution of hydrochloric acid. These results led Murlin and Sweet (1916–17) to study the course of pancreatic diabetes after the extirpation of the stomach. They found a much milder form of diabetes appeared when both stomach and pancreas were removed, than when the pancreas alone was extirpated. They believe their results indicate that the influence of the pancreas upon carbohydrate metabolism consists, at least in part, in neutralizing the acid produced by the stomach, thereby

[1] The literature on the effects of administering acids to animals is reviewed in the papers of Elias and of Elias and Kolb.

protecting the liver from acid intoxication. Their theory of the function of the pancreas is as follows:

"The results of this study . . . . suggest that the internal function of the pancreas may be closely akin to its external function in that, on the one hand (external), it provides for the complete neutralization of the acid contents of the stomach, thereby protecting the liver in its glycogenic function, and on the other (internal), it somehow preserves the proper concentration of hydrogen ions in the tissues for the combustion of glucose. It is not impossible that the pancreatic hormone, through which the latter function is discharged, may prove to be a peculiarly adapted alkali produced by the islands of Langerhans."

EXPERIMENTAL.

If the gastric juice were not completely neutralized in the intestine after the removal of the pancreas, it would react with the carbonates and phosphates of the blood in the portal system causing a reduction in the alkali reserve in the whole blood, inasmuch as the blood from the portal system is mixed with the blood from the rest of the body in the heart, lungs, and, to a less extent, in the liver. Since hyperglycemia and glucosuria appear very soon after the removal of the pancreas, it was thought worth while to study the alkali reserve of the blood as measured by the power of the plasma to combine with carbon dioxide, and compare this with the change in the concentration of glucose in the blood, and its excretion into the urine. The capacity of the plasma for carbon dioxide was determined by the method of Van Slyke (1917), Maclean's (1919) method for blood sugar was used, and the glucose in the urine was determined by titrating against Fehling's solution. Dr. Sweet removed the pancreas from the dogs by his usual technique.

DISCUSSION.

Our results from five dogs are given in Table I. We found that the alkali reserve of the blood, as measured by the carbon dioxide capacity of the plasma, showed no decrease until diabetes, as shown by the G:N ratio and increase in blood sugar, had been established for at least 24 hours. The earliest appearance of a decrease in the carbon dioxide capacity of the blood came about

TABLE I.

Time after operation.	CO_2 per 100 cc. plasma.	Glucose.*	Glucose in urine.	Nitrogen in urine.	G:N	Remarks.
			No. 1. Pregnant female.			
days	*cc.*	*per cent*	*gm.*	*gm.*		
Before.	41.5	0.063				
1	56.1	0.1998	17.70	7.18	2.47	Fasted.
2	59.6	0.257	18.93	8.59	2.2	
3	51.5	0.321	24.62	9.41	2.62	
			No. 2. Female.			
Before.	50.4	0.059				Fasted.
1	54.6	0.177	Urine lost.			
2	44.2	0.161	30.66	10.83	2.83	Urine may not have been properly preserved.
3	48.9	0.163	19.3	6.87	2.83	Animal died during the night of 4th day after operation.
			No. 3. Male.			
Before.	45.3	0.055				Fasted.
1	47.6	0.118	15.93	6.71	2.37	
2	56.3	0.119	28.64	10.4	2.75	
3	42.2	0.143	23.52	9.2	2.53	No evidence of peritonitis found.
6	30.9	0.120	22.6	7.37	3.07	Found dead on 7th day.
			No. 4. Male.			
Before.	51.2	0.073				Urine not preserved.
1	58.0	0.150				
2	41.5	0.123	23.8	9.45	2.52	
3	42.0	0.148	17.09	9.04	2.33	
4	41.1	0.243				Animal fed ½ lb. meat per day. Refused food on 4th day. Found dead on 5th day. No evidence of peritonitis was found.

* Computed on whole blood.

TABLE I—*Concluded.*

Time after operation.	CO_2 per 100 cc. plasma.	Glucose.*	Glucose in urine.	Nitrogen in urine.	G:N	Remarks.
			No. 5. Female.			
days	*cc.*	*per cent*	*gm.*	*gm.*		
Before.	51.7	0.075				
1	51.4	0.098	33.91	15.19	2.23	Urine of 1st and 2nd days.
2	52.8	0.293				
3	49.9	0.241	24.19	9.64	2.51	Animal fed ½ lb. meat per day.
4	39.5	0.270	26.18	8.69	3.01	
5	39.6	0.238	17.34	8.47	2.05	
7	38.9	0.270	16.17	7.31	2.21	
8	34.7	0.280	16.46	7.20	2.28	
9	29.8	0.315	24.52	10.05	2.44	Killed.

48 hours after the removal of the pancreas, while there was always a definite increase in the blood glucose within 1 day after the operation. It is well known that glucose appears in the urine within a few hours after pancreatectomy. It is of interest to note the more or less marked increase (1 to 20 per cent by volume of CO_2) in the alkali reserve on the 1st or 2nd day after the operation. In most cases, this increase is comparatively small and its significance is not clear.

It would seem that if acid is taken up from the intestine by the portal blood after the removal of the pancreas, it must be neutralized by some base other than bicarbonates or phosphates of the blood, or cause a simultaneous liberation of alkali from some part of the body. It may be pointed out in this connection that the formation of a pancreatic fistula, so that the pancreatic juice escapes to the exterior, does not cause any disturbance in the carbohydrate metabolism of the animal. In such cases, the acid from the gastric juice would be as likely to reach the liver as when the pancreas had been removed from the body. It is questionable, also, whether fragments of the pancreas, which are sufficient to prevent diabetes, can produce enough pancreatic juice to be of any importance in neutralizing the acid produced in the stomach.

Acidosis in depancreatized dogs undoubtedly develops as shown by Nos. 3 and 5. In No. 3, the carbon dioxide capacity of the plasma fell to about 31 volumes per cent, and in No. 5, to about 30 volumes per cent.

SUMMARY.

1. A decrease in the alkali reserve of the blood, as shown by the carbon dioxide capacity of the plasma, is not simultaneous with the appearance of hyperglycemia and glucosuria in pancreatectomized dogs.

2. A slight increase in the carbon dioxide capacity of blood plasma was observed on the 1st or 2nd day after the removal of the pancreas, later decreasing and gradually reaching about 30 volumes per cent in two cases.

3. Definite acidosis appears comparatively late in pancreatic diabetes in dogs.

4. No observations which support Murlin and Sweet's theory of the causal relation of the acid of the gastric juice to pancreatic diabetes were made.

We wish to take this opportunity to thank Dr. J. E. Sweet very kindly for doing the surgical work.

BIBLIOGRAPHY.

Elias, H., *Biochem. Z.*, 1912–13, xlviii, 120.
Elias, H., and Kolb, L., *Biochem. Z.*, 1913, lii, 331.
Jensen, V. W., *J. Biol. Chem.*, 1918, xxxiii, p. viii.
Maclean, H., *Biochem. J.*, 1919, xiii, 135.
Murlin, J. R., and Kramer, B., *J. Biol. Chem.*, 1916, xxvii, 481.
Murlin, J. R., and Sweet, J. E., *J. Biol. Chem.*, 1916–17, xxviii, 261.
Van Slyke, D. D., *J. Biol. Chem.*, 1917, xxx, 347.

THE GLOBULIN OF THE COHUNE NUT, ATTALEA COHUNE.

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Plate 1.

(Received for publication, September 30, 1920.)

The cohune palm (1, 2) is indigenous to the British Honduras (3) and the other countries of the Caribbean coastal region, and its nut is shown whole, opened, and in cross-section in Fig. 1.[1] Most of the nuts have but one kernel, a few two.

It is found mostly in damp lands, growing best in the deep forest (4). The nuts are produced in large clusters of about 75 pounds each, a single tree having an average yearly production of four clusters. They vary in size from $1\frac{1}{2}$ to 3 inches in length and from 1 to 2 inches in diameter, the shell being hard and dense with an average thickness of $\frac{1}{4}$ to $\frac{1}{2}$ inch. In certain districts the cohune palm constitutes 20 to 30 per cent of the vegetation (3). The kernel averages about 10 per cent by weight of the whole nut.

The nut was not commercially exploited until the World War when the shells were carbonized and used in gas masks, the kernels being pressed for their oil, which is of a very high quality and is similar to coconut oil (3–5).

A search has not yet disclosed literature bearing on the utilization of the press-cake. The nuts are now being pressed for oil in the British Honduras, and experiments are in progress in this country to produce the oil on a commercial scale. The press-cake will be suitable for use in feeds, its value as such being indicated by the analysis of the isolated protein described herein.

[1] This information and photograph were furnished by Mr. O. F. Cook of the Office of Crop Acclimatization, United States Department of Agriculture, Washington.

TABLE I.

Comparison of Analysis of Coconut and Cohune Nut Globulins.

Moisture- and ash-free basis.

	Coconut globulin.		Cohune nut globulin.	Distribution of nitrogen.‡		
	*	†		N	Coconut globulin.‡	Cohune nut globulin.
	per cent	*per cent*	*per cent*		*per cent*	*per cent*
C	50.88	51.23	51.75	Amide	1.36	1.35
H	6.82	6.90	6.92	Humin	0.14	0.17
N	17.89	18.40	17.82	Basic	6.06	7.14
S	1.03	1.06	1.35	Non-basic	10.92	9.34
O§	23.38	22.41	22.16			
Ash	0.98	0.25	1.83			

* Ritthausen (6).
† Chittenden (7).
‡ Osborne and Harris (8).
§ Calculated by difference.

TABLE II.

Comparison of Analysis of Coconut and Cohune Nut Globulins.

Analyses by the Van Slyke method. Total N regained corrected for solubility of bases (9).

	Coconut globulin.*	Cohune nut globulin.
	per cent	*per cent*
Amide N	7.99	7.50
Humin N adsorbed by lime	1.41	0.84
" N in amyl alcohol extract	0.11	0.11
Cystine N	0.96	0.53
Arginine N	29.50	30.87
Histidine N	3.68	2.61
Lysine N	6.41	7.94
Amino N of filtrate	45.44	47.87
Non-amino N of filtrate	4.60	2.28
Total N regained	100.10	100.55
Distribution of the basic amino-acids.		
Cystine	1.44	0.81
Arginine	15.92	17.17
Histidine	2.42	1.72
Lysine	5.80	7.42
Tryptophane	Present.	Present.

* Johns, Finks, and Gersdorff (10).

This analysis shows that the protein contains all the basic amino-acids necessary to produce normal growth. The protein also gives a strong qualitative test for tryptophane.

The press-cake used in this investigation was prepared in this Laboratory from nuts furnished by the United States Food Administration. It contained 20.63 per cent of protein (N × 6.25).

The principal protein extracted by means of sodium chloride solution is a globulin. This was obtained by dialyzing the saline extracts and could also be precipitated from the extracts by means of ammonium sulfate. This globulin is quite similar to that obtained from the coconut, no marked differences being observed in any of the analyses. These comparative results are given in Tables I and II.

Tests for albumin showed that a trace was present. This albumin coagulates between 60° and 68°C. while the globulin coagulates at near the boiling point of water.

EXPERIMENTAL.

Preparation of the Press-Cake.—Much difficulty was experienced in removing the husk and shell. This was accomplished by cracking each nut individually between a steel plate and the head of a steel power drill. The kernels which ran mostly one to a nut, seldom two, were cold-pressed, using a press of the oil expeller type. The granular press-cake was freed from residual oil by extracting twice with petroleum ether, the excess of ether being removed by drying *in vacuo* over sulfuric acid. The press-cake was then ground to a fine meal which contained 20.63 per cent of protein (N × 6.25).

Preliminary Experiments.—Extraction experiments were made with different concentrations of sodium chloride in water, with 70 per cent alcohol and with 1 per cent hydrochloric acid, extracting for 1 hour in each case. The percentages of protein extracted by the various solvents are given in Table III. Time extractions were then made with the solvent which extracted the maximum quantity of protein, and the data are given in Table IV.

All extractions described in this paper were made with a 10 per cent sodium chloride solution in distilled water. This solvent extracted the maximum quantity of protein, 16.09 per cent, in 1 hour.

TABLE III.

Preliminary Extraction Experiments.

Solvent to 1 gm. of meal.	Solvent.	Protein extracted (N × 6.25).
cc.		*per cent*
10	70 per cent alcohol.	0.52
10	1 " " HCl.	12.66
10	Distilled water.	4.04
10	0.5 per cent NaCl.	7.74
10	1.0 " " NaCl.	11.52
10	1.5 " " NaCl.	11.08
10	2.0 " " NaCl.	12.39
10	2.5 " " NaCl.	14.51
10	3.0 " " NaCl.	14.59
10	3.5 " " NaCl.	15.13
10	4.0 " " NaCl.	15.03
10	5.0 " " NaCl.	15.21
10	7.0 " " NaCl.	15.30
10	8.5 " " NaCl.	15.91
10	10.0 " " NaCl.	16.09

TABLE IV.

Time Extraction Experiments.

Solvent: 10 per cent sodium chloride solution, 10 cc. to 1 gm. of meal.

Time extracted, *hrs*	½	1	2	3	20	45	69
Protein extracted (N × 6.25), *per cent*	13.71	16.09	14.16	14.07	13.71	13.14	12.84

TABLE V.

Globulin Precipitated by 0.7 of Saturation with Ammonium Sulfate.

Calculated on moisture- and ash-free basis.

	I	II	Average.
	per cent	*per cent*	*per cent*
C	52.77	52.62	52.70
H	7.03	7.06	7.05
N	17.80	17.68	17.74
S	1.21		1.21
O*			21.30
Moisture		6.87	
Ash		0.53	

* Calculated by difference.

Globulin Extracted by Sodium Chloride and Precipitated by Ammonium Sulfate.—An extraction was made, using 10 cc. of solvent to each gram of meal. The quantity of meal used for a preparation ranged from 125 to 210 gm. The meal was mixed with the solvent, all lumps being disintegrated by hand, and the mixture allowed to stand at room temperature for 1 hour. The mixture was then poured into a large Buchner funnel fitted with a circular piece of closely woven cotton cloth, and the extract recovered by suction, the residue being washed twice with 10 per cent sodium chloride solution. The extract so recovered was nearly clear and was easily filtered through a mat of paper pulp on a Buchner funnel. The extract was then dialyzed in parchment bags suspended in running chilled water until practically free from chlorides. The dialyzed protein was washed and then redissolved in a volume of 10 per cent sodium chloride solution equal to about one-third the volume of the solvent originally used in extracting the meal. It was filtered clear through a mat of paper pulp on a Buchner funnel. The clear extract was made 0.7 saturated with ammonium sulfate and allowed to stand over night. The precipitated protein was filtered on a folded soft filter paper, redissolved in distilled water, filtered clear, and dialyzed for 12 days. The protein was removed from the mother liquor in the dialyzing bags, washed free from chlorides and sulfates, and dried. The water was removed by treating the protein with absolute alcohol, removing the alcohol by suspension in absolute ether, filtering, and drying, first over sulfuric acid *in vacuo* and then in a vacuum drying oven, gradually raising the temperature to 110°C. It was then ground to a fine powder in a mortar, the finished product being an amorphous white powder. The yield based on the weight of the meal used was 5.6 per cent. In order to facilitate the weighing of samples, all preparations of protein were exposed to the air to absorb moisture until an equilibrium was reached.

The analyses of this preparation, calculated on a moisture- and ash-free basis, are given in Table V.

Globulin Extracted by Sodium Chloride and Fractionally Precipitated by Ammonium Sulfate.—A sodium chloride extract was made as described above and the clear solution was gradually made from 0.1 to 0.7 saturated with ammonium sulfate, first

experimenting with a small portion of the extract. No precipitation occurred until the 0.3 saturation point was reached, and then only a slight turbidity was obtained which very gradually increased on adding each successive fraction of ammonium sulfate to make the solution 0.05 more saturated, until the 0.5 saturation point was reached, when the precipitate became flocculent after standing 18 hours. This precipitate was filtered off and washed several times with 10 per cent sodium chloride solution made 0.5 saturated with ammonium sulfate. It was redissolved in 10 per cent sodium chloride solution, filtered and dialyzed for 12 days, washed, and dried as previously described. This is Preparation II. The yield based on the meal used was 3.2 per cent.

The 0.5 saturated ammonium sulfate filtrate was then made successively 0.05 more saturated until the 0.7 of saturation was reached, after which no more precipitate was obtained. A slight turbidity occurred at 0.55 of saturation which increased as each successive fraction was added, becoming flocculent at 0.7 of saturation. This fraction was filtered on a folded filter paper and redissolved in distilled water. It was filtered clear and dialyzed for 12 days. The dialyzed protein was washed and dried as previously described. The yield based on the meal used was 1.6 per cent. This is Preparation III.

The analyses of Preparations II and III calculated on the moisture- and ash-free basis appear in Table VI.

We were unable to fix on a decisive separation point and are inclined to believe that but one globulin is present in the cohune nut. Our belief is strengthened by the analyses of these two fractions as compared with the analyses of the globulin obtained in Preparations I, IV, and V, the same difference in carbon content being evident in the preparations of the globulin as appears in the two preparations made by fractional precipitation with ammonium sulfate. We were unable to make a further study along this line owing to a lack of material.

Globulin Extracted by Sodium Chloride and Precipitated by Direct Dialysis.—Two sodium chloride extracts were made as before, and the clear extract was dialyzed in parchment bags against running chilled water for 12 days. The dialyzed protein was washed and dried as described previously in this paper. The

yield of protein in each case was 8.57 per cent, based on the meal used.

TABLE VI.

Fractional Precipitation of the Globulin by Ammonium Sulfate.

Calculated on moisture- and ash-free basis.

	Preparation II. 0.5 saturated ammonia sulfate precipitate.			Preparation III. 0.7 saturated ammonia sulfate precipitate.		
	I	II	Average.	I	II	Average.
	per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
C	50.70	50.73	50.72	52.87	52.73	52.80
H	6.77	6.84	6.81	7.18	6.99	7.09
N	17.64	17.76	17.70	17.63	17.77	17.70
S	1.43		1.43	1.23		1.23
O*			23.34			21.18
Moisture		6.46			7.92	
Ash		1.00			0.90	

* Calculated by difference.

The analyses of these two preparations (Preparations IV and V) calculated on a moisture- and ash-free basis appear in Table VII.

TABLE VII.

Direct Dialysis Preparations.

Calculated on moisture- and ash-free basis.

	Preparation IV.			Preparation V.		
	I	II	Average.	I	II	Average.
	per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
C	52.13	52.07	52.10	50.42	50.47	50.45
H	6.75	6.90	6.83	6.90	6.77	6.84
N	18.08	17.99	18.04	17.86	17.93	17.90
S	1.45		1.45	1.42		1.42
O*			21.58			23.39
Moisture		7.90			6.93	
Ash		3.65			3.05	

* Calculated by difference.

A summary of the analyses of the five preparations is given in Table VIII.

TABLE VIII.

Summary of the Analyses of the Cohune Nut Globulin.

Calculated on moisture- and ash-free basis.

	Preparation No.					Average.
	I	II	III	IV	V	
	per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
C	52.70	50.72	52.80	52.10	50.45	51.75
H	7.05	6.81	7.09	6.83	6.84	6.92
N	17.74	17.70	17.70	18.04	17.90	17.82
S	1.21	1.43	1.23	1.45	1.42	1.35
O*	21.30	23.34	21.18	21.58	23.39	22.16

* Calculated by difference.

TABLE IX.

Analyses by the Van Slyke Method.

Total N regained corrected for solubility of bases (9).

	I	II	I	II	Average.
	gm.	*gm.*	*per cent*	*per cent*	*per cent*
Amide N	0.0379	0.0367	7.47	7.53	7.50
Humin N adsorbed by lime	0.0042	0.0041	0.83	0.85	0.84
" N in amyl alcohol extract	0.0006	0.0004	0.12	0.09	0.11
Cystine N	0.0026	0.0026	0.51	0.54	0.53
Arginine N	0.1562	0.1506	30.77	30.96	30.87
Histidine N	0.0132	0.0127	2.60	2.61	2.61
Lysine N	0.0417	0.0373	8.21	7.66	7.94
Amino N of filtrate	0.2427	0.2331	47.81	47.92	47.87
Non-amino N of filtrate	0.0140	0.0088	2.75	1.81	2.28
Total N regained	0.5131	0.4863	101.07	99.97	100.55

TABLE X.

Distribution of Basic Amino-Acids in Cohune Nut Globulin.

	I	II	Average.
	per cent	*per cent*	*per cent*
Cystine	0.79	0.82	0.81
Arginine	17.12	17.22	17.17
Histidine	1.72	1.72	1.72
Lysine	7.67	7.16	7.42
Tryptophane			Present.

Analysis of the Globulin by the Van Slyke Method.—A globulin obtained by direct dialysis was analyzed in duplicate, some of the determinations on each sample also being done in duplicate. The samples weighed 3.1505 and 3.0190 gm. equivalent to 2.8361 and 2.7177 gm. of moisture- and ash-free samples, respectively, containing 17.90 per cent of nitrogen. Sample I contained 0.5077 gm. and Sample II contained 0.4865 gm. of nitrogen.

The protein was dissolved in 100 cc. of 20 per cent hydrochloric acid and hydrolyzed by boiling the solution under a condenser for 36 hours. The phosphotungstates of the bases were decomposed by the amyl alcohol-ether method (11). The results are given in Tables IX and X. The figures for cystine are undoubtedly low since this amino-acid is slowly decomposed in boiling hydrochloric acid.

TABLE XI.

Free Amino Nitrogen of Cohune Nut Globulin Compared with the Lysine Nitrogen.

Total N in 2 cc.	N gas from 2 cc.	Pressure.	Temperature.	Amino N in 2 cc.	Ratio of amino N to total N.	One-half lysine N by Van Slyke method.
mg.	*cc.*	*mm.*	*°C.*	*mg.*	*per cent*	*per cent*
13.28	0.75	761.2	24.0	0.419	3.16	3.97
13.28	0.83	761.2	24.5	0.463	3.48	

Free Amino Nitrogen of the Globulin Compared with the Lysine Nitrogen.—In order to determine the ratio between the free amino nitrogen of the unhydrolyzed globulin and the lysine nitrogen content as determined by the Van Slyke method, about 2 gm. of the wet globulin used in the Van Slyke analysis were dissolved in 0.1 N sodium hydroxide solution and made up to 25 cc. Two 2 cc. portions of the solution were used for the free amino nitrogen determinations, using Van Slyke's micro apparatus (12). Total nitrogen was determined on 5 cc. aliquots and the results were calculated to the basis of 2 cc. Diphenyl ether was used to prevent foaming and corrections were made for the reagents used. The free amino nitrogen found agrees fairly well with one-half the lysine nitrogen as determined by the Van Slyke method. The results will be found in Table XI.

Distribution of the Nitrogen in the Globulin.—The distribution of the nitrogen in the globulin was calculated from the results obtained by the Van Slyke method, the calculation being comparable with analyses obtained by Hausmann's modified method (8). The distribution of the nitrogen is shown in Table XII.

TABLE XII.

Distribution of Nitrogen in Cohune Nut Globulin.

N	Globulin precipitated by dialysis.		
	I	II	Average.
	per cent	*per cent*	*per cent*
Amide	1.34	1.35	1.35
Humin	0.17	0.17	0.17
Basic	7.18	7.10	7.14
Non-basic	9.41	9.27	9.34
Total	18.10	17.89	18.00

SUMMARY.

1. The globulin of the cohune nut has been isolated and analyzed, and a trace of albumin has been shown to be present in the nut.

2. The analyses of the globulin reveals a close similarity between it and the globulin of the coconut.

3. The basic amino-acids of the globulin were determined by the Van Slyke method and the distribution of the nitrogen (Hausmann's) was calculated from these results.

4. The analyses indicate that the cohune nut globulin contains all the basic amino-acids known to exist in proteins, being high in arginine and lysine. Tryptophane is also present.

5. The free amino nitrogen in the globulin has been determined and has been found to agree fairly well with one-half the lysine nitrogen as determined by the Van Slyke method.

BIBLIOGRAPHY.

1. Wehmer, C., Die Pflanzenstoffe, Jena, 1911, 74.
2. Lewkowitsch, J. I., Chemical technology and analysis of oils, fats and waxes, London, 5th edition, 1914, ii, 609.

3. Harris, G., *U. S. Dept. Com., Daily Consular and Trade Reports, No. 303*, 1914, 1323.
4. Willard, C. N., *U. S. Dept. Com., Bureau Foreign and Domestic Com., Commerce Reports, No. 132*, 1919, 1230.
5. *Scientific American*, 1918, cxix, 357.
6. Ritthausen, H., *Arch. ges. Physiol.*, 1880, xxi, 96.
7. Chittenden, R. H., *Med. Rec.*, 1894, xlv, 450.
8. Osborne, T. B., and Harris, I. F., *J. Am. Chem. Soc.*, 1903, xxv, 323.
9. Van Slyke, D. D., *J. Biol. Chem.*, 1911–12, x, 15.
10. Johns, C. O., Finks, A. J., and Gersdorff, C. E. F., *J. Biol. Chem.*, 1919, xxxvii, 149.
11. Van Slyke, D. D., *J. Biol. Chem.*, 1915, xxii, 281.
12. Van Slyke, D. D., and Birchard, F. J., *J. Biol. Chem.*, 1913–14, xvi, 539.

EXPLANATION OF PLATE 1.

FIG. 1. The cohune nut, *Attalea cohune* Martius. Natural size.

FIG. 1.

(Johns and Gersdorff: Globulin from the cohune nut.)

CONTRIBUTIONS TO THE BIOCHEMISTRY OF IODINE.

III. THE COMPARATIVE EFFECTS OF THYROID AND IODIDE FEEDING ON GROWTH IN WHITE RATS AND IN RABBITS.*

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(Received for publication, October 13, 1920.)

INTRODUCTION.

This investigation was carried out during the winter and spring of 1919–20. It was commenced with the idea of determining definitely whether thyroid feeding had any effect on growth in young animals,[1] and the earlier experiments dealt only with this one problem, and the comparative effect of iodide. In subsequent experiments observations were also made on the degree of hypertrophy produced in certain organs.

E. R. Hoskins (1916) has summarized the earlier literature relating to both problems. In determining the effect on gross weight the majority of earlier observations are on adult animals only. The following refer to growth in young animals:

Moussu (1899) found that small doses of thyroid stimulate the rate of growth in young dogs, but that large doses are toxic. Bircher (1910) fed thyroid to young rats, and obtained a retardation in growth and body weight, but an acceleration in the process of ossification. Schafer (1912) noted in young white rats fed thyroid gland an increased food consumption, increased metabolism, and acceleration of growth. Fordyce (1912) observed a decrease in rate of growth. Gudernatsch (1912–13, 1913–14) found that administration of thyroid to tadpoles retarded growth, but hastened metamorphosis.

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* A preliminary note, outlining the results arrived at in this investigation, has been communicated to the Physiological Society (England).

[1] Some preliminary and not very exact experiments by A. T. Cameron and T. D. Wheeler in this laboratory in 1914–15 had not yielded definite results.

Hoskins himself used white rats for his experiments. His results, based on average figures for a number of rats, do not show any definite effect of thyroid feeding on the rate of growth; the growth curves were approximately the same as those from the average figures for a series of controls from the same litters. He pointed out that the normal growth of the albino rat shows material variation, not only in different strains and under different conditions of environment, but even among litters when all conditions are kept as nearly constant as possible, so that in all such tests experimental and control animals should be selected of the same sex and from the same litter. He used both fresh gland and recently desiccated gland without any noticeable difference in result. The dose given every 2nd day varied from 10 mg. of desiccated (or 40 mg. of fresh) to 200 mg. of desiccated calf thyroid gland in different experiments.

Herring (1917, *b*) used white rats, carefully comparing thyroid-fed and control animals from the same litter and of the same sex. Fresh ox thyroid (0.1 to 0.2 gm.) was fed daily. A slight decrease was observed in the rate of growth of male rats. With females, more variable results were obtained. They appeared to be more susceptible to the treatment, but the average gain in weight differed very little from that of the controls (*cf.* also Herring (1919)).

Hewitt (1920) has just published the results of two series of similar experiments. One was on adult rats. In the second he claims to have obtained a decrease in growth rate, but gives no details of experimental observations on controls, although he indicates that such were made.

(Compare also the results of Iscovesco with a specific preparation, noted below.)

The discrepant results just recorded are in our opinion due to two sources of error: the first, non-comparison of every experimental animal with a control from the same litter kept rigidly under the same conditions (this source of error was excluded by Herring); the second, the variability of dosage. It seems natural to infer that a fixed dose, given over a long period to a rat of constantly changing weight, and to a series of rats of different initial weights, would not produce comparable effects; this method of dosage has been employed, however, by almost all the investigators of this problem. We have used a dose based rigidly on and bearing a constant ratio to the body weight of the animal at the time of administration, and have achieved concordant results.

The literature concerned with the second problem will now be outlined.

R. G. Hoskins (1910) fed daily for 15 days varying amounts (5 to 15 mg.) of thyroid to eighteen young guinea pigs. The adrenal glands averaged 25 per cent heavier than those of the same number of controls. Iscovesco (1913), using a lipoid preparation from thyroid (the part of the petroleum-ether extract insoluble in acetone), and experimenting with young rabbits, claims to have obtained a marked acceleration of general growth with young animals (initial age 45 days), and a slight decrease in growth rate with older animals (initial age $4\frac{1}{2}$ months). 2 months treatment with a dose of this preparation of 0.01 to 0.02 gm. per kilo of animal per day caused marked hypertrophy of adrenals, spleen, thyroid, testes, uterus and ovaries, a distinct effect on the heart and kidneys (the latter in females only), and no effect on the liver. His results are not in good agreement with those of subsequent workers, and Fenger's (1916) work on the phosphatide content of thyroid throws doubt on the lipoid nature of his preparation.

E. R. Hoskins (1916), in the experiments already described, found that thyroid feeding produced a decided hypertrophy of the heart, liver, spleen, and adrenal glands (especially in males), a somewhat more uncertain and less extensive increase in the weight of the alimentary canal and pituitary (male), and possibly in the skeleton, testes, and epididymi, and a decrease in the weight of the (female) pituitary. On the other hand, feeding with thymus, pineal, or pituitary produced no marked or constant change upon the growth rate of the body or organs.

Similarly R. G. and A. D. Hoskins (1916) found that the only effect of this nature from feeding adrenals to white rats was hypertrophy of testes, and in some cases hypertrophy of ovaries.

Kojima (1916) found that the pancreas hypertrophies; he described the occurrence of numerous mitotic figures in the nuclei and alterations in the granules of the cells.

Herring generally confirms Hoskins' results. Thyroid feeding produces enlargement of the adrenals, heart, liver, spleen, testes, and ovaries, no constant change in the thymus, and a decrease in size of the (female) pituitary. The pancreas is enlarged (confirming Kojima), while the thyroid glands are decreased in weight and show signs of relative inactivity. Herring especially draws attention to the marked hypertrophy which may occur with heart and kidneys. While Hoskins thought that the hypertrophies may be ascribable to the general increase in metabolism in the body, Herring takes the view that the adrenin output is compensatory to the thyroid, more especially in its action on the circulation, and the combination of these factors along with the increased metabolism may be the cause of the great hypertrophy of heart and kidneys.

Herring (1915–16, 1917, *a*) had previously shown that the adrenin content of the adrenals was increased. Kuriyama (1918) was unable to find an increase either in the size of the gland or in the adrenin content. Hewitt (1920) criticizes his method, and confirms Hoskins and Herring with respect to the adrenals, heart, liver, and spleen, and Herring with respect to the diminution in size of the thyroid. He finds that the pitui-

tary hypertrophies in the majority of cases (male rats). He uses for comparison figures Donaldson's (1915) collected data, a method open to some objection, as Hoskins has pointed out. He considers further that his results indicate that the abnormal condition is probably temporary, and that a return, perhaps incomplete, to normal proportions is indicated after cessation of thyroid feeding.

The loss of fat as a result of thyroid feeding is well known. Both Hoskins and Herring noted it in all their experiments, and this loss was considered to balance the organ hypertrophy and so mask any total weight changes.

Cramer and Krause (1912–13) found that, if cats and rats were fed thyroid, in 2 or 3 days glycogen completely disappeared from the liver, even on a rich carbohydrate diet, and this observation has been confirmed by Kuriyama and others. Kuriyama was unable to observe this loss of glycogen in the frog.

We are unaware of any experiments with iodides similar to those described above with thyroid.

It has been repeatedly shown that the iodine content of the thyroid is easily influenced by iodine in the diet, whether fed as inorganic or organic iodine, or as thyroid itself.[2] Fordyce showed that after thyroid feeding the vesicles of the thyroid of the rat were distended with colloid. Kojima (1917) obtained this result both after administration of thyroid and of sodium or potassium iodide. The epithelial cells lining the vesicles are flattened. Herring's findings are not exactly in agreement.

EXPERIMENTAL RESULTS.

The animals employed were white rats and rabbits. In all, forty male and seventeen female rats were used. Of these, thirty-one males, and sixteen females were from nine litters; seven controlled experiments were carried out with thyroid, and four with sodium iodide. The initial age of feeding varied from 40 to 60 days. The remaining ten animals were of different ages and from different litters, and were used in a specific experiment to induce tetany by feeding large thyroid doses. Most of the litters were born and reared in the Department; some were purchased locally when still quite young.

Previous to treatment, the rats were isolated in small cages, groups of such cages being kept side by side. The rats were weighed to the nearest 0.5 gm. each morning before food was given. Except in the first experiment, unlimited bread and

[2] See, for example, Baumann and Goldmann (1896), Roos (1899), and Simpson and Hunter (1911).

milk were fed throughout. The dried thyroid was weighed to the nearest milligram, mixed with flour (except where indicated), made up into a thick paste with water, and fed on a watch-glass before other food and immediately after weighing. After the 1st day it was always eaten greedily and completely, so that the error from wastage can only amount to a small per cent. The dosage was, in different experiments, 1 mg. of dried thyroid to 20, 10, 5, and 2 gm. of the actual body weight at the time of feeding (1:20,000; 1:10,000; 1:5,000; 1:2,000).

Three thyroid preparations were used: the first a Merck (Darmstadt) preparation at least 9 years old, containing 0.39 per cent of iodine (termed below Thyroid A); the second a hog thyroid preparation containing 0.34 per cent of iodine (termed Thyroid B); and the third a sheep thyroid preparation containing 0.18 per cent of iodine (termed Thyroid C). The two latter were prepared in December, 1919, and were obtained from the Armour laboratories through the kindness of Dr. F. Fenger.

In order to control any toxic effects of the thyroid preparations, not due to the specific nature of the tissue, ox liver, purchased locally, and dried at 100°C. without any previous aseptic or antiseptic precautions, was fed in amounts equal to the thyroid dose in a number of experiments, until it became evident that no different effect was observable with these controls, and with controls fed solely on bread and milk.

Sodium iodide was used on account of the non-activity of the sodium ion. Dilute solutions were employed of such strength that not more than 1 or 2 cc. were required to give the requisite dose. This was measured with a graduated pipette, mixed with flour to a thick paste, and given on a watch-glass as usual.

At the conclusion of each experiment the animals were killed with chloroform, the organs dissected, the larger weighed immediately, and the smaller transferred to closed weighed glass vessels for subsequent weighing. Where indicated below, one thyroid lobe was transferred to corrosive sublimate, and after hardening was sectioned and stained with hematoxylin-eosin for histological examination. The other lobe, or the whole thyroid, was dried at 100°C. for dry weight determinations. Attempts to estimate the small amounts of iodine in the single lobes by the Kendall (1914) method were not very successful and were abandoned.

In some of the later experiments Kendall's recent modification (1920) was used with successful results.

The tables show the body weights on every 3rd day. The daily weighings give somewhat less regular curves, probably largely owing to room temperature variations from the extreme climatic conditions. Since these were the same for control as for treated rats, they can be neglected.

In a few cases, where thyroid-fed rats died suddenly, and the controls were being used for other treated rats from the same litter, organ weights are given in the tables for comparison, taken from Donaldson's averages.

In one experiment five rabbits from one litter, born in the Department, were used. They were isolated into separate cages, handled, and weighed daily for some time before treatment commenced, as it was found that initially this handling appeared to affect them considerably. Their diet consisted of unlimited oats, hay, and green stuff. When their growth rate became relatively constant they were fed each morning dry thyroid mixed with dry ground oats, immediately after weighing and before other food was given. They soon became habituated to this treatment, and the thyroid was consumed with very little loss. At the end of the experiment they were killed and examined in the same way as the rats.

Experiment 1.—Thyroid A; 1:20,000; rats. Litter of four rats, two male and two female, born August 12th, 1919. Previously to the 39th day the diet consisted of oatmeal and household scraps. On the 39th day thyroid was fed with ground oatmeal to one male and one female, and the diet of all four was restricted to oatmeal and water. The diet showed a vitamine deficiency. On the 45th day green food was given in addition, and on the 49th day the diet was changed to bread and milk and 20 mg. of oatmeal in all cases. The control male rat was fed an amount of dried meat equal to thyroid from the 39th to the 70th days, and subsequently dried powdered ox liver. The control female rat was fed only the 20 mg. of oatmeal corresponding to that mixed with thyroid, in addition to the bread and milk. The figures for body weight are given in Table I.

Experiment 2.—Thyroid A; 1:20,000; rats. A litter of eight, five male and three female, born September 12th, 1919. Diet, unlimited bread and milk. Thyroid feeding was commenced on the 39th day. Two male controls were fed on liver corresponding in amount (1:20,000), and one male and one female control were given 20 mg. of oatmeal. All thyroid and liver doses were given with 20 mg. of oatmeal. The body weights are given in Table II.

The figures for dry thyroid in parentheses were calculated from those found for a single lobe. No. 1 was found dead on the 69th day. Macroscopic examination showed no obvious cause.

TABLE I.

Age.	Male.		Female.	
	Thyroid-fed.	Control.	Thyroid-fed.	Control.
days	*gm.*	*gm.*	*gm.*	*gm.*
39	60.5	65	55	55.5
42	51.5	62	52	55.5
45	54.5	61.5	49.5	53.5
48	54.5	60.5	50.5	56
51	61	76	57	66
54	75.5	80	67	76.5
57	84	82.5	70	81
60	92.5	95	73.5	80
63	96.5	98.5	80	86.5
66	102	113.5	85	90
69	104	118	89	96
72	108.5	125	88	102
75	111	130	96	102.5
78	116	137	90	104.5
81	124	144.5	95.5	108
84	126	143.5	105	111.5
87	136.5	148	103.5	116
90	146	158	114.5	117
93	144	166.5	113	121
96	148	169	115	125.5
99	153	171	120	129
102	156.5	177	126	133.5
105	159	186	134.5	135
108	162	190	136	135.5
111	163	191	136	132
114	174	194	144	135
115	170	198	146	139
Weight increase.				
In 18 days	23.5	17.5	15	25.5
" 36 "	50.5	65	41	47
" 72 "	102.5	126	81	76.5
Thyroid, fresh	0.0099	0.0203	0.0100	0.0126
" dry	0.0035	0.0044	0.0031	0.0028

TABLE II.

Age.	Male.					Female.		
	No. 1. Thyroid.	No. 2. Thyroid.	No. 3. Liver.	No. 4. Liver.	No. 5. Oatmeal.	No. 6. Thyroid.	No. 7. Thyroid.	No. 8. Oatmeal.
days	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*
36	49	48.5	48.5	49	51.5	49	45.5	45.5
39	57	58.5	56.5	60	62.5	55	53	54.5
42	66	69.5	69.5	75	73.5	62	62.5	63.5
45	76.5	76.5	77.5	85	81.5	66.5	76	71
48	80	77.5	82	94.5	92	70.5	78	79
51	85	82	89.5	101.5	100.5	78	81.5	86
54	88	86	97	109	107	80	81	91
57	97	91	102.5	119	115	89	89	97
60	99.5	91	111.5	125.5	123.5	93.5	90	103.5
63	106	97	120	133	129	96	96	106.5
66	115	101	123	140	139.5	103	102	114
69	112	102.5	129	146.5	144	106.5	104	120
72		106	133.5	152	151	110	112	125
75		116	144	162	159	118	116	129
78		122.5	148	172	163.5	121	122	133
81		131	157	177.5	168.5	126.5	124	135
84		142	163	181	175	131	128	139.5
87		142	171	188	183	131	131	146
90		150	178.5	192.5	183	138.5	136.5	147.5
93		161	182	199	189	141	144	148
96		161	188	202.5	196	141.5	143	145
99		160	190	208	201	144	145	157.5

102		170	192	212	199	146	146	157
105		168	197	222	207	149	151	155
108		170	202	224	215	155.5	156	156
111		168	207	226	215	154	156	160
114		168	207	226	218	159	159	158
115		166	212	232	219	157.5	159	161
Weight increase.								
In 18 days	40	32.5	46	59	52.5	34	36	42.5
" 36 "		57.5	87.5	102	96.5	63	63	74.5
" 72 "		109.5	150.5	166	152.5	99	103	105.5
Thyroid, fresh		0.0130	0.0178		0.0190	0.0121	0.0151	0.0144
" dry		(0.0044)	(0.0050)		0.0046	(0.0034)	0.0042	(0.0043)

Nos. 6 and 8 at the end of the experiment were killed, minced, and an ether extract was made. No. 6 yielded 8.5 and No. 8 14 gm. of ether-soluble material.

Experiment 3.—Thyroid A; 1:10,000; rats. A litter of two female rats, born October 11th, 1919. Thyroid fed with 20 mg. of oatmeal commencing on the 39th day; control animal fed 20 mg. of oatmeal. The body weights are given in Table III.

TABLE III.

Age.	Female. Thyroid-fed.	Female. Control.
days	*gm.*	*gm.*
36	45	45.5
39	53	53
42	55	57
45	60.5	67
48	63	70
51	68	75.5
54	71	78
57	74	84
60	76	88.5
63	88.5	94.5
66	95	101.5
69	100	107
72	99	108.5
75	103	110
78	107	118
79	103	119
Weight increase.		
In 18 days	21	31
" 36 "	50	57
Thyroid, fresh	0.0086	0.0087
" dry	0.0024	0.0022

Experiment 4.—Thyroid A; comparison of different dosages. A litter of three males and three females, born November 16th, 1919. Thyroid (mixed with flour) was fed from the 58th day. The controls were fed on bread and milk only. The body weights are given in Table IV and the organ weights in Table V.

The male rat fed a large thyroid dose was found dead on the 76th day (18th day of treatment). No definite cause was apparent. The female rat fed similar dose was found dead on the 80th day (22nd day of treatment). The cecum contained a small pebble. The rat had escaped from its cage for a few hours 3 days previously.

Experiment 5.—Comparison of different thyroid preparations. The litter consisted of five males and two females, born on January 5th, 1920. The females were used for iodide tests (see below). All thyroid doses were one-five thousandth of body weight, and were fed with flour. One control rat was fed equivalent amounts of dried liver, also given with flour. A second control was given bread and milk only. Treatment was commenced on the 46th day. The body and organ weights are given in Table VI.

TABLE IV.

Age.	Male.			Female.		
	Thyroid 1:5,000.	Thyroid 1:20,000.	Control.	Thyroid 1:5,000.	Thyroid 1:20,000.	Control.
days	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*
55	85	81	82	78	74	77
58	92	89	88	85	80	83
61	98.5	95	95	86	85.5	91
64	103	103	103	88	90	97
67	104	103	106	86	94	97
70	105	108	114	88	100	106
73	108	114	122	88	105	115
76	107	119	128	87	107	117
79		119.5	133	84	106	116
82		126	136		106	122
85		126	136		106	130
88		132	146		112	128
91		135	152		114	131
94		129	160		119	135
Weight increase.						
In 18 days.....	15	30	40	2	27	34
" 36 "		40	72		39	52

Experiment 6.—Comparison of different thyroid preparations. The litter, born on February 7th, 1920, consisted of six males. Two were used for iodide tests. The remainder were treated as in Experiment 5. The control was fed bread and milk only. Treatment was commenced on the 56th day. Dose 1: 5,000. The body and organ weights are given in Table VII.

No. 1 developed marked and unmistakable tetany symptoms on the 71st day (15th day of treatment). The spasms were accompanied by typical flexing of the fore limbs and paws and dragging of the hind limbs. Similar attacks were noticed during the 72nd, 74th, 75th, and 78th days, and others were suspected to have occurred at night, from the condition of the animal in the morning. During this period the rat exhibited a con-

TABLE V.

	Male. Thyroid; 1:5,000.	Control figures from Donaldson's tables for males.			Female. Thyroid; 1:5,000.	Control figures from Donaldson's tables for females.		
		Same age.	Same length.	Same weight.		Same age.	Same length.	Same weight.
	gm.	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*
Body weight	107	121.1	83.9	106.7	79	121.3	84.4	79
Liver	8.05	6.94	5.31	6.33	5.14	6.92	5.33	5.11
Kidneys	2.25	1.112	0.824	1.002	1.78	1.108	0.828	0.791
Heart	0.85	0.545	0.414	0.496	0.78	0.544	0.416	0.399
Lungs	1.17	0.778	0.585	0.704	1.12	0.776	0.587	0.562
Spleen	0.31	0.334	0.239	0.298	0.308	0.333	0.240	0.228
Adrenals	0.043	0.0250	0.0200	0.0231	0.054	0.0338	0.0249	0.0237
Thyroid	0.0097	0.0220	0.0166	0.0200	0.0104	0.0219	0.0167	0.0160
Body length, *mm*	153	173	153	166	150	169	150	147

	Male.		Female.		Male.		Female.	
	Thyroid; 1:20,000.	Control.	Thyroid; 1:20,000.	Control.	Thyroid.	Control.	Thyroid.	Control.
	gm.	*gm.*	*gm.*	*gm.*	*per cent**	*per cent**	*per cent**	*per cent**
Body weight	129	160	119	135				
Liver	9.91	7.12	8.41	7.12	7.68	4.45	7.07	5.27
Kidneys	1.82	1.56	1.90	1.37	1.40	0.97	1.67	1.37
Heart	0.85	0.79	0.78	0.75	0.66	0.50	0.65	0.55
Lungs	0.85	0.92	1.01	0.71	0.66	0.57	0.84	0.53
Spleen	0.398	0.419	0.447	0.423	0.31	0.26	0.37	0.31
Thyroid, fresh	0.0084	0.0127	0.0096	0.0108	0.0065	0.0079	0.0080	0.0080
" dry	(0.0026)	(0.0037)	(0.0033)	(0.0033)	(0.0020)	(0.0023)	(0.0028)	(0.0025)
Body length, *mm*	170	182	160	169				

* Computed on body weight.

TABLE VI.

Age.	Rat 1. Thyroid A; 0.38% I.	Rat 2. Thyroid B; 0.34% I.	Rat 3. Thyroid C; 0.18% I.	Rat 4. Liver. Control.	Rat 5. Control.
days	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*
40	73	74	68	66	60
43	76	81	73	70	65
46	79	82	76	71	66
49	81	85	84	82	75
52	84	89	88.5	88	83
55	89	92	90.5	95	86
58	91.5	94.5	94.5	98	92
61	95	100.5	99.5	105.5	101.5
63	95	101	103.5	107	106
Weight increase.					
In 17 days.....	16	19	27.5	36	40
Body length, *mm*....	170	170	180	175	175
	Weight of organs.				
Liver.....	8.79	7.90	6.99	5.71	4.99
Kidneys.....	1.58	1.39	1.40	1.10	1.13
Heart.....	0.72	0.66	0.60	0.52	0.61
Lungs.....	0.64	0.73	0.58	0.63	0.68
Testes.....	1.86	1.93	1.96	2.06	1.88
Spleen.....	0.323	0.316	0.328	0.250	0.281
Adrenals.....	0.025	0.025	0.021	0.018	0.017
Thyroid, fresh.....	0.0084	0.0081	0.0077	0.0138	0.0100
" dry.....	(0.0021)	(0.0015)	(0.0018)	(0.0035)	(0.0027)
	per cent	*per cent*	*per cent*	*per cent*	*per cent*
Liver.....	9.25	7.82	6.75	5.33	4.71
Kidneys.....	1.66	1.38	1.35	1.04	1.07
Heart.....	0.76	0.65	0.58	0.49	0.57
Lungs.....	0.68	0.73	0.56	0.59	0.59
Testes.....	1.96	1.92	1.89	1.93	1.77
Spleen.....	0.34	0.31	0.32	0.23	0.26
Adrenals.....	0.026	0.025	0.020	0.017	0.016
Thyroid, fresh.....	0.0089	0.0081	0.0074	0.0129	0.0094
" dry.....	(0.0022)	(0.0015)	(0.0017)	(0.0033)	(0.0025)

tinuous very rapid heart beat, rapid respiration, was restless, and developed a rough coat. There was no exophthalmos. There was a gradual protusion in front and above the thyroid cartilage, external to the muscular coat; pus developed and a scab formed. The tissue surrounding this was examined histologically, and appeared to be abnormal lymphatic tissue.

TABLE VII.

Age.	Rat 1. Thyroid A; 0.38% I.	Rat 2. Thyroid B; 0.34% I.	Rat 3. Thyroid C; 0.18% I.	Rat 4. Control.
days	*gm.*	*gm.*	*gm.*	*gm.*
50	50	50	46	50
53	58.5	56	50.5	56
56	61.5	61	58.5	63
59	65.5	63	64	72.5
62	68.5	66	67	80
65	75	71	76	88
68	78	75	81.5	96
71	79	77	83	102
74	76	85.5	92.5	111
77	79	90	98	122
80	76	90	103	129
82	75	93	109	134.5
Weight increase.				
In 18 days	14.5	24.5	34	48
" 26 "	13.5	32	50.5	71.5
Body length, *mm*	146	165	165	185
Weight of organs.				
Liver	7.72	7.64	8.05	8.01
Kidneys	2.05	1.39	1.41	1.26
Heart	0.61	0.72	0.68	0.60
Lungs	0.60	0.65	0.73	0.80
Testes	1.67	1.89	2.08	2.17
Spleen	0.230	0.402	0.398	0.330
Adrenals	0.048	0.025	0.022	0.019
Thyroid, fresh	0.0058	0.0074	0.0085	0.0125
" dry	(0.0015)	(0.0021)	(0.0028)	(0.0035)
	per cent	*per cent*	*per cent*	*per cent*
Liver	10.29	8.23	7.39	5.94
Kidneys	2.73	1.49	1.29	0.94
Heart	0.81	0.77	0.62	0.45
Lungs	0.80	0.70	0.67	0.59
Testes	2.23	2.03	1.91	1.61
Spleen	0.31	0.43	0.37	0.25
Adrenals	0.064	0.027	0.020	0.014
Thyroid, fresh	0.0077	0.0080	0.0078	0.0093
" dry	(0.0020)	(0.0022)	(0.0025)	(0.0026)

The muscular development was small, compared with the normal animal of the group. No. 2 had a rapid heart beat, was restless, developed a rough coat, and a doubtful exophthalmos. Towards the end of the experiment a marked protuberance appeared in a position similar to that in the first rat (a pus tumor). The appearance and condition of the other two rats were normal until they were killed.

Experiment 7.—Comparison of different thyroid preparations. An experiment with a litter of four female rats, born March 15th, 1920. The same treatment was given as in the two previous experiments. Thyroid feeding was commenced on the 39th day. Dose 1:5,000. The body and organ weights are given in Tables VIII and IX.

TABLE VIII.

Age.	Rat 1. Thyroid A; 0.38% I.	Rat 2. Thyroid B; 0.34% I.	Rat 3. Thyroid C; 0.18% I.	Rat 4. Control.
days	*gm.*	*gm.*	*gm.*	*gm.*
39	47	40	43.5	36
42	49	42.5	48	42
45	55.5	46	54.5	49
48	62	53	59	54.5
51	67.5	61	66	63.5
54	74	65	73	70
57	85	73	80	79.5
Weight increase.				
In 18 days.....	38 (81%)	33 (82%)	36.5 (84%)	43.5 (121%)
Body length, *mm.*	156	148	155	150

Experiment 8.—An endeavor to induce tetany by thyroid feeding. Ten young rats from different litters were fed thyroid over a long period. An unlimited bread and milk diet was given. The initial ages were not known. Feeding commenced May 8th, 1920. The first five were fed at the rate of 1:2,000; the second five at 1:5,000.

No. 1, female, initial weight 51 gm. On the 8th day of feeding thyroid, the rat showed symptoms of incipient tetany, on the ninth day appeared normal, and on the 10th day had an attack of tetany lasting 3 hours, with rapid pulse and respiration, high temperature, and fatal termination. The weight at death was 70 gm. The animal was autopsied. The liver presented a peculiar mottled appearance, the heart was empty (hard), and the blood was chiefly in the periphery. The other organs appeared normal.

No. 2, male, initial weight 57 gm. On the 18th day of treatment the animal appeared abnormal. The coat was ragged. There was no distinct symptom of tetany. Death occurred on the 19th day. For several days prior to death there was a permanent stricture of the penis. Weight at death 67 gm.

TABLE IX.

	Rat 1.	Rat 2.	Rat 3.	Rat 4.	Rat 1.	Rat 2.	Rat 3.	Rat 4.
	gm.	*gm.*	*gm.*	*gm.*	*per cent*	*per cent*	*per cent*	*per cent*
Liver	5.70	5.50	5.15	4.02	6.71	7.53	6.44	5.03
Kidneys	1.17	1.04	1.13	0.75	1.38	1.42	1.41	0.94
Heart	0.65	0.55	0.55	0.49	0.76	0.75	0.69	0.61
Lungs	0.78	0.60	0.71	0.62	0.94	0.82	0.89	0.78
Spleen	0.462	0.395	0.407	0.308	0.54	0.54	0.51	0.39
Adrenals	0.026	0.023	0.026	0.019	0.030	0.031	0.032	0.024
Thyroid, fresh	0.0060	0.0074	0.0065	0.0062	0.0070	0.0101	0.0080	0.0078
" dry	0.0020	0.0027	0.0022	0.0017	0.0024	0.0037	0.0027	0.0021

TABLE X.

	Rat 5. Thyroid A; 1; 2,000.	Control figures from Donaldson.		Rat 9. Thyroid A; 1: 5,000.	Control figures from Donaldson.		Rat 10. Thyroid A; 1: 5,000.	Control figures from Donaldson.	
		Same length.	Same weight.		Same length.	Same weight.		Same length.	Same weight.
	gm.	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*
Body weight	133	181.2	**132.3**	160	169.1	**160.5**	112	157.7	**112.6**
Body length, *mm.*	196	**196**	178	192	**192**	189	188	**188**	169
Liver	11.47	9.33	7.40	13.87	8.86	8.53	11.60	8.42	6.58
Kidneys	2.05	1.568	1.198	2.35	1.477	1.412	2.15	1.391	1.047
Heart	0.97	0.742	0.584	1.19	0.704	0.676	0.89	0.667	0.517
Lungs	1.15	1.083	0.836	1.31	1.022	0.979	1.14	0.965	0.735
Testes	1.91	2.051	1.654	2.16	1.964	1.898	2.35	1.876	1.450
Spleen	0.535	0.487	0.363	0.699	0.456	0.435	0.371	0.428	0.313
Adrenals	0.062	0.0321	0.0264	0.035	0.0307	0.0297	0.038	0.0294	0.0239
Thyroid	0.0139	0.0299	0.0235	0.0168	0.0284	0.0272	0.0099	0.0269	0.0208

No. 3, male, initial weight 134 gm. Died on the 27th day of treatment. Final weight 96 gm. The weight fell steadily throughout the treatment. There was occasional stricture of the penis after the 17th day. No distinct cause of death was noted.

No. 4, male, initial weight 130 gm. Death occurred on the 30th day, from no distinct cause. There was a steady slow fall in weight, the final weight being 112 gm.

No. 5, male, initial weight 115 gm. There was a slow gain in weight. On the 32nd day the animal (weight 133 gm.) was killed and examined (see Table X).

No. 6, male, initial weight 78 gm. The weight kept practically constant (maximum noted was 84 gm.). The animal died on the 17th day (weight 78 gm.). There were no symptoms of tetany and no distinct cause of death.

No. 7, male, initial weight 97 gm. The weight varied between 93 and 100 gm. for 16 days, and then fell slowly. The animal died on the 28th day from no observed cause. Final weight 88 gm. Occasional stricture of the penis was noted.

No. 8, male, initial weight 89.5 gm. Weight kept fairly constant (maximum reached, 97 gm.). Death occurred on the 29th day from no definite cause. Weight 84 gm.

No. 9, male, initial weight 115.5 gm. There was a slow increase in weight to 160 gm. on the 32nd day when the animal was killed and examined (see Table X). The appearance remained normal throughout, except that bald patches developed around the eyes.

No. 10, male, initial weight 115.5 gm. This increased to 128 gm. (11th day) and then fell slowly to 112 gm. (32nd day) when the animal was killed and examined (see Table X). The hair was somewhat rough.

It should be remarked further that the examination of No. 5 showed the development of numerous small lymphoid glands, especially in the neighborhood of the axilla. Several of the animals showed a marked inflammation (granulation) of the eyelids.

Experiment 9.—Sodium iodide. Two male rats from the same litter, born September 8th, 1919. The iodide dosage was made equivalent to the amount of iodine in the Thyroid A preparation fed in the ratio of 1:5,000 (1 part of iodine to 1,250,000 of body weight). The initial weights of the iodide and control rats were respectively 102 and 99 gm. (66 days old); the weights after feeding 36 days were 171 and 169 gm. At no time was the difference in weight greater than 7 gm. and it varied in both directions.

The weights of the following organs were respectively for the iodide and control rats: liver, 8.03 and 6.91 gm.; kidneys, 1.45 and 1.28 gm.; heart, 0.76 and 0.72 gm.; lungs, 0.92 and 0.83 gm.; spleen, 0.53 and 0.45 gm.; thyroid, fresh, 0.0122 and 0.0125 gm.; dry, calculated from one lobe, 0.0037 and 0.0043 gm.

Experiment 10.—Sodium iodide. One of two female rats from the litter born January 5th, 1920, was given sodium iodide in the ratio of 1 part of

iodine to 500,000 of body weight. The iodide was fed from the 46th day. Compare Experiment 5. The results are given in Tables XI and XII.

TABLE XI.

Age.	Iodide rat.	Control rat.
days	*gm.*	*gm.*
40	61	60
43	65	63
46	67	61
49	77	68
52	80.5	70
55	85.5	73
58	90	74
61	94.5	79
63	100	82
Body length, *mm*.....	165	160

TABLE XII.

	Iodide rat.	Control rat.	Iodide rat.	Control rat.
	gm.	*gm.*	*per cent*	*per cent*
Liver.....	5.01	3.88	5.01	4.73
Kidneys.....	1.12	0.89	1.12	1.08
Heart.....	0.60	0.45	0.60	0.55
Lungs.....	0.65	0.55	0.65	0.67
Spleen.....	0.254	0.227	0.25	0.28
Adrenals.....	0.023	0.019	0.023	0.023
Thyroid, fresh.....	0.0090	0.0092	0.0090	0.0112
" dry.....	(0.0019)	(0.0018)	(0.0019)	(0.0022)

The control rat during this period did not appear to be perfectly normal, and did not take food well.

Experiment 11.—Sodium iodide. Two of the six rats born February 7th, 1920 (see Experiment 6), were fed different doses of iodide, and the same control was used as for those fed thyroid. Feeding was commenced on the 56th day. The results are given in Tables XIII and XIV.

Experiment 12.—Sodium iodide. A litter of eight rats, males, born April 6th, 1920. Two rats (Nos. 1 and 2) were used as controls, two were fed iodide at the rate of 1 part of iodine to 50,000 of body weight (Nos. 3 and 4), two at the rate of 1 of iodine to 25,000 (Nos. 5 and 6), and two at the rate of 1 of iodine to 12,500 (Nos. 7 and 8). Feeding was commenced on the 41st day. The results are given in Table XV.

The thyroid material from each pair of rats was united and the iodine estimated by Kendall's (1920) method. The results are given in Table XVI.

TABLE XIII.

Age.	Rat 1. Iodine 1.5:500,000.	Rat 2. Iodine 3:500,000.	Control rat.
days	*gm.*	*gm.*	*gm.*
50	54.5	51.5	50
53	59.5	56.5	56
56	68	62	63
59	75	71	72.5
62	83	74.5	80
65	92	85	88
68	98	93	96
71	105	97	92
74	114	105	111
77	120	113.5	122
80	126.5	117.5	129
82	131	123	134.5
Weight increase.			
In 18 days	46	43	58
" 26 "	63	61	71.5
Body length, *mm*	170	180	185

TABLE XIV.

	Rat 1.	Rat 2.	Control rat.	Rat 1.	Rat 2.	Control rat.
	gm.	*gm.*	*gm.*	*per cent*	*per cent*	*per cent*
Liver	6.44	6.54	8.01	4.92	5.32	5.94
Kidneys	1.19	1.10	1.26	0.91	0.89	0.94
Heart	0.54	0.58	0.60	0.41	0.47	0.45
Lungs	0.81	0.68	0.80	0.62	0.55	0.59
Spleen	0.299	0.308	0.330	0.23	0.25	0.25
Testes	2.27	2.22	2.17	1.73	1.80	1.61
Adrenals	0.027	0.024	0.019	0.021	0.020	0.014
Thyroid, fresh	0.0129	0.0140	0.0125	0.0098	0.0114	0.0093
" dry	(0.0043)	(0.0042)	(0.0035)	(0.0033)	(0.0034)	(0.0026)

Experiment 13.—Rabbits; Thyroid A; 1:5,000. Litter born March 29th, 1920. Five survived; three males, and two females. Thyroid feeding commenced on the 50th day. The thyroid was mixed with oatmeal. The control female appeared abnormal on the 83rd day, and died on the 87th day. There was apparent paralysis of the hind limbs. Examination after death showed an abnormally distended bladder, very distended stomach, and an apparently abnormal spleen. The other animals appeared normal. They were killed on the 87th day. The results are given in Tables XVII and XVIII.

TABLE XV.

Age.	Rat 1; control.	Rat 2; control.	Rat 3; 1:50,000.	Rat 4; 1:50,000.	Rat 5; 1:25,000.	Rat 6; 1:25,000.	Rat 7; 1:12,500.	Rat 8; 1:12,500.
days	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*
41	41	43	45	50	45	41	45	40
44	50	49	53	57	55	48	50	43
47	53	55	59	60	62	56	58	49
50	59	63	64	70	67	58	62	53
53	64	70	70	79	74	63	68	63
56	69	75	77	86	83	69	74	70
59	78	80	86	95	94	74	79	75
62	85	86	98	106	102	82	86	85
65	93	93	105	118	118	85	91	90
68	100	106	115	125	124	93	98	99
71	104	110	118	132	133	100	105	102
74	116	121	131	143	144	106	112	110
77	121	128	137	152	150	112	119	119
80	132	139	145	163	161	115	128	131
Weight increase.								
In 18 days.....	37	37	41	45	49	33	34	35
" 36 "	80	85	92	102	105	71	74	79
" 39 "	91	96	100	113	116	74	83	91
Body length, *mm*.....	190	184	184	185	196	180	185	183

Weight of organs.

Liver	7.73	7.66	8.11	7.79	7.70	5.55	6.72	5.98
Kidneys	1.25	1.28	1.17	1.31	1.26	0.97	1.06	0.97
Heart	0.54	0.64	0.62	0.70	0.66	0.48	0.56	0.54
Lungs	0.86	0.85	1.13	1.11	0.96	0.87	0.91	0.95
Testes	1.92	2.13	2.14	2.25	2.24	1.98	2.09	1.98
Spleen	0.427	0.426	0.454	0.524	0.446	0.325	0.367	0.404
Adrenals	0.023	0.023	0.023	0.023	0.022	0.021	0.020	0.017
Thyroid, fresh	0.0151	0.0123	0.0125	0.0145	0.0155	0.0117	0.0112	0.0111
" dry	0.0038	0.0036	0.0038	0.0042	0.0045	0.0034	0.0032	0.0032
	per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
Liver	5.85	5.51	5.59	4.78	4.78	4.83	5.25	4.56
Kidneys	0.95	0.92	0.81	0.80	0.78	0.84	0.83	0.74
Heart	0.41	0.46	0.43	0.43	0.41	0.42	0.44	0.41
Lungs	0.65	0.61	0.78	0.68	0.60	0.76	0.71	0.72
Testes	1.45	1.53	1.48	1.38	1.39	1.72	1.63	1.51
Spleen	0.32	0.31	0.31	0.32	0.28	0.28	0.29	0.31
Adrenals	0.017	0.016	0.016	0.014	0.014	0.018	0.016	0.013
Thyroid, fresh	0.0114	0.0088	0.0086	0.0089	0.0096	0.0102	0.0087	0.0085
" dry	0.0029	0.0026	0.0026	0.0026	0.0028	0.0030	0.0025	0.0024

TABLE XVI.

Material.	Amount taken.	Iodine found.	
	gm.	*gm.*	*per cent*
Thyroids from Rats 1 and 2.....	0.0074	0.0000061	0.08
" " " 3 " 4.....	0.0080	0.0000258	0.32
" " " 5 " 6.....	0.0079	0.0000214	0.27
" " " 7 " 8.....	0.0064	0.0000170	0.27

Histological Examination.

Where in the tables figures for dried thyroid are given in parentheses, one lobe was transferred fresh to corrosive sublimate, sectioned, and examined histologically. Professor Swale Vincent kindly reported on these sections. He found that in every case not only had colloid distended the vesicles, but that the lymphatic vessels outside the vesicles, and the intervesicular tissue were also distended with colloid; that, in fact, the colloid had overflowed. Small doses over long periods produced effects comparable with larger doses over a short period, and the effects of administration of sodium iodide were the same as those of administration of thyroid.

In Experiment 5, liver, kidney, heart, lung, testes, spleen, and adrenal sections were made from No. 2, fed thyroid containing 0.34 per cent of iodine. These showed no observable difference from sections from the corresponding organs of the control rat.

DISCUSSION.

Results for Normal Rats.

The gross weights and body lengths show in individual cases marked differences from Donaldson's figures, though the average results agree moderately well. The organ weights, where measured, show distinct deviations from Donaldson's tables, as shown in Table XIX.

The differences for the spleen, which indicate variations both above and below the averages recorded for animals of the same age, weight, and body length, can be taken as typical also of the figures for liver, heart, kidneys, and lungs. In the case of the testes our figures appear to be distinctly higher than Donaldson's.

TABLE XVII.

Age.	Rabbit 1; male. Thyroid.	Rabbit 2; male. Thyroid.	Rabbit 3; male. Control.	Rabbit 4; female. Thyroid.	Rabbit 5; female. Control.
days	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*
38	297	324	335	313	267
44	307	363	345	330	298
50	330	379	351	339	309
56	335	374	420	320	383
62	355	395	463	260	409
68	425	472	535	344	469
74	455	498	559	379	482
80	488	511	603	390	517
86	554	561	639	410	524
87	543	567	658	415	(490)
Weight increase.					
In 18 days	95	93	184	5	160
" 36 "	224	182	288	71	215
Body length, *mm*	300	315	330	325	280
Weight of organs.					
Liver	29.29	25.94	26.57	25.94	21.40
Kidneys	5.44	5.44	5.56	4.46	5.15
Heart	2.35	2.67	1.66	2.35	2.35
Lungs	3.86	3.93	3.92	2.67	2.35
Testes	0.082	0.061	0.168		
Ovaries				0.030	0.033
Spleen	0.301	0.294	0.298	0.239	0.102
Adrenals	0.174	0.134	0.189	0.124	0.190
Pancreas	1.071	0.907	1.078	0.743	0.582
Thyroid, fresh	0.0313	0.0450	0.0456	0.0304	0.0325
" dry	0.0133	0.0203	0.0174	0.0124	0.0130
	per cent	*per cent*	*per cent*	*per cent*	*per cent*
Liver	5.39	4.58	4.04	6.25	4.04
Kidneys	1.00	0.96	0.84	1.07	0.97
Heart	0.43	0.47	0.25	0.56	0.44
Lungs	0.71	0.71	0.60	0.64	0.44
Testes	0.015	0.011	0.025		
Ovaries				0.0072	0.0062
Spleen	0.055	0.052	0.045	0.058	0.019
Adrenals	0.032	0.024	0.029	0.030	0.036
Pancreas	0.20	0.16	0.16	0.18	0.11
Thyroid, fresh	0.0058	0.0079	0.0069	0.0073	0.0061
" dry	0.0025	0.0036	0.0026	0.0030	0.0025

The discrepancy becomes more marked with the adrenals,[3] all of lower weight, and still more marked with the thyroids, the weights of which are only from one-third to one-half those of Donaldson's rats. These discrepancies may to some extent be due to differing diets. In regard to the adrenals it is now definitely proved (McCarrison (1919), Vincent and Hollenberg (1920)) that inanition causes marked hypertrophy. Such hypertrophy is also very distinct with thyroid feeding, and it is possible that other diet modifications may also cause changes. The thyroid discrepancies are more difficult to explain. Hoskins and Herring also obtained lower normal figures.

TABLE XVIII.

Estimation of Iodine Content of Thyroids.

Material.	Amount taken.	Iodine found.	
	gm.	*gm.*	*per cent*
Thyroids of Rabbit 1	0.0133	0.0000293	0.22
" " " 2	0.0203	0.0000642	0.32
" " " 3	0.0174	0.0000682	0.39
" " " 4	0.0124	0.0000350	0.29
" " " 5	0.0130	0.0000476	0.37

No clue is afforded from these figures as to whether comparisons should be made more appropriately with rats of the same age, weight, or body length. It is quite evident that wherever possible control rats of the same age from the same litter must always be used. Even with such animals distinct variations are evident, and it is also obvious that where there is a marked slowing of growth comparison of the actual weights of organs may lead to an incorrect conclusion. We consider that the comparisons least likely to lead to error are those of figures expressing percentage of organ weight to total body weight for animals of the same age although there will be with these an

[3] The adrenal figures are not included in the table. The following, for the control rat from Experiment 12, can be taken as typical: weight 0.023 gm.; Donaldson's figures, same age 0.0264 gm., same weight 0.0264 gm., same length 0.0300 gm.

TABLE XIX.

Experiment No.	Rat.	Weight.	Age.	Length.	Spleen.	Donaldson's figures for spleen.			Testes.	Donaldson's figures for testes.			Thyroid.	Donaldson's figures for thyroid.		
						Same age.	Same weight.	Same length.		Same age.	Same weight.	Same length.		Same age.	Same weight.	Same length.
		gm.	*days*	*mm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*
5	Control male.	106	63	175	0.281	0.247	0.298	0.345	1.88	1.125	1.382	1.586	0.0100	0.0171	0.0200	0.0226
12	" "	132	80	190	0.427	0.363	0.363	0.442	1.92	1.654	1.654	1.920	0.0151	0.0235	0.0235	0.0276
6	" "	134.5	82	185	0.330	0.375	0.369	0.407	2.17	1.698	1.675	1.809	0.0125	0.0242	0.0238	0.0258
12	" "	139	80	184	0.426	0.363	0.363	0.400	2.13	1.721	1.721	1.787	0.0123	0.0245	0.0245	0.0255
4	" "	152	91	182	0.419	0.414	0.414	0.388					0.0084	0.0262	0.0262	0.0248
7	" female.	79.5	57	150	0.308	0.208	0.228	0.240					0.0062	0.0148	0.0160	0.0167
10	" "	82	63	160	0.227	0.236	0.236	0.285					0.0092	0.0164	0.0164	0.0193
4	" "	131	91	169	0.423	0.375	0.356	0.333					0.0108	0.0244	0.0_32	0.0219

error of a small per cent for thyroid-fed animals from fat loss (compare Experiment 2). No great stress can be laid on the comparisons with Donaldson's figures in Experiment 8 since the ages of the animals were not known.

Effect of Thyroid Feeding on Total Body Weight.

Under the conditions of experiment adopted, in each of the seven experiments on rats, and that on rabbits, a distinct and marked decrease of growth rate is shown *without exception*, when animals are fed thyroid gland. This decrease is greater than any variation between animals in the same litter. The effect is usually perceptible after 2 or 3 days feeding. With prolonged feeding of small doses (Experiments 1 and 2, females) the body weight tends to become normal again. This is probably due to the fat loss (and perhaps the total loss through the decreased growth rate) becoming balanced by the hypertrophy of certain organs (Experiment 2 indicates a fat loss of about 4 per cent of the body weight). Large doses tend to inhibit growth completely (Experiment 8).

The same decrease in growth rate occurs in rabbits.

This decrease is not to be attributed to any toxic effect of thyroid other than that specific to thyroid tissue itself, at any rate with doses not greater than one-five thousandth of the body weight, since the equivalent amount of liver tissue, dried by methods certainly not more careful to exclude autolysis etc., produces no such effect.

The decrease in growth rate is definitely greater the greater the amount of thyroid fed (Experiment 4), but in order to show this it is necessary to use animals from the same litter (compare Experiments 2 and 3).

Analysis of the results detailed above does not indicate marked difference between females and males in susceptibility to thyroid feeding (*cf.* Herring).

The decrease in growth rate is greater the greater the iodine content of the thyroid fed (Experiments 5 and 6; Experiment 7 does not show this so accurately for the gross changes, but this can almost certainly be accounted for by the greater differences in initial weight; the percentage increases in weight are not in disagreement with the conclusion).

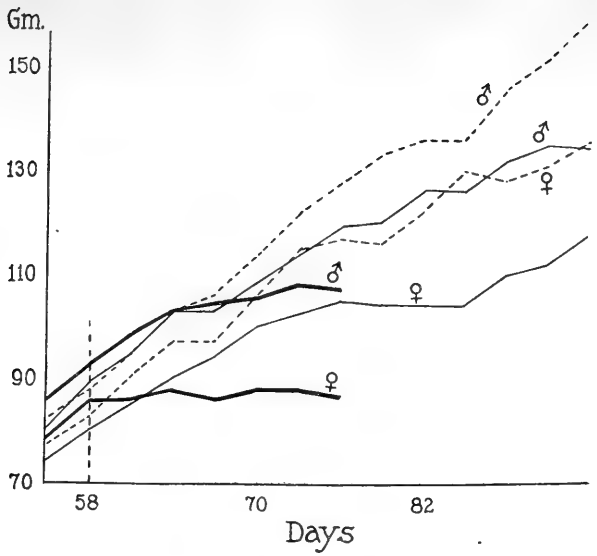

FIG. 1. Experiment 4. The heavy lines represent weights of rats dosed in the ratio of 1:5,000, the light lines those for the ratio 1:20,000, and the dotted lines those for control rats.

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

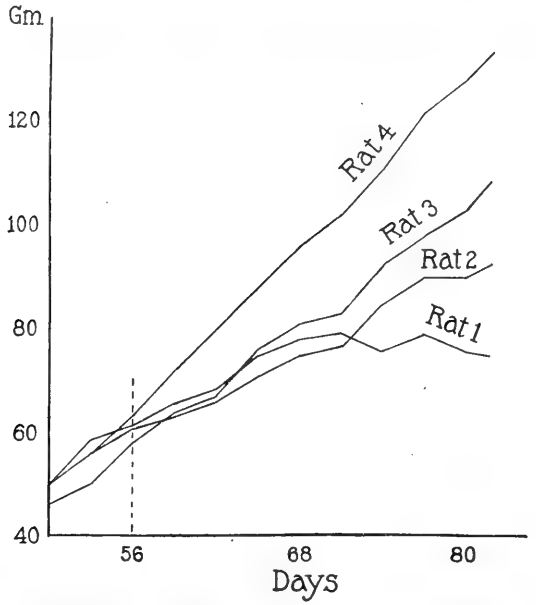

FIG. 2. Experiment 6. Rat 1, fed thyroid containing 0.38 per cent iodine; Rat 2, fed thyroid containing 0.34 per cent iodine; Rat 3, fed thyroid containing 0.18 per cent iodine; Rat 4, control rat.

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

Fig. 1 illustrates the effect of dosage on the extent of decrease, and Fig. 2 the effect of iodine content of the thyroid fed.

Effect of Thyroid Feeding on Body Length (Snout to Anus).

There is usually, but not invariably, a decrease in body length.

Effect of Thyroid Feeding on the Liver, Kidneys, Heart, Spleen, Lungs, Testes, and Adrenals.

The livers of nine rats out of eleven (Experiments 4, 5, 6, and 7) show a distinct hypertrophy from comparison of the actual weights. Expressed as percentages, the figures emphasize the hypertrophy, and those of the remaining two rats conform. The figures (actual weight) for kidneys show an increase in every case. There is one exception for heart, and one for spleen; expressing these in percentages brings them into agreement. The adrenals invariably show an actual increase (nine rats, Experiments 5, 6, 7; no accurate observations were made in Experiment 4). The figures for testes and lungs are less in accordance, but the percentage figures in most cases indicate a relative hypertrophy. The extent of the hypertrophy is evidently less for these two organs. The somewhat greater extent of general hypertrophy obtained by Herring would appear to be due to longer feeding with larger doses. Our figures, correlated with those of Hoskins, of Herring, and of Hewitt, lead to the general conclusion that the extent of hypertrophy depends on the size of dose and the length of dosage period, although variations with individual litters are marked.

Hewitt's results are open to criticism, since apparently Donaldson's figures alone were used for comparison. Nevertheless his conclusion that these organs tend to resume normal proportions after resumption of ordinary diet is valid, since it is based on observations on rats of the same litter.

It is probable that Herring's contention that part at least of the thyroid effect is produced through the medium of the hypertrophied adrenal may be correct in as far as the testes are concerned, since Hoskins (1916) noted that adrenal feeding produced hypertrophy of these organs. Since Hoskins' results indicated no effect on other organs, it is probable that the effects on heart,

pancreas, adrenals, kidneys, and liver are due directly to thyroid action. Herring has pointed out that Hatai (1915) has induced similar hypertrophies in rats subjected to a large amount of exercise, a treatment which may logically be compared with the increase of general metabolic activity now usually regarded as the chief function of the thyroid secretion.

Rabbits showed a relative hypertrophy except for testes, ovaries, and adrenals. There was no constant decrease in thyroid. The water content of the thyroid, both in normal and treated animals, was unusually low.

The percentage hypertrophies recorded in Experiments 5 and 6 point to greater hypertrophy with greater iodine content of thyroid fed. Experiment 7 only partly confirms this. While further experiments are desirable to exclude individual variations we conclude that the results indicate that this dependency on iodine content exists. The proportionality of the action on both growth rate and organ hypertrophy to iodine content, in harmony with other observations on the physiological and metabolic action of thyroid preparations, is to be expected if thyroxin is the essential constituent of the thyroid, since, according to Kendall (1915), this compound occurs combined in the gland in amount invariably corresponding to one-fourth of the total iodine content.

Effect of Thyroid Feeding on Size of Thyroid.

Our results confirm Herring's, that in growing young rats fed thyroid the thyroid shows relatively less growth. We obtained a greater effect with males than with females. The thyroids as a rule were paler than normal glands. Their histological appearance has been described. The figures for dry thyroid tissue also show a similar decrease.

Effect of Thyroid Feeding on Body Fat and Muscular Development.

Like most of the previous observers we have noted an almost complete disappearance of fat tissue in all animals fed thyroid. In one or two cases, *e.g.* No. 1, Experiment 6, the muscular development was distinctly less than normal.

Effect of Feeding Sodium Iodide.

Examination of the figures in Experiments 9 to 12 reveals no decrease in growth rate, no relative hypertrophy of body organs, and no effect on weight of thyroid. No disappearance of fat tissue was observed. The actual figures probably indicate fairly accurately the extent of variation in normal rats of the same litter. It has already been noted that histological examination of the thyroid shows the same changes after iodide as after thyroid feeding.

Other Effects from Thyroid Feeding.

The occasional deaths noted during thyroid feeding by previous observers, and occurring in our experiments especially with the larger doses, are presumably to be regarded as a definite result of thyroid action. Kendall (1919) has noted the same effect with thyroxin. The non-occurrence of such results when thyroid medication is given in diseases such as myxodema and cretinism over prolonged periods is explicable, since the thyroid deficiency is merely corrected, and the effect of a hypersecretion is not produced. Other hypersecretion effects (rough coat, increased heart beat, etc.) have been observed by us in but few cases, and then chiefly with young animals. These seem distinctly more susceptible to the same relative dose. The two cases of tetany that we observed were with young animals. While we have no explanation to offer for this occurrence, we would point out that the observations of Collip and Backus (1920), and of Grant and Goldman (1920), that prolonged forced breathing may cause symptoms of tetany in man, may have some bearing, since rapid respiration and heart beat were concomitant factors in the cases we observed.

Effect of Thyroid and of Iodide Feeding on the Iodine Content of the Thyroid.

Experiment 12 shows that there is a marked increase in iodine content after administration of iodide, in agreement with numerous previous observations. The amount of iodide fed, within the limits of this experiment, did not affect the amount of increase.

No such increase was observed in rabbit thyroids after thyroid feeding. This suggests that with the relatively small dose employed no iodine was stored in the thyroid, but that the iodine-containing compounds in the thyroid fed were entirely used up in producing the systemic effects, and the iodine was subsequently excreted.

Effect of Thyroid Preparations of Different Age.

The very similar effects produced by the 9 year old Merck, and the recently prepared hog thyroid preparation (Armour) indicate that age has no deteriorating effect on carefully prepared desiccated thyroid. Hoskins has already shown that desiccated and fresh glands produce the same results, in confirmation of the observations of previous workers on other thyroid effects.

SUMMARY.

The chief results described in this paper are:

1. Continued small doses of desiccated thyroid gland fed to young white rats produce (*a*) a definite and invariable decrease in growth rate; (*b*) hypertrophy of the organs concerned with increased metabolism—heart, liver, kidneys, adrenals, etc. (confirmatory of Hoskins and Herring); (*c*) disappearance of fat (confirmatory of Hoskins and Herring).

2. The decrease in rate of growth is proportional to (*a*) the amount of thyroid fed and (*b*) the iodine content of the thyroid fed.

3. The hypertrophy varies with dose and length of application of dose, and appears to be proportional to the iodine content of the dose.

4. Sodium iodide fed in quantities varying from amounts equal in iodine content to the thyroid doses to amounts 100 times as great produces no effect on growth rate, and no hypertrophy.

5. The effects produced are not due to protein feeding, autolysis products, or any similar cause, but specifically to thyroid tissue, or some constituent of it.

6. Both thyroid and iodide feeding increase the colloid in the thyroid (confirmatory of Fordyce, and of Kojima). Thyroid feeding inhibits the growth rate of the thyroid (confirmatory of Herring). Iodide does not produce this effect.

7. Similar effects from thyroid feeding on growth rate and organ hypertrophy were obtained with rabbits.

Since of numerous tissues tested only thyroid produces a definite effect (Hoskins) and since iodide does not produce this effect, we suggest that decreased growth rate, organ hypertrophy (especially of the liver, heart, kidneys, and adrenals), and relatively decreased thyroid can be used as discriminatory tests for preparations alleged to be the essential thyroid secretion.

We desire to acknowledge our indebtedness to Professor Swale Vincent for the interest that he has taken in this work, and for the valuable suggestions that he has made to us in the course of it.

BIBLIOGRAPHY.

Baumann, E., and Goldmann, E., *Münch. med. Woch.*, 1896, xliii, 1153.
Bircher, E., *Med. Klin.*, 1910, vi, 391, 1741; *Arch. klin. Chir.*, 1910, xci, 554.
Collip, J. B., and Backus, P. L., *Am. J. Physiol.*, 1920, li, 568.
Cramer, W., and Krause, R. A., *Proc. Roy. Soc. London, Series B*, 1912–13, lxxxvi, 550.
Donaldson, H. H., The rat, Philadelphia, 1915.
Fenger, F., *J. Biol. Chem.*, 1916, xxvii, 303.
Fordyce, A. D., *Edinburgh Med. J.*, 1912, ix, 55.
Grant, S. B., and Goldman, A., *Am. J. Physiol.*, 1920, lii, 209.
Gudernatsch, J. F., *Arch. Entwicklngsmechn. Organ.*, 1912–13, xxxv, 457; *Am. J. Anat.*, 1913–14, xv, 431.
Hatai, S., *Anat. Rec.*, 1915, ix, 647.
Herring, P. T., *Quart. J. Exp. Physiol.*, 1915–16, ix, 391; 1917 (*a*), xi, 47, (*b*) 231; 1919, xii, 115.
Hewitt, J. A., *Quart. J. Exp. Physiol.*, 1920, xii, 347.
Hoskins, E. R., *J. Exp. Zool.*, 1916, xxi, 295.
Hoskins, R. G., *J. Am. Med. Assn.*, 1910, lv, 1724.
Hoskins, R. G., and Hoskins, A. D., *Arch. Int. Med.*, 1916, xvii, 584.
Iscovesco, H., *Compt. rend. Soc. biol.*, 1913, lxxv, 361.
Kendall, E. C., *J. Biol. Chem.*, 1914, xix, 251; 1915, xx, 501; 1920, xliii, 149; *Endocrinology*, 1919, iii, 156.
Kojima, M., *Proc. Roy. Soc. Edinburgh*, 1916, xxxvi, 240; *Quart. J. Exp. Physiol.*, 1917, xi, 339.
Kuriyama, S., *J. Biol. Chem.*, 1918, xxxiii, 193, 207.
McCarrison, R., *Indian J. Med. Research*, 1919, vi, 275; vii, 188.
Moussu, G., *Compt. rend. Soc. biol.*, 1899, li, 242.
Roos, E., *Z. physiol. Chem.*, 1899, xxviii, 40.
Schafer, E. A., *Quart. J. Exp. Physiol.*, 1912, v, 203.
Simpson, S., and Hunter, A., *Quart. J. Exp. Physiol.*, 1911, iv, 257.
Vincent, S., and Hollenberg, M. S., *Endocrinology*, 1920, iv, 408.

RELATIONSHIP BETWEEN THE PLASMA BICARBONATE AND URINARY ACIDITY FOLLOWING THE ADMINISTRATION OF SODIUM BICARBONATE.

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(Received for publication, October 9, 1920.)

Palmer and Van Slyke (1) have shown that the urine of normal men approaches the alkalinity of the blood (pH = 7.4) when the plasma bicarbonate CO_2 exceeds 71 ± 5 volumes per cent, while the urines of many pathological cases, particularly cases with a high grade of renal disease and diabetes mellitus, do not approach the alkalinity of the blood until a higher plasma bicarbonate than in normal individuals has been reached. In certain instances the level of blood bicarbonate was well within the range where tetany is known to occur. Attention was called to the danger of using the urine reaction, pH = 7.4, as a safe criterion in the therapeutic use of sodium bicarbonate, but preferably in view of the evidence then available, to control alkali therapy by frequent determinations of the plasma bicarbonate. It was suggested that a more acid reaction of the urine than pH = 7.4, for instance a pH of 7.0, might be employed with advantage to control clinically the use of sodium bicarbonate in pathological cases. Furthermore, it was shown that sodium bicarbonate administered by mouth is readily absorbed and distributed in approximate uniformity to the body fluids in general as well as to the blood. The effect of a given amount of sodium bicarbonate, g, on the volume per cent of CO_2 of plasma bicarbonate, b, may be estimated from the formula, $b = \frac{38g}{W}$,

when W is the weight of the individual in kilos.[1] Estimation in this manner of the amount of sodium bicarbonate necessary to replenish the depleted alkali reserve as shown by plasma bicarbonate CO_2 determinations serve a useful purpose in clinical practice.

In view of the importance of the use of sodium bicarbonate in combating severe grades of acidosis, more especially in diabetes mellitus, nutritional diseases of children, and methyl alcohol poisoning, further observations have been made in the hope of extending the clinical applicability and reliability of the urinary reaction in controlling the administration of alkali. Although the reaction pH = 7.4 of the urine elaborated by kidneys, whose function of regulating the acid-base equilibrium of the body is impaired, is an insufficient, indeed, may be an unsafe indication that the proper amount of alkali has been given to restore the level of plasma bicarbonate to within normal limits, it was thought possible that the first significant effect of alkali administration on the urinary reaction (pH) might be employed as a safe clinical guide. That our expectations in this respect were justified is apparent from the following results.

EXPERIMENTAL.

Sodium bicarbonate in 2 gm. amounts in 100 cc. of water was given by mouth every $\frac{1}{2}$ hour to subjects in whom the initial plasma bicarbonate revealed no severe grade of acidosis; that is, not less than 50 volumes per cent. In cases with a moderately severe acidosis (a plasma bicarbonate between 40 and 50 volumes

[1] The calculation in terms of plasma CO_2 is made as follows: 1 gm. of $NaHCO_3$ contains 267 cc. of CO_2 measured at 0°, 760 mm. If the body fluids are estimated at 700 cc. for each kilo of body weight, then the distribution of 1 gm. of bicarbonate among them would raise the CO_2 content in cc. per 100 cc. of fluid by $\frac{267}{7W} = \frac{38}{W}$ cc., W representing the body weight in kilos. If g gm. of sodium bicarbonate were taken into the fluids, the rise in volume per cent of CO_2 would be $\frac{38g}{W}$. Conversely, the amount of bicarbonate necessary to raise the CO_2 by b volume per cent would be $g = \frac{bW}{38}$. Hence the formula $b = \frac{38g}{W}$.

per cent), the individual doses of sodium bicarbonate were increased to 5 gm. each and when a more marked reduction of the alkali reserve (below 40 volumes per cent) was found, the dose was increased to 10 gm. It became desirable, especially in cases with severe acidosis, to give the bicarbonate every hour instead of every $\frac{1}{2}$ hour. The hydrogen ion concentration of the urine was estimated immediately before the administration of each dose of alkali. Blood bicarbonate determinations were made before the sodium bicarbonate was given, and again at the point when the first significant depression of the urinary acidity was observed. It has been shown repeatedly in cases where a distinct acidosis exists and without alkali administration that, during the short interval of time of the experiments here reported, the reaction of the urine remains quite stationary. For this reason, "the first significant depression of the urinary acidity" was considered as a change corresponding to one interval in the scale of standard solutions used for the hydrogen ion concentration determinations. The standard solutions employed represented a pH of 7.4, 7.0, 6.9, 6.3, 6.0, 5.7, 5.3, 5.0, and 4.7, respectively.

The pH of the urine was determined by the colorimetric method described by Palmer and Henderson (2) with the exception that for pH values between 7.4 and 6.3 phenolsulfonephthalein was used; between 6.3 and 4.7 methyl red. A further slight modification was employed. Instead of introducing 10 cc. of urine into Florence flasks, 1 cc. of urine was pipetted into test-tubes (25 × 200 mm.) of clear glass and diluted to 25 cc. with distilled water. Under these conditions 10 drops of 0.04 per cent phenolsulphonephthalein and 10 drops of a saturated solution of methyl red produce a satisfactory color for purposes of comparison. Plasma bicarbonate estimations were made by the method described by Van Slyke and Cullen (3).

We have arranged our results in Table I in order of the grams per kilo necessary to produce a significant change in the pH of the urine. In Experiments 7, 19, and 27, before any significant change in the pH of the urine took place, the patients became nauseated, hence the experiments were discontinued.

TABLE I.

Normal and Pathological Individuals.

Experiment No.	Hospital No.	Weight.	Blood pressure.	Blood urea.	Phenolsulfonephthalein excretion in 2 hrs.	Reaction of urine.		Combined CO_2 in plasma.		Amount of $NaHCO_3$ given.		Time between taking of first and second blood samples.	Calculated increase in CO_2, $\frac{38g}{W}$.	Observed increase in CO_2.	Difference between calculated and observed CO_2 increase.	Remarks.
						Before $NaHCO_3$.	After $NaHCO_3$.	Before $NaHCO_3$.	After $NaHCO_3$.							
		kg.	*mm. Hg*	*gm. per liter*	*per cent*	*pH*	*pH*	*vol. per cent*	*vol. per cent*	*gm.*	*gm. per kg.*	*hrs.*	*vol. per cent*	*vol. per cent*	*vol. per cent*	
1		90				6.3	7.1	74.7	76.1	4	0.044	1	1.7	1.4	+0.3	Normal.
2		73				6.8	7.1	62.0	64.3	4	0.055	1	2.1	2.3	−0.2	"
3	PH 40433	61				6.4	7.6	70.9	71.6	4	0.066	1	2.5	0.7	+1.8	Chronic cardiac valvular disease, mitral disease, cardiac insufficiency.
4	JHH 43445	59	$\frac{270}{180}$	0.30	47	6.4	7.1	61.7	66.9	4	0.068	1	2.6	5.2	−2.6	Arteriosclerosis, hypertension, chronic nephritis.
5	PH 40439	91	$\frac{140}{100}$	0.40	46	5.8	6.3	61.0	61.0	7	0.079	2	2.9	0.0	+2.9	Subacute nephritis.
6	PH 40366	51				5.7	7.2	69.9	69.4	4	0.078	1	3.0	−0.5	+3.5	Chronic cardiac valvular disease, mitral disease, cardiac insufficiency.

7	PH 40204	75				5.2	5.3*	67.8	68.7	6	0.080	6	3.1	0.9	+2.2	Pneumonia.
8	PH 40176	95				5.5	6.8	70.7	76.0	8	0.084	4	3.3	5.3	−2.0	Chronic nephritis, hypertension.
9	PH 40266	59				5.4	7.2	61.1	66.8	5	0.085	1	3.2	5.7	−2.5	Cardiac insufficiency.
10		70				6.4	6.8	66.7	74.6	6	0.086	1½	3.3	7.9	−3.5	Normal.
11	PH 38054	70	135/100	0.32	38	5.8	7.3	60.4	61.4	6	0.086	1½	3.3	1.0	+2.3	Subacute nephritis.
12		41				6.9	7.6	65.3	70.9	4	0.098	1	3.7	5.6	−1.9	Empyema following pneumonia.
13	JHH 43556	40				6.3	6.7	76.0	76.3	4	0.100	1	3.8	0.3	+3.5	Diabetes insipidus.
14		36				6.3	7.3	58.4	66.3	4	0.111	1	4.2	7.9	−3.7	Chronic cardiac valvular disease, aortic disease.
15	PH 40278	57				6.9	7.6	69.7	78.8	7	0.123	4	4.7	9.1	−4.4	Diabetes mellitus, no ketonuria.
16	PH 39997	51	190/120	0.25	5	5.0	6.7	63.9	69.0	8	0.157	2	6.0	5.1	+0.9	Chronic nephritis, hypertension, arteriosclerosis.
17	PH 32084	58				5.5	6.9	59.9	60.0	10	0.172	1	6.5	0.1	+6.4	Cirrhosis of the liver.
18	JHH 43556	40				6.3	6.9	59.5	67.2	7	0.175	7	6.7	7.7	−1.0	Diabetes insipidus.
19	PH 41183	72				5.7	5.7*	67.4	70.3	13	0.181	6	6.9	2.9	−4.0	Syphilis, syphilitic aortitis.

* Nauseated and the experiment was discontinued.

TABLE I—*Concluded.*

Experiment No.	Hospital No.	Weight.	Blood pressure.	Blood urea.	Phenolsulfonephthalein excretion in 2 hrs.	Reaction of urine.		Combined CO_2 in plasma.		Amount of $NaHCO_3$ given.		Time between taking of first and second blood samples.	Calculated increase in CO_2, $\frac{38\ g.}{W}$.	Observed increase in CO_2.	Difference between calculated and observed CO_2 increase.	Remarks.
						Before $NaHCO_3$.	After $NaHCO_3$.	Before $NaHCO_3$.	After $NaHCO_3$.							
		kg.	*mm. Hg.*	*gm. per liter*	*per cent*	*pH*	*pH*	*vol. per cent*	*vol. per cent*	*gm.*	*gm. per kg.*	*hrs.*	*vol. per cent*	*vol. per cent*	*vol. per cent*	
20	PH 40268	60	$\frac{300}{145}$	0.50	0	5.2	7.0	59.1	70.0	15	0.250	2	9.5	10.9	−1.4	Chronic nephritis, hypertension, arteriosclerosis.
21	PH 40360	73				5.4	6.5	52.6	62.5	22	0.303	6	11.4	9.9	+1.5	Chronic cardiac valvular disease, mitral disease, cardiac insufficiency.
22		59				5.1	6.3	53.9	70.1	20	0.338	2	12.9	16.2	−3.3	Chronic nephritis.
23	PH 40389	89	$\frac{190}{130}$	0.62	16	5.4	6.0	45.3	72.4	31	0.347	5	13.3	27.1	+13.8	" " hypertension.
24	PH 39039	51				7.4	8.2	58.1	76.2	20	0.392	3	14.9	18.1	−3.2	Diabetes mellitus, no ketonuria.
25	PH 32304	33	$\frac{210}{145}$	0.68	10	5.4	7.3	45.3	66.9	14	0.428	$1\frac{1}{2}$	16.1	21.6	−5.5	Chronic nephritis.

26	RH 2961	69		2.54	0	5.3	7.0	52.0	70.0	30	0.435	3½	16.5	18.0	−1.5	Chronic nephritis.
27	PH 40457	95	260/125	3.90		5.4	5.4*	31.2	44.8	42	0.445	6	16.8	13.6	+3.2	" " hypertension, uremia.
28	JHH 43600	55	190/110	0.97	0	6.8	7.2	46.3	67.8	28	0.510	4	19.3	21.5	−2.2	" " "
29	PH 37492	89	195/140	0.95	15	5.4	5.9	50.6	67.2	46	0.517	7	19.6	16.6	+3.0	Chronic nephritis, cardiac insufficiency, hydrothorax, ascites.
30	PH 40323	91	210	0.92	9	5.5	6.8	47.9	68.1	49	0.538	7	20.5	20.2	+0.3	Chronic nephritis, uremia.
31	RH† 2941	42				5.4	5.9	32.5	61.4	37	0.880	4	33.5	28.9	+4.6	Diabetes mellitus, marked acidosis.
32	JHH 43658	95				5.6	6.0	34.5	74.2	90	0.950	9	36.0	39.7	−3.7	" " "
33	JHH 43722	43				5.7	5.8‡	41.8	81.5	60	1.395	5	53.0	39.7	+13.3	" " "
Average									68.7							

† Cases taken from the cases reported by Palmer and Van Slyke (1).

‡ At midnight the pH of the urine was 8.2.

DISCUSSION.

The results in Table I clearly demonstrate the reliability of employing the early effect of alkali administration on the pH of the urine as an indication of the restoration of the bicarbonate level of the blood plasma to within normal limits. A variety of pathological cases, particularly the clinical conditions known to have impaired renal function and diminished alkali reserve, were studied. For the thirty-three cases the average plasma bicarbonate CO_2 at which an appreciable depression of the acidity of the urine occurs is 68.7 volumes per cent, a value well within normal limits. The average change in the pH was 0.97. Disregarding for the moment Case 33 and Cases 19 and 27 which were discontinued on account of nausea, the maximum plasma bicarbonate CO_2 at which depression of urinary acidity occurs is 78.8 volumes per cent in Case 15, while the minimum plasma bicarbonate CO_2 value is 60.0 volumes per cent in Case 17, a variation of approximately ± 10 volumes per cent. It should be explained that in Case 33 large amounts of alkali were given in a relatively short time. Although the plasma bicarbonate CO_2 reached the high level of 81.5 volumes per cent the kidneys were excreting a urine the reaction of which was the same as that at the beginning of the experiment. Later, however, the urine became very alkaline, pH = 8.2. Similar phenomena have been observed in patients not reported in this paper. The evidence as far as it exists suggests that, in severe acidosis in diabetes mellitus, there is a disturbance of the acid-base regulatory mechanism of the kidneys. This disturbance of renal function appears to be temporary, and does not prevent the use of the pH as an index of sufficient alkali administration. In Cases 31 and 32, diabetes mellitus with severe acidosis, in which the alkali was given more slowly the plasma bicarbonate CO_2 was 61.4 and 74.2 volumes per cent, levels not to be considered dangerous. A safe manner of administration in instances of severe diabetic acidosis appears to be at the rate of not more than 10 gm. of sodium bicarbonate per hour if given by mouth. In the more severe grades of acidosis occurring in diabetes mellitus, the administration of large amounts of sodium bicarbonate by mouth may be unwise. The regulation of the diet in many instances may be sufficient to control the

situation. Where there is impending coma (plasma bicarbonate CO_2 below 30 volumes per cent) the introduction of sodium bicarbonate (25 gm. in 5 per cent solution) intravenously is desirable. Additional alkali may be given by mouth but should be discontinued at the first complaint of nausea.

In clinical practise the series of standard pH solutions may be eliminated. With the suitable indicators available, the urine obtained before the administration of alkali may be reserved as a guide to the degree of depression of the urinary acidity on subsequent observations. Such a procedure is reliable only if the physician is familiar with the use of the standard solution series.

The quantative relationships between the alkali retained in the pathological cases and the degree of acidosis is discussed by Palmer and Van Slyke (1). Since the rise in volume per cent of plasma bicarbonate CO_2 caused by absorption of g gm. of sodium bicarbonate is approximately $\frac{38g}{W}$, it should be possible to estimate the original plasma bicarbonate CO_2 in volumes per cent as follows, $68.7 - \frac{38g}{W}$. The level of the plasma bicarbonate CO_2 at which the urinary pH shows its first depression is 68.7 ± 10 volumes per cent. The $\pm$ 10 volumes per cent variation, therefore, excludes the use of the above method for estimating very accurately the initial plasma bicarbonate or the degree of acidosis from the amount of sodium bicarbonate given to produce an effect on the pH of the urine.

There is no constant relationship between the degree of injury to the renal function and the level of plasma bicarbonate CO_2 at which the first effect on the pH of the urine occurs following alkali administration. We have assembled the cases of nephritis, (1) in order of the severity of renal damage as shown by the phenolsulfonephthalein excretion and (2) in order of the magnitude of the blood urea, comparing the level of plasma bicarbonate CO_2 at which the urine showed the first effect of the alkali, as shown in Table II.

From the facts available there appears to be little or no parallelism between either the phenolsulfonephthalein excretion or the blood urea increase and the plasma bicarbonate CO_2 level at which the urinary acidity is decreased. Much more data are

necessary before a definite statement regarding the relationship of these renal functions can be made. In further study of this phase of the subject it would be necessary to separate the cases of nephritis into groups from the standpoint of the effect of disease on the several acid factors described by Palmer and Henderson (4). The acid factors in question were not determined in our cases.

TABLE II.

Experiment No.	Phenolsulfonephthalein excretion in 2 hrs.	Combined CO_2 in plasma after $NaHCO_3$.	Experiment No.	Blood urea.	Combined CO_2 in plasma after $NaHCO_3$.
	per cent	*vol. per cent*		*per cent*	*vol. per cent*
4	47	66.9	16	0.26	69.0
5	46	61.0	4	0.30	66.9
11	38	61.4	11	0.32	61.4
23	16	72.4	5	0.40	61.0
29	15	67.2	20	0.50	70.0
25	10	66.9	23	0.62	72.4
30	9	68.1	25	0.68	66.9
16	5	69.0	30	0.92	68.1
20	0	70.0	29	0.95	67.2
26	0	70.0	28	0.97	67.8
28	0	67.8	26	2.54	70.0

Our observations afford further confirmation of the validity and usefulness of the formula $b = \frac{38g}{W}$ in alkali therapy. In two instances, Cases 23 and 33, there occur rather wide variations (13.8 and 13.3 volumes per cent, respectively) between the calculated and observed increase in the plasma bicarbonate CO_2. The explanation for the discrepancy in Case 33 is not unlikely to be found in the incomplete absorption and distribution of the sodium bicarbonate among the body fluids resulting from too rapid administration. In Case 23 the large difference between the calculated and observed CO_2 is not clear. In the other cases the theoretical and actual increases in plasma bicarbonate CO_2 correspond with previous observations and are well within the limits of error for the experimental conditions.

SUMMARY.

1. In normal and pathological cases, following the administration of sodium bicarbonate, the plasma bicarbonate CO_2 approaches 68.7 ± 10 volumes per cent at the time the first depressing effect of the alkali on the urinary acidity is noted. The use of the first significant effect on the pH of the urine, therefore, in controlling the therapeutic use of sodium bicarbonate is safe and reliable.

2. Although the pH of the urine serves as a safe guide to the control of the therapeutic use of sodium bicarbonate, the inconstancy of the level of the plasma bicarbonate CO_2 at which a change in the pH of the urine occurs does not admit of estimating with sufficient accuracy the degree of diminished alkali reserve to be of practical value.

3. Confirmation of the observation of Palmer and Van Slyke that "absorbed sodium bicarbonate is distributed in approximate uniformity. . . . to the blood" and "the effect of a given dose in raising the plasma bicarbonate may be calculated by assuming that the body contains 700 cc. of fluid per kilo and that the bicarbonate absorbed is distributed therein uniformly" is afforded.

4. The variability among the several levels of plasma bicarbonate CO_2 at which a significant depression in the pH of the urine occurs in normal and pathological cases is probably explained by the varying degrees of injury to the acid-base regulatory functions of the kidney. There appears to be no close relationship between this function and the ability of the kidney to excrete phenolsulfonephthalein or the blood urea.

BIBLIOGRAPHY.

1. Palmer, W. W., and Van Slyke, D. D., *J. Biol. Chem.*, 1917, xxxii, 499.
2. Palmer, W. W., and Henderson, L. J., *Arch. Int. Med.*, 1913, xii, 153.
3. Van Slyke, D. D., and Cullen, G. E., *J. Biol. Chem.*, 1917, xxx, 289.
4. Palmer, W. W., and Henderson, L. J., *Arch. Int. Med.*, 1915, xvi, 109.

DETERMINATION OF CHLORIDES IN BLOOD.

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(Received for publication, October 13, 1920.)

At the present time the methods for the quantitation of chloride in the blood most generally used in clinical laboratories are those of Van Slyke and Donleavy (1), Rappleye (2), and Foster (3). Their methods give good results when properly performed. However, all of them are complicated by various pitfalls, inherent either in the method or in the collection of blood samples, which render the obtaining of accurate results, especially in the clinical laboratory, unduly difficult. For this reason a method has been devised which obviates these difficulties. It has the advantages that the same reagents are used for determination of chlorides in the whole blood and in the plasma. Furthermore, chlorides may be determined in blood samples collected for the analyses of the system of Folin and Wu, which is an indispensable part of the work of the clinical laboratory.

The method proposed is based on the precipitation of protein by copper hydroxide; on the precipitation of the oxalate and part, if not all, of the phosphate by an excess of calcium hydroxide; and on the titration of the chlorides by the procedure used by Rappleye. The copper hydroxide reagent employed to precipitate the protein has been successfully used by Harding and Mason (4) for the blood. The filtrates obtained by this means remain clear after the addition of the tungstic acid reagent of Folin and Wu (5).

Procedure.

The sample of blood is collected in dry potassium oxalate. Dry syringes should be used, as small amounts of water introduce a source of error, especially when small amounts of blood are collected.

Reagents.—1. 5 per cent copper sulfate solution, C.P.
2. 0.1 N sodium hydroxide.
3. Calcium hydroxide powder, C.P.
4. Standard silver nitrate solution:

Silver nitrate	7.2653 gm.
Nitric acid (concentrated)	150 cc.
Distilled water to	1,000 "

(1 cc. of silver solution = 2.5 mg. of NaCl)

5. 10 per cent iron alum solution.
6. Potassium thiocyanate solution: Make 1.6 gm. of potassium thiocyanate up to approximately 800 cc. and determine its strength by titrating against the silver nitrate solution. Then dilute the potassium thiocyanate solution until 12.5 cc. of it react with 5 cc. of the standard silver nitrate solution. If the precipitating reagent contains traces of chlorides then a correction must be applied to the potassium thiocyanate solution. (Most of the chemically pure articles on the market are sufficiently pure to use without making corrections.) (1 cc. of thiocyanate solution = 1.0 mg. of NaCl.)

Precipitation in the Plasma.

In a 50 cc. volumetric flask place 2 cc. of plasma accurately measured, 15 cc. of water, 4 cc. of the copper sulfate solution, and 10 cc. of 0.1 N NaOH. Heat in boiling water for about 1 minute. Keep the contents of the flask rotating while heating to insure complete precipitation of the protein. Cool the flask in running water and make up to 50 cc. mark. Shake thoroughly and filter through a chloride-free filter paper. To about 35 cc. of the filtrate add 0.5 gm. of calcium hydroxide powder and shake a few times. Allow to stand 1 minute and filter.

Precipitation in Whole Blood.

In a 50 cc. volumetric flask place 2 cc. of whole blood accurately measured, 10 cc. of water, 8 cc. of the copper sulfate solution, and 20 cc. of 0.1 N NaOH. Heat in boiling water for about 1 minute. Keep the contents of the flask rotating while heating to insure complete precipitation of the protein. Cool the flask in running water and make up to 50 cc. mark. Shake thoroughly and filter through a chloride-free filter paper. To about 35 cc. of the filtrate add 0.5 gm. of calcium hydroxide powder and shake a few times. Allow to stand 1 minute and filter.

Determination.

To 25 cc. of the filtrate from either whole blood or plasma add 5 cc. of the standard silver nitrate solution, 2 cc. of the iron alum solution, and stir until the silver chloride separates out. Titrate the excess silver nitrate with the potassium thiocyanate solution. The first reddish tinge is the end-point.

Subtract the number of cc. of potassium thiocyanate used in the titration from 12.5; the difference will be the gm. of NaCl per 1,000 cc. of whole blood or plasma.

DISCUSSION.

By the use of the method described above, sodium chloride was recovered quantitatively after its addition to the precipitating reagent. It was also quantitatively recovered after its addition to whole blood or plasma, as is shown in Tables I and II.

TABLE I.

Quantitative Recovery of NaCl Added to Plasma.

No. of sample.	NaCl in plasma.	NaCl added.	NaCl recovered.	Theoretical value.
	gm.	*gm.*	*gm.*	*gm.*
1	5.65	0.50	6.15	6.15
2	5.70	0.50	6.20	6.20
3	5.90	1.00	6.85	6.90
4	5.55	1.50	7.05	7.05
5	5.80	0.50	6.25	6.30

TABLE II.

Quantitative Recovery of NaCl Added to Whole Blood.

No. of sample.	NaCl in whole blood.	NaCl added.	NaCl recovered.	Theoretical value.
	gm.	*gm.*	*gm.*	*gm.*
1	4.65	0.50	5.15	5.15
2	4.50	1.00	5.45	5.50
3	4.55	1.50	6.05	6.05
4	4.70	0.50	5.20	5.20
5	4.30	0.50	4.75	4.80

Results obtained by the method described check with those obtained by the Austin-Van Slyke method for whole blood, and the Van Slyke-Donleavy method for plasma, as is shown by Table III.

TABLE III.

Comparison of Results Obtained with Methods of Van Slyke and the Author.

NaCl in whole blood.		NaCl in plasma.	
Van Slyke-Austin.	Author.	Van Slyke-Donleavy.	Author.
gm.	*gm.*	*gm.*	*gm.*
4.65	4.60	5.65	5.65
4.30	4.30	5.70	5.65
4.60	4.55	5.80	5.85
4.50	4.50	5.50	5.50
4.60	4.55	5.95	5.90

To check further the accuracy of the method the chloride content of whole blood and plasma was determined by a modification of the Gutmann and Schlesinger method (6) devised by the author. The method is carried out in the following manner.

In a small crucible place 1 cc. of the substance under examination and add 0.5 gm. of sodium carbonate dissolved in a small amount of water. Evaporate to dryness on a water bath or in a hot air oven. The destruction of the organic matter is carried out slowly with a microburner. It is well at short intervals to allow the crucible to cool and add a few drops of distilled water to the residue; this helps to destroy the carbonized matter without excessive heating. When the organic matter is completely destroyed the crucible is transferred to a 200 cc. beaker and a small amount of hot distilled water added. To the contents of the beaker add nitric acid until effervescence ceases. The crucible is held over the top of the beaker with a pair of tongs and washed thoroughly with distilled water. The contents of the beaker are filtered through a chloride-free filter paper, the beaker and filter paper being washed several times with hot distilled water.

To the filtrate add 5 cc. of the standard silver nitrate solution and 2 cc. of the iron alum solution; stir until the silver chloride separates out. Titrate the excess silver nitrate with the potassium thiocyanate solution.

The results obtained by the Gutmann and Schlesinger method and the method here described are shown in Table IV.

Results obtained in plasma by the method described check with those obtained by the use of the methods of Van Slyke-

Donleavy, and of Rappleye, provided the plasma samples are free from hemolysis. In the presence of even a slight degree of hemolysis results obtained by the latter two methods are of questionable accuracy, since the values for the chlorides are often too great. Van Slyke states in connection with his method for whole blood that only a protein-free filtrate is to be used for determining the chloride content, as some component of the laked cells other than the chloride combines with some of the silver nitrate, making the results too high. The same may be said of the Van Slyke-Donleavy method for chlorides in the

TABLE IV.

Comparison of Results Obtained with Methods of Gutmann and Schlesinger and the Author.

Whole blood.		Plasma.	
Gutmann and Schlesinger.	Author.	Gutmann and Schlesinger.	Author.
gm.	*gm.*	*gm.*	*gm.*
4.75	4.80	5.85	5.90
4.65	4.70	6.00	6.00
3.85	3.90	5.00	5.00
4.60	4.60	6.00	6.05

plasma when the latter is slightly colored from hemolysis. Hemolyzed plasma does not affect the accuracy of the results obtained by the method here described.

A method for the determination of chlorides in the blood has been devised by Harding and Mason (4) which is somewhat similar to the method here described. The chief objection to their method is the titration with potassium iodide, which in the author's experience is a more difficult procedure for the average clinical laboratory worker than is the titration with potassium thiocyanate.

The normal chloride threshold has been set by McLean (7) at 5.62. By the author's method normal plasmas have varied from 5.65 to 6.00.

The method described can be modified by using potassium thiocyanate, each cc. of which contains 0.5 mg. of sodium chloride; however, the end-point is not so clear as when the stronger thiocyanate is used.

BIBLIOGRAPHY.

1. Van Slyke, D. D., and Donleavy, J. J., *J. Biol. Chem.*, 1919, xxxvii, 551.
2. Rappleye, W. C., *J. Biol. Chem.*, 1918, xxxv, 509.
3. Foster, G. L., *J. Biol. Chem.*, 1917, xxxi, 483.
4. Harding, V. J., and Mason, E. H., *J. Biol. Chem.*, 1917, xxxi, 55.
5. Folin, O., and Wu, H., *J. Biol. Chem.*, 1919, xxxviii, 106.
6. Gutmann, S., and Schlesinger, F., *Biochem. Z.* 1914, lx, 283.
7. McLean, F. C., *J. Exp. Med.*, 1915, xxii, 212, 366.

VITAMINE STUDIES.

VI. THE INFLUENCE OF DIET OF THE COW UPON THE NUTRITIVE AND ANTISCORBUTIC PROPERTIES OF COW'S MILK.*

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(Received for publication, October 21, 1920.)

During the winter of 1918 experiments were in progress, in this laboratory, in which guinea pigs were fed a basal diet of 20 cc. of autoclaved milk and oats *ad libitum*. The milk used in this study was obtained from the University dairy herd which had received a grain mixture consisting of corn, barley, oats, middlings, bran, and oilmeal. The roughage consisted of silage and a poor grade of alfalfa hay. The milk was fed to the guinea pigs with the view of supplementing the oat proteins and salts, and was autoclaved to lower the antiscorbutic potency of the milk. It was found that guinea pigs which received the oats and autoclaved milk developed scurvy and died in the time usually expected on this type of diet.

However, during the spring months of 1919, it was observed that some of the animals which had been placed upon experiment late in the winter had ceased to lose in weight, scurvy symptoms were less pronounced, and the animals presented a much improved physical appearance. New groups of animals lived longer on the basal diet containing 20 cc. of autoclaved spring milk than animals receiving the same quantity of autoclaved winter milk. While scurvy developed in the new groups of animals during the spring, the symptoms were less severe and many of the animals actually

* Published with the approval of the Director as Paper No. 220, Journal Series, Minnesota Agricultural Experiment Station.

gained in weight when a loss of weight and death would have been expected.

Inquiry revealed the fact that the University herd had been turned out to spring pasture about 10 days before we had begun to notice the improved condition of the experimental animals just described. In view of the fact that the antiscorbutic vitamine is supposed to be very susceptible to heat, we postulated at the time[1] that the improved nutritive properties of the milk were due, in all probability, to an increased amount of the fat-soluble vitamine in the milk; the last mentioned vitamine having shown greater stability toward heat than either the antineuritic or antiscorbutic vitamines. Consequently, experiments were immediately initiated using 20 and 30 cc. of whole and skimmed raw summer milk. It was found that 20 and 30 cc. of summer milk protected guinea pigs from scurvy for 90 to 100 days when other investigators had often failed to obtain protection with much larger quantities of milk. We were convinced, therefore, that the disagreements which had existed in previous studies, where milk had been fed as the antiscorbutic material, could be explained upon dietary grounds and that the vitamine content of milk is dependent upon the vitamine content of the diet of the lactating animal.

Similar suggestions had already been made by other investigators, but in the majority of cases these suggestions had not been based upon experimental evidence. Previous to our announcement,[1] it had been observed that the antineuritic vitamine in human milk is dependent upon the diet of the nursing mother.[2] McCollum and coworkers,[3] experimenting with rats, came to the same conclusion. The last mentioned experiments, while undoubtedly correctly interpreted, appear somewhat lacking in concrete proof, due to the fact that it was not possible to determine whether or not the milk flow of the experimental mother rats was sufficient for the dietary needs of the young. Obviously, experiments which involve the use of dairy cattle are much to be preferred, in this regard, for although milk production may fall (as

[1] Dutcher, R. A., Pierson, E. M., and Biester, A., *Science*, 1919, l, 184.

[2] Andrew, V. L., *Philippine J. Sc., Section B*, 1912, vii, 67.

[3] McCollum, E. V., Simmonds, N., and Pitz, W., *J. Biol. Chem.*, 1916, xxvii, 33. McCollum, E. V., and Simmonds, N., *Am. J. Physiol.*, 1918, xlvi, 275.

it did in the study described in this paper) the amount of milk obtained is amply sufficient for feeding in any desired quantity. It is also evident that results obtained by the use of dairy cows should also be of much more practical significance. Osborne and Mendel describe experiments[4] in which they were unable to find that summer milk was superior to winter milk as far as the amount of water-soluble (B) vitamine is concerned. It has been pointed out[5] that the average winter ration is undoubtedly fairly adequate with regard to this vitamine due to its general distribution in the grains and also due to the fact that it does not seem to be injured appreciably during the drying and curing process. There is reason to believe, however, that if rations are fed which are sufficiently poor in this vitamine, the milk will also prove to be equally deficient.

During the same month (August) that we announced our observations regarding the nutritive superiority of summer milk, Barnes and Hume[6] reported that they had also noted a seasonal variation in the antiscorbutic properties of cow's milk.

In order that we might obtain more conclusive experimental evidence concerning the influence of diet upon the vitamine content of milk, we have fed vitamine-poor and vitamine-rich rations to dairy cows and the milk obtained from these animals has been subjected to investigation by means of laboratory animals. We have included in this study a biological investigation of the fat-soluble, the water-soluble (B), and antiscorbutic vitamines. In this paper we shall describe our experiments with regard to the antiscorbutic properties of milk produced under conditions of low and high vitamine feeding. In a later paper we shall submit data to show that the fat-soluble vitamine is markedly influenced by the diet of the cow, while the same is true for the water-soluble vitamine but to a lesser degree.

While our experiments were under way Hart, Steenbock, and Ellis[5] published the results of their studies of the same problem. These authors had an unusual opportunity to study this phase of nutrition inasmuch as there was available, at the University of

[4] Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1920, xli, 515.

[5] Hart, E. B., Steenbock, H., and Ellis, N. R., *J. Biol. Chem.*, 1920, xlii, 383.

[6] Barnes, R. E., and Hume, E. M., *Lancet*, 1919, ii, 323.

Wisconsin, a herd of eighteen dairy cows all of which had been "kept year after year on air-dried roughages and grains." It was found that the milk obtained from this herd was much inferior, in antiscorbutic properties, to summer milk *derived from other cows* in the University herd.

The experiments described in this paper differ from the Wisconsin study inasmuch as *the milk was derived from the same cows throughout the experiment, thereby eliminating any possible question of differences in milk from individual cows.* We have attempted, also, to obtain information regarding the length of time required for the vitamine-poor and vitamine-rich rations to produce an effect upon the nutritive properties of the milk. This has been done by adding new groups of guinea pigs from time to time as the experiment progressed.

EXPERIMENTAL.

Period 1; the Vitamine-Poor Period.

The Cows.—Two cows (a Jersey and a Holstein) were fed a vitamine-poor ration for a period of $4\frac{1}{2}$ months (January 17th, 1920, to June 1st). From June 1st to October 1st (4 months) they received the same grain ration and were given constant access to pasture grass. The Jersey and Holstein breeds were chosen on account of the fact that the former produces a milk with a high percentage of butter fat, while the Holstein milk is characterized by a relatively low content of butter fat. By mixing these milks it was thought that the resulting mixture would be fairly representative of average herd milk in chemical composition. Both of these animals had calved during the latter part of December, and were placed upon the vitamine-poor ration on the 17th day of the following month. The cows were milked morning and evening and samples of the mixed milk for the 24 hours were taken for the feeding experiments.

The Ration.—The constituents of the vitamine-poor ration were selected to satisfy the nutrient requirements of the animals and at the same time maintain a low supply of the fat-soluble and antiscorbutic vitamines.

The supply of the water-soluble (B) vitamine was kept as low as was practicable without going outside of the list of ordinary

cattle feeds, by the use of gluten feed (as a high protein feed), wheat middlings, and straw. The animals received what they would consume of a roughage consisting of equal parts of chopped timothy hay and oat straw. The grain rations consisted of equal parts of wheat middlings, gluten feed, ground oats, and ground barley. The ration was adjusted, in quantity, to satisfy the requirements of the animals, according to Armsby's feeding standard. While this ration is undoubtedly poor in the fat-soluble and antiscorbutic vitamines, on the whole, it is a better ration than is often fed on many farms during the winter months, and a fair flow of milk was obtained during the period that it was fed.

Records were kept of feed consumed and of milk produced, while fat tests were made at regular intervals throughout the experiment. On June 1st the cows were changed abruptly to pasture, while the feeding of the same grain ration was continued but in smaller amounts.

The Guinea Pigs.—Healthy, normal guinea pigs were used in all the feeding experiments. Most of the guinea pigs weighed between 200 and 300 gm.; only in a few cases did we use animals weighing as much as 400 gm. All the experimental groups contained at least three (and often four) guinea pigs which were chosen with regard to body weight. Each group contained one heavy animal, one light animal, and one or two which fell between these two extremes. This method placed all the groups of animals on a comparative basis, in case the size of the animal should become an influencing factor in the development of scurvy. Each animal was confined in a cage by itself and the cages were cleaned twice each week and washed with hot water. The cages were sprayed with a cresol preparation at each cleaning period.

Feeding.—The guinea pigs were allowed all the oats that they would eat. The milk, which was fed in varying amounts, was fed twice daily in clean porcelain dishes, which were introduced at each feeding. The animals were under observation at all times and weighings were made every 5 days until scurvy symptoms appeared, and at 3 day intervals thereafter.

We have described the symptomatology and postmortem appearances in guinea pig scurvy in a previous publication.[7]

[7] Dutcher, R. A., Pierson, E. M., and Biester, A., *J. Biol. Chem.*, 1920, xlii, 301.

On February 1st (2 weeks after the cows had been placed on the vitamine-poor diet), five groups of guinea pigs (totaling twenty animals) were placed upon experiment. The quantities of milk fed to the respective groups were as follows: 30, 40, 50, 70, and 80 cc.

On March 1st (1 month later), a second series of animals was placed upon the oats and winter milk diet in order to ascertain, if possible, whether or not the milk was becoming poorer in nutritive properties as the experiment progressed. This series consisted of five groups of guinea pigs (totaling fifteen animals). These groups received the following amounts of milk respectively: 20, 30, 40, 50, and 60 cc.

The third series of guinea pigs was started on April 1st (2 months later). This series was also made up of five groups of three animals each (totaling fifteen animals). These groups were not fed the larger quantities of milk on account of the short time remaining before the cows were to be turned out to pasture. The amounts of milk fed to the respective groups in this series were as follows:

Group 1, 20 cc. Jersey milk.
" 2, 30 " " "
" 3, 20 " Holstein "
" 4, 30 " " "
" 5, 30 " mixed "

The Jersey and Holstein milks were fed separately at this point to determine whether or not breed differences existed with regard to the nutritive properties of winter milk.

Period 2. The Vitamine-Rich Period.

On June 1st the cows were changed abruptly to pasture and the grain ration was continued. Three groups of guinea pigs (four pigs to the group) were placed upon experiment on June 1st, the day that the cows were given access to grass. These groups were fed 20, 30, and 40 cc. respectively of the mixed summer milk. 2 months later (August 1st) three additional groups of guinea pigs were added. These also received 20, 30, and 40 cc. of milk per animal in the respective groups. Observations and weighing were made as in Period 1.

DISCUSSION.

Examination of the data concerning the groups of guinea pigs receiving 20 cc. of milk (Chart 1) shows quite graphically the superiority of the summer milk over that produced in the winter on the vitamine-poor ration. In the remainder of this paper we shall use the terms "winter milk" and "summer milk" to differentiate the milk produced on the vitamine-poor and vitamine-rich rations. All the animals (Nos. 120, 121, and 122) which were placed on the winter milk on March 1st died within 10 weeks with marked evidence of scurvy. (S = first scurvy symptoms observed.) Two of the animals (Nos. 135 and 136) which were started on April 1st died with scurvy, while No. 137 lived on into Period 2 and responded to the summer milk almost immediately. Animals 135, 136, and 137 were fed Jersey milk, while Animals 141, 142, and 143 were fed Holstein milk. All the other animals in Chart 1 were fed mixed milk. No breed differences are apparent in these two groups. It should be noted that Animals 137 and 142 (which showed improvement after beginning the summer milk) had shown pronounced scurvy symptoms in Period 1, and when autopsied on the 1st of August no pronounced scurvy symptoms or lesions could be observed.

20 cc. of summer milk protected Animals 200, 201, and 203 for periods of 16, 4, and 8 weeks respectively, while of the four animals which were placed on summer milk in August (Nos. 260, 261, 262, and 263) three remained healthy and normal throughout the 8 weeks that they were on experiment.

Much to our surprise the first groups of animals (Nos. 103, 104, and 105), which received 30 cc. of winter milk (Chart 2), did not die or fall off in body weight although mild scurvy symptoms developed. However, Animals 123, 124, and 125 developed scurvy and died. No outstanding difference can be noted in the group receiving Jersey milk (Nos. 147, 148, and 149) and those receiving the same quantity of Holstein milk (Nos. 144, 145, and 146). Nos. 139 and 145 responded very soon (2 weeks) when changed to summer milk. Animals 204, 205, 206, and 207 which were started on summer milk on June 1st remained in excellent condition and did not develop scurvy throughout the 16 weeks feeding period, and the same holds true for Animals 264, 265, and 267

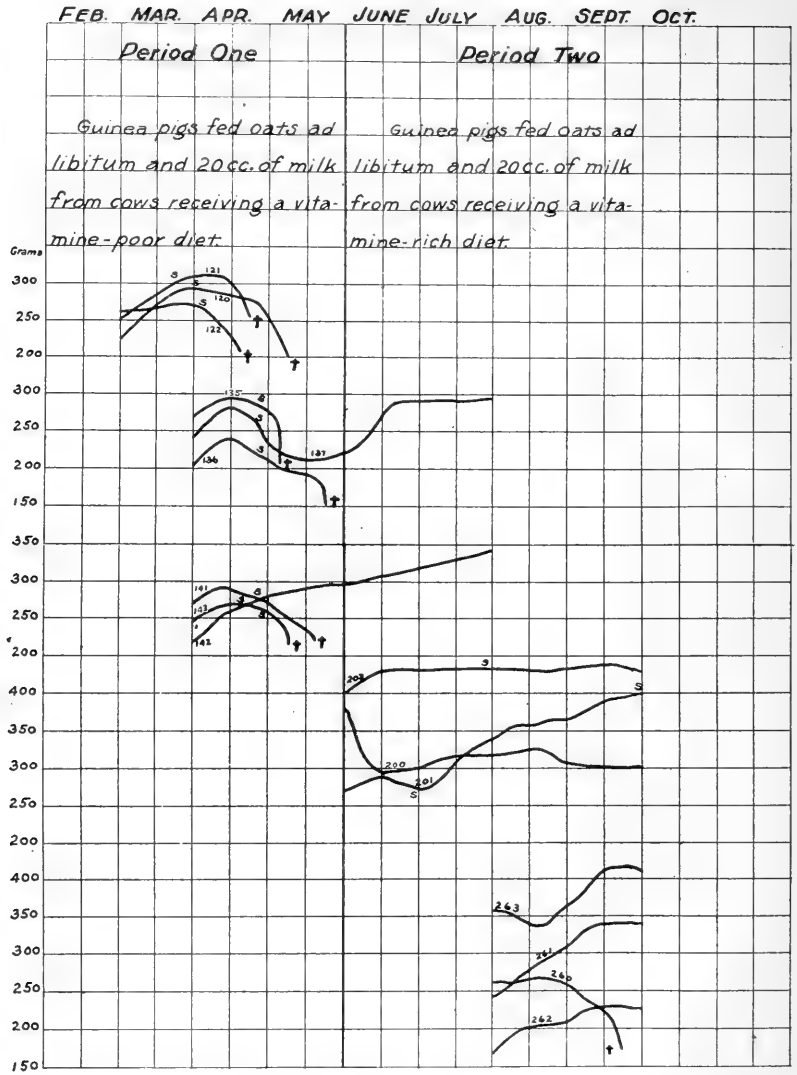

CHART 1. Showing the superiority of 20 cc. of summer milk over the same quantity of winter milk.

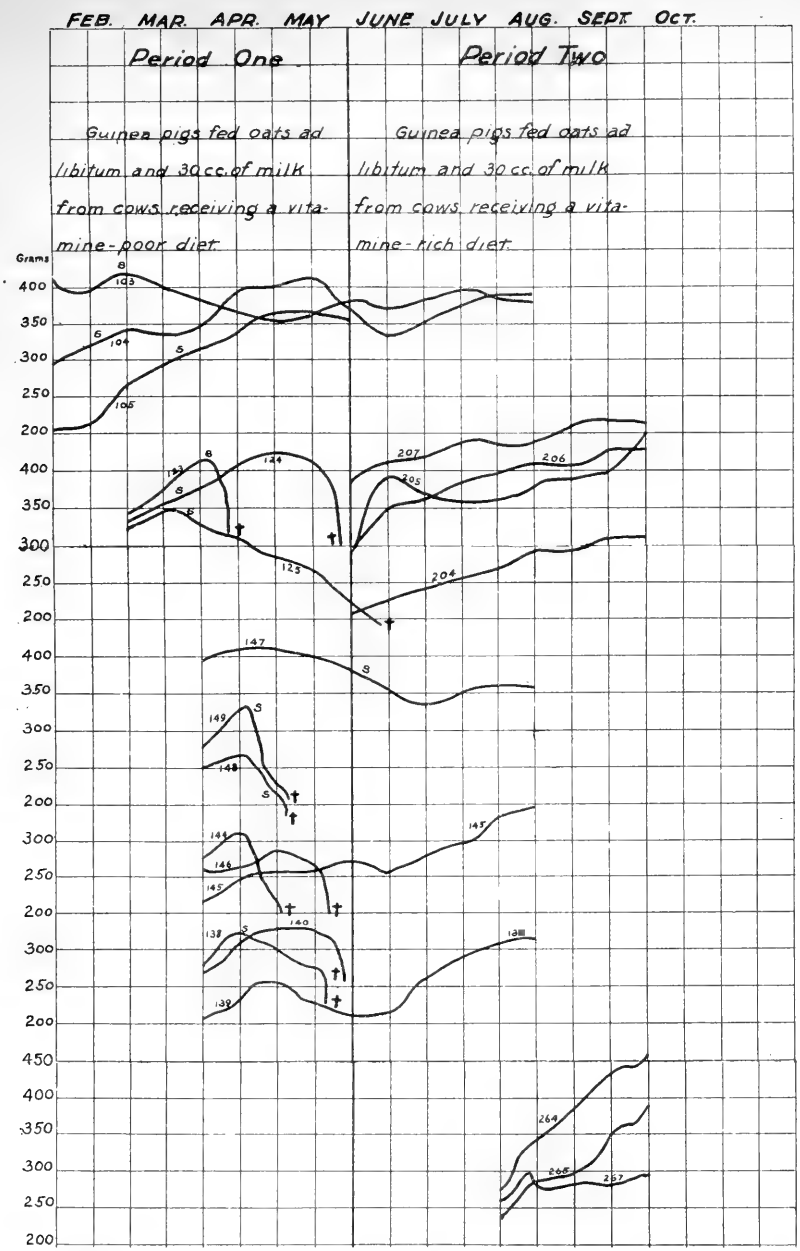

CHART 2. The group which was placed upon experiment in February grew well on 30 cc. of milk. 1 month later the milk had become so poor that all the animals died. 2 months later the milk was of such poor quality that six of the nine experimental animals died. Those which survived improved in physical appearance and showed fair growth when 30 cc. of summer milk were fed. All the animals receiving 30 cc. of summer milk grew well and no scurvy symptoms developed.

which were started on summer milk on August 1st. The superiority of the summer milk is quite evident from the curves of the last mentioned groups.

The animals which received 40 cc. of milk reacted in practically the same manner as the 30 cc. groups (see Chart 3). Those which received winter milk beginning February 1st grew well and did not develop scurvy. This might be explained upon the assump-

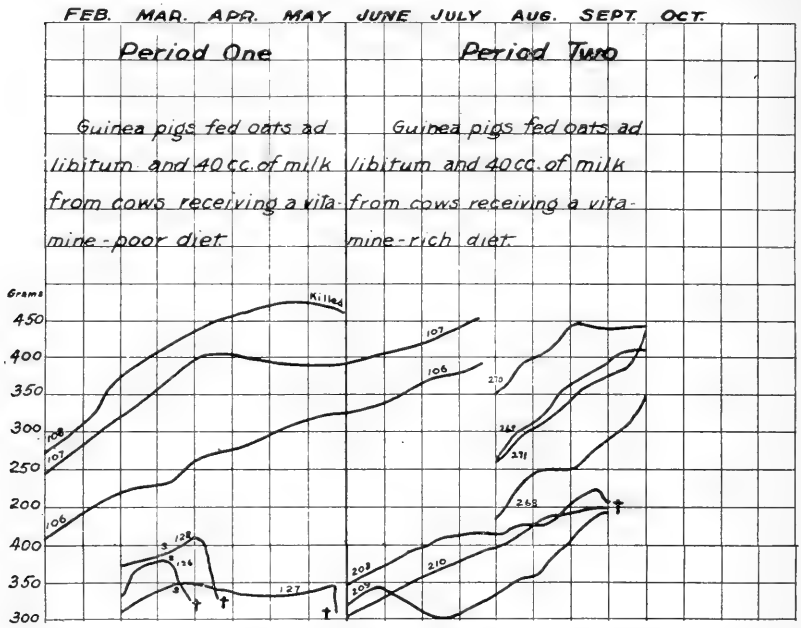

Chart 3. All animals placed on experiment in February lived and grew well. 1 month later the winter milk was of such poor quality that all the animals died. All the animals receiving 40 cc. of summer milk grew well and did not develop scurvy symptoms.

tion that the cow and the guinea pig, together, stored sufficient vitamines to carry the guinea pigs over the winter period. The fact that the March and April groups did not do so well probably lies in the fact that the milk was becoming progressively poorer in vitamines, and after the supply stored by the guinea pig was exhausted the deficiency disease developed. The groups receiving 40 cc. of summer milk grew well and did not develop scurvy.

No. 208 died suddenly during September having developed no scurvy symptoms. The cause of death is unknown.

The February groups of guinea pigs receiving 50 cc. of milk grew well and did not develop scurvy (see Chart 4). Animal 110

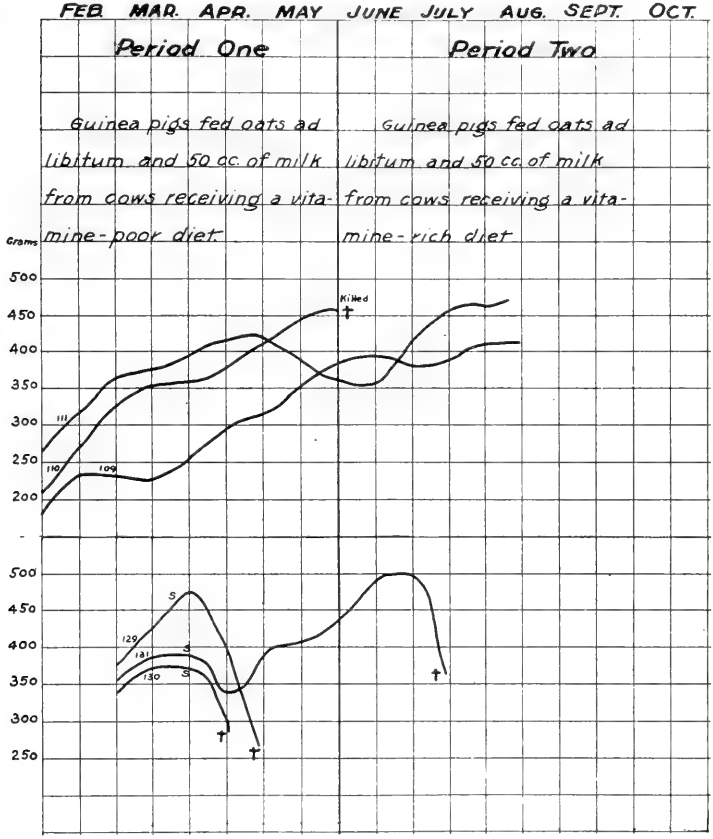

Chart 4. 50 cc. of winter milk did not protect the animals against the development of scurvy and all the animals died with the exception of the group which was placed on diet before sufficient time had elapsed for the milk to become poor.

was killed on June 1st and was found to be in excellent physical condition. The group which was started March 1st (1 month later) developed scurvy and died. Animal 131 did not respond

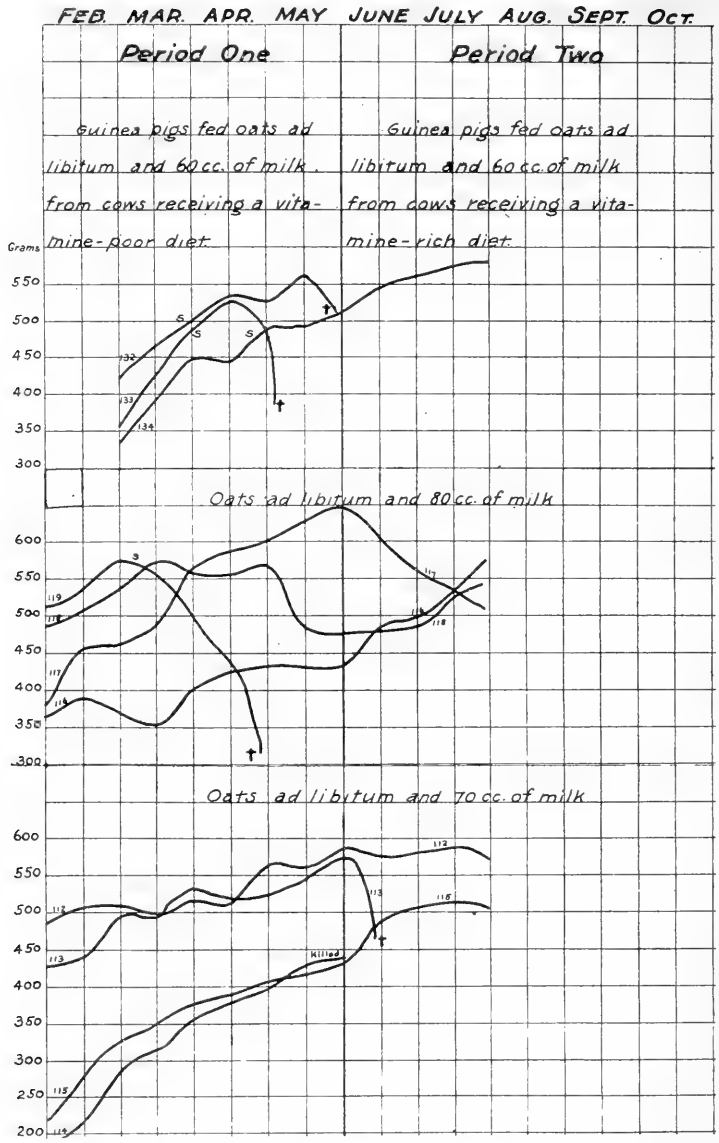

CHART 5. All guinea pigs receiving 60 cc. of winter milk developed scurvy and two of the animals died. After 2 months on a diet of 60 cc. of summer milk no scurvy symptoms could be detected in the animal which survived the winter period, although scurvy symptoms had been very pronounced in the winter months.

to the summer milk and died with very pronounced scurvy lesions. Chart 5 shows that 60 cc. of the "late" winter milk did not protect the animals against the development of scurvy. The scurvy symptoms (swollen joints) disappeared, in the case of No. 134, after 60 cc. of winter milk were replaced by 60 cc. of summer milk.

None of the groups receiving 70 cc. of winter milk (Chart 5), beginning the 1st of February, developed scurvy. The same may be said for the 80 cc. group (Chart 5) with the exception of Animal 119 which did not take the full amount of milk at the beginning of the feeding period.

When the charts are compared as a whole, it is evident that 20 cc. of summer milk were superior in nutritive value and in antiscorbutic potency to 60 cc. of winter milk. A study of the winter milk groups seems to show that the milk does not become poor rapidly; in fact, it is not until the 5th to the 8th week that the effect of the diet becomes manifest in the growth curves and physical condition of the experimental animals. On the other hand, it seems equally certain that the effect of the vitamine-rich ration is noticeable within 2 weeks after the cows were turned out to pasture. This is rather to be expected for it is but natural that the mother will draw upon the vitamines stored in her tissues in order to protect the offspring, even to the sacrifice of her own physical well being. Nor is it surprising to find that the milk becomes rich in vitamines almost immediately upon the ingestion of a vitamine-rich diet.

SUMMARY AND CONCLUSIONS.

Two cows, a Jersey and a Holstein, were placed upon a vitamine-poor ration in the middle of January. Mixed milk was fed in varying quantities to several groups of guinea pigs, new groups of guinea pigs being added from time to time as the experiment progressed. Beginning June 1st the cows were given a vitamine-rich ration and the former experiment was repeated. From the data obtained it was concluded that the vitamine content of cow's milk is dependent upon the vitamine content of the ration ingested by the cow. 20 cc. of summer milk were superior in nutritive value and in antiscorbutic potency to 60 cc. of winter milk. It was found that there is a tendency for the milk to become poor

slowly, when the diet of the cow is low in vitamines, while the milk becomes of higher nutritive value almost immediately upon the ingestion of a vitamine-rich ration. In other words, there seems to be a tendency for milk to become poor slowly and rich rapidly as far as the vitamines are concerned.

In conclusion, the writers wish to acknowledge the assistance of Mr. D. Bessessen in performing a number of the autopsies recorded in this paper.

ABSORPTION AND ELIMINATION OF MANGANESE INGESTED AS OXIDES AND SILICATES.

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(Received for publication, October 28, 1920.)

In 1919 Edsall, Wilbur, and Drinker (1) reviewed the older literature and described cases of manganese poisoning in the United States, occurring among mill employees who were working in a dust containing manganese as oxides and silicates. They point out that the chief portals of entry for the dust are the gastrointestinal tract and the lungs. Up to the present time, owing to lack of reliable methods of analysis, these clinical findings have never been supplemented by adequate analytical data on the manganese content of blood, tissues, or excreta. With the development of the method for detection and quantitation of minute amounts of manganese in biological material, published recently from this laboratory (2), the following experiments were carried out to study the absorption, distribution, and excretion of ingested manganese in the form of oxides and silicates.

Solubility in Gastric Juice.

The extent to which swallowing a manganese-containing ore involves a possible risk depends primarily upon its solubility in the gastric juice. This point was tested with franklinite, rhodenite, and manganese dioxide, representative manganese ores.[1]

[1] Franklinite is a mixed oxide of manganese, iron, and zinc. Analysis of the specimen used by us shows manganese 9.49, zinc 14.53, iron 27.87, calcium oxide 10.74, magnesium oxide, 1.07, silicon dioxide 5.79, and carbon dioxide 8.30 per cent. Rhodenite when pure is manganese silicate. In the sample employed calcite was the chief impurity and the ore contained 24 per cent of manganese as silicate. Chemically pure manganese dioxide containing 48 per cent of manganese was used.

1 gm. of the various ores, ground to 200 mesh, was incubated for different lengths of time in 50 cc. of fasting stomach contents obtained in the usual manner from hospital patients. In one case, in order to approach more nearly to normal conditions, C. K. D. took 8 gm. of franklinite with a test meal which was removed and the stomach washed after 30 minutes. Filtration and analysis were done without incubation. The complete removal of the finely divided dust from a water suspension by filtration proved difficult. The filter finally used was repeatedly proved to be efficient and consisted of an alundum thimble which had been previously dipped in collodion and dried. This was encased in a brass chamber and filtration carried out under about 60 pounds pressure.

A few typical figures for solubilities found are as follows: 14.13 mg. of manganese dissolved after 96 hours at 37°C. in juice with total acidity of 74; 5.56 mg. of manganese dissolved after 22 hours at 37°C. in juice with total acidity of 40; 1.10 mg. of manganese dissolved, in case of test meal (C. K. D.), in contents with total acidity of 21. Unquestionably the ores tested are soluble in gastric juice to an extent depending upon the acidity and duration of contact, but sufficient under normal conditions so that workmen who continually swallow small amounts of such ore dusts must continually furnish dissolved manganese for absorption into the blood stream.

Since the various ores showed practically the same solubility, franklinite was used in most of our feeding experiments.

Effect of Ingested Manganese Ores on the Manganese Content of the Blood.

We were fortunate in being able to obtain blood specimens from employees of the New Jersey Zinc Company at Franklin, New Jersey.[2] Before the installation of the present dust removal systems and methods of periodical physical examination of employees, several cases of manganese poisoning had occurred at this plant. The blood samples were taken by one of us, choosing as subjects some of the men who had been in the employ of the

[2] This company very kindly further cooperated with us by supplying and preparing the franklinite and rhodenite used in these experiments.

company for a long time and whose work kept them a part of the time in some of the dusty places, such as the bag house connected with the dust removal system. The blood was removed from one of the large veins of the arm during the working hours of the men, employing an all glass syringe and using the greatest care to prevent contamination. The figures obtained by analysis are presented in Table I.

TABLE I.

*Manganese Content of Bloods of Franklin Employees.**

Name.	Mn per 100 gm. blood.	Name.	Mn per 100 gm. blood.
	mg.		*mg.*
S. D.	0.008	T. F.	0.010
J. W.	0.008	R. L. McC.	0.005
P. H.	0.019	J. B.	0.021
J. P.	0.013	W. A. M.	0.016
C. S.	0.005	M. E. P.	0.023
O. G.	0.009	W. D.	0.016
G. B.	0.020	H. S.	0.010
C. C.	0.010	A. M.	0.006
L. B. D.	0.013	R. D.	0.006
H. V. A.	0.023	W. C.	0.010
R. M.	0.006	G. M.	0.022
C. D.	0.019	J. J.	0.020
C. B.	0.013	L. B.	0.016
J. K.	0.010		
Average			0.013

* 30 gm. samples of blood were analyzed in every case.

We had hoped to find variations from the normal content which at least in the case of certain individuals would be sufficiently marked to show a connection between the manganese concentration in the blood and susceptibility to poisoning. A study of the results, however, shows that in no case is the concentration unusually high, and that the mean result as well as the variations from the mean are very close to the normal figure (0.012 mg. per 100 gm. of blood) reported in an earlier paper (2). In view of findings to be reported later in this paper this is not surprising. In the case of the workmen whose bloods have been studied the intake of manganese, though continuous, is very small,

and the normal rate of excretion, as will be shown later, is more than adequate to keep the concentration in the blood at a normal level.

This leads directly to the question of whether larger amounts of ore dust taken at one dose may not temporarily increase the manganese of the blood to a higher level. To prove this point, volunteers in the laboratory submitted to the following experiment which in several instances was repeated with identical results. The subjects in every case drank a water suspension containing 8 gm. of franklinite. Analyses were made of blood samples taken before and 1, 3, 6, and 24 hours after taking the ore. In the experiment of September, 1920, in order to show whether conditions tending to affect the acidity of the gastric juice would alter the amount of manganese in the blood, 50 gm. of cooked meat were eaten immediately after swallowing the ore dust. As these results are in no way different from those of previous experiments when the dust was taken without food, the data are all presented together in Table II.

In almost every case the manganese concentration increased slightly during the first hour or two and then fell rapidly back to normal. It is apparent that one or more of the following three factors tends to maintain this low level: (1) the rate of solution of the ore by the gastric juice is very slow, or (2) the rate of absorption into the blood stream is equally slow, or (3) the rate of removal from the blood is too rapid to allow any considerable rise of the manganese content of the blood. The literature upon the behavior of foreign crystalloids in the blood stream and experiments of our own in which soluble manganese has been injected into the blood stream cause us to favor the last possibility as the controlling factor, since a few minutes after a considerable intravenous injection of manganous chloride the manganese content of the blood is practically normal.

Whatever the controlling factor, since identical results have repeatedly been obtained in the same subject and varying results with different individuals, it is evident that in some subjects the manganese content of the blood can temporarily be doubled by ingestion of manganese compounds while in others the concentration remains constant. It seems probable that individuals falling into the first group would be more susceptible to man-

ganese poisoning than those in whom the concentration does not rise.

TABLE II.

*Manganese Concentration of Blood after Eating Franklinite.**

Name.	Date.	Mn per 100 gm.				
		Control.	1 hr. after.	3 hrs. after.	6 hrs. after.	24 hrs. after.
	1919	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*
C. K. D.	Dec. 19	0.012	0.024	0.017	0.019	0.014
	1920					
"	Jan.	0.011	0.023	0.010		
"	Sept.	0.010	0.022	0.012	0.013	
C. K. R.	Jan. 20	0.008	0.017	0.010	0.010	
"	Sept.	0.010	0.028	0.021	0.013	
R. M. T.	Jan. 15	0.010	0.014	0.011	0.011	0.010
"	Sept.	0.012	0.011	0.010	0.010	
L. A. S.	Jan. 15	0,010	0.017	0.012	0.008	0.010
"	Sept.	0.008	0.013	0.010	0.010	
A. S. M.	Jan. 20	0.014	0.012	0.017	0.015	
"	Sept. 27	0.013	0.013	0.016	0.010	
W. O. F.	Jan. 15	0.011	0.010	0.011	0.010	0.011
L. F.	" 20	0.009	0.014	0.007		
W. H.	" 20	0.008	0.009	0.012	0.008	
K. R. D.	Sept. 27	0.013	0.020	0.017	0.013	
H. P. C	" 27	0.011	0.014	0.012	0.011	

* 8 gm. franklinite containing 0.77 gm. manganese eaten in every case. 30 gm. samples of blood taken in every case.

Elimination of Manganese.

The rapid return of the blood to a normal manganese content raises the question of the manner of elimination. Wichert (3), Kobert (4), Cahn (5), and Harnack (6) have stated that manganese is excreted in the bile and intestines but give very little analytical data. A more elaborate feeding experiment than those described above was carried out, in which C. K. D. ate 25 gm. of franklinite in the course of 3 days. During that time and for several days after, the total 24 hour amounts of urine and feces were collected and carefully sampled. 500 cc. of urine and 2 gm. of feces were used for analysis and the total manganese excreted in 24 hours was calculated.

Like many of the heavy metals, manganese is excreted chiefly by way of the intestines. In no case was more than a trace found in the urine, while approximately complete recovery was made in the feces (Table III), a finding which has been repeatedly corroborated by later work on both men and animals. The figures for blood in this case are remarkable only because they again show the identical level of manganese concentration reached by the same subject after eating dust in the experiment in Table II.

TABLE III.

Elimination of Ingested Manganese.

Date.	Mn per 100 gm. blood.	Total Mn in urine for 24 hrs.	Total Mn in feces for 24 hrs.	Remarks.
1919	*mg.*	*mg.*	*mg.*	
Nov. 4–5	0.012	0.000	2.59	Control.
" 5–6		0.014	1.72	"
" 6–7		0.008	5.99	"
" 7–8		0.021	12.58	5 gm. franklinite Nov. 7, 10 a.m.
				5 " " " 7, 4.30 p.m.
" 8	0.024			Blood drawn Nov. 8, 10 a.m.
" 8–9		0.012	748.1	5 gm. franklinite Nov. 8, 10 a.m.
				5 " " " 8, 4.30 p.m.
" 9–10		0.009	1,830.5	5 " " " 9, 10 a.m.
" 10	0.010			Blood drawn Nov. 10, 10 a.m.
" 10–11		0.000	772.6	Control.
" 11–12		0.000	169.9	"
" 12–13		0.000	11.55	"

Following out the suggestion that manganese is eliminated from the blood stream in the bile, the occurrence of the metal in this fluid was studied after feeding franklinite to three persons with biliary fistulas. All the subjects were hospital patients with constant drainage of bile following bile duct operations. Control analyses before giving the ore show that the normal manganese content of bile is very low while after eating the ore it was very markedly increased, especially in Cases 1 and 3 (Table IV). No quantitative comparison can be made between the total amounts of eaten and excreted manganese, for only a fraction of the ore was dissolved and absorbed in the stomach and intestines and not all the bile secreted was obtained through the fistula. Never-

theless, the results do show that the bile is at least one important path of elimination of ingested and absorbed manganese.

TABLE IV.

Elimination of Manganese in Bile.

Date.	Amount ingested.	Mn per 100 gm.
	1. A. M.	
1919		*mg.*
Dec. 13	Control.	0.010
" 14	"	0.013
" 15	"	0.013
" 16	"	0.010
" 16	Ate 5 gm. franklinite	
" 17	in 3 doses.	0.111
" 18		0.100
" 19		0.054
	2. E. L.	
Feb. 16	Control.	0.003
" 16	Ate 3.3 gm. franklinite	
" 17	in one dose.	0.011
" 18		0.006
" 19		0.005
" 20		0.005
" 21		0.003
	3. M. M.	
Feb. 17	Control.	0.003
" 17	5 gm. franklinite in one	
" 18	dose.	0.108
" 19		0.120
" 20		0.046
" 21		0.027
" 22		0.033
" 23		0.018
" 24		0.018

Effect on Blood and Tissues of Prolonged Feeding of Large Amounts of Manganese Ores to Dogs.

Up to this point, the work on human subjects, while it proved the absorption and prompt elimination of manganese ore dust when swallowed, obviously could not be carried on for long

enough periods to be in any way comparable to the long continued exposure in the mills; neither has it been possible to obtain autopsy material from cases of poisoning in order to study the distribution of absorbed manganese in the body. Accordingly, animal experimentation was undertaken to obtain such data.

Six healthy dogs were chosen as subjects and for a long period they were well cared for and fed a diet of bread and meat with which had been mixed their daily ration of a manganese ore. Blood specimens were taken from the jugular vein at intervals of 1 month, and both the manganese and non-protein nitrogen content was determined. The nitrogen determinations were made by Dr. Folin by the method of Folin and Wu (7). As there was no change in nitrogen level, these figures were omitted.

Dog 6 was killed after 11 months because of poor condition due to an attack of distemper rather than to any effects that could be ascribed to manganese feeding. Dog 7 developed an extensive cellulitis and died after removal of a pus sac from his neck. The other four dogs were apparently in as good condition at the end of the experiment as at the beginning, an observation further substantiated by the fact that Dog 3 gave birth to four normal healthy puppies during the experiment.

The figures in Table V show no significant change in manganese concentration in the blood during the entire course of the experiment. The fluctuations may be explained by the fact that the specimens were taken at varying intervals after feeding, and the different values simply further illustrate the fact that the manganese content of the blood is temporarily increased after eating manganese ores. The metal must, then, either be completely eliminated or perhaps stored in some insoluble non-toxic form in certain tissues of the body.

When it became apparent that it was impossible by feeding to produce any changes demonstrable in living animals, or to produce the slightest symptom of manganese poisoning, the dogs were killed and a complete analysis of the tissues was made. A comparison of the figures in Table VI with the normal values obtained by Lund, Shaw, and Drinker (8) for various dog tissues shows that the manganese content of all tissues in the dogs fed with ores is somewhat higher, the increase being more marked in the liver and bile as the results given in Table IV would lead one

TABLE V.

Manganese in Blood of Dogs in Prolonged Feeding of Manganese-Containing Ores.

Legends.	Mar., 1919.	May, 1919.	July, 1919.	Sept., 1919.	Nov., 1919.	Jan., 1920.	Feb., 1920.	Mar., 1920.	May, 1920.	July, 1920.
	mg.	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*
Dog 1. 6.7 gm. franklinite per day. Mar. 26, 1919–July 5, 1920. 477 days. Total 3,200 gm. = 304 gm. Mn.	0.028	0.035	0.033	0.026	0.011	0.030	0.018	0.010		0.016
Dog 2. 6.7 gm. franklinite per day. Mar. 26, 1919–July 5, 1920. 477 days. Total 3,200 gm. = 304 gm. Mn.	0.017	0.022	0.022	0.020	0.036	0.042	0.019	0.023	0.016	0.024
Dog 3. 3.5 gm. Baker's 82.5 per cent MnO_2 per day. 477 days. Total 1,600 gm. = 768 gm. Mn.	0.028	0.020		0.018	0.018	0.020	0.018	0.036	0.016	0.010
Dog 5. 3.5 gm. Baker's 82.5 per cent MnO_2 per day. May 15, 1919–Mar. 30, 1920. 455 days. Total 1,450 gm. = 696 gm. Mn.	0.020		0.013	0.016	0.015	0.022	0.012	0.010	0.020	0.036
Dog 6. 3.5 gm. rhodenite per day. May 15, 1919–Mar. 30, 1920. 320 days. Total 1,120 gm. = 269 gm. Mn.		0.040	0.024	0.022	0.010	0.039	0.010	Tr.		
Dog 7. 3.5 gm. rhodenite per day. May 15, 1919–Feb. 8, 1920. 270 days. Total 950 gm. = 228 gm. Mn.			0.024	0.020	0.028	0.022	0.010			

to expect; that is, the manganese in these tissues was in the process of being eliminated into the intestine. There is no special local storing up of the metal in any organ. The chemical analyses were supplemented by histological examinations which revealed nothing abnormal in any tissue.

TABLE VI.

Manganese Content of Tissues after Prolonged Manganese Ingestion.

Form and total amount of Mn ingested	Dog 1. Franklinite. 304 gm. Mn.	Dog 2. Franklinite. 304 gm. Mn.	Dog 3. Baker's c.p. MnO_2. 768 gm. Mn.	Dog 5. Baker's c.p. MnO_2. 696 gm. Mn.	Dog 6. Rhodenite. 269 gm. Mn.	Dog 7. Rhodenite. 228 gm. Mn.	Average normal manganese content.*
Duration of experiment, *days*...	477	477	477	455	320	270	
Tissue.	Mn per 100 gm.						
	mg.	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*
Liver	0.700	0.660	0.276	0.545	0.54	0.90	0.238
Kidney	0.216	0.120	0.170	0.188	0.133	0.11	0.087
Spleen	0.025	0.112	0.032	0.043	0.046	0.031	0.022
Pancreas	0.220	0.284	0.137	0.100	0.089	0.170	
Stomach	0.115	0.211	0.081	0.710†	2.14†	0.059	0.043
Duodenum	0.377	0.088	0.051	0.203	0.175	0.072	
Small intestine	0.300	0.420	0.114	0.211	0.168	0.062	0.028
Colon	0.314	1.56	0.310	0.396	0.307	0.060	0.080
Lung	0.024	0.050	0.030	0.056	0.040	0.047	0.023
Heart	0.025	0.067	0.071	0.040	0.300	0.028	
Brain hemispheres	0.030	0.023	0.153‡	0.042	0.039	0.031	0.062§
Basal ganglia	0.042	0.046		0.138	0.057	0.049	
Cord	0.040	0.024	0.049	0.082	0.031	0.088	0.044
Omentum	0.006	0.130	0.009	0.017			
Muscle	0.020	0.042	0.025	0.023	0.016	0.034	0.020
Bone marrow	0.043	0.055	0.225	0.045			0.101
Bile	1.60	3.68		1.08	0.666	1.70	

* Lund, Shaw, and Drinker (8).

† Dog had eaten recently.

‡ Brain analyzed as a whole.

§ Whole brain.

SUMMARY AND CONCLUSIONS.

Ores containing manganese as oxides and silicates are soluble in gastric juice. Manganese is absorbed in the blood stream causing in most cases a slight temporary rise in manganese concentration followed by a quick return to normal. In none of the cases studied was the manganese content of the blood increased by the ingestion of manganese ores to a value more than double the normal level, and in some of the subjects no increase was noted. We suggest that individuals of the first group would probably be more susceptible to manganese poisoning than those of the latter.

Even prolonged feeding of large amounts of manganese ore to dogs failed to produce significant changes in manganese content of blood and tissues or to cause any pathological symptoms. Manganese ores are thus very non-toxic and in order to produce symptoms of poisoning must be ingested by individuals who are peculiarly susceptible. Clinical experience (1) has demonstrated that such persons are extremely rare.

BIBLIOGRAPHY.

1. Edsall, D. L., Wilbur, F. P., and Drinker, C. K., *J. Ind. Hyg.*, 1919, i, 183.
2. Reiman, C. K., and Minot, A. S., *J. Biol. Chem.*, 1920, xlii, 329.
3. Wichert, E., Inaugural dissertation, Dorpat, 1860.
4. Kobert, R., *Arch. exp. Path. u. Pharmakol.*, 1883, xvi, 361.
5. Cahn, J., *Arch. exp. Path. u. Pharmakol.*, 1884, xviii, 129.
6. Harnack, E., *Arch. exp. Path. u. Pharmakol.*, 1901, xlvi, 372.
7. Folin, O., and Wu, H., *J. Biol. Chem.*, 1919, xxxviii, 81.
8. Lund, C. C., Shaw, L. A., and Drinker, C. K., *J. Exp. Med.*, in press.

GROWTH ON DIETS POOR IN TRUE FATS.*

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(Received for publication, October 21, 1920.)

The reason why there is no available information respecting the actual requirement of the healthy mammal for fat is attributable to the experimental difficulties heretofore inherent in its solution.[1]

In a previous paper[2] we employed the word "fat" to designate the simple glycerides of fatty acids. Attention was specifically directed to the possible independent significance of "that heterogenous group of substances designated 'lipoids'." At the same time we reported that rats even doubled or tripled their body weight upon fat-free rations consisting of a purified protein (casein or edestin), corn-starch, sucrose, and either natural protein-free milk extracted with anhydrous ether or "artificial protein-free milk" composed of a mixture of inorganic salts and lactose. Despite this apparent initial success all the rats in the reported trials and many others conducted at that time failed to reach a large size and sooner or later began to decline in weight. Some of them died at this stage; others were restored by the use of milk food containing fat. In referring to this outcome we[3] remarked that "this failure to attain complete growth involves

* The expenses of this investigation were shared by the Connecticut Agricultural Experiment Station and the Carnegie Institution of Washington, Washington, D. C.

[1] A discussion of earlier attempts in this direction will be found in our monograph, Osborne, T. B., and Mendel, L. B., Feeding experiments with isolated food-substances, *Carnegie Inst. Washington, Pub. No. 156*, pts. i and ii, 1911.

[2] Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1912, xii, 81.

[3] Osborne and Mendel,[2] p. 84, foot-note 10.

some factor in nutrition other than the fat and is at present under investigation."

At that time the significance of the vitamines had not been clearly formulated. It was in part the outcome of these experiments which led us to the discovery of the fat-soluble vitamine.[4] The nutritive failure of the animals in the series of 1912 was undoubtedly attributable in part at least to a deficiency in this vitamine. The fact that the foods, though extracted with ether, had not been extracted with hot alcohol, and hence could not be regarded as *lipoid*-free, may explain the not inconsiderable growth obtained, for the so called "lipoids" include substances soluble in hot alcohol which may not dissolve in ether.[5] Evidence since accumulated indicates that the so called fat-soluble vitamine is not always removed by ether unless the product has previously been treated with alcohol.[6]

The recent controversy[7,8] regarding the necessity for fats in the diet fails to emphasize adequately the distinction in the significance of fats as sources of energy, carriers of vitamine, and of lipoids regarding the rôle of which we are still largely uninformed.

After citing the ability of rats to grow on a diet of casein, wheat bran, wheat starch, and butter in contrast with the failure to thrive in the absence of the butter, Aron[7] has insisted that food fats have a specific nutrient value, "Sondernährwert," which cannot be replaced by other foodstuffs. Thus he says: "The special nutritive value of food fats probably involves the presence of certain lipoids."[9] Aron's thesis is essentially another plea for the recognition in some natural fats of nutritive factors not expressed in terms of calories alone.

[4] Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1913, xv, 311.

[5] Osborne and Mendel,[2] p. 84.

[6] A discussion of this problem is being prepared for publication.

[7] Aron, H., *Biochem. Z.*, 1918, xcii, 211.

[8] Aron, H., *Biochem. Z.*, 1920, ciii, 172. Salkowski, E., *Biochem. Z.*, 1919, xciv, 205. von Gröer, F., *Biochem. Z.*, 1919, xcvii, 311.

[9] Aron's actual experiments are not entirely convincing in so far as the benefits of the butter are ascribed entirely to the *fat* component. Butter carries a not negligible portion of non-fat milk solids which may include water-soluble vitamine, etc., in which his basal diet may have been low.

This by no means answers the fundamental question as to whether true fat is indispensable in the diet. Among the various explanations of the genesis of so called war edema has been the shortage of fats and of vitamines in the diet. Thus Schittenhelm and Schlecht[10] conclude from their extensive observations:

"Die Erfahrungen, welche wir heute über den Einfluss fettfreier Nahrung haben, beschränken sich völlig auf Tierexperimente. Die Tiere sind in ihrem Wachstum geschädigt und gehen schliesslich zugrunde. Besonders hervorstechende Krankheitserscheinungen scheinen dabei nicht aufzutreten. Wie sich die Wirkung andauernder fettfreier Ernährung beim Menschen äussern würde, müsste erst erwiesen werden. Man kann aber wohl annehmen, dass die Lipoidverarmung des Körpers zu Störungen Veranlassung geben kann, wenn man die lebenswichtige Funktion in Betracht zieht, welche den Lipoiden als mutmasslichen Bestandteil der Plasmahaut für die Aufnahme der Stoffe in die Zelle zukommt."

The experimental studies of Kohman[11] tend to make the fat deficiency explanation of nutritional edema untenable since they show that even in the presence of fats in the diet "a combination of low calories, low protein and excessive fluid intake will lead to a marked dropsy corresponding to war dropsy in all respects. The importance of specific vitamines seems to be excluded by these experiments."[12]

Maignon[13] supposes that the fats play an important rôle in the utilization of protein, a rôle which carbohydrates are powerless to fill. His experiments, conducted by feeding mixtures of more or less isolated food materials without due consideration to the now well recognized need of accessory food factors or vitamines, need not be further reviewed at this time.

Attention has recently been concentrated anew on this question by a publication of von Gröer[8] from Pirquet's clinic in Vienna. He has succeeded in attaining practically normal increments of weight in two infants who were fed during their first half year of life on a diet practically devoid of fat. This consisted of

[10] Schittenhelm, A., and Schlecht, H., Die Ödemkrankheit, Berlin, 1919; also *Z. ges. exp. Med.*, 1919, ix, 1.

[11] Kohman, E. A., *Am. J. Physiol.*, 1920, li, 378.

[12] Maver, M. B., *J. Am. Med. Assn.*, 1920, lxxiv, 934.

[13] Maignon, F., Recherches sur le rôle des graisses dans l'utilisation des albuminoïdes, Lyon, 1919.

highly centrifugated milk[14] supplemented with an energy equivalent of cane sugar. Later a porridge containing skimmed milk and cane sugar and having twice the fuel value of whole milk was fed. According to a personal communication from Professor von Gröer to one of us (M.) this diet was adhered to strictly. He concludes that "the theoretical fat minimum of the human suckling as shown by these two nutrition experiments is in any event of such a small magnitude that, contrary to the current views, it may practically be disregarded."

It might appear as if this apparent success contravened the experience of numerous investigators, including ourselves, regarding the indispensability of the fat-soluble vitamine for the normal nutrition of growing individuals.[15] Several considerations, however, must be taken into account. First, centrifugated milk is not *entirely* devoid of fat or of the fat-soluble vitamine.[16] We[17] have seen young rats grow to considerable size on diets of isolated foods in which the added daily supply of fat-soluble vitamine consisted of only 0.1 gm. of butter fat, and our early seemingly successful experiments with fat-free foods have already been alluded to. Second, it is by no means certain yet whether or not young animals have a certain store of this food factor which only gradually becomes exhausted.[18] Third, the period of growth in von Gröer's two clinical observations was comparatively short. 6

[14] Fat content about 0.01 per cent.

[15] Mendel, L. B., *New York State J. Med.*, 1920, xx, 212.

[16] *Cf.* McCollum, E. V., and Davis, M., *J. Biol. Chem.*, 1915, xxiii, 233.

[17] Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1920, xli, 549.

[18] The question is discussed in some detail in a paper by Osborne and Mendel, soon to appear; see also *Med. Research Comm., Nat. Health Insurance, Special Rep. Series, No. 38*, London, 1919, 16, which states:". . . . for a short time the young animals are able to grow when the fat-soluble factor is deficient in their diet. The explanation of this is probably supplied by the assumption that the animal organism normally contains reserve supplies o the factor 'A' in its own body. If this hypothes is correct it s reasonable to assume that these reserves are mobilized for use when a deficiency occurs in the diet, but as soon as they are exhausted growth is immediately inhibited. During the period of temporary growth, throughout which it has been assumed that the reserve supplies are being utilized, the animals show a normal appearance and there does not appear to be any decline in their health, but when the reserves are exhausted and the deficiency becomes felt, not only do they cease to grow but they become highly susceptible to bacterial infection." See also Mendel.[15]

months of human growth corresponds to a few days only of rat growth. In von Gröer's experiments both children showed signs of interrupted growth in the course of the feeding periods (one of them after 3 months) and were attacked by illness which he ascribes to intercurrent infection during a prevailing epidemic of grip. The suspicion is raised that a lowered nutritive condition may have contributed to this interruption of growth—a conclusion in harmony with experiments on animals living on diets poor in fat-soluble vitamine.

In discussing von Gröer's experiments Aron[8] writes: "Young sucklings can develop apparently unharmed for several months on a diet practically fat-free; subsequently, however, disturbances in their well being arise."

He thereupon adds: "Children nourished on a diet poor in fat, as has been demonstrated on other occasions,[19] are not very resistant toward infections and are affected by them with exceptional severity."

Here again there is a confusion between the possible effects of a lack of fats *per se* and a deficiency of the vitamine factor which is associated with them in milk fat and elsewhere.

The ideal plan for ascertaining experimentally whether true fats are dispensable in nutrition would consist in adding the isolated fat-soluble vitamine to an absolutely fat-free otherwise adequate diet as the ration of growing animals. We have "concentrated" the fat-soluble vitamine by preparing "butter oil"[20] as well as by extracting potent fractions of "oils" from certain plant products.[17, 21]

The appreciation of the distribution of the fat-soluble vitamine in considerable concentration among plant products, notably green leaves, not ordinarily classed as fat foods has made it possible to approach a reasonably successful method of investigation. Consequently we have conducted new feeding experiments on young rats with diets presumably containing adequate amounts of both the fat-soluble and water-soluble vitamines, but exceedingly poor in true fats; *i.e.*, compounds soluble in pure

[19] Niemann, *Berl. klin. Woch.*, 1919, 777.

[20] Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1915, xx, 379.

[21] Osborne, T. B., and Mendel, L. B., *Proc. Soc. Exp. Biol. and Med.*, 1918–19, xvi, 98. Zilva, S. S., *Biochem. J.*, 1920, xiv, 494.

ether. The fat-soluble factor was supplied by dried alfalfa,[22] the water-soluble by dried yeast. The foods were prepared as follows.[23]

The starch was first mixed with cold water and then gelatinized by pouring into boiling water. The meat residue and salts were added and the mixture was dried in air at about 70°.

In the first series (Rats 6369, 6367, 6383) the food mixture consisted of meat residue 23, starch 72, and salt mixture 5 per cent. To furnish the necessary vitamines tablets containing 0.4 gm. of brewery yeast, 0.2 gm. of alfalfa, and 0.2 gm. of cane sugar were fed daily apart from the rest of the food which was offered *ad libitum*. Rats 6367 and 6383 received additional doses of alfalfa, as indicated on Chart 1, during the latter period of their growth.

The meat residue[24] yielded 1.6 per cent of ether extract. The diet can at best be described as exceptionally low in true fats. Nevertheless the animals grew on it with vigor, reaching approximately 300 gm. when this report was prepared. In two cases (Rats 6367, 6383) the daily supply of alfalfa was increased to 1 and 0.4 gm. respectively at a time when it seemed as if the amount of fat-soluble vitamine might be too small. This change increased very slightly the amount of true fat available for the animals.

In order to reduce still further the fat component of the diet which, except in the very small fixed daily portions of alfalfa and yeast, increased with the augmented food intake of the large animals, a food mixture was prepared for which the thoroughly dried meat residue was extracted five times with ether containing a little alcohol.

Extracting with anhydrous ether removed from the non-vitamine portion of the food mixture 0.24 per cent of solids. On the basis of these data the largest daily intake of ether extract (fat(?))

[22] The preparation and potency of this from young alfalfa leaves is described by Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1919, xxxvii, 187. Its content of ether-soluble material was 5.06 per cent. 0.2 gm. of this dried alfalfa, as supplied daily to the rats, would thus contain only 0.01 gm. of fat (ether extract).

[23] The general conduct of such experiments by us has been described by Ferry, E. L., *J. Lab. and Clin. Med.*, 1920, v, 735.

[24] For the preparation of this, see Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1917, xxxii, 369.

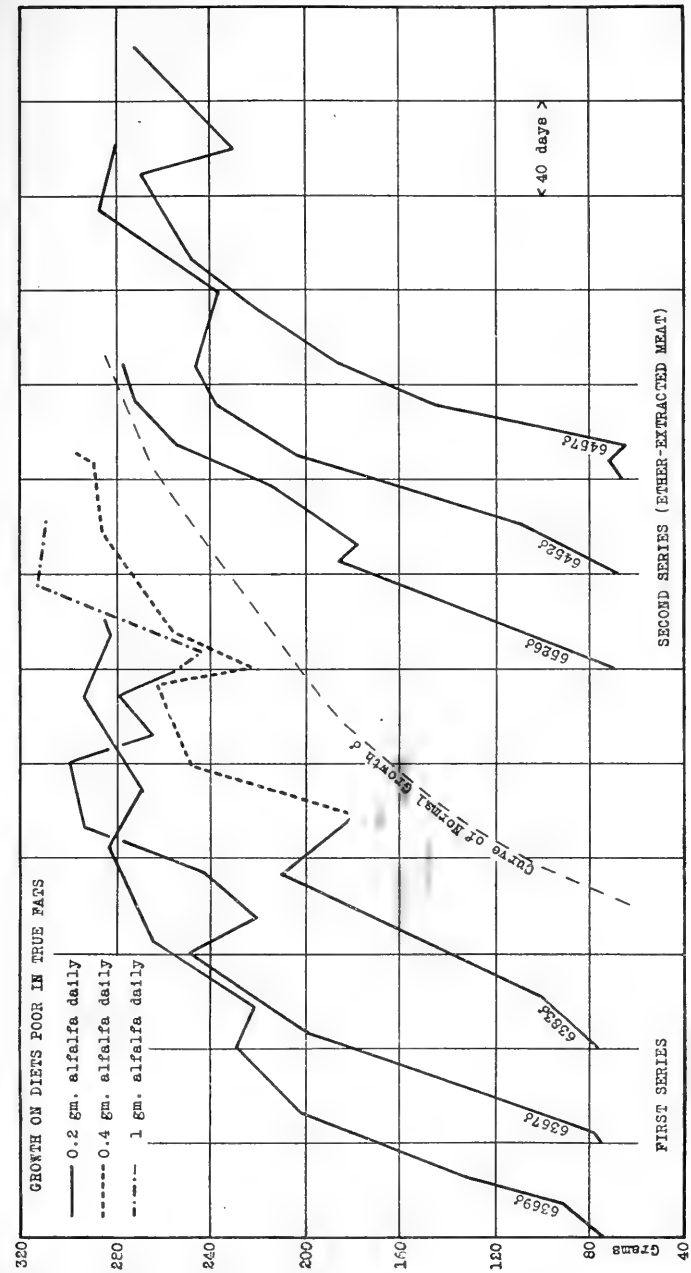
GROWTH ON DIETS POOR IN TRUE FATS
0.2 gm. alfalfa daily
0.4 gm. alfalfa daily
1 gm. alfalfa daily
Curve of Normal Growth
FIRST SERIES
SECOND SERIES (ETHER-EXTRACTED MEAT)
< 40 days >
6369d
6367d
6368d
6366d
6452d
6457d
Grams
320
280
240
200
160
120
80
40

CHART 1.

for any of the animals on this series was 0.078 gm., and this during a period when the weekly food intake reached the exceptional amount of 174 gm. These rats (Nos. 6526, 6452, 6457), like those of the first series, have shown growth to large size (Chart 1). Inasmuch as all the animals starting on the diet with a body weight of approximately 70 gm. have quadrupled their weight within the usual time, and appear as well nourished as companion rats on diets containing liberal portions of butter fat or lard, we cannot avoid the conclusion that *if true fats are essential for nutrition during growth the minimum necessary must be exceedingly small.*

Hindhede,[25] whose results with young men correspond to observations on laboratory animals, has contended that fat is not required in the diet if a sufficient amount of fresh fruits and vegetables is eaten daily to supply vitamines.

The 80 to 90 gm. of the fat-free diets (about 3.8 calories per gm.) voluntarily eaten by the rats compared with the 60 to 70 gm. of our standard experimental diets containing 28 per cent of fat (about $5\frac{1}{2}$ calories per gm.) usually eaten by rats of similar size represents the adjustment to the energy needs of the animals.

As they grew larger the difference in the consumption of the "fat-free" over the "standard" ration became even more striking. Thus at a body weight of 270 gm. the animals frequently ate 125 to 160 gm. or more of fat-free food per week in contrast with an average of 82 gm. on the standard diet. Incidentally the large intakes have resulted in the ingestion of large quantities of protein. This has evidently not been detrimental to the rate of growth of the animals. On the other hand, in view of the very rapid gains made by these animals during the early weeks of the experiments, it may even be debated whether it has not represented an actual advantage in the nutrition of growth.

[25] Hindhede, M., *Molkerei-Ztg.*, 1918, xxviii, 152; abstracted in *Chem. Zentr.*, 1918, ii, 745; *Skand. Arch. Physiol.*, 1920, xxxix, 78.

THE PHYSIOLOGICAL EFFECTS OF SHORT EXPOSURES TO LOW PRESSURE.

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(Received for publication, September 27, 1920.)

Although it has been recognized that prolonged sojourns at high elevations tend to upset the physiological equilibria and result in a permanent stimulation of the hematopoietic apparatus, the time required for these changes still remains a matter of conjecture. In regard to exposures to low pressure of short duration such as are met with in aeroplane flights, the current opinion is that the mainly respiratory and circulatory effects are of a transitory character, and that the cellular life of the body is left undisturbed. If it could be demonstrated that a few hours stay in high altitudes is sufficient to stimulate the blood-forming organs to increased activity, such a discovery would be, from a therapeutical point of view and taking into consideration the ease in reaching high elevation that is offered by modern aviation, of a far reaching interest. It must be expected, however, considering the difficulties that for a long time kept the blood changes during prolonged sojourns at high elevations open to argument, that the crucial test of a stimulation of the bone marrow occurring from only a short exposure to low pressure would be still more difficult to produce.

The older data on the subject, obtained from observations during balloon ascensions, are contradictory. While Gaule (1) claimed that he found evidence of a stimulated formation of red cells, von Schroetter and Zuntz (2) were unable to confirm his observations; they admit, however, that the blood-forming organs may be hyperemic after a few hours stay at an altitude of 4,000 meters. The permanent increase of erythrocytes Kuhn (3) and others have observed from use of the "suction mask" has been attributed by them to a direct effect on the bone mar-

row, and, although it must remain doubtful whether their procedure can be compared with an exposure to low pressure, their extended time of observation seems to warrant their conclusion that the bone marrow may be sensitive to a deficient oxygen supply of very short duration.

Gregg, Lutz, and Schneider (4) in a recent piece of research, in which they interned a great number of men in a low pressure chamber for short periods of time, found in the majority of cases an increase in the number of red cells, but they are inclined to the view that an "increased production of hemoglobin and erythrocytes by the bone marrow is improbable" and that the change is due either to a concentration of blood plasma or to a dormant supply of old blood corpuscles.

The emphasis Abderhalden (5) has laid upon the necessity of combining blood countings with biochemical methods for reaching an understanding regarding the blood changes during prolonged sojourns in high altitudes, seems to hold true also in the controversy whether the increase of red cells, occasionally observed after short exposures to low pressure, is caused by a stimulation of the erythropoietic organs or not. It is evident that the biochemical methods one of us (6) has employed to test the increase of erythrocytes in mountain trips would be useless for our present problem; that, however, the biochemical equilibria of the body may be profoundly affected by short exposures to low pressure may be learned from investigations of Haldane and coworkers (7) in which they found changes of the acid-base equilibria, similar to those that have been demonstrated by one of us (8) to take place during prolonged stay at high elevation.

Masing (9) has suggested the use of the phosphorus content of the erythrocytes as a criterion of the presence of newly formed red cells; a higher percentage would then indicate a stimulation of the erythropoietic organs. Considering the presence, according to the hematologists, of nuclear remnants in the circulating red cells, Masing's idea seems to be worthy of adoption, in the light of recent investigations of Bloor (10). It is, however, doubtful whether any decision can be reached as to the average age of the erythrocytes from analysis of the red corpuscles for phosphoric acid. Furthermore as it has been found that the corpuscle volume may vary within wide limits, the rough figure

for the phosphorus content of a unit volume of packed corpuscles is misleading for our present purpose. The average phosphorus content of an individual red cell, which for our inquiry would be instructive, is affected with such an excessive experimental error as in most cases to make its use imaginary.

Recognizing the obstacles referred to above, but anyhow hoping to arrive at results that might throw some light on the question whether any stimulation of the blood-forming organs occurs when they are subjected to a decreased oxygen supply, we decided to combine estimations of the phosphorus content of the blood with blood countings in animals kept for a few hours at artificially decreased pressure. We were the more induced to undertake the investigation as no data are available regarding the phosphorus content of the blood in high altitudes; and in addition as such a research would furnish a great number of data with reference to the normal variations of the total phosphoric acid and its different fractions. One of us (6) has speculated whether the constant phosphorus retention he found, combined with a lowered bicarbonate content of the blood, could possibly depend on the participation to a greater extent of the phosphates in the buffer value of the blood in high altitudes. It may be said in this connection that our present investigation wholly disproves this suggestion, which was formulated because of lack of information of the high phosphorus content of the red cells, which fully explains the positive phosphorus balances observed.

We employed for our experiments a low pressure chamber that had been constructed by one of us (E. S. S.) for artificial high altitude experiments on small animals. The apparatus consists of a cylindrical vessel made of heavy galvanized sheet iron (height 50, diameter 65 cm.) with concave bottom and top, and with twelve vertical wrought iron braces branching off to support the top. In the center of this is a round window, 35 cm. in diameter, surrounded by a rim for a rubber gasket. On the rubber gasket is placed a piece of rubber tubing on which rests a piece of plate glass, $\frac{5}{8}$ inch thick. By this simple arrangement a hermetical sealing is accomplished when the pressure inside the apparatus is decreased. The evacuation of air is done with a suction pump, driven by a $\frac{1}{4}$ horse power electrical motor. A constant pressure is attained by adding to the constant air inlet an intermittent one, which is controlled by a mercury manometer.

When the pressure reaches a certain point a contact is made, closing a circuit from two dry cells, which in turn by means of a relay closes the main circuit and opens a valve in a modified gas faucet, connecting the chamber for a moment with the outside air. This automatic device can be relied upon to keep the pressure inside the apparatus constant for any length of time, even at a vacuum of 650 mm. For experiments extending over longer periods of time the apparatus is supplied with a device for feeding the animal, with an arrrangement to collect urine and feces, which consists of an inner enameled metal funnel around the wall to half its height and passing through the bottom of the chamber. The feces are collected on a wire net suspended above the bottom of the funnel. A water-sprinkling device of simple construction is further added in order to rinse off the urine at the end of certain periods without necessitating the lowering of pressure.

As experimental animals rabbits were chosen, as their quiet habits minimize the errors that might in a work of this kind result from excessive struggling. The blood samples were drawn from the heart, one before putting the animal into the apparatus, the other immediately after restoring normal pressure. To enable quicker handling the animal was tied down on its back in a box in as comfortable a position as possible and it soon became quiet, especially when the pressure went down. While thus in the low pressure rabbits were comparatively quiet; the control animals, which were also tied down and otherwise treated exactly as the former ones, occasionally tried to pull themselves loose. The experiments were started in the morning before the rabbits had been fed. The reduction of pressure was rapid, requiring only 2 to 3 minutes, and the restoration of normal pressure took place in about the same length of time.

In the first ten experiments 25 cc. of blood were drawn, in other experiments only 15 cc. were taken. 0.5 per cent of powdered potassium oxalate was added to prevent clotting. The same hemocytometer was used throughout the work and only duplicate countings that were nearly identical were accepted.

In order to test out whether the peripheral blood differs in its red count from the blood from central organs we did a few parallel countings in the blood from the heart and from the ear vein as well. The figures agreed with each other within the limits of experimental error.

The methods for the determination of phosphoric acid constituents in blood have been described previously (11) and certain modifications of the methods which have been used in this work are discussed in the following paper (10). A special form of hematocrit was used for the work and since it has proved accurate and satisfactory a brief description is desirable. The apparatus consists of the hematocrit proper and a container. In making the hematocrit two sections of capillary tubing of even bore, 1 mm. inside, 5 mm. outside diameter, and 150 mm. long, were cut off and the sharp edges of the ends beveled a little so that they would not break when used in the centrifuge. The container consists of a length of glass tubing about 120 mm. long, 15 mm. inside diameter, and with walls at least 1 mm. thick. This was fitted with a 10 mm. section of solid rubber stopper at one end and with a stopper perforated to admit the hematocrit tube at the other.

In use the well mixed blood is drawn up into the hematocrit tube to a height of 12 or 13 cm., held there by a finger over the upper end, the excess blood wiped off, and the lower end sealed by a disk of gummed label cut to the size of the tube. The hematocrit containing the blood is then placed in the container through the perforated stopper at the top, pushed firmly against the stopper in the bottom, and the container placed in the metal tube of the centrifuge. After centrifugation the length of the column of total blood and of corpuscles is measured with an accurate scale and the percentage of corpuscles calculated. The variation in values of duplicate samples using these tubes is generally within 1 per cent of the total value. Values obtained with the hematocrit are always lower, generally one or two points, than values obtained from ordinary graduated centrifuge tubes under the same conditions.

Of thirty-one experiments in all, nineteen were low pressure experiments. In three experiments the pressure was reduced to to 350 mm., in eleven to 400, and in five to 450 mm. Twelve were control experiments at normal pressure. One attempt was made to bring the pressure down to 300 mm., but the rabbit died after $\frac{1}{2}$ hour. In twenty-one experiments the animals were exposed to low pressure 3 hours, in the rest the time varied from $1\frac{1}{2}$ to $5\frac{1}{2}$ hours.

TABLE I.

Red Corpuscles, Hematocrit Value, Corpuscle Volume, and Phosphoric Acid Content—Total, Inorganic, Organic, and Lipoid—in Corpuscles and Plasma per 100 Cc.

								Phosphoric acid in corpuscles.				Phosphoric acid in plasma.			
1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
No. of experiment.	Weight.	Pressure.	Time.	Series.	No. of red corpuscles.	Hematocrit value.	Corpuscle volume.	Total.	Inorganic.	Organic.	Lipoid	Total.	Inorganic	Organic.	Lipoid.
	gm.	*mm.*	*hrs.*		*millions*		μ^3	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*
1	4,230	400	4	A	6.09	44.8	73.6	289	20	231	38	21.4	10.3	0.4	10.4
				B	6.52	40.7	62.4	324	13	268	37	16.5	8.3	0	7.5
2	4,425	400	$3\frac{1}{3}$	A	6.03	34.2	56.7	283	19	205	68	23.5	8.3	1.9	16.5
				B	6.33	37.3	58.9	283	14	200	60	19.0			
3	3,500	350	2	A	5.32	33.1	62.2	400	9	309	70	21.0	11.3		9.4
				B	5.91	30.0	50.8	442	7	339	80	18.0	9.0		8.7
4	4,430	400	$2\frac{1}{3}$	A	6.96	40.5	58.2	338	14	266	63	26.0	11.9		13.9
				B	6.42	39.3	61.2	359	13	277	68	21.1	10.0		11.5
5	3,500	350	$1\frac{1}{2}$	A	6.23	42.2	67.7	350	15	256	60	24.1	10.2	3.0	11.5
				B	6.09	40.0	65.7	355	14	267	52	20.0	8.5	2.5	8.6
6	3,100	350	2	A	7.19	39.7	55.2	414				24.0			
				B	6.20	29.0	46.8	470				17.3			
7	4,425	400	$5\frac{1}{2}$	A	5.67	34.0	60.0	333		240	82	34.0	9.7	1.6	25.0
				B	5.29	35.2	66.5	351		252	82	36.0	10.8	1.1	25.0
8	4,425	400	$2\frac{1}{3}$	A	6.62	41.2	62.2	342		293	65	27.0	11.1	6.2	8.7
				B	6.45	36.4	56.4	393		310	68	20.0	10.3	3.8	5.2
9	3,000	400	$1\frac{1}{2}$	A	5.55	34.9	62.9	350	9.0			19.3	10.3	4.0	5.0
				B	5.90	37.7	63.9	350	8.3		62	15.0	7.8	2.2	5.0
10	3,100	400	2	A	6.54	37.6	57.5	358				21.1			
				B	6.91	37.1	53.7	342				16.1			
11	4,400	450	3	A	6.21	35.1	56.5	350			66	20.0	9.5		11.5
				B	5.36	33.2	62.0	350			64	14.0	4.8		9.7

TABLE I—*Continued.*

								Phosphoric acid in corpuscles.				Phosphoric acid in plasma.			
1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
No. of experiment.	Weight.	Pressure.	Time.	Series.	No. of red corpuscles.	Hematocrit value.	Corpuscle volume.	Total.	Inorganic.	Organic.	Lipoid.	Total.	Inorganic.	Organic.	Lipoid.
	gm.	*mm.*	*hrs.*		*millions*		μ^3	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*
12	3,375	450	3	A	6.35	32.5	51.1	350			57	32.0	10.0		20.0
				B	5.43	30.6	56.4	350			57	23.0	6.6		17.0
13	4,340	450	3	A	4.68	30.0	64.1	425			70	29.5	11.4		17.5
				B	4.34	28.6	65.9	395			70	22.0	7.5		15.0
14	3,360	450	3	A	5.49	35.3	64.3	343			66	30.0	14.0		12.0
				B	5.39	30.5	56.6	365			72	24.3	11.0		10.0
15	3,300	450	3	A	6.27	32.8	52.3	455	405*		69	23.0	10.0		11.5
				B	6.44	31.3	48.6	460	400*		68	24.0	11.8		10.0
16	4,100	400	3	A	4.92	30.2	61.4	401	300*		98	27.4	10.5		17.0
				B	4.34	28.3	65.2	450	360*		98	24.2	6.8		15.0
17	3,580	400	3	A	5.27	36.3	68.9	330	10	270		17.5	6.8		11.3
				B	5.22	34.7	60.7	352	10			16.3	6.0		10.4
18	3,640	400	3	A	4.84	29.5	61.0	345	28	280	67	18.4	6.7		11.1
				B	4.47	26.2	58.6	340	25	290	60	14.0	4.5		9.1
19	3,950	400	3	A	4.91	30.4	61.9	350		270	70	22.0	9.4		11.5
				B	5.14	29.4	57.2	378		280	75	14.0	5.0		9.5
20	4,100	760	3	A	5.28	29.8	56.4	385	18.9	265	85	24.2	11.5	1.0	10.1
	Same as No. 16.			B	5.86	30.0	51.2	390	20.6	264	92	19.7	7.5	2.5	12.0
21	3,580	760	3	A	5.54	36.6	66.0	325	26.0	230	72	21.7	6.7	2.3	11.1
	Same as No. 17.			B	5.88	33.3	56.6	320	26.0	224	75	21.0	7.5	1.5	10.1
22	3,070	760	3	A	5.46	32.2	59.0	320	6.0	219	63	26.2	12.2		10.7
				B	5.12	31.9	62.3	320	11.1	214	66	26.2	10.0		10.0

* Acid-soluble.

TABLE I—*Concluded.*

								Phosphoric acid in corpuscles.				Phosphoric acid in plasma.			
1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
No. of experiment.	Weight.	Pressure.	Time.	Series.	No. of red corpuscles	Hematocrit value.	Corpuscle volume.	Total.	Inorganic.	Organic.	Lipoid.	Total.	Inorganic.	Organic.	Lipoid.
	gm.	*mm.*	*hrs.*		*millions*		μ^3	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*
23	3,950	760	3	A	5.02	30.4	60.6	391	10.7	274	67	28.6	11.2	2.8	13.5
	Same as No. 19.			B	5.06	30.0	59.3	390	10.7	275	65	23.3	9.2	2.6	13.5
24	2,780	760	3	A	5.07	27.5	54.2	336	20.0	236	85	22.5	9.1	1.9	11.0
				B	3.90	24.7	63.3	330	22.0	233	90	16.0	5.0	2.5	8.5
25	3,040	760	3	A	3.34	21.7	65.0	470	20.0	315	110	27.4	10.5	5.5	10.0
				B	3.86	26.0	67.4	490	16.0	339	120	30.0	12.0	2.0	14.3
26	3,830	760	3	A	5.00	31.9	63.8	500	8.3	292	65	27.4	7.5	2.8	13.0
				B	4.90	30.5	62.2	445	10.0	310	90	27.4	7.5	2.5	13.0
27	4,260	760	3	A	5.47	34.9	63.8	330	±	255	70	21.7	10.0	3.4	8.9
				B	5.71	37.9	66.4	330	±	255	75	17.0	5.9	2.2	8.8
28	3,460	760	3	A	5.75	36.4	63.3	280	8.0	232	50	21.5	10.0	2.5	10.0
				B	5.51	34.6	62.8	317	11.5	264	48	19.0	6.4	3.0	9.4
29	3,150	760	3	A	5.81	38.0	65.4	300	14.0	251	53	26.2	12.0	4.0	10.2
				B	5.51	33.3	60.4	325	15.6	254	53	24.2	11.0	3.0	10.2
30	3,440	760	3	A	4.97	33.3	67.2	250	8.0	200	53	19.5	5.0	5.5	9.5
				B	4.46	30.8	69.1	250	8.8	202	53	18.5	4.0	5.0	9.7
31	2,880	760	3	A	6.34	35.0	55.2	360	19.0	273	65		6.2	2.8	12.2
				B	6.03	32.9	54.6	350	16.0	259	63		6.0	3.8	12.2

In Table I the results of the experiments are recorded respectively for red count, hematocrit value, corpuscle volume (obtained by dividing the volume of red corpuscles in 1 c.mm. of blood as expressed by the hematocrit value in one-thousand millionth of a c.mm., or as we prefer to express it in μ^3 as unit, by the number of red cells in 1 c.mm. of blood), and phosphoric acid content in blood

corpuscles and plasma, both the directly determined total amount and its fractions—inorganic, organic, and lipoid. In the table, A designates the series at the beginning and B the series at the end of each experiment.

The complete analysis of the blood of twenty-eight normal rabbits for phosphoric acid constituents as given in Table I makes it possible to establish tentative average values and variations for these constituents in normal rabbits.

Blood Count.—Thirty samples (including extra samples at different times from Rabbits 16, 17, and 19 and excluding No. 25 which was definitely anemic). Average 5.66; highest 7.19; lowest 4.68 millions.

Hematocrit Value.—On the same animals. Average 34.67; highest 48; lowest 27.5 per cent.

Plasma Phosphate.—Inorganic Phosphate.—Twenty-six animals. Average 9.76; highest 14; lowest 5.0 mg. per 100 cc.

Lipoid Phosphate.—Twenty-five animals. Average 11.46; highest 25; lowest 5.0 mg.

Corpuscle Phosphate.—Inorganic Phosphate.—Sixteen animals. Average 14.2; highest 28; lowest less than 3.0 mg. per 100 cc. of corpuscles.

Organic Phosphate.—Nineteen animals. Average 257; highest 315 (in the anemic rabbit); lowest 200 mg. per 100 cc.

Lipoid Phosphate.—Average for 27 animals 68.4; highest 110; lowest 38 mg. per 100 cc.

Since for our present study the absolute figures are of minor importance, the interest centering around the changes taking place in the blood from the exposure to the low pressure, we have in Table II calculated the percentage changes of the number of erythrocytes, corpuscle volume, corpuscle phosphates, and plasma phosphates, total, inorganic, and lipoid. The experiments are arranged according to pressure and time of exposure.

It is regrettable that our experimental methods involved a loss of 5 to 6 per cent of the blood of the rabbit at the commencement of each experiment. It is well known that large hemorrhages themselves may stimulate the blood-forming organs. *A priori* it is impossible to tell to what extent the hemorrhage might have interfered with the effect of the internment in the low pressure chamber. On the other side the objection may be raised that the awkward position of the animal during the experiment in some way may affect the composition of the blood. As already mentioned real struggling of the animal very seldom

TABLE II.

Changes in the Number of Red Corpuscles, Corpuscle Volume, Total Phosphorus of Red Cells, and Total, Inorganic, and Lipoid Phosphorus in Plasma.

No. of experiment.	Pressure.	Time.	No. of red cells.	Corpuscle volume.	Phosphorus.			
					Total in corpuscles.	Total in plasma.	Inorganic in plasma.	Lipoid in plasma.
	mm.	*hrs.*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
"Ideal" controls.								
22	760	2½	−6.2	+4.2	0	0	−18.0	−6.5
26	760	3	−2.0	−2.5	−11.0	0	0	0
27	760	3	+4.4	+4.1	0	−21.7	−41.0	−1.1
28	760	3	−4.2	−0.8	+4.5	−11.6	−36.0	−6.0
29	760	3	−5.2	−7.6	+8.5	−7.6	−8.3	0
30	760	3	−10.3	+2.8	−3.8	−5.1	−20.0	+2.1
31	760	3	−4.9	−1.1	−5.1	+1.0	−3.2	0
Controls, second exposure.								
20	760	3	+11.0	−9.2	+1.3	−18.6	−34.8	+18.8
21	760	3	+6.1	−15.2	−1.5	−3.2	+11.9	−9.0
22	760	3	+0.8	−2.1	−0.2	−18.5	−17.9	0
Control.								
25*	760	3	+15.6	+3.7	+4.3	+9.5	+14.3	+43.0
Control.								
24†	760	3	(−22.9)	(+16.8)	−1.8	−28.9	−45.1	−22.3
Low pressure experiments.								
5	350	1½	−2.2	−2.9	+1.4	−17.0	−16.7	−25.2
3	350	2	+11.1	−18.3	+10.5	−14.3	−20.4	−7.4
6	350	2	−13.8	−15.2	+13.5	−27.9		
7	400	1½	+6.3	+1.6	0	−22.3	−24.3	0
10	400	2	+5.7	−6.6	−4.5	−27.9		
4	400	2⅓	−7.8	+5.2	+6.2	−18.8	−16.0	−17.3
8	400	2⅓	−2.6	−9.3	+14.9	−25.9	+7.2	−3.8
16	400	3	−11.8	+6.2	+12.2	−11.7	−35.2	−11.8
17	400	3	+8.5	−11.9	+6.7	−6.9	−11.8	−8.0
18	400	3	−7.6	−3.9	−1.5	−23.9	−32.8	−18.0
19	400	3	+4.7	−7.6	+8.0	−36.4	−46.8	−17.3
2	400	3⅓	+5.0	+3.9	0	−19.1		
1	400	4	+7.1	−15.2	+12.1	−23.0	−19.4	−27.9
7	400	5½	−6.7	+10.8	+5.4	+5.9	+11.3	0
11	450	3	−13.7	+9.7	0	−30.0	−49.5	−15.7
12	450	3	−14.5	+10.4	0	−28.1	−34.0	−15.0
13	450	3	−7.3	+2.8	−7.0	−25.4	−34.2	−14.2
14	450	3	−1.8	−12.0	+7.0	−19.0	−21.4	−16.7
15	450	3	+2.7	−7.0	+1.1	+4.3	+18.0	−13.1

* Anemic; red corpuscles 3.34 millions.

† Very small rabbit, 2.78 kilos.

took place. By adding a great number of control experiments, in which the rabbits were treated in exactly the same manner as in the low pressure experiments, we believe that we have overcome the above named difficulties. As will be seen some changes occurred to about the same degree in both the main series of experiments but it would be difficult to understand why other changes were met with only in the low pressure series, if it were not that these changes are attributable only to the new environmental factors of the low pressure apparatus.

Of the twelve controls the first seven (in Table II) were in all respects ideal ones. In the next three the rabbits had 3 weeks previously been used for low pressure experiments and presumably because of this earlier "sensitizing" these experiments present differences from the other controls. In the next experiment the rabbit was anemic at the beginning and consequently may be supposed to have reacted more strongly to the hemorrhage than the others; while in the last of the controls the rabbit was unusually small.

Turning our attention to the percentage changes in the ideal controls we recognize that they are, with sole exception of the red count and inorganic phosphates, small and in most cases within the limits of experimental error. The decrease of the number of red corpuscles varies with exception of only No. 26 between 2.0 and 10.3 per cent, with an average of 5.5 per cent. When we calculate the blood volume from the body weight and compare the amount of drawn blood with this hypothetical blood volume, we arrive at approximately the same figure. It thus appears as if the decrease of red count bears a relation to the amount of hemorrhage, which seems to indicate that no dormant supply of erythrocytes was drawn upon.

The changes in the corpuscle volume vary between +4.2 and −7.6 per cent and as the experimental error of the corpuscle volume equals the superposed errors of two different estimations, we are inclined to the view that no change of the corpuscle volume took place in the ideal controls.

The total phosphorus content of the packed corpuscles varies in the seven experiments between +8.5 and −11.0 per cent. As the figures in five of the cases are well within experimental error, it is doubtful whether any significance can be attached to the

two somewhat larger deviations. Moreover, the problem, that for our present inquiry would be more important than the phosphorus content of the mass of mixed corpuscles, namely the average phosphorus content of an individual red cell, is difficult to approach because of the trebled experimental error. We have, however, satisfied ourselves as to this matter, but we have failed to discover anything that would point in the direction of a change of the phosphorus content of the single erythrocyte. The deviations were equally distributed around the average.

Regarding the change of phosphorus content of the plasma we notice a decided difference between the two main fractions, inorganic and lipoid phosphorus. While the change of the inorganic phosphates is, in all cases except one, strongly negative the lipoid fraction remains remarkably constant, the total phosphates occupying an intermediate position. The average decrease of inorganic phosphates is 18.1 per cent, of lipoids 1.6 per cent. It seems possible, therefore, that the lipoids were little if at all affected by the restoration of the blood to its normal volume.

Postponing the discussion of the remaining control experiments at normal pressure till later, we pass over to a consideration of the low pressure experiments. The number of red corpuscles (Table II) does not allow us to draw any sweeping conclusions, as the percentage changes vary between +11.1 and −14.5. Since the positive deviations occur mostly at the lower pressures, although the amount of blood drawn was in these cases somewhat larger, it is, however, probable that increase of red cells, notwithstanding the loss of blood, was due to the lowered atmospheric pressure. If 5 per cent of the negative changes is considered as due to the loss of blood we find that in the majority of cases at 350 and 400 mm. an increase of erythrocytes had taken place. But even the negative changes are noteworthy, as in high altitude researches it has often been found that a decreasing effect on the number of erythrocytes may precede the increasing one, making it probable that lability of the number of red corpuscles is a typical feature. On the other hand, although naturally a stimulation of the erythropoietic organs most frequently is accompanied by an increase of circulating red corpuscles, an absence of this latter phenomenon does not seem to exclude a

greater activity of the bone marrow, as it is possible that the newly formed red cells may linger there for some time before they enter the circulation.

The change of the corpuscle volume varies between +10.8 and −18.3 per cent. The maximal negative changes, which undoubtedly exceed the experimental error, are found after exposures to very low pressure. The largest positive change is reached after an exceptionally long exposure ($5\frac{1}{2}$ hours) to 400 mm. We are therefore inclined to the view that the present study confirms the results reported by one of us (12), that low pressure tends to alter the size of the erythrocytes. Whether these changes bear any relation to changes in the hydrogen ion concentration of the blood, as suggested, we are not in a position to answer, although the determination of the dissociation curve of the oxyhemoglobin in some of the experiments apparently pointed in this direction.

While very little weight can be laid upon averages, when the number of cases is small, it occurred to us that it might be of some interest to calculate the average change of corpuscle volume at different pressures.

Pressure. *mm.*	Corpuscle volume. *per cent*
350	−12.1
400	−3.8
450	+0.8
760	−0.8

The changes in the total phosphoric acid content of the corpuscles vary between +14.9 and −7.0 per cent. We further recognize that most of the changes are to the positive side and that the maximal ones of these occur at the lowest pressures. We would attach more importance to the prevalence of positive changes and believe that they indicate an increase of the phosphorus content of the corpuscles at high altitudes if we had succeeded in confirming them by calculating the phosphorus content of individual red cells. The maximal positive change is 19.1 per cent, and the maximal negative change is 11.1 per cent; the average change is +1.0 per cent. In the first seven control experiments the corresponding figures are 12.4, 13.2, and +1.2. In only two experiments is the positive change of the individual

corpuscle content of phosphorus safely beyond the limits of experimental error. In one of the cases the duration of the experiment was twice the average duration and it is possible that if we had extended the duration in other experiments still longer we would have been able to discover an increase of the individual corpuscle H_3PO_4 content proportional with the length of time.

The estimation of the lipoid phosphorus content in individual red cells revealed that only in three cases was the change beyond the limits of the experimental error. All these changes were negative (15.8, 17.0, and 13.9) and, as will be seen, coincided with maximal negative changes of the plasma lipoids.

If we turn our attention to the plasma phosphates in the low pressure experiments there seems to be no doubt that a decrease has taken place and this applies equally well to the inorganic and lipoid phosphorus and to the total phosphorus. The average decrease of the inorganic phosphorus is at 350 mm. 18.6, at 400 mm. 20.2, and at 450 mm. 24.2 per cent, with a total average of 21.3 per cent. The average decrease of the inorganic plasma phosphorus in the seven controls is 18.16 per cent. It therefore appears as if the decrease of the inorganic phosphates in plasma were attributable to the bleeding alone.

The decrease in the low pressure experiments for lipoid phosphorus is at 350 mm. 16.3, at 400 mm. 11.6, and at 450 mm. 14.9 per cent, with a total average of 13.2 per cent. When compared with the constancy of the lipoid phosphorus in the seven control experiments (average 1.6 per cent) these figures lead us to the conclusion that the decrease of lipoid phosphorus is caused by the low pressure treatment. The fact that no change of the lipoid phosphorus occurred in the two experiments of the shortest and longest duration seems to indicate that the greatest decrease takes place between $1\frac{1}{2}$ and $5\frac{1}{2}$ hours.

It now remains to seek an explanation for the decrease of plasma lipoids, which seems to be confined to the low pressure experiments. Effects of digestion and work are excluded as the animals were fasting and were tied down. Complete muscular inactivity could, naturally, not be obtained, but as to possible effects of struggling it has been pointed out that the control animals were more restless than the others. It seems certain that an enrichment of the circulating red cells with lipoids, that would

result in an impoverishment of the plasma in these substances, did not occur, as it was found that the greatest decreases of the plasma lipoids were accompanied by proportional decreases of the corpuscle lipoids. Thus the three maximal changes of plasma lipoids to the negative side (27.9, 25.2, and 18.2 per cent) corresponded to the three greatest changes in the same direction of the individual corpuscle lipoids (17.2, 15.8, and 13.9 per cent). Instead of compensating for the loss of lipoids from plasma by storing up lipoids, it is probable that even the circulating red cells may be compelled to contribute some of their own lipoids. But where are all these phospholipoids that probably consist mainly of lecithin deposited? While the inorganic phosphates are easily eliminated from the body through the kidneys (although such an explanation of their decrease in the plasma is doubtful in the light of the fact that a retention of phosphates is a constant phenomenon in high altitudes) such an output of the lipoids is out of the question. A dilution of the blood would likewise be contrary to the repeated observation that the blood plasma tends to become concentrated at high elevations. The most probable alternative is that the lipoids have been taken up by some part of the body itself.

A number of investigators have tried to elucidate the effect of small doses of lecithin on metabolism. In all the cases where the experiments have been well controlled (13) a retention of nitrogen and phosphorus and partly also of sulfur has been found, indicating a formation of new body protein. Other observers (14) have paid attention to the effect of injections of lecithin on the number of red corpuscles and have been able to find a rather constant increase. That lecithin probably plays some rôle in the function of the erythropoietic organs is evident from the fact that the bone marrow contains comparatively large amounts of lecithin and that this percentage is higher in the red marrow of young animals (15). Whether the hypothesis that the lecithin acts as a bridge to the nucleins, these proteins obtaining their phosphorus supply from the lipoids, will prove correct is impossible to tell; however, if that is the case, the beneficial effect of lecithin injections on the number of red cells would be satisfactorily explained. The lecithin requirements for the formation of new erythrocytes would, furthermore, throw light on the changes of the plasma

lipoids at low pressure. A part of these would be taken up by the blood-forming organs. If future investigations prove that this is true, the almost immediate reaction of the bone marrow to lowered pressure in the direction of a stimulation, which we are now able to offer only as a hypothesis, will at the same time obtain a firmer foundation.

As already mentioned, in the control experiments, Nos. 20, 21, and 23, the animals had 3 weeks previously been exposed to low pressure. One of us has directed attention to the sensitizing effect of sojourns in high altitudes (16) to very small changes in atmospheric pressure. It seems possible that the difference the three above named rabbits presented from the other controls can be explained in the same way; namely, that these animals reacted more strongly to the hemorrhage than the others. In spite of the loss of blood of all the rabbits, their blood contained a higher number of red cells after than before the experiment. The effect on the corpuscle volume was also more marked and in a negative direction. The effect on the plasma phosphates varied to a great extent; *e.g.*, in one animal the lipoid phosphorus increased by nearly 19 per cent. It deserves mentioning that the initial number of red corpuscles in the second experiment on the same animals exceeded the initial counting in the first series by 2.2 to 7.3 per cent. A greater number of comparative red counts would, however, be necessary to ascertain whether the stimulation of the bone marrow, that we have indicated, lasted for such a long period of time.

We have found that in normal rabbits a loss of about 5 per cent of the total blood volume does not seem to stimulate the bone marrow to greater activity during the first hours after the hemorrhage. It would have been interesting to study the effect of the bleeding of animals that at the onset of the experiment were anemic and of the effect of a somewhat proportionally larger hemorrhage. Only one experiment of each of these kinds is available in our series; namely, Experiments 25 (anemic rabbit) and 26 (smallest rabbit in all the series, therefore a proportionally greater loss of blood). In the former animal all the recorded blood changes are to the positive side. Expecially the increase of the number of red corpuscles and of the lipoid phosphorus of the plasma is noteworthy, but it would be futile in a single case

to speculate as to the coincidence of these two changes. In the latter experiment we are the more prevented from drawing any conclusions as the incongruence of the blood count and hematocrit value makes us suspect some error in the sampling of the blood for the counting. In such a case the figures for the red count and the corpuscle volume would be incorrect. However, the great decrease of all the main phosphorus fractions would then remain unexplained. It is possible that our estimations are correct and that the low body weight of the rabbit and therefore resulting disproportionally large loss of blood are responsible for the changes in question.

SUMMARY.

1. A self-regulating and inexpensive apparatus for animal experiments at low pressure is briefly described.

2. A convenient and accurate method for obtaining the hematocrit value by use of a high power centrifuge is given.

3. The variations of the content of phosphoric acid and its fractions—inorganic, organic, and lipoid—of the corpuscles and plasma of normal rabbits are discussed.

4. From comparisons of the percentage changes in the number of erythrocytes, of the corpuscle volume, and of the phosphorus content of corpuscles and plasma of rabbits that have been subjected to a small hemorrhage and then exposed for a short period of time on the one hand to normal pressure and on the other hand to pressures from 350 to 450 mm. it is concluded that:

(*a*) While at normal pressure the change of the number of red cells corresponds to the loss of blood from the experimental hemorrhage, the greater alteration of the number of erythrocytes at lower pressures is due to the low pressure.

(*b*) At very low pressures a decrease in the size of the red corpuscles occurs.

(*c*) The phosphorus content of the red cells does not undergo any marked change. In a few cases the decrease of the lipoid phosphorus is probably beyond experimental error.

(*d*) The change in the inorganic phosphorus of the plasma is negative, and of the same amplitude in control and low pressure series.

(*e*) The most characteristic feature of the low pressure experiments, when compared with the controls, is a constant decrease in the former of the lipoid phosphorus of the plasma.

5. It is suggested that the decrease of the lipoid phosphorus of the plasma at low pressure indicates an enrichment of the erythropoietic organs with lipoid material and that this may be the first phase of a stimulation of these organs resulting from a lowering of the atmospheric pressure.

BIBLIOGRAPHY.

1. Gaule, J., *Arch. ges. Physiol.*, 1902, lxxxix, 119.
2. von Schroetter, H., and Zuntz, N., *Arch. Physiol.*, 1902, xcii, 479.
3. Kuhn, E., *Münch. med. Woch.*, 1907, liv, 1713.
4. Gregg, H. W., Lutz, B. R., and Schneider, C. E., *Am. J. Physiol.*, 1919–20, l, 216.
5. Abderhalden, E., *Med. Klin.*, 1905, i, 210.
6. Sundstroem, E. S., *Univ. California Publ. Physiol.*, 1919, v, 87.
7. Haldane, J. S., Kellas, A. M., and Kennaway, E. L., *J. Physiol.*, 1919–20, liii, 181.
8. Sundstroem, E. S., *Univ. California Pub. Physiol.*, 1919, v, 121.
9. Masing, E., *Arch. Exp. Path. u. Pharmakol.*, 1911, lxvi, 71.
10. Bloor, W. R., *J. Biol. Chem.*, 1920, xliv, 171.
11. Bloor, W. R., *J. Biol. Chem.*, 1918, xxxvi, 33.
12. Sundstroem, E. S., *Univ. California Pub. Physiol.*, 1919, v, 149.
13. Slowtzoff, B., *Beitr. chem. Physiol. u. Path.*, 1906, viii, 370. Yoshimoto, S., *Z. physiol. Chem.*, 1910, lxiv, 464.
14. Danilewsky, B., *Arch. ges. Physiol.*, 1895, lxi, 264. Serono, C., *Arch. Ital. biol.*, 1897, xxvii, 349. Stassano, H., and Bilon, F., *Compt. rend Acad.*, 1902, cxxxiv, 318.
15. Otolski, S. W., *Biochem. Z.*, 1907, iv, 124. Glikin, W., *Biochem. Z.*, 1907, iv, 235. Bolle, A., *Biochem. Z.*, 1910, xxiv, 179.
16. Sundstroem, E. S., *Univ. California Pub. Physiol.*, 1919, v, 133.

BLOOD PHOSPHATES IN THE LIPEMIA PRODUCED BY ACUTE EXPERIMENTAL ANEMIA IN RABBITS.

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(Received for publication, September 27, 1920.)

It is probable that lecithin or a similar phospholipoid is an intermediate step in the utilization of fat in animals, since, aside from the close chemical relationship, it has been shown on the one hand that accumulation of fat in the liver in various conditions is probably followed by a transformation into phospholipoid in the organ (1), that during fat absorption lipoid phosphorus increases in the blood (2), and that in diabetic lipemia (3) the high values for fat are always accompanied by high lecithin values. On the other hand phospholipoid has been found to decrease in blood plasma during the formation of fat in milk secretion (4). In general it has been found that wherever there are variations in the fat of the blood they are accompanied by corresponding changes in the phospholipoid (3, 5).

In continuing the study of the relation of the lipoid phosphorus to fat metabolism, it seemed desirable to include a study of other phosphoric acid compounds in the blood so as to discover what relation, if any, existed between them and the lipoid phosphorus and what form of phosphoric acid combination participated in the formation of phospholipoid from fat. For the purpose of the study, the lipemia produced by acute experimental anemia described by Boggs and Morris (6) was chosen as a beginning, since this type of lipemia is easily produced in rabbits, reaches remarkable heights, and has been recently the subject of a study with regard to the variations in the blood lipoids during the course of the lipemia by Horiuchi (7). Moreover, as the result of some experimental work in this laboratory it seems likely that the condition is not easily produced in dogs, which offers a means

of differentiating between changes referable to the lipemia and those due to regeneration of blood corpuscles.

In the lipemia which follows repeated large bleedings in rabbits, Boggs and Morris found total fat values up to 4.5 per cent with fatty acids of high iodine value, and increase of lecithin, but no cholesterol. Horiuchi found similar high values for fat (total fatty acids) in plasma up to twenty-five times the normal value (4.4 per cent), increases of lecithin up to seven, and cholesterol up to eight times the normal values. The changes in the corpuscles were relatively much less, at no time rising much over twice the normal values for any of these constituents. Horiuchi found that the lipemia occurred whether the animal was on a high or low fat diet but was produced more readily and lasted longer on the high fat. In the work to be reported below a study has been made of the same condition from the point of view of the phosphoric acid compounds of the blood, both with the idea of inquiring into the relation of these compounds to the metabolism of fat and also of following the changes in these substances during the bleeding, in their relation to blood regeneration. It was desirable to find out for example which group of these compounds took part in the formation or resulted from the decomposition of lecithin and whether the organic phosphorus present in small amounts in plasma and in relatively very large amounts in corpuscles bore any relation to the loss of the nucleus in red cells during their formation.

A full discussion of the various phosphoric acid compounds of the blood was given in an earlier paper (8) but a short summary of the available knowledge on the subject will not be out of place here. In blood plasma, phosphoric acid is present as inorganic phosphate, as phospholipoid, and in small amounts as an unknown form soluble in acids; *i.e.*, organic phosphorus. In most samples of blood plasma the sum of the three—inorganic, lipoid, and organic phosphorus—is equal to the total phosphate within the limit of error of the determinations. In occasional samples another form of phosphoric acid combination is found which is insoluble in either acid ammonium sulfate or alcohol-ether, and which is probably nucleoprotein.

In blood corpuscles the same groups of compounds are found and again the sum of inorganic, lipoid, and organic is found to be

equal to the total phosphate in practically all normal samples examined. Nucleoprotein is therefore not normally present. In amounts these forms are generally much larger than in the plasma, the organic phosphorus being relatively very large and forming the main phosphoric acid constituent of the corpuscles. Corpuscle values tend to be much more constant than those of plasma and the determination of phosphates in whole blood is therefore not to be recommended when enough blood is available that plasma and corpuscles may be examined separately.

The methods used for the determination of the various phosphoric acid compounds have already been described in full (9), but certain modifications have been found desirable and are given below. For reasons noted above, plasma and corpuscles were analyzed separately, the separation being made in all cases by centrifugation at about 3,800 revolutions per minute for 10 minutes.

Total Phosphate.—1 cc. of plasma and 0.048 cc. of corpuscles were found to be the most suitable amounts for the determination in rabbit blood, in which the phosphoric acid content of the plasma is lower and that of the corpuscles higher than in human blood. In the digestion the second stage (boiling of the concentrated acid for about 8 minutes) has been omitted since it has been found that nothing is gained by the continued heating while there is a real danger of loss of phosphoric acid either mechanically or by vaporization. Digestion is carried only to the stage where the water is completely driven off, then if the mixture is colorless it is cooled, sugar added, and then the mixture is treated further as directed. If not colorless after the heatings, 1 or more drops of the reagent are added as required and the mixture is again heated. After the final heating the sulfuric acid solution should be clear and colorless. In occasional samples a yellowish tint persists in the hot solution, disappearing on cooling, which is impossible to remove, but since it does not appear to affect the accuracy of the results it has been neglected.

Standards Used.—For plasma 5 cc. of a standard containing 0.84 mg. of H_3PO_4 in 100 cc. of solution; for corpuscles 5 cc. of a standard containing 0.6 mg. of H_3PO_4 per 100 cc. (see note on standards below).

Lipoid Phosphate.—In normal rabbit plasma, alcohol-ether extract corresponding to 0.9 cc. of plasma was required. To obtain this amount 3 cc. of plasma were made up in 50 cc. of alcohol-ether as directed and 15 cc. used for the determination. As a standard 5 cc. of a solution containing 0.36 mg. of H_3PO_4 per 100 cc. were used. In many cases a weaker standard (0.24 mg.) is desirable.

In normal corpuscles the sample taken corresponded to 0.18 cc. of corpuscles in 10 cc. of alcohol-ether extract (see note below on corpuscle solutions). For a standard, 0.6 mg. of H_3PO_4 in 100 cc. was used.

Acid-Soluble Phosphate.—Total Acid-Soluble Phosphate.—For plasma 5 cc. of extract corresponding to 0.6 cc. of plasma have been found most suitable with a standard of 5 cc. of solution containing 0.36 mg. of H_3PO_4 per 100 cc. (properly adjusted as to salt content, etc.). In the digestion with H_2SO_4-HNO_3 the sugar treatment has been found unnecessary and has been omitted.

For corpuscles 2 cc. of extract corresponding to 0.0384 cc. of corpuscles have been used with a standard containing 0.36 mg. of H_3PO_4 per 100 cc. of solution.

Inorganic Phosphate.—For plasma 2 cc. of extract corresponding to 0.24 cc. of plasma were used with 2 cc. of a standard containing 1.2 mg. of H_3PO_4 per 100 cc.

Corpuscles.—The determination of inorganic phosphate in rabbit corpuscles has been found somewhat unsatisfactory. Values are widely variable and in one case (Rabbit B) protein was not completely removed by the acid ammonium sulfate. There is also uncertainty as to what extent the organic fraction is decomposed during the treatment, giving incorrectly higher values for inorganic phosphate. However, results are given of the determinations, which are at least of comparative value. Ordinarily 2 cc. of the extract, corresponding to 0.096 cc. of corpuscles, were used with 2 cc. of a standard containing 1.2 mg. of H_3PO_4 in 100 cc.

Standards.—Correction of the nephelometric readings has been found unsatisfactory and, in order to avoid the necessity of correction, a variety of standard solutions has been used. By selecting a standard solution not more than 30 per cent stronger or weaker than the solution to be measured the need for correc-

tion is avoided. The standards are conveniently made up in 100 cc. portions containing phosphate and salts as below. For total and lipoid phosphate in plasma and corpuscles, standards containing 0.84, 0.6, 0.36 mg. of H_3PO_4 in 100 cc. were used. In making these standards the phosphate solution is measured into a 100 cc. graduated flask, alkali equal to four times the amount used in neutralizing a single digestion mixture, ordinarily 18 cc., is added, then 4 drops of 0.3 per cent phenolphthalein are added, after which the solution is titrated to neutrality with the 1:1 sulfuric acid. In order to prevent the growth of mould about 12 drops more of the acid are added, the solution is cooled, and made to the mark. Moulds grow slowly in the neutral solution and soon remove most of the phosphate from it. Addition of excess acid as above has been found to prevent their growth in all but an occasional instance. If mould is detected the solution must be thrown out and a new standard made.

For acid-soluble phosphate in plasma, standards containing 0.24 and 0.36 mg. of phosphoric acid per 100 cc. were used. In making these solutions the phosphate is measured into a 100 cc. graduated flask, 8 drops of the phenolphthalein solution, 20 cc. of the alkali, and 18 cc. of the acid ammonium sulfate are added, and the solution is titrated to neutrality. 12 more drops of acid are added and the solution is cooled and made up to the mark. Moulds do not grow in this solution.

For acid-soluble phosphate in corpuscles, standards containing 0.36 and 0.6 mg. of H_3PO_4 were found to cover the range of values found. In preparing these standards the phosphate is measured into the graduated flask, 8 drops of phenolphthalein, 8 cc. of acid ammonium sulfate, and 18 cc. of the alkali are added, the solution is neutralized, and 12 drops of extra acid are added as before. The solution is then cooled and made up to the mark.

For inorganic phosphate in both corpuscles and plasma the standard as recommended (9) was found suitable except that, since 5 cc. of the standard and test solutions gave suspensions too concentrated for accurate reading, 2 cc. of each were used.

For these determinations and for nephelometric work in general, exactly parallel conditions in composition of solutions, time of standing after addition of reagent, temperature, amount of shaking, etc., must be preserved. It is desirable to establish a

routine procedure which should be as closely followed as conditions will permit.

Sampling of Corpuscles.—In experiments which involve extensive bleeding, as in those described below, where the percentage of corpuscles falls to a low level it is necessary to practice some economy so as to get enough material to complete the various analyses. The following scheme has been found satisfactory for rabbit blood: 3 cc. of the separated corpuscles are measured into a 10 cc. graduated flask, the pipette is rinsed out, and the washings and warm water are added to fill the flask to the mark. The whole is well shaken. After standing for at least 10 minutes for hemolysis to take place (in the ordinary routine from $\frac{1}{2}$ to 1 hour is allowed) and after again shaking, 4 cc. of the mixture are measured into acid ammonium sulfate in a 25 cc. flask for determinations of inorganic and acid-soluble phosphate. 3 cc. are measured into alcohol-ether for determination of lipoid phosphate and 2 cc. into water in a 25 cc. flask of which, after filling to the mark, 2 cc. are used for total phosphate.

Direct determinations were made of total, lipoid, inorganic, and acid-soluble phosphate. Organic phosphate is found by subtracting inorganic from acid-soluble.

The rabbits used in these experiments were on their ordinary diet of alfalfa and rolled barley which is a diet low in fat although containing more than the low fat diet used by Horiuchi. Blood samples were taken from the marginal ear vein, approximately 10 per cent of the blood volume (calculated as 8 per cent of the body weight) being taken daily. If the animal is previously exercised until the general circulation is stimulated and then the ear massaged a free and rapid flow of blood is obtained, yielding the desired amount in a few minutes. Clotting was prevented by the use of 1 per cent of saturated sodium citrate. The bleeding was continued daily until a satisfactory lipemia was established, then less frequently and taking only enough blood to follow the conditions during recovery. (About 15 cc. of blood were required to furnish material for a complete analysis.) Results are given in Table I. Six animals in all were studied of which complete reports are given of two while the others giving similar and less striking results are reported more briefly.

TABLE I.

Blood Phosphates in the Lipemia of Acute Experimental Anemia.

Day.	Blood taken.	Hematocrit.	Red count.	Corpuscle volume.	Phosphates per 100 cc.								Remarks.
					Plasma.				Corpuscles.				
					Total.	Inorganic.	Lipoid.	Organic.	Total.	Inorganic.	Lipoid.	Organic.	
	Rabbit A. Weight 8¼ lbs.												
	cc.	*per cent*	*millions*	*c.mm.* $\times 10^{-8}$	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	
1	30	35.9	6.21	5.9	20.0	7.7	9.6	3.8	365	24	65	276	Plasma clear.
2	30	28.1	5.94	4.8		8.5	10.0	6.0	390	43	80	261	" "
3	30	21.5	5.23	4.2	28.5	11.0	11.5	3.0					" "
4	30	19.3	3.17	6.0	37.0	8.8	23.5	5.2	465	50	100	305	" moderately milky.
5	30	18.0	2.38	7.5	40.0	7.8	26.0	3.1	453				" "
6	30	16.9	1.68	10.0	45.0	10.0	26.0	4.0	562	62	140	332	Plasma milky.
7	30	16.5	2.07	8.0	47.0	11.0	30.5	5.0	532	88	156	287	" very milky.
8	15	16.1	1.87	8.6	50.0	17.6	30.0	2.4	560	95	155	280	" "
9	15	19.5	2.09	9.3	55.0	11.1	38.5	4.9	570	107	160	283	" "
10	15	20.0	2.53	8.0	48.0	9.4	37.5	2.1	555	83	162	297	Plasma milky.
11	15	21.0	2.93	7.2	48.0	11.0	33.3	3.4	555	50	113	301	" clear.
12	15	23.5	2.48	7.6	37.0	11.5	25.0	0.5	490	90	140	260	" "
13	15	23.6	3.04	7.8		9.0	17.2	5.0	550	50	130	340	" "
15	15	26.5	3.64	7.3	24.2	11.1	12.5	3.3	486	53	120	322	" "
16	15	30.2	3.25	9.3	33.5	15.5	14.3	4.5	465	57	106	283	" "
17	15	30.1	4.40	6 9	25.2	12.5	12.2	1.6	470	52	120	308	" "
27	15	34.4	5.30	6 5	23.0	12.5	8.0	1.5	475	57	95	303	
36	15	34.4	5.48	6.3	23.3	13.5	8.3	1.5	402	51	95	240	
57	15	38.4	6.84	5.6	18.0	8.6	7.3		345	41	65	234	
	Rabbit B. Weight 8½ lbs.												
1	30	32.4	5.17	6.3	21.7				375	28	83	287	Plasma clear.
2	30	23.3	3.57	6.5	20.3	9.7	8.0	1.3	365	29	80	281	" "
3	30	20.7	3.36	6.2	18.5	9.5	8.0	1.5	370	23	80	257	" "
4	30	18.4	3.19	5.8	23.0	12.0	10.9	±	430	14	109	316	Faint milkiness.
5	30	17.8	2.46	8.0	33.0	13.6	20.0		402	19	90	325	Plasmamoderately milky.

TABLE I—*Continued.*

Day.	Blood taken.	Hematocrit.	Red count.	Corpuscle volume.	Phosphates per 100 cc.								Remarks.
					Plasma.				Corpuscles.				
					Total.	Inorganic.	Lipoid.	Organic.	Total.	Inorganic.	Lipoid.	Organic.	
Rabbit B—*Concluded.*													
	cc.	*per cent*	*millions*	*c.mm.* $\times 10^{-8}$	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	
6	30	19.6	2.46	7.2	35.0	11.1	28.6	2.3	450	38	135	277	Plasma moderately milky.
7	35	17.3	1.71	10.0	40.0	10.0	29.0	±	422	21	121	284	Plasma milky.
8	30	17.5	2.32	7.5	50.0	11.1	37.5		470	32	145	288	" "
9	30	18.0	2.41	7.4	48.0	11.5	33.0	+	480	42	170	288	" "
10	15	16.1	2.20	7.4	57.0	6.0	43.0	4.8	475	43	155	317	" "
11	15	17.6	1.84	9.5	34.0	10.0	23.0	2.9	440	30	140	290	" "
12	15	20.2	2.37	8.5	45.0	13.0	32.0	±	430	57	150	233	" "
14	15	25.0	2.5	10.0	30.0	11.5	18.0	1.0	475	36	160	314	Faint milkiness.
15	15	24.1	2.52	9.6	30.0	12.0	19.0	0.8	430	20	170	260	" "
17	15	25.7	4.07	6.3	26.2	13.5	12.5		485	27	200	263	Very faint milkiness.
19	15	25.8	3.91	6.5	20.3	9.4	8.0	2.0	445	50	170	240	Plasma clear.
37	15	36.5	5.56	6.6	17.5	9.0	7.0	2.0	345	13	75	242	" "
Rabbit C. Weight 7½ lbs.													
1	20	36.0	5.40	6.6	14.0	7.5	7.5	1.3	315	8.5	63	262	Plasma clear.
4	25	22.5	3.96	10.6			10.0						Faint lipemia.
5	25	18.4	2.16	8.5	16.9	9.3	7.0	1.0	350	35.0	66	235	Plasma clear.
7	30	18.1	2.8	6.5	22.5	7.6	13.9	3.4	405	7.8	80	332	" faintly milky.
9	30	19.5	2.3	8.5	30.0	7.0	20.0	2.2	460	7.1	107	308	Plasma milky.
12	20	28.8	3.9	7.4	20.0	12.5	10.0	1.1	400	20.0	125	277	" faintly milky.
19	18	34.5	6.0	5.8	24.2	10.0	7.0	4.5	380	12.0	100	288	Plasma clear.
Rabbit E. Weight 8½ lbs.													
1	35	27.5	3.8	7.2	23.0	9.5	13.0	0.5	347	25.0	57	272	Plasma muddy but no lipemia.
3	35	25.3	2.74	7.2	21.0	6.6	12.5	3.1	325	9.3	55	261	" "
5	35	16.7	2.20	7.6	20.4	7.0	13.6	0.2	350	15.4	66	275	Plasma faintly milky.

TABLE I—*Concluded.*

Day.	Blood taken.	Hematocrit.	Red count.	Corpuscle volume.	Phosphates per 100 cc.								Remarks.
					Plasma.				Corpuscles.				
					Total.	Inorganic.	Lipoid.	Organic.	Total.	Inorganic.	Lipoid.	Organic.	
Rabbit E—*Concluded.*													
	cc.	*per cent*	*millions*	*c.mm.* $\times 10^{-}$	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	
7	35	15.3	2.36	6.5	25.2	8.7	15.7	2.4	430	16.0	97	304	Plasma milky.
10	18	22.2	3.09	7.2	23.5	11.1	10.0	4.9	550	17.2	140	353	" clear.
16	18	29.9	4.77	6.3	22.5	14.3	8.5	2.2	465	16.5	97	333	" "
Rabbit F. Weight 5 lbs.													
1	20	36.0			33.1	17.5	14.3	2.0	415	65	63	285	Plasma muddy but no lipemia.
3	18	24.5	3.76	6.6	30.0	13.6	15.5	0.4	415	56	76	276	" "
5	20	21.3	2.87	7.6	40.0	13.7	21.5	4.3	470	56	104	266	Plasma faintly milky.
7	20	20.4		7.8	36.0	12.0	19.0	4.0	485	66	120	284	" "
15	14	30.8	3.97	7.8	30.0	15.0	11.1	2.3	470	47	100	343	Plasma clear.
Rabbit G. Weight 8 lbs.													
1	25	30.0	5.5	5.4	31.7		15.7		301		54		Plasma clear.
6	25	23.0	3.06	7.5	35.1		15.9		355				" "
7	20	26.4	2.80	9.4			25.4		364		94		" milky.
8	30	17.7	2.52	7.0			18.0		493		103		" "
11	25	28.3	3.76	7.5	33.8		12.8		374		100		" clear.

DISCUSSION.

Lipoid Phosphorus.

The most characteristic change is in the lipoid phosphorus. In the plasma it is always very much increased (up to five times its normal value), while in the corpuscles the increase is less marked, in only three cases rising to over twice the normal value. In the plasma the highest values for lipoid phosphorus are generally found at the height of the lipemia and diminish as the

visible milkiness of the plasma diminishes. But even when the plasma has become clear, high values for lipoid phosphorus persist for some days. In the corpuscles the highest values for lipoid phosphorus are not generally reached until some time after the height of the visible milkiness, or even after the plasma has been clear for a time and the values do not become normal until some days after the plasma values have become normal.

The plasma begins to show milkiness at about the time the blood corpuscles are reduced to half their original number. This generally persists throughout the period of low corpuscle values, although in one case the plasma cleared after the first milkiness although the number of corpuscles was lower than when milkiness first appeared.

It was found in earlier work (2) that lipoid phosphorus increased in the blood corpuscles during absorption of fat in dogs, and it seemed likely that the corpuscles took an active part in fat metabolism by the transformation of fat into lecithin.[1] In human anemia (Bloor and MacPherson (5)) it was found that abnormalities in the blood lipoids were present only when the blood corpuscles were reduced to below half their normal number, the abnormalities being ascribed to inadequate functioning of the red blood cells, due to their reduced number. In the results given above, increase of fat and its metabolites is found associated with low corpuscle values. Whether the same explanation holds—that the disturbance in fat metabolism, which shows itself in the lipemia, is directly the result of the inability of the smaller number of corpuscles to perform this function in fat metabolism—or whether the lipemia is indirectly connected with the corpuscles in some other way, must be left for future work to decide. The results obtained in the work on the above type of lipemia indicate again, however, the probability of a participation of the corpuscles in the metabolism of fat.

[1] Further unreported work in this laboratory has not, however, entirely borne out these findings, since certain dogs have been found, in which the increase of lipoid phosphorus took place in the plasma and not in the corpuscles.

Inorganic Phosphorus.

Plasma.—In Experiment A, with strong lipemia, inorganic phosphate increased to twice the normal value and remained high throughout. In Experiment B, also with marked lipemia, the inorganic increased, but not to more than 50 per cent above normal values. In Experiment C, with mild lipemia, increases of 70 per cent were found while in Experiment E, phosphate first diminished and then increased above the original value. In Experiment F, with mild lipemia, the inorganic phosphate diminished. In this case, however, it was unusually high to start with.

It thus appears that in severe lipemia increases of inorganic phosphates are to be found, while in the mild lipemia a slight increase or a decrease may be present. In none of the experiments was a parallelism between inorganic and lipoid phosphate to be noted. In a study of fat formation during milk production, Meigs and coworkers (4) found that with the decrease of lipoid phosphorus in the blood during its passage through the mammary gland there was a corresponding increase in inorganic phosphate resulting from the setting free of phosphoric acid from the lecithin in its change into fat. In cases where there was much increase of lecithin, as in the above, one might expect a decrease in inorganic phosphate in the plasma. Instead, there is found an increase. Inorganic phosphate in plasma must be subject, however, to constant change; loss through the excretions, and addition from food or from phosphate stores, and the same cause which produced an increase of lipoid phosphate might also act to increase the inorganic phosphate, either by retention or by supply from the stores, in order to provide the material for formation of lecithin from fat.

Another reason for expecting low rather than high values for inorganic phosphorus in the plasma is the formation of new blood cells, which are relatively very rich in phosphoric acid compounds. Diminution of inorganic phosphorus has actually been found in cases of a single withdrawal of blood (10). The same reasoning might be applied in this case as in the above—that with increased demand there is increased supply, and, as is usual in living beings, the supply is in excess of what was origi-

nally present. The only case in which the plasma values remained below the beginning value was in Rabbit F, where the beginning level was unusually high.

Corpuscles.—In the experiment with Rabbit B, the acid ammonium sulfate did not completely precipitate the protein of the corpuscles, hence the values of inorganic phosphate are doubtful. In all other cases protein was completely removed so that the figures given are probably correct. In Experiments A and B, with high lipemia, the inorganic phosphate increases more or less parallel with the lipoid phosphorus. In Rabbit C, with moderate lipemia, inorganic increased but irregularly. In Rabbit E, it diminished; in Rabbit F, it diminished slightly. It thus appears that unless the lipemia is marked the changes in values for inorganic phosphate in the corpuscles are slight or negative, while with marked lipemia the inorganic phosphate increases more or less parallel with the increase in lipoid phosphorus, and both are closely bound up with the appearance of the lipemia. An examination of Table I will show that in most cases high values for inorganic phosphorus in the corpuscles are accompanied by high values in the plasma, but there are many exceptions and nothing like a parallelism can be observed.

These results bring into discussion the whole question of the presence of free phosphates in blood. Taylor and Miller (11) found only traces which confirmed the earlier observation of Gürber (12). No water-soluble phosphate is extracted by the treatment of blood with alcohol-ether in the determination of lipoid phosphorus above, although phosphoric acid and water-soluble phosphates are measurably soluble in that solvent. In the results given above, there is rarely to be observed a balance or parallelism between inorganic phosphate in plasma and corpuscles. In rabbit as in human blood, the inorganic phosphate in the corpuscles is generally much higher than in the plasma. These observations render improbable any free exchange by diffusion of phosphate between corpuscles and plasma, such as would be expected if phosphate were present in the free form. On the other hand, Greenwald (13) and later Feigl (14), by treatment of the blood to remove protein with hot trichloroacetic acid, and the writer, by faintly acid, saturated ammonium sulfate, were able to get protein-free extracts which undoubtedly

contained free phosphate. Whatever objection may be raised to the use of hot trichloroacetic acid as being likely to break up unstable organic compounds of phosphoric acid cannot be urged as regards the cold treatment with the mildly acid ammonium sulfate and the inference is that inorganic phosphate must be present in the blood although not in a water-soluble, diffusible form. As was pointed out previously, there is enough calcium and magnesium in blood to combine with all the phosphate present, and at the prevailing reaction of the blood to form insoluble compounds with it. Another explanation which is in line with modern ideas is that the phosphate is combined with blood protein in such a way as to hinder or prevent its free diffusion. When the blood is deproteinized with reagents which readily dissolve phosphate—as with either of the acid reagents mentioned above—it is loosed from its protein combination and passes into solution. The fact that acid reagents dissolve phosphate while neutral reagents, as alcohol-ether, do not renders it probable that the phosphoric acid is present in a form insoluble in neutral solvent—as would be the case if combined with calcium or magnesium or with protein at the prevailing reaction of the blood.

Attention may be called to the fact that the amount of phosphate passing through the blood plasma daily is much greater than the amount constantly present. Thus the normal human being will excrete and normally consume with the food about 3 gm. of H_3PO_4 per day, while the amount present in the whole blood plasma would not be more than about one-eighth that amount.

Organic Phosphorus.

Corpuscles.—Organic phosphorus in corpuscles is subject in general only to small and irregular variations, indicating that this constituent is constant in the corpuscle from the time of formation, even under the stress of severe hemorrhage. The amount is also remarkably constant for all the animals reported here. The same may be said of the values obtained from a study of rabbit blood for another purpose, which included the examination of about twenty normal rabbits (10). Organic phosphorus in this amount appears to be characteristic of rabbit corpus-

cles. In the series reported above, notably in the case of Rabbit E and, to a certain extent, in Rabbits C and F, values of 15 or 20 per cent above the beginning values are found, but these serve only to emphasize the stability of values for this constituent. Low values are practically never found, and the high values may come at any time in the experiment after the anemia is established.

Plasma.—Organic phosphorus in the plasma is subject to great and irregular variations, so that very little can be said about it. In general, values are reached in the course of the bleeding which are much higher than those found at the beginning of the experiment. On the other hand, very low values are also found. No relation can be discovered between this constituent and the lipoid fraction, on the one hand, or the inorganic fractions, on the other. Normally, organic phosphorus has been found in the plasma of all the animals examined. During bleeding, it generally increases, but may disappear altogether at times, as in Rabbit B. It is possible that this constituent is connected on the one hand with the loss of the nucleus in the red cells, and with the presence of organic phosphorus in urine on the other.

Nucleoprotein, Etc.—In the normal animal, the sum of acid-soluble (inorganic and organic) and lipoid phosphorus is about equal (within the limits of error of the determinations) to the value of the separately determined total phosphate, so that the presence of notable amounts of other combinations of phosphorus—as, for example, nucleoprotein—is doubtful. In the course of the experiments it will be noted, however, that the sum of these fractions in both corpuscles and plasma is generally below the value for total phosphates, and sometimes markedly so, and it is therefore probable that some nucleoprotein is present during the anemia.

On the other hand, the sum of acid-soluble and lipoid sometimes gives a value notably higher than the total phosphate, which would indicate at those times some overlapping; *i.e.*, some unusual substance is present which dissolves in both the acid ammonium sulfate and the alcohol-ether.

Corpuscle Volume during Hemorrhage.—It is well known that in anemia corpuscles irregular both in shape and size appear and the abnormality in size is well illustrated in Column 5 of

Table I. The value "Corpuscle volume" was obtained by dividing the percentage volume of the corpuscles as shown by the hematocrit by the number, as obtained in the count. For example, in 1 c.mm. of blood, the number of corpuscles is known from the count while the volume which they occupy would be the fraction of 1 c.mm. represented by the percentage. Thus, with a count of 5.0 millions, and a corpuscle percentage of 31.0, the average volume of the individual corpuscle would be 0.31/5,000,000, or 6.2×10^{-8} c.mm. As may be seen from the table, the average corpuscle volume invariably rises during the course of the bleeding, sometimes to over 50 per cent above the beginning volume. As recovery takes place, the volume falls to the normal level for the animal or below it. Whether the increase in size is due to swelling of normal corpuscles, or whether some of the corpuscles are abnormally large when formed cannot be definitely determined from the data at hand, but the relative constancy of the value for organic phosphorus would indicate the latter.

Is there anything in the composition of the corpuscles to indicate whether they are young or old? Masing (15) from the results of similar experiments on rabbits and geese is of the opinion that the lipoid phosphorus content of the erythrocytes may be taken as a criterion of the age of the cells. In the work above the most marked difference between the composition of the corpuscles at the beginning and during the experiment, which might be taken as a criterion of the presence of newly formed corpuscles, is in the lipoid phosphorus content, which always rises during the hemorrhage and is the slowest of all constituents to return to normal values. The next most notable constituent is the organic phosphorus fraction, which also generally rises during the anemia. The increase above beginning values of this constituent is, however, relatively small and irregular. Whether these changes are due to the lipemia or are referable to the newly formed corpuscles cannot be stated. Present evidence on the dog indicates that in this animal, which apparently does not develop lipemia from bleeding, neither of these constituents is affected, which would lead one to the conclusion that changes in these values in the rabbit are referable to the lipemia.

While no exact relation has been found between the lipoid phosphorus and the other phosphorus compounds of the blood, it may be seen that in severe lipemia there is a marked increase of both inorganic and lipoid phosphorus in both plasma and corpuscles. Slight changes in lipoid phosphorus are not accompanied by corresponding changes in inorganic phosphorus.

SUMMARY AND CONCLUSIONS.

Of the phosphoric acid compounds of the blood, the one most markedly affected by anemia and consequent lipemia, is the lipoid phosphorus. Values up to five times the normal have been found in plasma, and to twice or over the normal value in the corpuscles.

When the lipoid phosphorus is markedly above normal in either plasma or corpuscles, there is an accompanying increase of inorganic phosphate, but no corresponding change in the other forms of phosphorus. Inorganic phosphorus thus appears to be the form most directly related to the lipoid phosphorus.

Organic phosphorus in the corpuscles remains remarkably constant throughout the experiments, the changes noted being relatively small, so that this constituent in the amounts noted appears to be characteristic of rabbit corpuscles from the time of formation. Organic phosphorus in the plasma varies greatly, sometimes being entirely absent. It generally increases during the bleeding.

The only form of phosphorus which is notably higher in newly formed corpuscles than in older ones is the lipoid phosphorus. Whether the higher values are characteristic of young corpuscles, or are due to the lipemia, cannot be stated.

BIBLIOGRAPHY.

1. Leathes, J. B., The fats, London, 1913.
2. Bloor, W. R., *J. Biol. Chem.*, 1916, xxiv, 447.
3. Bloor, W. R., *J. Biol. Chem.*, 1916, xxvi, 417.
4. Meigs, E. B., Blatherwick, N. R., and Cary, C. A., *J. Biol. Chem.*, 1919, xxxvii, 1.
5. Bloor, W. R., *J. Biol. Chem.*, 1917, xxxi, 575. Bloor, W. R., and MacPherson, D. J., *J. Biol. Chem.*, 1917, xxxi, 79.
6. Boggs, T. R., and Morris, R. S., *J. Exp. Med.*, 1909, xi, 553.

7. Horiuchi, Y., *J. Biol. Chem.*, 1920, xliv, 345,363.
8. Bloor, W. R., *J. Biol. Chem.*, 1918, xxxvi, 49.
9. Bloor, W. R., *J. Biol. Chem.*, 1918, xxxvi, 33.
10. Sundstroem, E. S., and Bloor, W. R., *J. Biol. Chem.*, 1920, xlv, 153.
11. Taylor, A. E., and Miller, C. W., *J. Biol. Chem.*, 1914, xviii, 215.
12. Gürber, A., *Verhandl. phys.-med. Ges. Würzburg*, 1894, xxviii, 129.
13. Greenwald, I., *J. Biol. Chem.*, 1915, xxi, 29.
14. Feigl, J., *Biochem. Z.*, 1918, lxxxiii, 218; lxxxiv, 331.
15. Masing, E., *Arch. Exp. Path. u. Pharmakol.*, 1911, lxvi, 71.

HEMATO-RESPIRATORY FUNCTIONS.

VII. THE REVERSIBLE ALTERATIONS OF THE H_2CO_3: $NaHCO_3$ EQUILIBRIUM IN BLOOD AND PLASMA UNDER VARIATIONS IN CO_2 TENSION AND THEIR MECHANISM.*

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(Received for publication, September 29, 1920.)

It has been universally assumed that alterations of the gaseous and acid-alkali equilibrium of blood under the influence of variations in the tensions of oxygen and CO_2 are readily and completely reversible. The simplest conception of the process involved is as follows:

Hydrogen and chlorine ions (HCl) pass back and forth between the plasma and the corpuscles in response to variations of CO_2 tension, each time that the blood makes the round of the circulation. Thus Na in the plasma is released from NaCl to form $NaHCO_3$ as the blood comes to the tissues; and this Na is again neutralized to NaCl as CO_2 passes off in the lungs. The higher the tension of CO_2, and the concentration of H_2CO_3 in solution, the more Cl is displaced; and, in the proportion expressed by the CO_2 dissociation curve, the more $NaHCO_3$ is formed.

It follows from this that the tension of CO_2—at the moment, for instance, that the corpuscles are centrifuged off—determines (along a plasma curve dependent upon that of the whole blood) the quantity of alkali bicarbonate in the plasma. The power of separated plasma to vary its content of bicarbonate through the mediation of its proteins, although greater than that of a mere solution of $NaHCO_3$, is much less than that of whole blood.

* This and the following papers are continuations of work published by us in this *Journal* some months ago under the same general title (1). All these investigations were performed at about the same time (1918) and under the same conditions and collaboration.

This in brief is the present state of knowledge (2–5).

Some investigators (6, 7) have recently inclined to the view that hemoglobin itself is the principal CO_2-transporting agent. The evidence indicates, however, that hemoglobin, at least when in unlaked corpuscles, plays its part chiefly by absorbing acids and thus unmasking alkali from neutral salt to form bicarbonate. It is this action of the corpuscles which enables the plasma to transport CO_2. Probably also the same reaction occurs in respect to the fluid within the corpuscles since, under variations of CO_2 tension within physiological limits, and apart from the influence of oxygen, the corpuscles in normal blood take up and give off again (*in vitro*) an amount of CO_2 of about the same order of magnitude as an equal volume of the plasma.

We here intentionally avoid the question as to whether hemoglobin acts in the blood as an acid or as a base. We have therefore made use of a phraseology, *e.g.* "acid-concealing power, HCl load, and alkali-producing capacity of the corpuscles," compatible with any intracorpuscular mechanism—whether it finally turns out that hemoglobin acts by adsorption, or by chemical combination, as an acid or as a base or both.

If at the C_H prevailing in normal blood hemoglobin acts as an acid, as present theory seems to indicate (8), the absorption and concealment of HCl by the corpuscles may consist in some such reaction as: $Hb.B + HCl = Hb.H + BCl$. If on the contrary it acts as a base then presumably $Hb.OH + HCl = HbCl + H_2O$ or else $Hb + HCl = Hb.HCl$. It is certain, however, that it is not alkali hydroxide, but acid (chiefly HCl) which passes in and out between corpuscle and plasma.

Present information and variety of opinion on this and related topics may be seen in the papers of Davies, Haldane, and Kennaway (9), Gray (10), Joffe and Poulton (11), Campbell and Poulton (12), Dale and Evans (13), and Mellanby and Thomas (14). All these papers reached us after our own were ready for press. Many of the observations of these writers agree, but one paper (14) disagrees decidedly in its interpretation, with ours.

The peculiar feature of our discussion is that we have thought of the blood as a system which, when it has no tension of CO_2, contains no alkali bicarbonate. Starting from this assumption (verifiable by the experiment of evacuating the blood in a vacuum) we are justified, we think, in treating any $NaHCO_3$ which occurs in blood as called forth by the existing tension of CO_2 by action on the corpuscles.

Thus hemoglobin is almost as essential for the transportation of CO_2 by blood as it is for the transportation of oxygen, but its modes of action in relation to the two gases are quite different.

The following experiments illustrate the reversible reaction under the influence of CO_2. They will serve us as a background for dealing in succeeding papers with an irreversible alteration of the CO_2 ratio, and its bearing upon the interaction of oxygen and CO_2 in blood and upon hemolysis. Our experiments on these topics are published now, not because we can as yet fully explain the phenomena, but because of the interest which this general field of work is exciting, and because investigators unaware of this peculiar irreversible effect may be led to quite erroneous conclusions regarding some phases of the hemato-respiratory functions.

The normal reversibility of the CO_2-alkali equilibrium may be illustrated as follows:

Experiment 1.—Blood was drawn through a cannula from the femoral artery of a dog under local anesthesia with cocaine, and was delivered under mineral oil in a test-tube, where it was mixed with 0.005 gm. of ammonium oxalate per cc.; *i.e.*, 0.5 per cent.

It was divided into two samples which were treated as follows:

Sample A was equilibrated at 37°C. with air containing 70 mm. of CO_2. A part was then analyzed for its CO_2 content. The remainder of this sample was then equilibrated with 40 mm. of CO_2 and analyzed.

Sample B was equilibrated in succession with the same tensions of CO_2, but in reverse order.

The analyses showed CO_2 contents as follows:

Sample A.	At 72 mm.	= 56	volumes per cent of	CO_2
	" 40 "	= 46	" " "	" CO_2
Sample B.	" 40 "	= 46	" " "	" CO_2
	" 72 "	= 56	" " "	" CO_2

(This demonstration of perfect reversibility may seem, now at least, so perfectly obvious as not to deserve publication, but it is essential as a control for comparison with the observations to be reported in succeeding papers.)

The Behavior of Plasma in Contact with Corpuscles and After Separation.

As long as a plasma forms part of a normal blood and is in contact with its corpuscles its content of bicarbonate rises and falls, within physiological limits of CO_2 tension and apart from the oxygen effect, in close parallel with the CO_2 content; that is, with the dissociation curve of the whole blood. Above normal limits of CO_2 tension the plasma takes up less than its share of

any increase of CO_2 content, and further absorptions of CO_2, aside from mere solution, are effected almost wholly by the corpuscles.

From any blood there may be obtained many plasmas of widely varied bicarbonate content, and each of these plasmas has, after separation from the corpuscles, its own particular form of behavior; that is, its own dissociation curve. The plasma proteins are evidently able to function like the corpuscles in providing alkali, but less effectively. Thus there is a primary plasma curve, which within certain limits of CO_2 tension closely parallels that of the whole blood when normal, but not when abnormal. This curve is obtained by centrifuging the blood at various tensions of CO_2 and analyzing the plasmas at these tensions. But each of these plasmas, when exposed to various tensions of CO_2 after separation from corpuscles has a curve of its own, and (as has been shown previously by Hasselbalch and Warburg (15)) these secondary curves are flatter than the primary curve and cross it at the CO_2 tensions at which the blood was centrifuged.

Experiment 2.—Oxalated dog blood was divided into two samples which were equilibrated at 37°C. to tensions of CO_2 of 40 and 58 mm. respectively. The CO_2 content of the blood was then in each case determined. The plasmas were separated by the centrifuge, at 37°C. and under the equilibrating atmospheres, and their bicarbonate contents were determined.

	CO_2 tension.		Rise in CO_2 content.
	40 mm.	58 mm.	
	vol. per cent	*vol. per cent*	*vol. per cent*
Bicarbonate content of 1 cc. of whole blood	46	52	6
" " " 1 " " plasma.....	58	64	6
Difference between blood and plasma.....	12	12	0

It is here seen that, within the limits of the experimental conditions, the CO_2 content of the plasma has a constant difference from the CO_2 content of the whole blood from which it is separated, and that the bicarbonate content of the plasma is controlled by the CO_2 tension in the blood at the moment of separation. In blood drawn from various individuals (dogs) we find that the plasma-blood difference has various values, the figures for the

plasma being sometimes lower and sometimes higher than for the whole blood. But in all normal bloods the type of relation here shown holds true; that is, within physiological limits of CO_2 tension the plasma-blood difference is nearly constant. This seems to indicate that, apart from the change in volume of the corpuscles, and apart from the oxygen effect, the plasma and the corpuscles, at all moderate tensions of CO_2, take an equal share in the absorption and release of CO_2. This accords fully with the observations of Van Slyke and Cullen (4) on this important point.

This relation of plasma and corpuscles holds true so generally that we have used it as a test that the sample under study is normal resting blood. Blood is easily altered in this respect; for example, by overaeration in the process of defibrination, by gaseous evacuation, and similar treatments involving the irreversible effect described in succeeding papers.

In the next two experiments it will be seen that the plasma not only varies in its CO_2 content according to the tension of CO_2 at which it is separated from its corpuscles, but also that when these plasmas—all from the same blood—are equilibrated with various tensions of CO_2 each has its own dissociation curve.

Experiment 3.—Oxalated dog blood was divided into three portions which were then equilibrated with air containing CO_2 at 21, 40, and 73 mm., respectively at 40°C. 1 cc. of each was analyzed and the remainder of each was centrifuged. The plasmas from the first and third (the second was lost) were then equilibrated with CO_2 at 21, 40, and 73 mm. at 40°C. and analyzed. The results obtained (after subtraction of dissolved CO_2) were as follows:

	CO_2 tension.		
	21 mm.	40 mm.	73 mm.
	vol. per cent	*vol. per cent*	*vol. per cent*
Whole blood	38	49	57
Plasma separated at 21 mm.	40	47	52
" " " 73 "	45	53	58

Experiment 4.—Oxalated dog blood was divided into three samples and treated as in the previous experiment. The (=) sign is used to signify "contained." The amount of combined CO_2 is stated after it.

Sample A. At 21 mm. CO_2 = 32 volumes per cent CO_2, its plasma = 35 volumes per cent CO_2, and at 21 mm. CO_2 = 35 volumes per cent CO_2
40 " CO_2 = 39 " " " CO_2
72 " CO_2 = 45 " " " CO_2

Sample B. At 40 mm. CO_2 = 40 volumes per cent CO_2, its plasma = 44 volumes per cent CO_2, and at 21 mm. CO_2 = 41 volumes per cent CO_2
40 " CO_2 = 44 " " " CO_2
72 " CO_2 = 51 " " " CO_2

Sample C. At 72 mm. CO_2 = 51 volumes per cent CO_2, its plasma = 54 volumes per cent CO_2, and at 21 mm. CO_2 = 47 volumes per cent CO_2
40 " CO_2 = 51 " " " CO_2
72 " CO_2 = 54 " " " CO_2

These data are expressed graphically in Fig. 1.

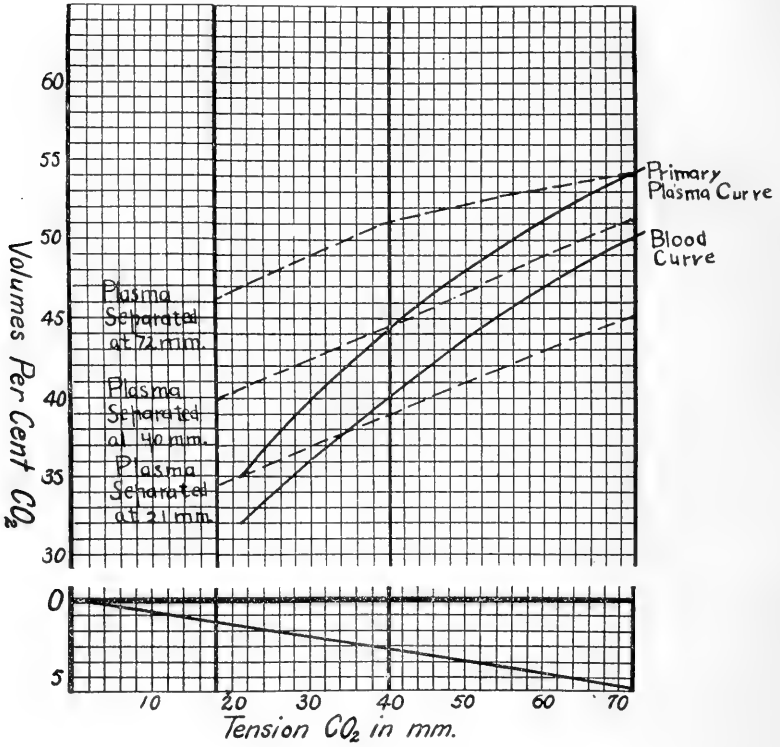

FIG. 1. From the data of Experiment 4.

The limit of the plasma-corpuscle interplay of chlorine is shown in the next experiment. Here the passage of chlorine into the

corpuscles and the unmasking of sodium in the plasma to form $NaHCO_3$ is seen to become inadequate between 74 and 140 mm. of CO_2. The curve for the whole blood and that for the plasma then become divergent, and the difference between the CO_2 contents of blood and plasma increases. The plasma at such tensions would do much less than its share in the transportation of CO_2. Up to 74 mm., the relation formulated in connection with Experiment 2 still holds true, however.

Experiment 5.—Oxalated dog blood was divided into four portions, which were equilibrated separately with tensions of CO_2 of 42, 74, 140, and 360 mm. CO_2 respectively. Part of each was analyzed; and the remainder was centrifuged and its plasma analyzed. The results were as follows:

	CO_2 tension.			
	42 mm.	74 mm.	140 mm.	360 mm.
	vol. per cent	*vol. per cent*	*vol. per cent*	*vol. per cent*
Combined CO_2 of whole blood	47	53	75	110
" CO_2 " plasma	49	55	70	93
Difference between blood and plasma	+2	+2	−5	−17

Experiment 6.—Oxalated dog blood was divided into seven samples and equilibrated at 40°C. at the tensions of CO_2 shown in the table. A part of each sample was analyzed directly and the remainder centrifugalized under oil and its plasma analyzed.

	CO_2 tension at 40°C.								
	21 mm.	41 mm.	72 mm.	146 mm.	210 mm.	360 mm.	440 mm.	504 mm.	719 mm.
	vol. per cent	*vol. per cent*	*vol. per cent*	*vol. per cent*	*vol. per cent*	*vol. per cent*	*vol. per cent*	*vol. per cent*	*vol. per cent*
Whole blood	30	42	54	66	72	77	80	79	80
Plasma	34	46	56	61	61	60	62	60	58

From Experiment 6 it appears that when blood is exposed to increasing tensions of CO_2 the amount of alkali produced in the plasma reaches a maximum between 72 and 146 mm. and that the amount of CO_2 combined by the corpuscles reaches its maximum somewhat below 440 mm.

Parallelism of Primary Plasma Curve and Whole Blood.

The parallelism of the primary plasma curve and that of the whole blood means simply that, under the influence of changes of CO_2 tension (but with uniform oxygen), the concentration of alkali bicarbonate rises and falls equally inside the corpuscles and in the plasma surrounding them. If this were not the case, the C_H inside the corpuscles and that in the plasma would be influenced unequally by variations of CO_2 tension. (We are here neglecting the influence of oxygen upon CO_2 capacity and we are assuming that the only condition determining C_H is the CO_2 ratio, H_2CO_3:$NaHCO_3$.)

In altered blood in which this parallelism does not hold, either such differences of C_H inside and outside the corpuscles are developed or the hemoglobin undergoes some alteration of its acid-absorbing power, such as an increased capacity for interaction of oxygen and CO_2.

Relation of Plasma Alkali to HCl Load of Corpuscles.

It appears also from the foregoing experiments that it is quite misleading to think of the plasma as having, independently of the corpuscles, a certain alkali value. Just as an organism dominates its environment, so the corpuscles control the concentration of bicarbonate in the fluid around them. They vary it under the influence of variations in CO_2 tension and C_H. When the plasma alkali is determined by analysis, what is revealed is fundamentally the alkali-producing power of the corpuscles and the load of HCl which they are capable of taking. As will be shown in the next paper, corpuscles which have the quality of inducing, at a certain tension of CO_2, a certain amount of alkali—be it large or small—induce nearly that amount of "alkaline reserve" even in a normal saline solution.

The enormous buffer value of hemoglobin endows the blood with a potential alkaline reserve which is much larger than the plasma alkali. The plasma alkali represents merely that fraction of the alkali-producing power which is called into use at a certain tension of CO_2, concentration of H_2CO_3, and C_H.

CONCLUSIONS.

Hemoglobin plays almost as large a part in the transportation of CO_2 as it does in that of oxygen. It does not itself combine directly with CO_2 to any great extent, however; but under pressure of CO_2 it absorbs HCl, and thus provides alkali, which in the plasma is chiefly Na from NaCl, to form bicarbonate. As CO_2 is given off in the lungs, NaCl is reformed from $NaHCO_3$.

From any blood, therefore, plasmas of widely varying content of alkali are obtained, depending upon the tension of CO_2 at the time of centrifuging. Each of these plasmas has various alkali values with varied tensions of CO_2. The relations of the primary and secondary dissociation curves of the plasma to each other and to the curve of the whole blood are here exemplified.

A quality of normal blood from a resting animal is that variations of CO_2 tension (in the presence of ample oxygen) call nearly the same increase of alkali into use in unit volume of the blood as in unit volume of its unseparated plasma. Thus the bicarbonate content of the corpuscles (apart from the effect of variations of oxygen) rises and falls with that of the plasma around them. But this does not hold at abnormally high tensions; and at very low tensions, as will be shown in later papers, the blood undergoes an irreversible alteration. The parallelism of alkali variations in corpuscles and plasma affords, therefore, a convenient test as to whether or not a given blood sample is normal.

It is probably by means of the equality of gain and loss of bicarbonate that the C_H inside the corpuscles and that outside them in the plasma are kept the same, or at least in uniform relation. The C_H indicated by the primary plasma curve at various tensions of CO_2 in the blood is particularly significant.

It thus appears that the corpuscles dominate the plasma so powerfully that the alkali of the (unseparated) plasma at a certain tension of CO_2 is essentially an expression of the alkali-producing power of the corpuscles. The plasma alkali is only a part of the total alkaline reserve of the blood. The chief buffer is the hemoglobin. The extent to which the corpuscles are loaded with HCl is indicated by the amount of alkali which they call into use out of NaCl at a certain tension of CO_2; that is, the CO_2 capacity of the blood.

The condition underlying the variations in this quality of the corpuscles will be considered in the next paper.

BIBLIOGRAPHY.

1. Haggard, H. W., and Henderson, Y., *J. Biol. Chem.*, 1919, xxxix, 163; 1920, xlii, 237; xliii, 3, 15, 29.
2. Zuntz, N., in Hermann, L., Handbuch der Physiologie, Leipsic, 1882, iv, pt. ii, 77.
3. Hamburger, H. J., *Biochem. Z.*, 1918, lxxxvi, 309.
4. Van Slyke, D. D., and Cullen, G. E., *J. Biol. Chem.*, 1917, xxx, 342.
5. Fridericia, L. S., *J. Biol. Chem.*, 1920, xlii, 245.
6. Buckmaster, G. A., *J. Physiol.*, 1917, li, 105, 164, p. i.
7. Bayliss, W. M., *J. Physiol.*, 1919, liii, 176.
8. Parsons, T. R., *J. Physiol.*, 1919, liii, 340.
9. Davies, H. W., Haldane, J. B. S., and Kennaway, E. L., *J. Physiol.*, 1920, liv, 32.
10. Gray, J., *J. Physiol.*, 1920, liv, 68.
11. Joffe, J., and Poulton, E. P., *J. Physiol.*, 1920, liv, 129.
12. Campbell, J. M. H., and Poulton, E. P., *J. Physiol.*, 1920, liv, 152.
13. Dale, H. H., and Evans, C. L., *J. Physiol.*, 1920, liv, 167.
14. Mellanby, J., and Thomas, C. J., *J. Physiol.*, 1920, liv, 178.
15. Hasselbalch, K. A., and Warburg, E. J., *Biochem. Z.*, 1918, lxxxvi, 410.

HEMATO-RESPIRATORY FUNCTIONS.

VIII. THE DEGREE OF SATURATION OF THE CORPUSCLES WITH HCl AS A CONDITION UNDERLYING THE AMOUNT OF ALKALI CALLED INTO USE IN THE PLASMA.

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(Received for publication, September 25, 1920.)

It is shown in the preceding paper that the alkali of the plasma, chiefly $NaHCO_3$, is not an independent property of that fluid, but is controlled and determined by the corpuscles. From this it follows that when a figure for the plasma alkali, for instance 40, 55, or 70, is found by analysis, its significance for the blood lies in large part in what it tells of the quality of the corpuscles.

In this paper we shall report experiments which throw some light on this quality of the corpuscles. What are the conditions which determine why one blood, when equilibrated with a certain tension of CO_2 (for instance 40 mm.), develops a plasma alkali of 40, while another blood at the same tension develops 55, and a third 70? The experiments given below indicate that one of the conditions in question is the extent to which the corpuscles are loaded with HCl.

It will here appear that the amount of alkali developed in the plasma of any blood equilibrated to 40 mm. of CO_2 tension is an index of the extent to which the corpuscles have been previously depleted of alkali-producing power. If they are heavily loaded already they can take additional HCl less readily, and thus develop less alkali in the plasma than if previously only lightly loaded. If on the contrary the load of the corpuscles is artificially decreased, by the treatment described below, they will then take HCl more readily and thus produce a higher plasma alkali than they were previously able to do. Thus, as we shall show, any corpuscles may be made to furnish any plasma alkali.

The underlying reasons for this behavior of corpuscles are probably to be found (1) in the capacity of hemoglobin to combine with large amounts of acid, or alkali, under slight change of C_H—in other words its extraordinary buffer value; and (2) in the tendency of normal corpuscles to maintain within certain limits the same C_H, or at least the same difference of C_H, in the fluid around about them and in their own intrinsic fluid under variations of CO_2 tension.

To Zuntz (1) and to Hamburger (2) we owe the clear recognition and demonstration of the fact that under the influence of CO_2 there is a passage of HCl between plasma and corpuscles. Gürber (3), Van Slyke and Cullen (4), and Fridericia (5) have proved by chloride analyses that the amount of HCl formed from NaCl and passing into the corpuscles corresponds to the greater part of the amount of alkali bicarbonate produced.

Starting from this point, our experiments show the really enormous amounts of HCl which the corpuscles are capable of taking up under slight increase of the CO_2 ratio and C_H. These observations illustrate the effectiveness with which the corpuscles stabilize this ratio ($H_2CO_3 : NaHCO_3$), and therefore the C_H, in the plasma. The conception of the "HCl load of the corpuscles," here introduced, suggests that the real source of the alkali of the plasma is not the alkali of the food, but the NaCl of the blood. It follows from this conception that, for instance, if two plasmas, separated in both cases under 40 mm. of CO_2, show CO_2 contents respectively of 50 and 60 volumes per cent, the HCl loads of the corpuscles in the two cases must differ by an amount very much greater than a quantity of HCl which would correspond to 10 volumes per cent of CO_2. From this conception we may safely infer that when the body is striving to call alkali into use in the plasma through depressed breathing and acidosis, or to cause alkali to disappear through acapnia and alkalosis, the variations induced in the HCl load of the corpuscles must be very large indeed—much greater than analyses of plasma CO_2 would directly indicate.

The method of experimentation was as follows: Samples of oxalated dog blood were equilibrated with various tensions of CO_2 and the plasmas separated by the centrifuge. The corpuscles were then washed once with normal saline solution (0.9

per cent NaCl solution with no tension of CO_2), recentrifuged, and then made up to the original blood volume with fresh saline solution. The resulting suspension was thoroughly mixed, and was then equilibrated with CO_2 at various tensions. Finally the saline solution was separated from the corpuscles by means of the centrifuge; and the bicarbonate which had been formed in the saline was determined.

All the operations of equilibration, centrifuging, and analyzing were carried out at a summer room temperature of 30°C.

Experiment 1.—

	Combined CO_2 in Sample A.		Combined CO_2 in Sample B.
	vol. per cent		*vol. per cent*
(1) Blood equilibrated with 41 mm. CO_2 and centrifuged.		(1) Another sample of same blood equilibrated with 712 mm. CO_2 and centrifuged.	
Its plasma at 41 mm...	48	Its plasma at 41 mm...	80
(2) Corpuscles (from A1) washed, mixed with saline, and equilibrated with 41 mm. CO_2.		(2) Corpuscles (from B1) washed, mixed with saline, and equilibrated with 41 mm. CO_2.	
Corpuscle-saline suspension	32	Corpuscle-saline suspension.....	22
Saline centrifuged off...	46	Saline centrifuged off....	29
(3) The same corpuscle-saline suspension (as A2) equilibrated at 712 mm. CO_2 and centrifuged.		(3) The same corpuscle-saline suspension (as B2) equilibrated at 712 mm. CO_2 and centrifuged.	
This saline at 41 mm. CO_2.....	77	This saline at 41 mm. CO_2.....	40
Sum of (1) and (3)	125		120

In Experiment 1 it is to be noted that (in Samples A1 and A2) the figures for the alkali called into use by these corpuscles at 41 mm. of CO_2 in the natural plasma and in a saline solution are nearly the same; namely, 48 and 46 volumes per cent of CO_2.

Note that similarly (in Samples A3 and B1) equilibration at 712 mm. of CO_2 also calls into use nearly the same amount of alkali from natural plasma and from saline; namely, 77 and 80 volumes per cent of CO_2.

Note on the other hand that after the corpuscles have received an increased load of HCl under the influence of a high tension of CO_2 (as in Sample B1) their power of calling alkali into use is diminished, for example from 46 (in Sample A2) to 29 (in Sample B2) and from 77 (in Sample A3) to 40 volumes per cent (in Sample B3).

We have found in other experiments, in which the CO_2 dissociation curve of whole blood was determined up to very high tensions (700 mm. and over), that above certain limits no more alkali is called into use, and additional CO_2 is taken up only in simple solution (see Experiment 6 of the preceding paper). From this it might be supposed that the total alkali-producing power of the corpuscles had been called into use, and that the combined CO_2 at, for instance, 450 mm. could be taken as a measure of it.

But from Experiment 1 it appears that such is not the case; for the same corpuscles which (in Sample B1) produced an alkali of 80 in their own plasma at 712 mm. had still the power to produce considerable additional amounts of alkali in saline (Samples B2 and B3). Evidently the alkali-producing force is balanced against the alkali (or the C_H) already in the fluid, either plasma or saline. (Quantitatively there are discrepancies in our data from whole blood, in the preceding paper, and from corpuscle-saline suspensions.)

Experiment 2.—Blood (from same dog as in Experiment 1) equilibrated with 712 mm. of CO_2, and centrifuged as in Sample B of Experiment 1.

The corpuscles were then mixed with saline to which a small amount of sodium bicarbonate had been added. The suspension was then equilibrated with 41 mm. of CO_2 and centrifuged.

Saline-bicarbonate solution at 41 mm. CO_2 before addition of corpuscles = 49 volumes per cent CO_2.

Saline-bicarbonate solution after centrifuging = 10 volumes per cent CO_2.

Experiment 3.—

Sample A. Arterial blood centrifugalized.

Plasma bicarbonate at 41 mm. = 36 volumes per cent CO_2.

Sample B. The corpuscles (from Sample A) mixed with saline and equilibrated with 41 mm. CO_2.

Saline centrifuged off at 41 mm. = 33 volumes per cent CO_2.

Sample C. The corpuscles (from Sample B) were again mixed with fresh saline, and again equilibrated to 41 mm. CO_2, and centrifuged.

This saline at 41 mm. = 28 volumes per cent CO_2.

Sample D. The corpuscles (from Sample C) were mixed with saline a third time, equilibrated to 712 mm. CO_2, and centrifuged.

This saline at 41 mm. = 54 volumes per cent CO_2.

Total of plasma and salines = 151 volumes per cent CO_2.

(The equal sign (=) is here used to signify that the saline or plasma was found on analysis to contain the indicated amount of combined CO_2; *i.e.*, $NaHCO_3$.

Experiment 4.—

	Combined CO_2 in Sample A.		Combined CO_2 in Sample B.
	vol. per cent		*vol. per cent*
(1) Blood rotated for a few minutes in a flask with room air and centrifuged.		(1) Another sample of same blood equilibrated at 712 mm. CO_2 and centrifuged.	
Plasma at 41 mm. CO_2..	35	Plasma at 41 mm. CO_2.	91
(2) Corpuscles (from Sample A1) washed, mixed with saline, and equilibrated at 41 mm. CO_2.		(2) Corpuscles (from Sample B1) washed, mixed with saline, and equilibrated at 41 mm. CO_2.	
Corpuscle-saline suspension.....	35	Corpuscle-saline suspension.....	22
Saline centrifuged off...	50	Saline centrifuged off..	36
(3) Corpuscles (from Sample A2) suspended in fresh saline, equilibrated with 712 mm. CO_2 and centrifuged.		(3) The same corpuscle-saline suspension (as Sample B2) equilibrated with 712 mm. CO_2 and centrifuged.	
This saline at 41 mm. CO_2.....	104	This saline at 41 mm. CO_2.....	91
Total of plasma and salines.	189	Sum of (1) and (3).....	182

Experiment 5.—Arterial blood was used. It contained 40 volumes per cent of CO_2. It was centrifuged, and the corpuscles were found to constitute almost exactly 50 per cent of the volume. The plasma contained 40 volumes per cent of CO_2.

The corpuscles were divided into four parts and suspended respectively in saline solution (0.8 per cent NaCl), (*a*) of equal volume, (*b*) of three volumes, (*c*) of six volumes, (*d*) of twelve volumes. All were equilibrated at 40 mm. of CO_2 at 37°C. and centrifuged; and the salines were analyzed.

	CO_2 in 1 cc.	Total combined CO_2 produced by 1 cc. corpuscles.	CO_2 ratio.	$C_H 7$	pH
	vol. per cent	*vol. per cent*			
Plasma	40	40	3:40	0.60	7.22
Saline (*a*), one volume	32	32	3:32	0.75	7.12
" (*b*), three volumes	27	81	3:27	0.88	7.05
" (*c*), six "	24	144	3:24	1.00	7.00
" (*d*), twelve "	19	228	3:19	1.26	6.91

From these data we see that under an increase of C_H from 0.6 to 1.26 $\times 10^{-7}$ (or pH 7.22 to 6.91) these corpuscles absorbed an amount of HCl, and produced an amount of alkali, corresponding to 228 volumes per cent of CO_2.

From Experiment 2 we see that when corpuscles have been loaded with HCl by exposure to a high tension of CO_2 and are then transferred to a saline solution of moderate alkalinity at a moderate tension of CO_2, they give off HCl and reduce the alkali of the solution, turning $NaHCO_3$ into NaCl.

From Experiment 3 we see that normal corpuscles have a very large capacity for HCl. The figures show only a slight decrease of alkali production in the second saline in which they were equilibrated at normal CO_2 tension. That they still had a large reserve capacity for HCl is shown by the even larger amount of alkali produced in a third saline in which they were equilibrated with a high tension of CO_2.

In Experiment 4 the contrasting figures for Samples A and B show that corpuscles may have their load of HCl decreased by equilibration to a low tension of CO_2, and increased by a high tension. The less loaded and the more loaded are then seen to produce quite different amounts of alkali in saline both at normal and at supernormal tensions of CO_2. But the increase of alkali called forth is nearly the same. Thus comparing Sample A2 with Sample B2, and Sample A3 with Sample B3 we find $50 - 36 = 14$, and $104 - 91 = 13$.

From these experiments it appears that the capacity of the corpuscles to absorb and give off HCl is very large, and that it is controlled by the tension of CO_2 through the concentration of H_2CO_3 in solution. Evidently the total alkaline reserve of the blood is not merely the $NaHCO_3$ of the plasma but the entire acid-absorbing and alkali-producing power of the corpuscles. The data above given indicate that this acid-concealing power of the corpuscles is five or ten times as great as the acid-neutralizing power of the $NaHCO_3$ in its plasma.

Regulation of Plasma C_H by Alkali-Producing Power of Corpuscles.

We thus see the enormously important part played by the corpuscles in the regulation of C_H in the plasma. Indeed, the intrasanguinary mechanism for the regulation of neutrality need involve little more than the capacity of the corpuscles to produce $NaHCO_3$, or to neutralize it to NaCl, almost in proportion to the concentration of H_2CO_3; for theory indicates that the C_H varies in proportion to the CO_2 ratio ($H_2CO_3 : NaHCO_3$).

Consideration of these experiments suggests that the placing of corpuscles in saline is virtually equivalent to adding to blood an amount of HCl just sufficient to convert all the $NaHCO_3$ of the plasma into NaCl. Presumably, if the blood were then equilibrated with 40 mm. of CO_2 the corpuscles would in large part (four-fifths to nine-tenths) restore the alkali in the plasma. But the C_H would be increased by 10 or 20 per cent.

Doubtless it is through its influence upon C_H that the tension of CO_2 and concentration of H_2CO_3 act. If so, the effects of the addition of acid or alkali to blood should be dealt with, not at a uniform tension of CO_2, as in the experiments above reported, but at a uniform C_H; that is, at a uniform CO_2 ratio. Thus if the normal level of the ratio $H_2CO_3 : NaHCO_3$ is 3 : 60, and an amount of HCl is added corresponding to 20 volumes per cent of CO_2, the plasma alkali would not fall from 60 to 40 (although $60 - 20 = 40$), but merely to an intermediate figure, as long as the tension of CO_2 is unchanged. But if the tension were reduced so that the concentration of H_2CO_3 is now 2 instead of 3, the alkali should then be 40. At the new level of 2 : 40 the C_H would again be normal. The load of HCl on the hemoglobin—or, if hemo-

globin acts as an acid, the amount of base combined with it—would then be the same as at the original 3 : 60 level. The whole change would consist in the neutralization of part of the alkali in use and a proportional reduction of H_2CO_3. The CO_2 ratio and C_H would be unaltered. This is, apparently, what occurs *in vivo* (6).

The same consideration would apply to a rise of level to 4 : 80 due to addition of alkali. The CO_2 ratio and C_H being normal, the load on the hemoglobin would be the same as at the levels 2 : 40 and 3 : 60.

We have performed some experiments *in vitro* which suggest that these expectations are correct, but they need to be repeated and extended before detailed publication.

CONCLUSIONS.

Centrifuged corpuscles suspended in saline solution and exposed to a tension of CO_2 produce in the saline solution an amount of alkali only a little (one-fifth or one-tenth) less than that called into play in the plasma before centrifugation at this CO_2 tension.

Corpuscles may have their load of HCl increased or decreased by suitable equilibrations. The alkali-producing power of the corpuscles is adjusted accordingly. When suspended in a saline solution, corpuscles already heavily loaded with HCl call forth less alkali at a given tension of CO_2 than less loaded corpuscles.

The total capacity of the corpuscles for acid is equivalent to an amount of alkali several times greater than that in the plasma of normal blood. This capacity is controlled immediately by the tension of CO_2 and concentration of H_2CO_3 and presumably the C_H, in the blood. When the H_2CO_3 is increased there is an almost proportionate increase of $NaHCO_3$ and the change in the CO_2 ratio ($H_2CO_3 : NaHCO_3$) and C_H is correspondingly slight.

When whole blood is equilibrated at a very high tension of CO_2 the alkali-producing power is not (as might be supposed) exhausted. The alkali called into use at such tensions is therefore not a measure of the total potential alkali-producing capacity of the corpuscles. It is merely a measure, or approximation, of what they can do in the existing plasma (high in $NaHCO_3$), against which they are balanced.

BIBLIOGRAPHY.

1. Zuntz, N., in Hermann, L., Handbuch der Physiologie, Leipsic, 1882, iv, pt. ii, 77.
2. Hamburger, H. J., *Biochem. Z.*, 1918, lxxxvi, 309 (quotes his earlier work).
3. Gürber, A., *Jahresb. Thierchem.*, 1896, xxv, 164.
4. Van Slyke, D. D., and Cullen, G. E., *J. Biol. Chem.*, 1917, xxx, 330, 342.
5. Fridericia, L. S., *J. Biol. Chem.*, 1920, xlii, 245.
6. Haggard, H. W., and Henderson, Y., *J. Biol. Chem.*, 1919, xxxix, 163.

HEMATO-RESPIRATORY FUNCTIONS.

IX. AN IRREVERSIBLE ALTERATION OF THE H_2CO_3:$NaHCO_3$ EQUILIBRIUM OF BLOOD, INDUCED BY TEMPORARY EXPOSURE TO A LOW TENSION OF CO_2.

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(Received for publication, September 25, 1920.)

Observations in this laboratory have pointed repeatedly to the occurrence of a more or less permanent and irreversible, or difficultly reversible, alteration in the blood under low tensions of CO_2. Henderson (1) observed such an alteration *in vivo* in 1908. Henderson and Haggard (2) estimated the critical level at 33 to 36 volumes per cent of CO_2 in the arterial blood, but we now think that, owing to neglect of temperature, these figures are somewhat too high.

We found that in dogs, when overventilation of the lungs was carried so far that the arterial blood was reduced below the critical level, an irreversible reduction in the CO_2-combining power of the blood occurred. Thereafter, restoration of CO_2 to the blood even by inhalation of CO_2 in air was ineffective, and death always followed.

In a more recent publication (3) we have called attention to the fact that in equilibrating blood with gases, for the determination of the CO_2 dissociation curve, care must be taken that the blood is not exposed to very low tensions of CO_2; *e.g.*, 20 mm. or less at body temperature. At such tensions we found that the same, or a closely similar, alteration of the blood occurs *in vitro* as that noted above *in vivo*.

We were led to this observation by the occurrence of discrepancies in our analytical results. In some experiments the CO_2 content deduced from the CO_2 dissociation curve of the blood at the alveolar CO_2 tension did not agree with the CO_2 content found

in the arterial blood by direct analysis. This indicated that in these cases the curve was erroneous, and that the error was due to alterations in the blood *in vitro*.

In Experiment 1 is to be seen an example of this type of analytical discrepancy. The arterial blood contained 46 volumes per cent of CO_2. Some of this same blood equilibrated with air containing 41 mm. of CO_2 likewise contained 46 volumes per cent of CO_2. But when a third sample of the same blood was equilibrated in succession to tensions of 18, 40, and 72 mm., the curve plotted from the analytical results indicated a CO_2 content of only 42 volumes per cent at 41 mm. Evidently the blood had lost CO_2 capacity in the course of the equilibrations; that is, it called less alkali into use at a CO_2 tension of 41 mm. than originally.

Experiment 1.—The femoral artery of a dog was cannulated under local anesthesia. Arterial blood was drawn under oil and onto a small amount of oxalate. It was divided into three samples of which one (*a*) was immediately analyzed, another (*b*) was equilibrated with air containing 41 mm. CO_2 at 37.5°C. and analyzed, and the third (*c*) was equilibrated in succession at 37.5°C. with air containing 18, 40, and 72 mm. CO_2, and an analysis made at each tension.

The analyses showed (*a*) = 46 volumes per cent CO_2; (*b*) = 46 volumes per cent CO_2; (*c*) at 18 mm. = 30 volumes per cent CO_2, at 40 mm. = 41 volumes per cent CO_2, and at 72 mm. = 50 volumes per cent CO_2.

From the dissociation curve plotted from the last three figures, (*c*) at 41 mm. would contain about 42 volumes per cent CO_2 in contrast to 46 in (*a*) and (*b*).

It needs to be distinctly noted that this indicates quite a different type of alteration of the corpuscles from the immediately reversible reaction to CO_2, or the adjustment of their load of HCl, discussed in the two previous papers.

Experiment 2.—Arterial blood from a dog was oxalated. It was then equilibrated at 18 mm. of CO_2, at 37°C. for ½ hour. At intervals samples were taken and analyzed for their content of CO_2. As 8 minutes is ample time for equilibrium, the following figures suggest a progressive loss of CO_2-combining power.

Time, *min*	0	8	15	20	30
CO_2 content, *vol. per cent*	45	26	24	22	21

Observations of the character shown in Experiment 2 also led us to suspect that exposure of blood *in vitro* to low tensions of CO_2 results in some fundamental alteration in its CO_2-combining

power. To test this hypothesis a sample of blood was equilibrated in succession with decreasing tensions of CO_2 and its dissociation curve plotted. With a second portion of the same blood equilibrations were made in the reverse order; that is, beginning with a very low tension but using the same gas mixtures as previously. The results obtained are shown in Experiment 3.

Experiment 3.—Oxalated arterial dog blood was used. One sample (*a*) was equilibrated at 37.5°C., for 15 minutes each time, with air containing 72, 40, 18, and 1 mm. CO_2, and an analysis for CO_2 content was made at each tension. A second sample (*b*) was similarly equilibrated with the same CO_2 tensions but in reverse order. The analysis showed:

Sample (*a*) at 72, 40, 18, and 1 mm. contained 56, 47, 32, and 14 volumes per cent CO_2 respectively.

Sample (*b*) at 1, 18, 40, and 72 mm. contained 14, 27, 38, and 49 volumes per cent CO_2 respectively.

(Previous experiments have demonstrated that the time, 15 minutes, here allowed, is quite sufficient for the complete attainment of equilibrium.)

It has been shown by Christiansen, Douglas, and Haldane (4) that the CO_2-combining power of defibrinated blood is sometimes initially greater than that of the undefibrinated, and that the defibrinated undergoes a progressive decrease of CO_2-combining power on standing. These authors, Buckmaster (5), and Bohr (6) refer to changes of blood alkalinity induced by defibrination as indicated by the work of 20 to 40 years ago in which the blood was titrated. But it is not easy to say just how much bearing these early observations have on the present problem.

Experiment 4.—From a cannula in the femoral artery of a dog three blood samples of 10 cc. each were drawn in rapid succession. The first (*a*) was treated with ammonium oxalate under oil. The second (*b*) was carefully defibrinated by moderate stirring with a feather under oil. The third (*c*) was collected in a paraffined flask. All three were immediately equilibrated with 40 mm. of CO_2 at 37.5°C. The analyses of these samples showed

Sample (*a*) = 48 volumes per cent CO_2
" (*b*) = 54 " " " CO_2
" (*c*) = 48 " " " CO_2

Experiment 5.—Two samples of arterial blood were drawn. One was defibrinated, the other oxalated. Both were equilibrated with 40 mm. CO_2 at 37.5°C. for 2 hours. At intervals samples were withdrawn and analyzed. The results were as follows:

Time, *min*	15	30	60	120
Defibrinated blood, *vol. per cent*	54	52	46	42
Oxalated blood " " "	47	47	48	47

Experiment 6.—Oxalated arterial dog blood was allowed to stand exposed to the air at room temperature in a beaker. One sample of the original blood was equilibrated with air containing 40 mm. CO_2 and a second with air of 21 mm. CO_2. At intervals additional samples were taken from the beaker, and divided into two parts, of which one was immediately analyzed for CO_2 while the other was equilibrated with 40 mm. CO_2 and analyzed.

Time, *min*	0	60	120	180	240	300
CO_2 content, *vol. per cent*	41	37	33	30	25	21
CO_2-combining power at 21 mm.	30					
CO_2-combining power at 40 mm.	40	40	39	40	38	36

Evidently this blood underwent no fundamental change until, at the end of 180 minutes, its content of CO_2 fell below 30 volumes per cent and its tension below 21 mm.

Experiment 4 indicates that for the purposes of blood gas equilibration and analysis oxalated blood is much nearer to normal blood than is that which has been defibrinated.

Experiment 5 in turn indicates that even when defibrinated blood is kept at a CO_2 tension of normal amount it tends to undergo a progressive loss of CO_2-combining power. Oxalated blood on the contrary within any reasonable time does not exhibit this change.

In Experiment 6 it is to be seen, however, that if oxalated blood is exposed to air it likewise undergoes a progressive loss of CO_2-combining power when, but not until, the CO_2 tension falls below 21 mm. at room temperature—as shown by the results of analyses of samples separated at intervals and equilibrated again at 40 mm. of CO_2.

In Experiment 7 it will be seen that an even more rapid loss of CO_2-combining power may be produced in oxalated blood by passing through it a continuous stream of air free from CO_2. In the performance of this experiment difficulty was at first encountered because of the concentration of the blood by evaporation as indicated by determinations of total solids. In the experiment here given a closed circuit apparatus was employed. The blood was placed in a paraffined vessel immersed in a water bath at 37°C. and by means of a small blower air was circulated through this vessel and through a wash bottle containing a solution of sodium hydroxide.

Experiment 7.—Oxalated dog blood was subjected to a stream of CO_2-free air circulated as described in the text. The equilibrations and analyses were similar to those of Experiment 6.

Time, *min*	0	30	45	60	120
CO_2 content, *vol. per cent*	52	10	10	8	9
CO_2-combining power at 40 mm	54	44		32	31

What the nature of the alteration in blood induced by overventilation may be we cannot as yet fully define. It appears, however, to be associated with several other phenomena among which the following may be mentioned.

Some of the corpuscles are ruptured and the plasma or saline is tinged with hemoglobin in solution, as is shown in Paper XI of this series.

Blood altered by overventilation does not thereafter meet the test for normality described in Paper VII of this series. Thus when two samples of such altered blood are exposed to different tensions of CO_2 (for instance, 40 and 50 mm.) and analyzed, and their plasmas are separated and analyzed, it is found that the plasmas do not show the same difference in CO_2 content as do the two samples of whole blood at the two tensions. This suggests that the alteration of the blood involves the development of abnormal differences of C_H between the fluid within the corpuscles and that surrounding them.

We are inclined to believe (but further work is needed) that the alteration induced by overventilation is also associated with the development in the corpuscles of an increased capacity for the interaction of oxygen and CO_2. Data bearing on this matter are given in the following paper.

CONCLUSIONS.

When blood is exposed to a tension of CO_2 below a critical level it undergoes an irreversible, or at least not readily reversible, change. Thereafter, if it is equilibrated with a normal tension of CO_2 it combines with distinctly less CO_2, and presumably produces correspondingly less $NaHCO_3$, than it did at this tension previously. Defibrinated blood is even more prone to such changes than oxalated blood, but oxalated blood likewise shows them.

Some associated phenomena are mentioned.

BIBLIOGRAPHY.

1. Henderson, Y., *Am. J. Physiol.*, 1908, xxi, 126; 1910–11, xxvii, 152.
2. Henderson, Y., and Haggard, H. W., *J. Biol. Chem.*, 1918, xxxiii, 345.
3. Haggard, H. W., and Henderson, Y., *J. Biol. Chem.*, 1919, xxxix, 163.
4. Christiansen, J., Douglas, C. G., and Haldane, J. S., *J. Physiol.*, 1914, xlviii, 244.
5. Buckmaster, G. A., *J. Physiol.*, 1917, li, 105, 164, p. i.
6. Bohr, C., in Nagel, W., Handbuch der Physiologie des Menschen, Braunschweig, 1909, i, 104.

HEMATO-RESPIRATORY FUNCTIONS.

X. THE VARIABILITY OF RECIPROCAL ACTION OF OXYGEN AND CO_2 IN BLOOD.

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(Received for publication, September 25, 1920.)

The opinion that variations of CO_2 content and of C_H in the blood influence the amount of oxygen absorbed or liberated by hemoglobin has won general acceptance.

If this view is correct, the converse is, on theoretical grounds, extremely probable; namely, that variations of oxygen tension and content influence likewise the capacity of the blood to hold CO_2.

Experiments directed by Ludwig (1) failed to show this effect, however; and Bohr (2) later had a similar negative result.

On the other hand Christiansen, Douglas, and Haldane (3) believe that they have proved the phenomenon; and L. J. Henderson (4) has recently based an extensive theoretical discussion upon their results.

We find, however, that the facts in the matter indicate much more complicated relations than any one has supposed. Thus the following experiments show that one and the same blood may have this property of gaseous reciprocity in marked degree, or may lack it, according to the way the blood has been treated. Our observations suggest that the same treatment which, as shown in Paper IX, induces a lowering of the CO_2 dissociation curve of the irreversible type also induces the capacity for reciprocal action of gases.

It is noteworthy that until recently, and to a large extent even now, most of the studies upon the blood gases have been made with defibrinated blood. In our hands, defibrinated blood commonly shows the property of interaction of oxygen and CO_2, while oxalated blood usually does not.

TABLE I.

Influence of Oxygen in Decreasing the CO_2 Capacity of Defibrinated Blood.

Sample No.	Equilibrating gas mixture.	CO_2 content of blood.		Average.
		vol. per cent		*vol. per cent*
1	Air + 40 mm. CO_2	53	52	52.5
	Hydrogen + 40 mm. CO_2	58	57	57.5
	Nitrogen + 40 " CO_2	58	57	57.5
2	Air + 40 mm. CO_2	50	48	49.0
	Hydrogen + 40 mm. CO_2	54	54	54.0
	Nitrogen + 40 " CO_2	55	54	54.5
3	Air + 40 mm. CO_2	48	46	47.0
	Oxygen + 40 mm. CO_2	47	44	45.5
	Hydrogen + 40 mm. CO_2	54	52	53.0
	Nitrogen + 40 " CO_2	52	53	52.5

For the experiments herewith tabulated fresh arterial dog blood was used. In some cases it was defibrinated; in others it was oxalated. In all cases the samples were equilibrated with the gas mixtures indicated in Tables I and II at a temperature of 40°C. and barometric pressures ranging between 755 and 765 mm.

TABLE II.

In Oxalated Blood the Presence or Absence of Oxygen Does Not Influence the Capacity for CO_2.

Sample No.	Equilibrating gas mixture.	CO_2 content of blood.						Average.
		vol. per cent						*vol. per cent*
1	Air + 40 mm. CO_2	42	43	42				42.3
	Nitrogen + 40 mm. CO_2	42	42	43	43			42.5
2	Air + 40 mm. CO_2	37	39	39	38	39	37	38.2
	Oxygen + 40 mm. CO_2	39	37	39				38.3
	Hydrogen + 40 mm. CO_2	39	36	38	39			38.0
	Nitrogen + 40 " CO_2	38	40	40				39.3
3	Air + 70 mm. CO_2	58	60					59
	Hydrogen + 70 mm. CO_2	56	58					57
	Nitrogen + 70 " CO_2	58	58					58

It will be seen that all the defibrinated bloods show the influence of oxygen in decreasing CO_2-combining power. With the oxalated bloods, on the contrary, none of the variations in the figures obtained lie beyond the range of analytical error.

CONCLUSIONS.

The action of oxygen upon the CO_2 capacity of blood is variable. It commonly occurs in dog blood which has been defibrinated, but not usually in that which has been oxalated. In the light of the results reported in previous papers bearing upon associated processes it appears to us probable that the distinction between the effects of the two methods of treating blood is in some way associated with the irreversible alteration discussed in Paper IX.

BIBLIOGRAPHY.

1. Ludwig., *Wien. med. Jahrb.*, 1865, 169, quoted from Christiansen, Douglas, and Haldane (3).
2. Bohr, C., in Nagel, W., Handbuch der Physiologie des Menschen, Braunschweig, 1909, i, 104.
3. Christiansen, J., Douglas, C. G., and Haldane, J. S., *J. Physiol.*, 1914, xlviii, 244.
4. Henderson, L. J., *J. Biol. Chem.*, 1920, xli, 401.

HEMATO-RESPIRATORY FUNCTIONS.

XI. THE RELATION OF HEMOLYSIS TO ALTERATION OF THE H_2CO_3: $NaHCO_3$ EQUILIBRIUM.

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In several of the previous papers of this series we have commented on the occurrence of hemolysis. From these observations it appears that there is a relation between hemolysis and the irreversible alteration of the blood discussed in Paper IX of this series.

When we first observed this relation we were inclined to think that the explanation might be that laked hemoglobin acts in plasma like an acid. Perhaps it does; but it now seems to us that the evidence on the whole indicates something more fundamental. One or the other, or an interaction of two processes may be involved. One is that excessive decrease of the load of HCl by exposure to abnormally low tensions of CO_2 may have effects within the corpuscles which include increased fragility. The other is that under conditions of abnormally low CO_2 ratio (relatively $H_2CO_3 < NaHCO$) and therefore low C_H there is a strain upon the surface of the corpuscles which tends to cause their rupture.

The matter is important as a possible hint regarding the physical chemistry of the hemolytic incompatibility of some bloods. It is, we think, quite certain that it affords the reason why plasma or serum obtained either by allowing blood to stand or by means of the centrifuge is often tinged with hemoglobin. Hemolysis usually occurs when the surface of the blood has been exposed to the air so that gaseous diffusion acts freely on even a thin layer; but the plasma is generally clear if the surface is protected with oil. In our experience the serum of defibrinated blood is generally

tinged with hemoglobin; and this, we believe, is largely due to the exposure of the blood.

In Table I are summarized the observations which occurred in experiments on the blood of a number of dogs. In each case a sample of arterial blood was oxalated, equilibrated with 40 mm. of CO_2, and analyzed. A second oxalated sample was equilib-

TABLE I.

Relation of Hemolysis to the Irreversible Alteration of CO_2-Combining Power Induced in Blood by a Preliminary Equilibration at an Abnormally Low Tension of CO_2.

Blood No.	First sample.		Second sample.		
	CO_2 content at 40 mm.	Hemolysis.	CO_2 content at 18 mm. and then at 40 mm.		Hemolysis.
	vol. per cent		*vol. per cent*		
1	45	–	32	44	–
2	41	–	25	39	+
3	33	–	24	30	+
4	36	–	20	26	++
5	30	–	21	29	–
6	30	–	19	30	–
7	39	–	26	36	+
8	37	–	24	31	++
9	31	–	23	29	+
10	33	–	20	33	–
11	28	–	20	28	–
12	27	–	19	27	–
13	40	–	32	36	+++
14	39	–	24	36	+
15	37	–	24	30	++
16	31	–	22	30	–
17	33	–	20	33	–
18	28	–	20	28	–
19	46	–	33	44	+
20	41	–	26	32	+

rated first with air containing only 18 mm. of CO_2, then with 40 mm. of CO_2, with analyses at each tension. As will be seen in the table, some degree of hemolysis was observed in more than half of the second samples but in none of the first. It is also to be seen in the table that the "irreversible effect" in reduction of CO_2-combining power occurred in exact parallelism to hemolysis.

Thus whenever the figure in Column 5 was distinctly lower than that in Column 2 hemolysis developed. Whenever the figures were virtually the same in these columns hemolysis was absent.

It is also to be seen in the table that hemolysis and the irreversible alteration of CO_2 capacity are not induced in all bloods at the same CO_2 tension or content. This is probably due to the fact that it is not only the HCl load of the corpuscles but also the previous alkali content of the plasma which determines the conditions, particularly the C_H, induced within and without the corpuscles by a low tension of CO_2 (for instance 18 mm.). If this view is correct, the data in the table suggest that by the methods used in Paper VIII of this series the resistance of the corpuscles to hemolysis of this type may be artificially increased or decreased. We hope to test this idea.

CONCLUSION.

The occurrence of hemolysis in connection with the irreversible alteration of CO_2 capacity induced by exposure of abnormally low tensions of CO_2 is demonstrated.

GASOMETRIC DETERMINATION OF NITROGEN AND ITS APPLICATION TO THE ESTIMATION OF THE NON-PROTEIN NITROGEN OF BLOOD.

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(Received for publication, September 25, 1920.)

A number of reactions between sodium hypobromite and various nitrogenous substances are known in which nitrogen is liberated as a gas. Among this number are the reactions with urea, ammonium salts, uric acid, creatine, etc. A method for the quantitative estimation of the first named has been based upon the reaction in point, though it has now been discredited to a large extent. As far as the writer is aware the reaction between sodium hypobromite and ammonium salts has never been utilized in the quantitative determination of nitrogen. According to Krogh[1] this reaction falls short of being quantitative as in the case of urea. She found 97.5 per cent of the nitrogen of ammonium chloride to be liberated by sodium hypobromite and obtained a qualitative test for nitric acid in the solution after the reaction.

The experiments carried out heretofore have been performed at ordinary pressures. Some time ago it occurred to the writer that it would be of interest to carry out several of the above reactions in the apparatus devised by Van Slyke[2] for the determination of the carbon dioxide content of blood plasma; that is, *in vacuo*. The urea reaction was tried first and the outcome was clear-cut to the extent that this is possible with an apparatus of the capacity of that in question. Instead of a smaller quantity of nitrogen which is obtained when the reaction occurs at

[1] Krogh, M., *Z. physiol. Chem.*, 1913, lxxxiv, 379.

[2] Van Slyke, D. D., *J. Biol. Chem.*, 1917, xxx, 347.

atmospheric pressure, trial showed that the theoretical quantity of nitrogen is liberated. It was observed incidentally that the quantity of carbon dioxide produced is also in accordance with theory. Ammonium sulfate was then substituted for urea and again the theoretical quantity of nitrogen was obtained. Whether Krogh's results were due to the concentration or pressure relations obtaining in her experiments is not elucidated here, nor is the result in the case of the urea reaction, but the analyses submitted in this communication show that the yield of nitrogen obtained in the Van Slyke apparatus is quantitative. Inasmuch as in the Kjeldahl method for total nitrogen the first step is the conversion of the nitrogen of the sample into ammonium sulfate, we have in the hypobromite method an alternative procedure for the long distillation process of the Kjeldahl method, which is considerably shorter and requires no standard solutions. As described here, using the ordinary Van Slyke apparatus, the method does not have the accuracy obtainable by the Kjeldahl method. This is due to the impossibility of reading the volume of nitrogen with so high a degree of precision as one might wish. Enlarging the apparatus would help, but whether this is feasible the author has not attempted to decide. However, the accuracy obtainable with the ordinary apparatus is ample for many purposes.

The procedure is as follows. The sample is digested with as small a quantity of sulfuric acid as possible and a small crystal of copper sulfate. After cooling, it is diluted and the solution made up to some definite volume, for instance 100 cc. A definite quantity, preferably not more than 10 cc., is then placed in the apparatus. The dissolved air is liberated by subjection to a vacuum and expelled. Strong alkali sufficient to neutralize the sulfuric acid is run into the apparatus followed by 2 cc. of the hypobromite solution.[3] The pressure in the reaction chamber is reduced and after shaking for about a minute the liberation of nitrogen is complete. Apparently, if the hypobromite is present in more than a slight excess, there is some liberation of oxygen. In order to eliminate the error which would result from its pres-

[3] Two stock solutions are prepared. One contains 28 gm. of sodium hydroxide per 100 cc.; the other 12.5 gm. of sodium bromide and 12.5 gm. of bromine per 100 cc. For use one volume of each and three volumes of water are mixed.

ence, 1 cc. of a sodium pyrogallate solution (5 gm. of pyrogallate acid in 100 cc. of 28 per cent sodium hydroxide) is run into the reaction chamber as soon as the decomposition of ammonium sulfate is complete. After the oxygen has been absorbed the solution is freed from dissolved gases and is then lowered into the proper chamber whereupon the nitrogen volume is ready to be measured. This can be done so that the contained gas is at atmospheric pressure or by making the volume exactly 1 cc. and then measuring the pressure conditions necessary to accomplish this. A somewhat higher degree of accuracy appears to be obtainable by the latter procedure. It has the advantage that one is adjusting the meniscus at a set mark instead of estimating between marks, and, what is more important, one is always using the maximum capacity of the apparatus. Since the error of reading the volume is the same at any point, it is obvious that the error of any determination introduced by this cause will be smaller the greater the volume measured. On the other hand, if the quantity of gas is so small that the pressure must be reduced to 40 or 50 mm. Hg, for example, then small errors made in estimating the pressure introduce a relatively large error, though perhaps not so large then as when the volume is read at atmospheric pressure. Correction must be made for the nitrogen content of the hypobromite solution. This is approximately 0.009 cc. (at 760 mm. Hg and 0°) per 1 cc. of solution. The nitrogen content of the strong alkali and pyrogallate is negligible.

The quantity of nitrogen dissolved in the final solution is worthy of consideration. If the quantity of solution amounted to 10 cc. and the amount of nitrogen liberated was about 1 cc., then, since the capacity of the apparatus is 50 cc., the minimum nitrogen pressure obtainable in the apparatus would be $\frac{1}{40}$ of an atmosphere. 1 cc. of water dissolves 0.01639 cc. of nitrogen at 20° and 760 mm. pressure. At $\frac{1}{40}$ of an atmosphere the amount would be $\frac{1}{40} \times 0.01639 = 0.00041$ cc. per 1 cc. of water, or 0.0041 cc. per 10 cc. The solvent here is not pure water, however, but a rather strong solution of sodium sulfate, sodium bromide, and sodium pyrogallate in which the nitrogen solubility is considerably less than in pure water. The exact concentration is a variable quantity and of course no solubility data exist which meet the present demand. If it is assumed that a 10 per cent solution

of sodium sulfate is approximately correct, then the correction per 10 cc. would be about 0.002 instead of 0.0041 cc. This may be 50 per cent from the true value without introducing an error greater than 0.1 per cent into the final result. If the volume of gas measured is 0.5 cc. instead of 1 cc., then the correction just suggested is halved. According to the accuracy desired the solubility factor may be taken into account or neglected.

The results obtained on various substances are given in Table I.

TABLE I.

Substance.	Found.	Theory or Kjeldahl.
	cc.	cc.
Sodium sulfanilate	5.92	6.07
" "	6.04	6.07
" "	6.15	6.07
" "	6.15	6.07
" "	6.15	6.07
Casein	13.6	13.9
"	13.7	13.9
"	13.8	13.9
"	13.8	13.9
"	13.8	13.9
Uric acid	32.5	32.4
" "	32.7	32.4
" "	32.8	32.4
" "	33.6	32.4
Dried milk	5.37	5.35
" "	5.42	5.35
Urea	46.5	46.7
"	46.7	46.7

The method for the determination of total nitrogen just described may be applied to the determination of the non-protein nitrogen of blood. The tungstic acid precipitation method described by Folin and Wu[4] for obtaining a protein-free blood filtrate is well adapted for the present method. 5 or 10 cc. of the filtrate are digested with 0.5 cc. of nitrogen-free sulfuric acid and 1 drop of a 5 per cent copper sulfate solution. Small Kjeldahl flasks (30 cc.), with necks bent almost at right angles about $1\frac{1}{2}$ inches from the end, serve well for the digestion. They are heated

[4] Folin, O., and Wu, H., *J. Biol. Chem.*, 1919, xxxviii, 81.

in a Bunsen flame before use to reduce bumping. If the heat from the microburner used in the digestion is controlled by hand manipulation, there is little danger of loss of material. After the contents of the flask have charred, the burner may be set under the flask with the flame reduced. At the first sign of an ominous stillness in the flask a piece of quartz sand is dropped in, and active though gentle boiling immediately occurs. This process is repeated as often as necessary until the digestion is complete. Digesting with the sulfuric-phosphoric acid mixture recommended by Folin and Wu hastens the process, but the method has disadvantages which outweigh the 2 or 3 minutes saved. After cooling the digest, it is diluted with 4 or 5 cc. of water and the sulfuric acid is partially neutralized with strong alkali. This is done merely to save the generation of unnecessary heat in the apparatus. One can determine how much strong alkali (55 per cent) is required for 0.5 cc. of sulfuric acid, and add about two-thirds of this quantity. The solution is again cooled and then transferred to the apparatus. The volume of liquid should be about 10 cc. (It is desirable, of course, to keep the volume as small as possible but if sufficient water is not present sodium sulfate crystals will form.) Dissolved air is then eliminated in the usual manner. If this is not well attended to a relatively large error will be introduced in the case of a blood normal with respect to its quantity of non-protein nitrogen inasmuch as the amount of gas liberated is small. Sufficient alkali to complete the neutralization of the sulfuric acid is now run into the vacuum chamber followed by 1 cc. of the hypobromite solution described. The apparatus is immediately evacuated and shaken for about a minute. As soon as the reaction is complete 1 cc. of pyrogallate solution is admitted and the apparatus is again shaken to insure the absorption of any oxygen. As a rule the heat of neutralization is sufficient to warm the apparatus several degrees. It is necessary, therefore, to allow it to stand for a time before reading the volume of nitrogen or it may be immersed in a cylinder of water for a few minutes. After the gas is at room temperature the volume is measured in the usual manner. If the blood were a normal specimen the volume of gas obtained from 5 cc. of filtrate would be in the neighborhood of 0.20 cc. The device of making the volume 1 cc. arbitrarily is

appropriate here. In reducing the volume to standard conditions substitution in the ordinary gas formula is suitable for an occasional determination, but for routine work the tables given in Landolt-Börnstein-Roth's "Tabellen" are a great convenience.

TABLE II.

Non-Protein Nitrogen, per 100 Cc. Blood.

Hypobromite method.	Kjeldahl method.
mg.	*mg.*
39.5	41.8
39.3	39.3
38.7	36.0
38.3	41.9

Results are easily duplicated by the method just described and to some who have difficulties with colorimetric methods the choice of a method based upon a perfectly clear-cut chemical reaction may be welcome. The results have been checked by the Kjeldahl method (Table II), though this could hardly be considered essential in view of the results given in Table I.

RELATION OF FODDER TO THE ANTISCORBUTIC POTENCY AND SALT CONTENT OF MILK.*

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(Received for publication, October 26, 1920.)

In 1914 Funk (1) raised the question as to whether milk varies in its vitamine content according to the fodder of the cow. Since this time several other investigators have referred to an interrelationship of this nature, notably Barnes and Hume (2) in a recent paper on the antiscorbutic content of milk. In an article treating of the fat-soluble vitamine, we suggested an insufficiency of this dietary factor in milk as the result of the winter fodder of cows (3). Indeed, in view of the probability that the animal organism is incapable of synthesizing vitamines, and is dependent on the food for its supply, one should expect the milk to be greatly affected by the dietary intake. The first experimental proof of the existence of this interrelationship has been furnished recently by the work of Hart, Steenbock, and Ellis (4). These investigators conclusively show that milk secreted by cows receiving dry fodder contains far less antiscorbutic vitamine than milk secreted by cows on summer pasture. The herd which they tested had never been given any "fresh green vegetable tissue," having been fed continuously on "air-dried roughages and grains." The summer pasture milk was derived from cows which grazed part of the day on grass and clover.

Our investigation, which was undertaken last spring, differs from that of Hart, Steenbock, and Ellis mainly in a delimitation of the duration of the feeding periods, and in the inclusion of a chemical examination of the two varieties of milk. The plan of the experiment was as follows: Five grade Holstein cows which had been freshened about 2 months previously were employed

* Presented before the Society of Experimental Biology and Medicine, New York, October 20, 1920.

for the entire test. These cows had been stall-fed throughout the winter on a normal winter ration of ensilage, hay, and concentrates. Beginning April 30th, 1920, and for 21 days thereafter, the cows were fed on a daily ration of 25 pounds of a concentrate mixture which consisted of one part of bean meal, two parts of oil meal (flaxseed extracted by pressure), two parts of hominy, two parts of gluten meal, and two parts of bran; they received also 8 pounds of kiln-dried beet pulp, 4 quarts of molasses, and 12 pounds of straw. The object of this dietary was to provide a food which was ample in all respects excepting in its quota of antiscorbutic vitamine. For the first few days the cows manifested a marked loss of appetite, but thereafter they consumed practically the entire amount of the dry ration. They had produced, prior to this experimental feeding, an average of about 40 pounds of milk per day. At first this change in fodder resulted in an average decrease in milk production of about 10 pounds per cow per day, but later the animals became more accustomed to the ration, so that at the end of the 21 day period there was an average decrease in milk flow of only 5 pounds per day. The general health of the cows had not been maintained. After 3 weeks of this antiscorbutic-free ration a mixed sample of milk from all five cows was collected and dried by means of the Just roller process, by which it is subjected to about 230° F. for a few seconds.

The cows were then placed on pasture for 3 weeks, a small amount of concentrate being given, in addition, for the first few days only. After 3 weeks pasturage, a day's milking of the five cows was collected and dried. Throughout this second period, excepting for the first few days, the cows received only fresh grass. The physical condition of three of the cows showed marked improvement, but two did not respond so quickly to the change in fodder. The production of milk did not increase materially during this period of pasturage, and at its close was still 5 pounds less per cow per day than before the concentrated feeding had been instituted.

The object of using dried rather than fluid milk for this experiment was that it permitted a test of both varieties of milk to be carried out synchronously and under identical conditions. It is evident that only by this means could dry fodder milk and pas-

ture milk from the same cows be tested at one and the same time. Furthermore, it made it possible to define sharply a feeding period—3 weeks—and thereby to judge the effect on the quality of the milk of a dietary given during this limited period. Unless dried milk were used, the pasture milk would represent a product varying according to the ever lengthening time that the cows had been grazing, whereas by using dried milk the entire quantity was obtained from 1 day's milking just 3 weeks after dry fodder had been discontinued.

The milk was kept tightly sealed in tin containers until required, and was then diluted with water to its original water content. Guinea pigs were fed the equivalent of 80 cc. *per capita* daily of fluid milk, in addition to oats and bran. This amount of milk was selected because previous tests with dried milk had shown that it contains what may be termed the borderline protective dose of antiscorbutic vitamine, and that when guinea pigs are given more than this quantity they fail to develop scurvy. Every animal received its full quota daily, supplementary hand feeding being resorted to when necessary.

Charts 1 and 2 illustrate the results of two sets of feeding tests. It is seen that all the guinea pigs fed with the dry fodder milk developed scurvy within a period of 21 days, and died of scurvy within 56 days. On the other hand, the animals which received pasture milk are still alive after more than twice this interval—120 days—two showing mild signs of scurvy. The contrast is striking, and definitely illustrates the marked difference in antiscorbutic content of the two varieties of milk.

When we bear in mind that this marked alteration in the quality of the milk took place in cows which had been on pasture but 3 weeks, it is evident that the antiscorbutic vitamine of the food is rapidly secreted into the milk by the lacteal glands, even in the extreme instance where the body has been absolutely starved of this vitamine. The tissues do not seem to replenish their deficiency of this vitamine or to hoard it before excreting any from the body by way of the milk. This result bears out some unpublished experiments which showed that guinea pigs are unable to store the antiscorbutic vitamine, that even though their diet has contained it in large excess such provision does not protect them from a subsequent period of antiscorbutic vita-

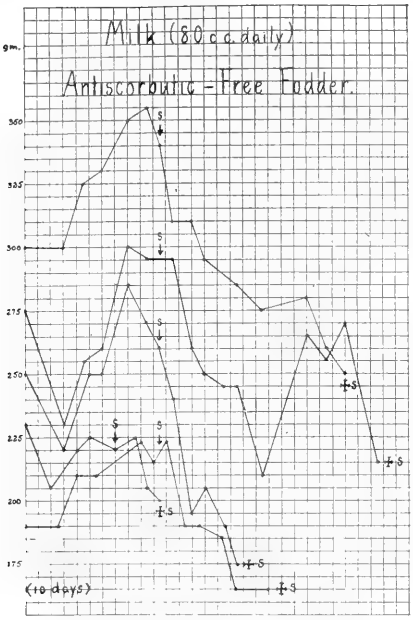

CHART 1. Guinea pig feeding experiment. The cows producing this milk had been fed on fodder containing almost no antiscorbutic vitamine for a period of 3 weeks.

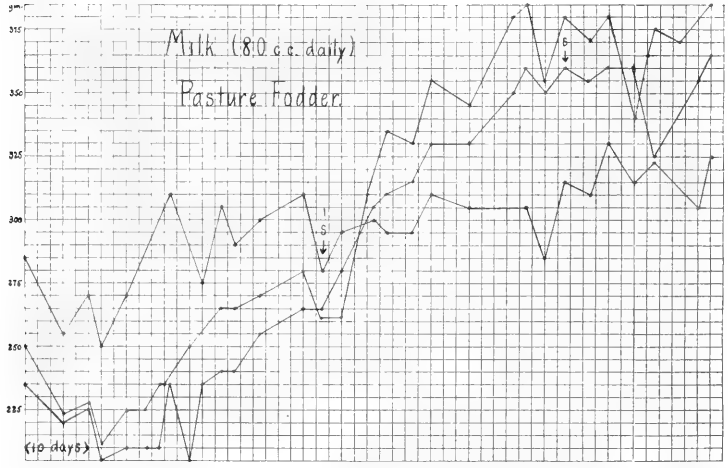

CHART 2. Guinea pig feeding experiment. Milk from the same cows as in Chart 1 after they had been pasture-fed for a period of 3 weeks.

mine starvation. In other words, these animals seem to be carrying on a precarious hand-to-mouth existence in relation to this essential dietary factor.

The feeding test with dry fodder milk demonstrates that it contained but little antiscorbutic vitamine. If we compare this result with that obtained when guinea pigs are fed an equivalent amount of milk which has been autoclaved at 110°C. for 1 hour in order to destroy its antiscorbutic vitamine, we find that there is little difference in the length of time it takes for scurvy to develop; the sole advantage enjoyed by the guinea pigs fed on the dry fodder milk is a somewhat longer duration of life. Such a result must be interpreted as indicating that at the end of the first stage of our test the cows were secreting milk which was almost devoid of the antiscorbutic vitamine, and that when the supply of this dietary factor is entirely cut off, the milk is rapidly depleted of its antiscorbutic content. It should be remembered, however, that the feeding conditions were exceptionally rigid, so severe as to be rarely encountered under natural circumstances in man or in animal.

Table I shows the composition of the milk as affected by the two kinds of fodder. A composite sample of the liquid milk from all five cows was used for the analyses, with the exception of the lecithin determination, for which dried milk was employed. A study of the table shows that the fat, protein, and sugar percentages were very similar, but that there were certain well defined differences in the composition of the ash of the two milks. Passing over minor variations, it is seen that the percentages of calcium and of phosphorus were significantly higher in the pasture milk and that its citric acid content was over 50 per cent greater.[1] On the other hand there was less sulfur in this milk, in spite of its somewhat higher protein content.

The percentages of calcium and of phosphorus in the antiscorbutic-free milk are below the normal; those of the pasture milk are within normal limits. The question of a causal relationship naturally suggested itself between the lack of antiscorbutic vitamine and the decreased excretion of these salts

[1] Citric acid was determined by the Bacon and Dunbar method (Bacon, F., and Dunbar, P. B., *U. S. Dept. Agric., Bureau of Chemistry, Circular 78*, 1911).

into the milk. A conclusive answer to this question must be postponed, however, until further data are available.

The increase in citric acid may be explained in part by the greater amount of citrates contained in the green fodder, but gains added interest in view of the close association between citric acid and many of the most potent antiscorbutic foodstuffs. In view of the marked variation of this constituent in the two

TABLE I.

Composition of Milk as Affected by Fodder.

	Antiscorbutic-free fodder.	Pasture.
	per cent	*per cent*
Water	88.38	88.19
Total solids	11.62	11.81
Fat	3.37	3.44
Total proteins (N × 6.38)	2.82	2.93
Casein (N × 6.38)	2.28	2.32
Albumin (N × 6.38)	0.42	0.49
Extractive nitrogen	0.02	0.02
Lecithin	0.069	0.7
Lactose	4.73	4.56
Citric acid	0.08	**0.13**
Ash	0.606	0.670
Potassium oxide	0.150	0.157
Sodium "	0.051	0.056
Calcium "	0.138	**0.165**
Magnesium "	0.009	0.005
Iron "	0.0001	0.0002
Phosphorus pentoxide	0.158	**0.190**
Sulfur trioxide	**0.023**	0.014
Chlorine	0.054	0.097

varieties of milk, normal figures which have been established without regard to the fodder of the cows must be regarded as incomplete (5). In fact a lesson to be derived from this chemical examination of antiscorbutic-free and of pasture milk is the danger of setting up rigid standards for milk constituents, and the necessity of prefacing such results with full data as to the diet of the lactating animal.

BIBLIOGRAPHY.

1. Funk, C., Die Vitamine, Wiesbaden, 1914.
2. Barnes, R. E., and Hume, E. M., *Biochem. J.*, 1919, xiii, 306; *Lancet*, 1919, ii, 323.
3. Hess, A. F., and Unger, L. J., *J. Am. Med. Assn.*, 1920, lxxiv, 217.
4. Hart, E. B., Steenbock, H., and Ellis, N. R., *J. Biol. Chem.*, 1920, xlii, 383.
5. Sommer, H. H., and Hart, E. B., *J. Biol. Chem.*, 1918, xxxv, 313.

CREATININE AND CREATINE IN THE BLOOD.

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(Received for publication, October 26, 1920.)

Few figures have been published for the creatinine and creatine in the blood of normal women. Hunter and Campbell (1) make the tentative suggestion that blood creatinine is lower for women than for men, but all the women they observed were either in late stages of pregnancy or were convalescent after childbirth, and were therefore very inactive. They also conclude that the creatinine is "lower in subjects deprived of exercise than in those leading an active life." It may be, therefore, as they themselves suggest, that the inactivity rather than the sex of the subjects is the cause of the difference. Their average plasma creatinine for forty-three males is 1.04 mg. per 100 cc. and for seventeen females 0.87 mg. Plass (2) also gives lower figures for creatinine concentrations in women than in men—1.0 to 1.2 mg. and 1.3 to 1.5 mg. per 100 cc. respectively—but he does not give a detailed report on this investigation.

Creatine, on the other hand, according to Hunter and Campbell (1) seems to be higher in the blood of females than of males, 6.81 mg. on the average (uncorrected) as opposed to 5.57 mg. per 100 cc. It is also higher in women in late stages of pregnancy than in normal women (3).

Considerable variation in creatine is to be expected both from individual to individual and in the same individual from day to day. The creatinine is a much less variable quantity. Hammett's (4) recent series of weekly analyses of blood from the same individuals shows this difference plainly. He divides the various blood constituents into three groups according to the amount of variation from day to day in the same individual. Creatinine is in the first group, the least variable of all; and creatine in the

second group, more variable than creatinine, total non-protein nitrogen, and total nitrogen, and less variable than uric acid, amino-acid, and urea nitrogen. Some of Hammett's subjects were women but he does not say which figures belong to the women and which to men.

TABLE I.

Experimenters.	Creatinine per 100 cc.	Creatine per 100 cc.
	mg.	*mg.*
Folin and Denis (5)	1.1 –1.4	5.2 – 8.4
Myers and Fine (6)	1.0 –2.0	5.0 –10.0
Gettler and Baker (7)	0.1 –0.5	3.0 – 6.5
Feigl (8, 9)	<1.0	
	>2.5	
Hunter and Campbell (1)	0.7 –1.3	2.7 – 3.5
		Average about 3.0.
Pemberton and Buckmann (10)	0.6 –1.3	3.7 – 5.0
Hammett (4)	1.08–1.67	3.00– 6.21

It is not surprising, therefore, that the many published figures for creatinine should be in fairly close agreement (with the exception of those of Gettler and Baker (7)) and that the creatine figures should show considerable variation. Also the older method for creatine was much more faulty than that for creatinine. Table I summarizes most of the published creatinine and creatine figures, the great majority of them being for men.

No work has been done on variation of creatinine and creatine in blood during menstruation. That such variation might be expected could be anticipated by the work of Krause (11) and that of Rose (12) on urine. The results of the former show that on a creatine- and creatinine-free diet creatine is invariably present in the urine after menstruation, while the latter states that the creatine in urine tended to disappear during menstruation in a number of the cases studied, though in most cases no regularity was observed.

It therefore seemed desirable to obtain more figures on the blood creatinine and creatine of normal women and especially to determine whether there is any regular variation during menstruation.

EXPERIMENTAL.

For our experiments we had as subjects twenty-four apparently normal women students or faculty members ranging in ages from 20 to 41 years and following the ordinary University regime with a good deal of laboratory work. They were, therefore, decidedly more active than those who served Hunter and Campbell as subjects.

TABLE II.

Intermenstrual and Menstrual Creatinine and Creatine per 100 Cc. of Blood.

Subject.	Age.	Preformed creatinine.		Creatine as creatinine.	
		Intermenstrual.	Menstrual.	Intermenstrual.	Menstrual.
	yrs.	*mg.*	*mg.*	*mg.*	*mg.*
E. V.	20	1.17	1.58	4.65	3.77
Z. T. W.*	21	0.96	1.20	3.45	3.22
B. W.	21	1.38	1.10	4.05	2.68
H. K.	22	1.36		2.23	
E. M.	22		1.42		2.56
B. W.	24	1.10		3.30	
P. R.	24	1.62	1.55	2.81	2.89
M. A. P.†	26	1.22	1.11	2.90	3.38
W. S.	26	1.10	1.13	2.80	2.45
L. M. D.†	28		1.37		3.34
M. O. L.	28	1.00	1.38	2.28	2.35
R. M.	28	1.33	1.33	3.08	2.99
G. B.	28	1.07	1.65	3.22	3.29
A. N.	28	1.18	1.36	3.20	3.14
S. W.	29		1.46		3.12
J. C.	33	1.39	1.36	3.35	4.01
M. M.	34	1.56	1.02	3.07	2.64
L. K.	36	1.27		3.23	
L. K.	36	1.01		4.52	
L. D.	37	1.27	1.35	2.80	3.19
R. L. R.	40	1.39	1.50	3.12	4.09
F. R.	41	1.28	1.34	3.47	3.74
Average		1.25	1.35	3.24	3.16

* Menstrual specimen 2nd day of period.

† Irregular menstrually; 3 weeks overdue.

The blood specimens were drawn on the first day of the menstrual period, if possible, but in a few instances on the second day (exceptions which are listed in Table II), and a second speci-

men during the intermenstrual period. No definite regularity as to the hour for drawing the samples was followed but in all but a very few cases the time was between 11 a.m. and 12 m. or late in the afternoon; that is, 3 or 4 hours after the last meal. The diet of the subjects was not controlled, but their general habit was to live on a rather low protein level.

In determining the creatinine and creatine Folin and Wu's (13) new method was exactly followed. The picric acid was purified according to the method of Folin and Doisy (3). The creatinine for the standard, obtained from the Central Scientific Company, was tested for purity by recrystallization from alcohol and comparison of the recrystallized and original substances in the colorimeter. No difference was observed.

The results of the analyses are given in Table II.

Discussion of Results.

We find no regular variation in the creatinine and creatine during menstruation. The average of all results for creatinine for the intermenstrual period is 1.25 mg., while for the menstrual period the average is 1.35 mg., an increase of only 7.4 per cent. For the creatine, we find an average of 3.24 mg. during the intermenstrual period and of 3.16 mg. during menstruation, which is a decrease of 2.5 per cent.

Of the fifteen individual cases with both menstrual and intermenstrual observations, nine show higher creatinine during the menstrual period, five lower, and one no difference. The greatest difference amounts to 0.58 mg. For creatine, seven show lower figures during menstruation and eight higher.

Thus, while the figures seem to show an occasional tendency toward increased creatinine and decreased creatine during menstruation, the proportion of the cases showing this tendency is not high enough to justify any definite conclusion. It is safer to conclude that the variation is not regular and is no greater than expected from day to day.

It is of interest to compare our figures with those of other investigators, summarized in Table I. Our ranges for creatinine, 0.96 to 1.65 mg., and for creatine, 2.23 to 4.65 mg., are within the limits of other investigators. All but five of our creatinine results fall within the limits set by Hammett (4). Our creatine

results are lower than his. Thirteen out of thirty-seven of our figures are below his lower limit and none reaches his upper limit, 6.21 mg.

Our results thus fail to substantiate the suggestion of Hunter and Campbell (1), that the creatinine appears in lower concentration in the blood of women than of men.

Like Hunter and Campbell (1) we fail to find figures in agreement with work by Feigl (9) indicating an increase of creatinine with age. Feigl worked with a large group of people ranging in age from under 10 years up to 80 years. We have only a small number falling in any of the three decades represented, and it is impossible to draw any definite conclusion that there is a greater concentration with increased age.

Influence of Sugar Diet on Creatinine and Creatine Determination.

We also had as a secondary problem the influence of sugar on blood creatinine and creatine. We made analyses on the blood of ten women on a normal diet, usually from 1 to 2 hours after a meal, and, again on a different day, 1 hour after the subject had eaten 70 gm. of sugar. Table III shows the comparative results.

TABLE III.

Results on High Sugar Diet per 100 Cc. of Blood.

Subject.	Age.	Preformed creatinine.		Creatine as creatinine.	
		Low sugar.	High sugar.	Low sugar.	High sugar.
	yrs.	*mg.*	*mg.*	*mg.*	*mg.*
B. W.	24	1.10	1.41	3.30	4.25
J. C.	33	1.39	1.59	3.35	3.67
H. K.	22	1.36	1.21	2.23	3.40
A. N.*	28	1.18	1.57	3.20	
L. K.	36	1.01	1.28	4.52	3.80
L. D.	37	1.27	1.54	2.80	2.78
E. V.	20	1.17	1.49	4.65	4.55
W. S.†	26	1.10	1.31	2.80	2.40
E. D.	22	1.48	1.56	3.81	3.72
L. G.†	22	1.03	1.10	3.45	4.36
Average		1.21	1.41	3.41	3.66

* High sugar taken on 2nd day of menstruation.
† " " " " 1st " " "

Discussion of Table III.

On the high sugar, with one exception, the creatinine was higher than without sugar. The greatest increase is 0.39 mg., and the least 0.07 mg. The average for all shows 1.21 mg. of creatinine on the normal diet and 1.41 mg. on the high sugar; an increase of 0.20 mg. or 17 per cent.

The creatine readings were not so noticeably influenced, probably because the possible increase due to glucose was masked by the usual day by day variation. The average without the sugar was 3.41 mg. and with the sugar was 3.66 mg.

SUMMARY AND CONCLUSIONS.

1. Observations were made on the creatinine and creatine of the blood of twenty-four normal women, fifteen of them being observed during both intermenstrual and menstrual periods.

2. No regular variation was found during menstruation for either creatinine or creatine.

3. In nine cases there was a slight creatinine increase during menstruation and in five a decrease. On the other hand, seven cases showed lower creatine during menstruation and eight higher.

4. The range for creatinine in the different subjects was from 0.96 to 1.65 mg. per 100 cc. The average content was found to be 1.30 mg. This falls within the range observed by other investigators, chiefly on men, and fails to substantiate the suggestion that blood creatinine is lower in women.

5. The range for creatine concentration is from 2.23 to 4.65 mg. per 100 cc., which is slightly lower than the range observed by other investigators.

6. No relation was observed between the age of the subject and increased creatinine.

7. The accuracy of creatinine determinations may be affected by high sugar diet, through the additional development of color by glucose.

The authors wish to thank Dr. Katharine Blunt for her helpful suggestions and her untiring assistance, and other members of the department for their interest in this work and their cooperation in acting as subjects.

BIBLIOGRAPHY.

1. Hunter, A., and Campbell, W. R., *J. Biol. Chem.*, 1918, xxxiii, 169.
2. Plass, E. D., *Bull. Johns Hopkins Hosp.*, 1917, xxviii, 137, 297.
3. Folin, O., and Doisy, E. A., *J. Biol. Chem.*, 1916–17, xxviii, 349.
4. Hammett, F. S., *J. Biol. Chem.*, 1920, xli, 599.
5. Folin, O., and Denis, W., *J. Biol. Chem.*, 1914, xvii, 487.
6. Myers, V. C., and Fine, M. S., *Post-Graduate*, 1915, xxx, 39.
7. Gettler, A. O., and Baker, W., *J. Biol. Chem.*, 1916, xxv, 211.
8. Feigl, J., *Biochem. Z.*, 1917, lxxxiv, 264.
9. Feigl, J., *Biochem. Z.*, 1918, lxxxvii, 1.
10. Pemberton, R., and Buckmann, T. E., *Arch. Int. Med.*, 1920, xxv, 335.
11. Krause, R. A., *Quart. J. Exp. Physiol.*, 1911, iv, 293.
12. Rose, M. S., *J. Biol. Chem.*, 1917, xxxii, 1.
13. Folin, O., and Wu, H., *J. Biol. Chem.*, 1919, xxxviii, 81.

STUDIES OF THE DISTRIBUTION OF CARBON DIOXIDE BETWEEN CELLS AND PLASMA.*

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(Received for publication, November 9, 1920.)

The manner in which CO_2 is transported from the tissues to the lungs by the blood has recently been the subject of considerable discussion (1–3). To throw further light on this sufficiently fundamental problem we have undertaken a study of the distribution of CO_2 between the cells and the plasma of arterial and venous blood both in normal individuals, and in various pathologic states.

Methods.

Samples of blood were drawn from the arm vein or artery (without stasis), under oil, and analyses were made of the CO_2 content both of the whole blood and, after centrifugation, of the plasma, by the method and with the apparatus of Henderson (4, 5). The blood was prevented from clotting by the addition of a few dry potassium oxalate crystals. All the analyses were made in duplicate.

At the same time the proportion of cells to plasma in each sample was determined by means of the hematocrit, and the relative amounts of CO_2 in the cells and in the plasma of a unit of blood were calculated therefrom. For example if the CO_2 content of a sample of whole blood is 50 volumes per cent and if that of the plasma of the same sample is 62 volumes per cent

* This paper is Study No. 7 in a series of studies of the physiology and pathology of the blood from the Harvard Medical School and allied hospitals. The expenses were in part defrayed by the Proctor Fund. A preliminary report on this work was read at the Annual Meeting of the American Society for Clinical Investigation, Atlantic City, New Jersey, May 3, 1920.

and if the hematocrit shows that the proportion of cells to plasma in that sample is as 40 : 60, then in 100 volumes of whole blood 37.2 volumes will be contained in the plasma thus

$$\frac{60}{100} \times 62 = 37.2$$

and 12.8 volumes will be contained in the cells thus

$$50 - 37.2 = 12.8$$

All the hematocrit observations were made in duplicate.

Findings in Normal Persons.

We first made a study of the distribution of CO_2 between the cells and plasma of normal venous blood. The average results are shown in Table I. The average CO_2 content of eighteen

TABLE I.

Average Figures for Venous Blood.

Series.	CO_2 in whole blood.	CO_2 in plasma.	CO_2 in plasma of a unit of blood.	CO_2 in cells of a unit of blood.
	vol. per cent	*vol. per cent*	*vol. per cent*	*vol. per cent*
Normal. 18 bloods from 13 subjects.				
Maximum	62	64	40.8	26.1
Average	57	61	36	21
Minimum	53	58	32.5	15.4
Anemia. 14 bloods from 14 patients.				
Maximum	64	66	60.0	10
Average	58	60	53	5
Minimum	48	48	42.2	1.4

samples of venous blood from thirteen normal persons was 57 volumes of CO_2 per 100 volumes of whole blood and, of these 57 volumes of CO_2, 36 volumes were in the plasma and 21 in the cells.

We next made a study of the differences in CO_2 distribution in arterial and venous blood. For this purpose samples of blood were drawn within a few minutes of each other from the arm vein and the radial artery. The results of these observations are given in Table II.

The average CO_2 content of eight normal arterial whole bloods was 50.4 volumes per cent and of the venous 58.7 volumes per cent. The parallel findings on the plasma were arterial 61.0 and venous 62.5 volumes per cent. These findings are similar to those in the literature. Thus Harrop (6) in ten persons with normal hearts and lungs found an average of 49.7 volumes per cent of CO_2 in the arterial and 54.7 volumes per cent in the venous blood. Peters and Barr (7) in three normal subjects found an average of 50.5 volumes per cent in the arterial and 59.1 volumes per cent in the venous whole blood. Our normal venous-arterial whole blood difference thus was 8.3, Harrop's 5.0, and that of Peters and Barr 8.6 volumes per cent.

A series of arterial and venous plasma CO_2 contents has been published by Stadie and Van Slyke (8). The average figures for thirty-one samples from twenty-one patients[1] were arterial plasma 60.4 and venous plasma 63.6 volumes per cent. These observations were chiefly on pneumonia patients with arterial anoxemia of varying grades, but some were on normal persons. Even so their figures are quite like our normal plasma figures, though their venous-arterial plasma difference is 3.2 volumes per cent as against ours of 1.5 volumes per cent. It is not shown in their table which are the normal subjects, but if we take those who had an arterial oxygen unsaturation of less than 8 volumes per cent we find the average venous plasma CO_2 content to be 67.0 and the arterial 63.5 volumes per cent making the venous-arterial plasma difference 3.5 volumes per cent.

When on the basis of the hematocrit observations we study the distribution of the CO_2 content of arterial and venous whole blood between the cells and the plasma, we get the interesting averages of 35.8 volumes of CO_2 in the plasma of 100 volumes of arterial blood, and 36.5 volumes in that of venous blood. At the same time the cells of 100 volumes of arterial blood contain on an average 14.6 volumes of CO_2 while those of venous blood contain 22.2 volumes.

In other words as blood passes from artery to vein, it gains on an average 8.3 volumes per cent of CO_2, but of this less than 1 volume per cent, 0.7 volume per cent to be exact, is gained by

[1] In these averages we have taken from their table only cases in which arterial and venous determinations were made.

TABLE II.

Observations on Arterial and Venous Blood.

Subject.	Observed.								Calculated.						Remarks.
	Hematocrit.				CO_2				CO_2				CO_2 of V plasma minus CO_2 of A plasma.	CO_2 of V cells minus CO_2 of A cells.	
	Cells.		Plasma.		Whole blood.		Plasma.		Plasma of a unit of whole blood.		Cells of a unit of whole blood.				
	A*	V*	A	V	A	V	A	V	A	V	A	V			
	per cent	*per cent*	*per cent*	*per cent*	*vol. per cent*	*vol. per cent*	*vol. per cent*	*vol. per cent*	*vol. per cent*	*vol. per cent*	*vol. per cent*	*vol. per cent*	*vol. per cent*	*vol. per cent*	
Dr. S.	42	42	58	58	50	56	62	62	35.9	35.9	14.1	20.1	0.0	6.0	Normal subject.
Miss M.	37	39	63	61	46	56	60	62	37.8	37.8	8.2	18.2	0.0	10.0	" "
Dr. M.	45	43	55	57	54	60	62	63	34.1	35.9	19.9	24.1	1.8	4.2	" "
" B.	44	44	56	56	48	56	58	60	32.4	33.6	15.6	22.4	1.2	6.8	" "
" S.	40	40	60	60	50	58	62	63	37.2	37.8	12.8	20.2	0.6	7.4	" "
Mr. A.	38	39	62	61	49	60	62	64	38.4	39.0	10.6	21.0	0.6	10.4	Surgical patient. Fracture; otherwise normal.
" B.	41	42	59	58	52	62	60	62	35.4	35.9	16.6	26.1	0.5	9.5	" " "
" C.	43	43	57	57	54	62	62	64	35.3	36.5	18.7	25.5	1.2	6.8	" " "
Normal average.	41.2	41.5	58.7	58.5	50.4	58.7	61.0	62.5	**35.8**	**36.5**	**14.6**	**22.2**	**0.7**	**7.6**	

Mrs. S.	11	11	89	89	56	60	60	62	53.4	55.1	2.6	4.9	1.7	2.3	Primary anemia. Hemoglobin 40 per cent. Red blood count 1.0 million.
" C.	13	13	87	87	52	56	58	58	50.4	50.4	1.6	5.6	0.0	4.0	Secondary anemia. Hemoglobin 52 per cent. Red blood count 4.0 millions.
Mr. B.	14	14	86	86	52	56	59	60	50.7	51.6	1.3	4.4	0.9	3.1	Primary anemia. Hemoglobin 27 per cent. Red blood count 1.7 millions.
" O.	12	12	88	88	44	48	48	48	42.2	42.2	1.8	5.8	0.0	4.0	Primary anemia. Hemoglobin 27 per cent. Red blood count 0.9 million.
Anemia average	12.5	12.5	87.5	87.5	51.0	55.0	56.2	57.0	**49.2**	**49.8**	**1.8**	**5.2**	**0.6**	**3.3**	
Mr. Cy.	45	45	55	55	66	72	98	100	53.9	55.0	12.1	17.0	1.1	4.9	Lymphatic leucemia. Hemoglobin 70 per cent. White blood count 39,000.
" Cd.	57	58	43	42	70	78	90	92	38.7	38.6	31.3	39.4	−0.1	8.1	Polycythemia vera. Hemoglobin 195 per cent. Red blood count 8.2 millions.
" F.	43	44	57	56	50	58	60	60	34.2	33.6	15.8	24.4	−0.6	8.6	Lobar pneumonia.
" P.	44	44	56	56	48	58	58	60	32.4	33.6	15.6	24.4	1.2	8.8	Cardiorenal disease.
Miscellaneous average	47.2	47.7	52.7	52.2	58.5	66.5	76.5	78.0	**39.8**	**40.2**	**18.7**	**26.3**	**0.4**	**7.6**	

* A = arterial; V = venous.

the plasma while 7.6 volumes per cent are gained by the cells. The minimum and maximum figures on this same series show that the plasma gains as it becomes venous from 0.0 to 1.8 volumes per 100 volumes of whole blood, while the cells gain from 4.2 to 10.4 volumes. The obvious conclusion from these figures is that the transport of CO_2 is accomplished mainly by the cells.

Findings in Disease.

When we turn to the findings in anemia we meet with much the same thing. In a series of fourteen venous bloods from as many patients with anemia (Table I) the average CO_2 content of the whole blood was 58 volumes per cent which is essentially the same as that of the normal series. As one might suppose, of this 58 volumes per cent relatively more is in the plasma, 53 volumes per cent, and less in the cells, 5 volumes per cent, than in the case of the normal subjects. This altered distribution appears to be merely the result of the reduction in the number of cells in anemia.

When we come to compare arterial and venous samples in anemia (Table II), we find that here as in health it is the cells that are chiefly concerned in CO_2 transport. When we calculate the quantity of CO_2 carried in the plasma of arterial blood we get an average figure of 49.2 and for venous blood 49.8 volumes per cent. The cells of arterial blood, however, contain an average of 1.8 and those of venous blood 5.2 volumes per cent. As the blood passes from artery to vein, therefore, it gains an average of 4 volumes per cent, but of this only 0.6 volume per cent is gained by the plasma, while 3.3 volumes per cent, or more than five times as much, are gained by the cells. In anemia then, as in health, the conclusion may be drawn that the cells are more important in CO_2 transport than is the plasma.

In Table II observations on four patients with other diseases are shown. In Mr. F. with lobar pneumonia, and Mr. P. with cardiorenal disease, all factors and relationships are essentially like those of the normal series. Mr. Cy. with leucemia, and Mr. Cd. with polycythemia vera, on the other hand, both present a marked increase in the CO_2 content of their whole blood. A study of the distribution shows that in the former the amount in the cells is normal but the amount in the plasma increased, while

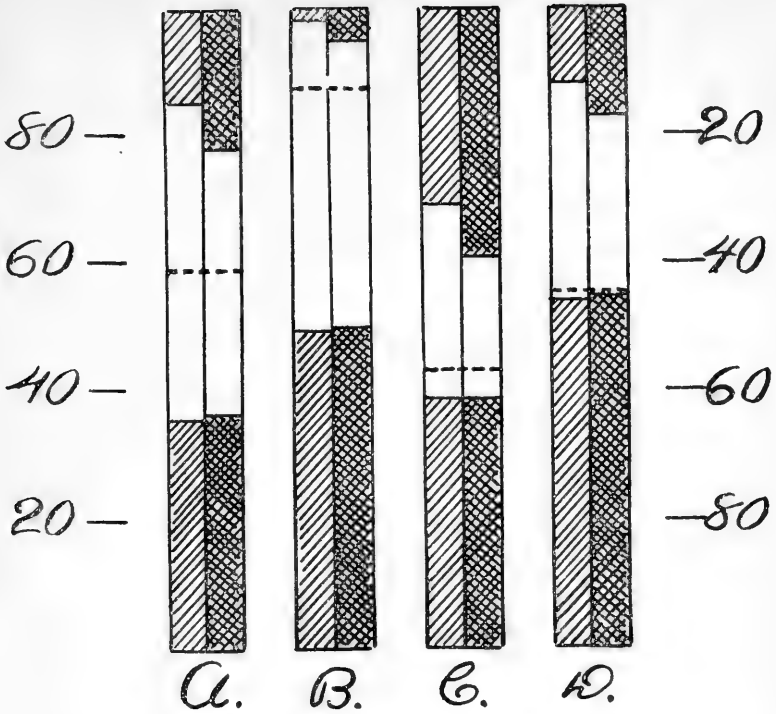

Fig. 1. Showing the distribution of CO_2 between cells and plasma in health and disease.

The entire column represents 100 cc. of blood. The portion above the horizontal interrupted line represents cells, that below, plasma, in accordance with the hematocrit findings. The upper shaded area shows the volume of CO_2 actually contained in the cells, and the lower shaded area that contained in the plasma of the given blood. Scales are provided so that these volumes may be read either from the top or bottom. The lightly shaded portions are for arterial, the heavily shaded, for venous blood.

Column A shows the relationships found in an average of eight normal bloods; Column B those of an average of four anemia bloods; Column C shows those of a patient with polycythemia vera; and Column D those of a patient with lymphatic leucemia.

It will be noted that in all four sets of columns the gain in CO_2 as blood passes from the arterial to the venous phase, is confined largely to the cells.

The difference in distribution of CO_2 between cells and plasma seems except in Column D to be dependent upon the relative amounts of cells and plasma in the several conditions. Anemic patients with a small proportion of cells carry less CO_2 in cells than normal persons and conversely the polycythemic patient carries more. In the leucemic patient (Column D), there is an actually greater quantity of CO_2 in the plasma which is not explainable on the ratio of cells to plasma.

in the latter the increase is in the load of the cells, the plasma load being normal. The increased amount in the cells in polycythemia is the converse of the condition in anemia and is therefore quite understandable. The cause for the increased amount of CO_2 in the plasma in the leucemia patient is less obvious. In both these patients in spite of the alteration in the distribution, the transport of CO_2 as in the other conditions studied is effected chiefly by the cells.

These relationships are shown graphically in Fig. 1.

DISCUSSION.

We do not propose to enter into an elaborate discussion of why the cells take a greater part in CO_2 transport than does plasma, nor of the actual method of its transport within the cells, whether as inorganic bicarbonate or in actual combination with hemoglobin as hemoglobin bicarbonate. Our purpose at the present time is merely to record certain phenomena as we found them.

It will not be out of place, however, to call attention to the greater and greater importance that, in the recent literature (1–3, 9), is being assigned to the rôle of hemoglobin in the matter of neutrality regulation and gas transport. It seems to be reasonably well established that hemoglobin is as important in the transport of CO_2 as it is in that of oxygen. Furthermore the recent work of Haggard and Henderson (10) seems to show that the corpuscles also regulate the bicarbonate reserve of the plasma.

Our findings, then, that both in health and disease the cells are chiefly concerned in CO_2 transport are not surprising. Our studies were on the actual relationships as they exist *in vivo*, but our results nevertheless seem to be essentially in agreement with those obtained with defibrinated blood *in vitro* by Joffe and Poulton (9). These writers found that the effect of oxygenation is to cause a loss of CO_2 which is more than twice as great for the corpuscles as for the plasma, and they draw the same conclusion, as do we, that "the transport of CO_2 in the blood is chiefly carried out by the corpuscles."

CONCLUSIONS.

1. As the blood passes from the arterial to the venous side of the circulation in normal man its cells gain from 4 to 11 volumes per cent of CO_2. At the same time the corresponding gain in the plasma is only from 0.0 to 1.8 volumes per cent. The conclusion is drawn, therefore, that the transport of CO_2 is accomplished mainly by the cells.

2. The same holds true in anemia and in certain other diseases, even though because of alterations in the cell volume: plasma volume ratio the actual distribution of CO_2 between cells and plasma is altered.

BIBLIOGRAPHY.

1. Parsons, T. R., *J. Physiol.*, 1919–20, liii, 42, 340.
2. Bayliss, W. M., *J. Physiol.*, 1919–20, liii, 162.
3. Henderson, L. J., *J. Biol. Chem.*, 1920, xli, 401.
4. Henderson, Y., and Morriss, W. H., *J. Biol. Chem.*, 1917, xxxi, 217.
5. Haggard, H. W., *J. Biol. Chem.*, 1920, xlii, 237.
6. Harrop, G. A., Jr., *J. Exp. Med.*, 1919, xxx, 241.
7. Peters, J. P., Jr., and Barr, D. P., personal communication.
8. Stadie, W. C., and Van Slyke, D. D., *J. Biol. Chem.*, 1920, xli, 191.
9. Joffe, J., and Poulton, E. P., *J. Physiol.*, 1919–20, liii, p. lxvi.
10. Haggard, H. W., and Henderson, Y., *J. Biol. Chem.*, 1919–20, xlv, 199.

RELATIONSHIP BETWEEN CHOLESTEROL AND CHOLESTEROL ESTERS IN THE BLOOD DURING THEIR ABSORPTION.*

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(Received for publication, November 1, 1920.)

In a previous paper (1) it was shown that during the absorption of a neutral fat (olive oil) there is a marked increase in the blood of the cholesterol esters but no increase in the total cholesterol. Following the same experimental technique it was thought that it would be advisable to determine the relationship between the total cholesterol and the cholesterol esters in the blood during the absorption of free cholesterol and of cholesterol esters respectively.

That cholesterol is absorbed from the food and that the absorption is followed by a rise in the blood cholesterol is considered as established.

The first work of any significance was reported by Pribram (2) in 1906 who found that by the feeding of pure cholesterol and cholesterol esters respectively an increase in the blood of rabbits resulted. The increase was in part at least due to free cholesterol, as the serum from such animals showed an increased inhibitory power toward the hemolytic action of saponin, the free cholesterol alone being active as an inhibitory agent. Kusumoto (3) also showed indirectly that cholesterol must be absorbed as he found that 30 per cent of the amount ingested failed of excretion through the intestine. Gardner and his coworkers (4) in extensive studies on the origin and destiny of cholesterol in the animal organism came to the conclusion that cholesterol and cholesterol esters are absorbed into the blood, that the cholesterol esters are hydrolyzed before absorption, and that there results an increase in both free cholesterol and cholesterol esters in the blood after feeding cholesterol. Lehman (5) found that feeding cholesterol to rabbits results in an increase of the cholesterol in the blood in a few hours. In a series of experiments on dogs with a fistula in

* This investigation was supported in part by a grant from the Elizabeth Thompson Science Fund.

the thoracic duct Mueller (6) found that cholesterol was readily absorbed in the chyle. When cholesterol was fed either in the free form or in the form of esters there resulted an increase in both of these in the chyle, the proportion between the two remaining approximately the same as normally. These results indicated that either in the intestine or in the walls of the intestine processes of esterifications or hydrolysis take place depending upon the character of the material fed.

In the present investigation dogs were given a diet containing pure cholesterol or esters of cholesterol and blood specimens taken at once and at 2 hour intervals for 8 hours. Previous to the experiment the dogs were fasted for 24 hours. The blood specimens were drawn from the jugular vein into a hypodermic syringe (containing a little sodium citrate) and run at once into a centrifuge tube containing a little citrate. 3 cc. of the whole blood were then extracted and made up to 100 cc. volume according to Bloor's procedure (7). Then the tube was centrifuged for 30 minutes at 2,000 revolutions per minute and relative volume of corpuscles and plasma noted. 3 cc. of the plasma were then extracted as was the whole blood.

Total cholesterol and cholesterol esters were determined on each specimen of whole blood and plasma. Cholesterol was determined by Bloor's method (8) and cholesterol esters were determined by Bloor and Knudson's method (9).

Nine experiments were carried out, five of which were on diets containing free cholesterol and four on diets containing esters of cholesterol. In all the experiments, fat was excluded from the diet. A brief description of the experiments follows.

Experiment 1.—Dog 4 was a healthy male weighing 11.3 kg. It was fed a diet consisting of 4 gm. of cholesterol, 30 gm. of cracker meal, and 100 cc. of water at 9.10 a.m. At 9.15 a.m. the first specimen of blood was taken. After taking a 3 cc. sample for whole blood, the remainder was centrifuged and corpuscles were found to be 50.5 per cent. The plasma was clear and a 3 cc. sample was taken for analysis. The other specimens were as follows.

Specimen No.	Time.	Corpuscle.	Plasma.
		per cent	
2	11.20 a.m.	47.2	Clear.
3	1.20 p.m.	46.2	"
4	3.22 "	45.0	"
5	5.20 "	45.5	"

Experiment 2.—Dog 4 as above, weight 11.3 kg. Fed a diet containing 4 gm. of cholesterol, 30 gm. of cracker meal, and 100 cc. of water at 8.40 a.m. Specimens as follows.

Specimen No.	Time.	Corpuscle.	Plasma.
		per cent	
1	8.47 a.m.	48.5	Clear.
2	10.42 "	48.0	Slight turbidity.
3	12.42 p.m.	45.3	" "
4	2.50 "	45.0	" "
5	4.45 "	45.0	" "

Experiment 3.—Dog 6 was a healthy male weighing 10.4 kg. Fed a diet containing 3 gm. of cholesterol, 25 gm. of bread, and 150 cc. of water at 8.50 a.m. Specimens as follows.

Specimen No.	Time.	Corpuscle.	Plasma.
		per cent	
1	8.50 a.m.	38.0	Clear.
2	10.50 "	35.8	"
3	12.50 p.m.	36.0	"
4	3.00 "	37.0	"
5	4.50 "	36.8	"

Experiment 4.—Dog 8 was a healthy female weighing 8.5 kg. Fed a diet containing 3 gm. of cholesterol, 100 gm. of lean chopped beef, and 150 cc. of water at 12 m. Specimens as follows.

Specimen No.	Time.	Corpuscle.	Plasma.
		per cent	
1	12.10 p.m.	41.8	Clear.
2	2.10 "	42.0	"
3	4.10 "	41.9	"
4	6.05 "	41.5	"
5	8.05 "	42.1	"

Experiment 5.—Dog 8 as above, weight 9 kg. Fed a diet containing 2.5 gm. of cholesterol, 100 gm. of lean chopped beef, and 200 cc. of water at 11.25 a.m. Specimens as follows.

Specimen No.	Time.	Corpuscle.	Plasma.
		per cent	
1	11.30 a.m.	40.5	Clear.
2	1.35 p.m.	42.5	Slight turbidity.
3	3.30 "	40.4	Turbid.
4	5.30 "	42.66	Slight turbidity.
5	7.30 "	42.9	Turbid.

Experiment 6.—Dog 4 as above, weight 12.5 kg. Fed a diet containing 2.5 gm. of cholesterol palmitate, 50 gm. of bread, and 150 cc. of water at 8.40 a.m. Specimens as follows.

Specimen No.	Time.	Corpuscle.	Plasma.
		per cent	
1	8.45 a.m.	39.0	Clear.
2	10.50 "	37.0	"
3	12.55 p.m.	35.1	"
4	2.55 "	37.0	"
5	4.55 "	36.0	"

Experiment 7.—Dog 6 as above, weight 10 kg. Fed a diet containing 2.5 gm. of cholesterol oleate, 25 gm. of lean chopped beef, and 100 cc. of water at 8.50 a.m. Specimens as follows.

Specimen No.	Time.	Corpuscle.	Plasma.
		per cent	
1	9.00 a.m.	39.5	Clear.
2	10.50 "	36.0	Slight turbidity.
3	12.50 p.m.	36.2	Clear.
4	2.50 "	37.3	Slight turbidity.
5	4.50 "	36.6	" "

Experiment 8.—Dog 8 as above, weight 9 kg. Fed a diet containing 2.5 gm. of cholesterol stearate, 100 gm. of lean chopped beef, and 200 cc. of water at 11.20 a.m. Specimens as follows.

Specimen No.	Time.	Corpuscle.	Plasma.
		per cent	
1	11.30 a.m.	42.6	Clear.
2	1.35 p.m.	40.6	Slight turbidity.
3	3.50 "	42.0	" "
4	5.30 "	42.3	Turbid.
5	7.30 "	42.0	Slight turbidity.

Experiment 9.—Dog 11 was a healthy female weighing 20.5 kg. Fed a diet containing 2.5 gm. of cholesterol palmitate, 25 gm. of sugar in 200 cc. of a 1 per cent gum tragacanth solution at 9.55 a.m. Specimens as follows.

Specimen No.	Time.	Corpuscle.	Plasma.
		per cent	
1	10.05 a.m.	57.8	Clear.
2	12.00 m.	57.4	"
3	2.05 p.m.	57.0	Slight turbidity.
4	4.00 "	57.3	Clear.
5	6.00 "	57.8	"

TABLE I.

Cholesterol and Cholesterol Esters During Their Absorption per 100 Cc.

No. of experiment.	Time.	Total cholesterol.			Cholesterol as esters.		
		Whole blood.	Plasma.	Corpuscles.	Whole blood.	Plasma.	Corpuscles.
	hrs.	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*
1	Before.	211	215	208	73	148	1
	2	253	195	318	72	149	4
	4	301	298	326	72	136	2
	6	306	309	302	71	135	6
	8	324	324	324	70	134	6
2	Before.	225	225	225	71	136	−2
	2	250	258	241	73	138	−2.6
	4	268	277	256	74	130	+5
	6	258	277	236	73	134	−1
	8	294	309	277	70	132	−5
3	Before.	170	168	173	70	111	+2.6
	2	190	192	187	69	107	0
	4	201	203	198	74	113	+4.2
	6	208	211	202	70	108	−5.4
	8	193	178	217	71	115	−4
4	Before.	193	185	204	70	125	−6
	2	219	215	225	71	124	−2.3
	4	238	220	264	70	126	−7
	6	208	192	231	69	123	−7
	8	263	250	280	72	131	+4.7
5	Before.	190	193	186	81	136	0
	2	205	200	211	80	140	−1
	4	223	206	247	78	134	−4.9
	6	245	213	287	78	132	+4.6
	8	219	200	245	80	140	0
6	Before.	167	160	178	78	125	+4
	2	192	174	223	80	125	+2.7
	4	203	178	251	78	120	0
	6	196	195	200	79	122	+5.4
	8	177	168	194	79	121	+4.1

TABLE I—*Concluded.*

No. of experiment.	Time.	Total cholesterol.			Cholesterol as esters.		
		Whole blood.	Plasma.	Corpuscles.	Whole blood.	Plasma.	Corpuscles.
	hrs.	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*
7	Before.	172	177	165	71	117	0
	2	179	185	170	72	114	−2.7
	4	217	214	221	75	116	+1.9
	6	205	208	201	73	115	+2.6
	8	183	194	164	70	111	−1.2
8	Before.	193	192	194	77	136	−2.4
	2	211	208	215	77	132	−3.7
	4	231	227	237	80	138	0
	6	250	238	262	79	132	+5.9
	8	250	234	271	83	140	+4.5
9	Before.	185	181	188	62	145	+1.3
	2	209	208	213	64	152	−1
	4	221	221	221	66	147	+4.7
	6	260	268	255	66	147	+5.2
	8	236	238	235	60	140	+1.7

The dogs used in these experiments were previously accustomed to handling, and did not struggle during the experiments or show other evidences of excitement, so those factors do not influence these results.

The analytical results are given in Table I. Direct determinations were made only on the whole blood and plasma from which, since the percentage of corpuscles in the blood was known, the composition of the corpuscles was calculated.

DISCUSSION.

The total cholesterol is found to be increased in all the experiments both in feeding cholesterol and cholesterol esters. This result is in accord with the work of other investigators (2–6). The increase in the whole blood varies from 21.5 per cent in Experiment 6 to 53.5 per cent in Experiment 1 and averages 32.3 per cent. The greatest increase occurs in most of the experiments either in the 6th or the 8th hour after feeding. Comparing

the increase of cholesterol in the plasma and corpuscles it is observed that the increase in the corpuscles is greater in seven of the nine experiments.

The values for cholesterol esters show that the cholesterol in combined form remains the same throughout the periods of observation. Even though the total cholesterol increases as much as 50 per cent in the whole blood the amount of cholesterol as esters does not show any appreciable change. The values for cholesterol esters in the corpuscles indicate that the corpuscles probably contain no combined cholesterol which bears out the assumption generally made. Of course it must be remembered that the values for cholesterol esters in the corpuscles are calculated from the amount of them in the whole blood and plasma and as a consequence are not so reliable as when determined directly. This will also explain why some values in the table are given in negative quantities.

The results of these experiments indicate then that when cholesterol or cholesterol esters such as palmitate, oleate, or stearate are fed in a diet there results a marked increase in the total cholesterol in the blood but no change in the amount of cholesterol as esters. The cholesterol esters must as a consequence be hydrolyzed before absorption in the intestine and apparently are not synthesized again before passing into the blood.

These results do not agree with the observations reported by Gardner and his coworkers (4) who found that when cholesterol was fed to rabbits there resulted an increase in the cholesterol esters as well as cholesterol.

The experiments of Gardner were carried out by feeding a diet to which cholesterol was added and after several days killing the animal and analyzing the blood. In these experiments it may be possible that the level for cholesterol in the tissues and blood was raised after feeding it for several days and as a result an equilibrium is established between free cholesterol and cholesterol esters in the blood and tissue resulting in an increase of cholesterol esters as well as free cholesterol. Recently Hueck and Wacker (10) have reported that cholesterol fed to rabbits causes an increase in the esters in the blood. Their experiments were similar to those of Gardner and his coworkers but were carried over a longer period. They fed rabbits for a period of 181 days in one experiment and 126 days in another and in that time the rabbits had received 220 and 130 gm. of chol-

esterol respectively. Due to the large amount of cholesterol fed it would be expected that there would be an accumulation of cholesterol in the tissues and blood and the relation between the free and bound cholesterol would tend in time to be constant. In neither of these investigations was the blood examined just before feeding of cholesterol diets and at short intervals during the period of absorption so that the results are not comparable with mine.

However, Mueller (6) found that there resulted an increase in both cholesterol and cholesterol esters in the chyle of dogs when either one was added to the diet. Mueller's results might be partially explained by the fact that in the diets containing cholesterol or cholesterol esters fat was also present. I have shown in a previous paper (1) that during absorption of fat alone there results a marked increase of the cholesterol esters in the blood so that the fatty acids resulting from the hydrolysis of the fat in Mueller's experiments are probably transported in part combined with the cholesterol. In all my experiments I excluded fat from the diets except very small amounts present in those experiments where I used some lean beef, bread, or cracker meal. By omitting the fat there was no excessive amount of fatty acid to be transported by the blood.

SUMMARY.

A series of experiments carried out on dogs feeding them cholesterol and cholesterol esters, respectively, shows that free cholesterol is increased considerably in all the experiments during the absorption of the diets but that there is no apparent change in the amount of cholesterol in the ester form.

BIBLIOGRAPHY.

1. Knudson, A., *J. Biol. Chem.*, 1917, xxxii, 337.
2. Pribram, H., *Biochem. Z.*, 1906, i, 413.
3. Kusumoto, C., *Biochem. Z.*, 1908, xiv, 411.
4. Dorèe, C., and Gardner, J. A., *Proc. Roy. Soc. London, Series B*, 1909, lxxxi, 109. Fraser, M. T., and Gardner, J. A., *Proc. Roy. Soc. London, Series B*, 1909, lxxxi, 230; 1909–10, lxxxii, 559.
5. Lehman, E. P., *J. Biol. Chem.*, 1913–14, xvi, 495.
6. Mueller, J. H., *J. Biol. Chem.*, 1915, xxii, 1.
7. Bloor, W. R., *J. Biol. Chem.*, 1916, xxiv, 227.
8. Bloor, W. R., *J. Biol. Chem.*, 1917, xxix, 437.
9. Bloor, W. R., and Knudson, A., *J. Biol. Chem.*, 1917, xxix, 7.
10. Hueck, W., and Wacker, L., *Biochem. Z.*, 1919, c, 84.

PENTOSE MONONUCLEOTIDES OF THE PANCREAS OF THE DOGFISH (SQUALUS SUCKLII).

PRELIMINARY COMMUNICATION.

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(Received for publication, October 6, 1920.)

Two mononucleotides in which a pentose, *d*-ribose, unites purine and phosphoric acid are known to occur in the animal body; guanylic acid and inosinic acid. The former contains guanine as purine base, the latter hypoxanthine. The corresponding compound with adenine has been shown to be one of the component mononucleotides of yeast nucleic acid by Jones and Kennedy (1) and by Levene (2); but it does not appear to have been found in the animal body. Guanylic acid, originally isolated by Hammarsten (3) from the β-nucleoprotein of the pancreas of the ox, has since been found widely distributed in mammalian glands (4, 5). Inosinic acid, first isolated by Liebig (6) from meat extract, is now known to be a constituent of the muscle of many mammals and of some birds and fishes (7). It has not, however, been identified as a gland constituent in any animal and the wide distribution of pentoses in animal tissues has generally been attributed to the presence of guanylic acid (8).

It is the object of the present communication to record evidence pointing toward the occurrence of adenosinphosphoric acid and inosinic acid, in addition to a guanylic acid, in the pancreas of the dogfish.

The essentials of Hammarsten's method of preparation of β-nucleoprotein from ox pancreas are rapid extraction of the material with boiling water and precipitation from the cooled extract by dilute acid. The crude material may be purified by dissolving in dilute alkali and reprecipitating with dilute acid repeatedly and yields an alkali albuminate and guanylic acid on heating at water bath temperature with 2 per cent potash

(9). According to Bang the acid is precipitated on neutralizing the alkaline digest and cooling. He points out, however, that it is soluble in water to the extent of about 0.3 per cent, but only sparsely soluble in dilute acetic acid. Levene and Mandel (5), working with liver nucleoprotein, obtained precipitation by concentrating the neutral solution strongly at low temperature, while Jones and Rowntree (4), utilizing material obtained from pigs' spleen, find it necessary to add acetic acid to cause the guanylic acid to separate.

Inosinic acid occurs either free or in readily broken down combination in the muscle tissues in which it has been found. There is no evidence that it forms any protein combination. It is readily extracted in water and cannot be precipitated from the extract either by boiling or acidification. Several methods have

TABLE I.

Experiment No.	Pentose (as xylose).		Total pentose (xylose) remaining in acetic acid filtrate.
	Extract.	Acetic acid filtrate.	
	per cent	*per cent*	*per cent*
1	0.0226	0.0114	50.4
2	0.0358	0.0217	60.6

been described for its isolation which, together with a new one, are summarized by Bennett (7). All, except the original method of Liebig, depend upon precipitation of the acid either as the lead or the silver salt. In a recent communication (10) attention was drawn by the writer to the occurrence of a substance, or substances, in boiling water extracts of various tissues of marine animals which yielded furfurol in relatively large quantity on heating with 12 per cent hydrochloric acid, and probably therefore contains a pentose, and was not precipitated on adding acetic acid to the extract.

The fraction of the furfurol-yielding substances in the extract which remained in solution after complete precipitation with acetic acid was by no means insignificant. The figures in Table I were, for instance, obtained from extracts of dogfish pancreas and the corresponding acetic acid filtrates.

Grund's modification of the method of Tollens and Kröbe for the determination of pentoses (11) was used and calculation made to xylose. It was necessary to concentrate the acetic acid filtrates in order to obtain a sufficiently large yield of furfurol from the prescribed volume of liquid. The samples taken for analysis were of such a volume that, after making up to 100 cc. with concentrated hydrochloric acid, the solutions contained the necessary 12 per cent of acid.

Interest was first aroused in the presence of these acetic acid-soluble furfurol-yielding compounds from the point of view of its possibly indicating pentosans in the tissues of the animals examined. Pentosans are largely represented in the algæ which, directly or indirectly, make up their diet; moreover it has been stated that such compounds occur in certain mollusks living on algæ (12). It was found, however, that in all the cases examined the pentose compounds could be completely precipitated from the acetic acid solution by alcohol and that the precipitates thus obtained invariably contained both phosphoric acid and purine in conjugation, so that it seemed more probable that the pentose was present in nucleotide combination.

The tissue selected for examination with a view to the more precise determination of the nature of the combination was the pancreas of the dogfish for two reasons; first, because it was the tissue richest in pentose of all those examined and, second, because the pancreas is the classical seat of guanylic acid in mammals and it seemed of special interest to determine whether the same compound occurs in the lower vertebrates of which the dogfish may be taken as a type.

EXPERIMENTAL.

The property of yielding furfurol, which is easily recognized by its reaction with aniline acetate, phloroglucin, orcinol, etc., on heating with 12 per cent hydrochloric acid, has been taken as evidence of the presence of combined pentose in the preparations under examination. The only other class of compounds which might have been present and which yield furfurol under the same conditions is the conjugated glucuronic acids. Practically all these compounds are, however, soluble in alcohol and ether (13) and the material used in this work was found to give up none

of its furfurol-yielding constituents by exhaustive extraction with both solvents. Starkenstein and Henze (12) consider that glucosamine, which occurs in the tissues combined as glucoprotein, is responsible for the furfurol reactions with orcinol and phloroglucin given by preparations from the livers of certain mollusks. It was found, however, that glucosamine chloride, prepared from crab shells, did not yield a trace of furfurol on heating with 12 per cent hydrochloric acid and concluded, therefore, that compounds of this class might reasonably be excluded from consideration. Bial's orcinol reagent was frequently used to detect pentose, but in every case where sufficient material was at hand to make comparisons possible it was found that a positive reaction with this reagent indicated a positive one on distilling with 12 per cent hydrochloric acid, in accordance with the Tollens method for pentose determination, and testing the distillate with aniline acetate or phloroglucin.

The test for purines has been carried out by the Krüger-Schmid procedure (14) followed by production of the silver compounds by precipitation with ammoniacal silver nitrate from the solution obtained by hydrogen sulfide decomposition of the copper salts. Purines were freed from combination by boiling with 5 per cent sulfuric acid. Free phosphoric acid was detected by magnesia mixture and the conjugated acid by ammonium molybdate after boiling with nitric acid.

The pancreas was excised from forty-nine freshly killed dogfish and freed as far as possible from adherent mesentery. The material weighed 495 gm. It was put through a mincer and immediately put into rapidly boiling water, boiled for 5 minutes and strained through close cloth, squeezed out, and extracted in the same way a second time. The residual material after this treatment gave only a trace of furfurol on distillation with 12 per cent hydrochloric acid. The united extracts measured 2.25 liters. After cooling, the liquid was decanted from solid material which had settled out and, after repeated filtration, was obtained in an only slightly turbid condition. 20 cc. of glacial acetic acid were added which produced a heavy precipitate. This was allowed to settle and left a perfectly clear fluid which was partly decanted and partly filtered off.

The precipitate was washed repeatedly by decantation with 1 per cent acetic acid and finally put upon a filter. It gave a strong pentose reaction and contained conjugated phosphoric acid, purine, and protein. An attempt was made to purify the material by dissolving it in cold 1 per cent sodium hydroxide and reprecipitating with acetic acid. It was found that the pentose-containing substance could not be completely reprecipitated by dilute acid or by adding a considerable excess and that, after repeating the operation twice, only protein and a trace of phosphoric acid could be detected in the precipitate, but no purine or pentose. The nucleoprotein was evidently split by treatment with alkali and the non-protein constituent remained in solution in acetic acid.

The acetic acid filtrates were accordingly neutralized and concentrated to small volume. The solution contained no protein and no free pentose, purine, or phosphoric acid. All were present in the conjugated condition. The solution was poured into a large volume of alcohol which produced a voluminous precipitate giving all the reactions of the original material and containing all the combined pentose. The precipitate was dissolved in 100 cc. of 5 per cent sulfuric acid and heated at water bath temperature for $2\frac{1}{2}$ hours. After cooling, the solution was neutralized and the purines were precipitated as cuprous compounds by the Krüger-Schmid procedure (14). The precipitate was suspended in hot water, decomposed with hydrogen sulfide, and, after boiling off excess hydrogen sulfide, the liquid with copper sulfide in suspension was boiled with sulfuric acid and filtered. The filtrate was neutralized with ammonia, 2 per cent excess added, and warmed. A precipitate separated immediately. After digesting on the water bath for 1 hour and standing over night this was filtered off, washed with 1 per cent ammonia and water, and purified by dissolving in 1 per cent sodium hydroxide and reprecipitating with acetic acid. The material so obtained was further identified as guanine by the crystalline form of its hydrochloride and the melting point of the picrate prepared from it (159–160°C.).

The ammoniacal filtrate from the guanine precipitation was treated with silver nitrate which produced a heavy precipitate. This was filtered off, washed, and decomposed with a small quantity of warm hydrochloric acid. The filtrate from silver

chloride gave no trace of precipitate on making 2 per cent ammoniacal and warming. Guanine was therefore absent. The solution was boiled till free from ammonia, made just acid with hydrochloric acid, and excess of a saturated solution of picric acid added. A characteristic light yellow precipitate of adenine picrate immediately separated which was filtered off, washed, recrystallized from boiling water, and further characterized as adenine picrate by its crystalline form and melting point (180–181°C.).

The filtrate from adenine picrate was freed from picric acid by means of sulfuric acid and ether, made ammoniacal, and tested with silver nitrate. Absence of precipitate indicated that the solution contained no further purines.

The precipitate produced by acetic acid from the boiling water extract of dogfish pancreas contains therefore, in addition to protein, pentose, and conjugated phosphoric acid, guanine and adenine as purine bases.

The acetic acid filtrate from this precipitate was neutralized and evaporated to 470 cc. at 60–70°C. It was cooled at this stage and filtered from a small amount of coagulated material. It contained conjugated purine, pentose, and phosphoric acid, but only a trace of phosphoric acid in the free condition. 1 per cent of acetic acid was added and the liquid allowed to stand over night. No precipitate was formed. It was further concentrated to 100 cc. and again treated with acetic acid, but still without result even after prolonged standing. Guanylic acid similar to that isolated from mammalian material does not, therefore, appear to be present. The solution was poured into a large volume of alcohol which produced a heavy gelatinous precipitate which gave a slight biuret reaction, but no xanthoproteic, and did not react with Millon's reagent. It contained conjugated phosphoric acid, purine, and all the combined pentose present in the original solution, but none of the compounds in a free condition.

In order to break down any protein combination present the material was dissolved in 100 cc. of 2 per cent caustic potash and heated on the water bath for ½ hour. The solution thus obtained was cooled, acidified with acetic acid, and allowed to stand for 18 hours. No precipitation took place. The solution was neu-

tralized and treated with saturated barium hydroxide until precipitation was complete to remove any free phosphoric acid or protein decomposition products present. The precipitate was filtered off and washed. The filtrate gave no biuret reaction. It was neutralized with acetic acid and treated with basic lead acetate solution until precipitation was just complete. The lead precipitate was thoroughly washed, suspended in water, and decomposed with hydrogen sulfide, excess hydrogen sulfide removed by an air current, and excess barium carbonate added. After boiling, the lead sulfide and barium carbonate were filtered off and the filtrate was evaporated to a small volume at as low a temperature as possible on a water bath. It was then poured into five volumes of alcohol which produced a bulky gelatinous precipitate containing all the pentose. This was filtered off with suction, dried at room temperature, and dissolved in hot water. On cooling, the solution deposited a white powdery substance which increased on standing over sulfuric acid in an exhausted desiccator for some days, but no definite crystalline form could be made out even after recrystallizing. After slow evaporation of the solution nearly to dryness this material was filtered off, washed with alcohol and ether, and dried. It gave positive tests for pentose, purine, conjugated phosphoric acid, and barium. It was redissolved in water, the barium removed with sulfuric acid, and the acid strength made up to 5 per cent. After heating the solution on a water bath for 3 hours it was neutralized and the same procedure followed for the separation of the purines as in the case of the acetic acid precipitate from the original extract. No guanine could be detected and only a small quantity of adenine, but the filtrate from adenine picrate gave a precipitate with ammoniacal silver nitrate indicating the presence of further purine compounds. The silver nitrate precipitate was accordingly decomposed with hydrochloric acid and the solution of chlorides thus obtained evaporated cautiously to dryness, moistened, and evaporated a second time. The residue gave no xanthine reaction with nitric acid and soda and was completely soluble in warm water. Xanthine was therefore not present. The copper precipitation was repeated and the residue obtained by evaporation of the solution resulting from hydrogen sulfide decomposition of the precipitate crystallized from 6 per cent nitric acid. Char-

acteristic whetstone-shaped crystals of hypoxanthine nitrate were thus obtained.

The filtrate from the acetic acid precipitation of the boiling water extract of the dogfish pancreas is thus shown to contain hypoxanthine and adenine as purine bases as well as conjugated phosphoric acid and pentose.

It seemed possible to interpret the results thus far obtained on the assumption that only ordinary α-nucleoprotein (the compound of protein with nucleic acid of the thymus nucleic acid type) and ordinary β-nucleoprotein (the compound with guanylic acid) were in question and that:

1. α-nucleoprotein had been extracted from the pancreas together with β-nucleoprotein and the adenine in the acetic acid precipitate was due to nucleic acid, the pentose being combined only with guanine.

2. Nucleic acid had been broken down by enzyme action leading to the production of adenine hexose nucleotide or nucleoside and a corresponding hypoxanthine compound, and both of these had remained in solution after treatment of the extract with acetic acid. Since, however, both combined guanine and free pentose are absent from the acetic acid solution the combined pentose present would in this case have to be attributed to the pentose-phosphoric acid compound split off from guanylic acid.

The first possibility seemed unlikely because the essence of the method of separation of α- and β-nucleoproteins is the insolubility of the former in boiling water;[1] the second because of the expeditiousness with which the tissues had been brought into boiling water. Moreover, the assumption made in this latter case to account for the presence of pentose necessitates a splitting off of free guanine from guanylic acid. However, no free purine was found either in the original acetic acid precipitate or in the filtrate from that precipitate, and there seemed no reason to anticipate that either boiling with water or the action of cold dilute acetic acid could have caused such a split.

[1] The only difference in the procedure used here and that usually described for extracting β-nucleoprotein from tissues is that the material has been placed directly in boiling water, in order to eliminate risk of enzyme action during extraction, instead of putting it into cold water and raising to the boiling point.

Jones (15) has shown in the case of the pig that the splitting off of guanine from guanylic acid is not brought about under normal conditions by pancreatic enzymes. Thus both purely chemical and (as far as it is justifiable to argue from the pig to the dogfish) biochemical considerations indicate that the presence of combined pentose in the acetic acid filtrate of the extract of dogfish pancreas cannot be attributed to breakdown of guanylic acid with the elimination of guanine.

The entire absence of xanthine from both the precipitates and filtrates obtained from the extract also argues against any enzyme action having intervened in the course of the operations if analogy is justifiable between ferment conditions in the pancreas of the dogfish and that of the pig, since, in the latter case, xanthine is invariably a product of autolysis (15).

There seems, therefore, no alternative but that the pentose in the acetic acid filtrate is combined with preformed adenine or hypoxanthine, or both.

It seemed probable that if more material were taken at the outset it might be possible to separate the compounds of guanine, adenine, and hypoxanthine more completely and thus determine with greater certainty with which of the purines the pentose was combined.

To this end the pancreas was excised from 140 dogfish and the material, which weighed 921 gm., ground, and extracted with boiling water as in the previous instance, and the extract cooled and filtered. About 5 liters of extract were obtained which were concentrated at a low temperature to 1,500 cc. During concentration a small amount of suspended material coagulated and was filtered off after cooling. The filtrate was only very slightly turbid. 30 cc. of glacial acetic acid were added and the precipitate produced was separated and washed as before. The precipitate behaved as in the previous instance on endeavoring to purify it by alternate treatments with alkali and acetic acid and the non-protein constituents were obtained in solution in acetic acid.

This solution was neutralized and a saturated solution of normal lead acetate added until precipitation was complete. The lead salt was converted into the barium salt in the manner previously described. The barium salt was crystalline and difficultly soluble

in water. It contained conjugated pentose, purine, and phosphoric acid. It was freed from barium, hydrolyzed with 5 per cent sulfuric acid, and the purines present were determined as before. Guanine and adenine were found in about equal quantities but no xanthine or hypoxanthine.

The filtrate from the normal lead acetate precipitate was treated with basic lead acetate and a further precipitate obtained. This was converted into the barium salt as before. It had the same appearance and gave the same reaction as that obtained from the normal lead acetate precipitate and contained the same purine bases; adenine was, however, present in larger quantity than guanine.

The filtrate from the basic lead acetate precipitate, after removal of lead, gave no pentose reaction and was not further examined.

The purine constituents of the acetic acid precipitate are thus shown to be guanine and adenine as in the experiment previously described.

The filtrate from the acetic acid precipitation of the extract was neutralized and a saturated solution of barium hydroxide added until precipitation was complete. The precipitate thus obtained contained the pentose nucleotide constituents. It was decomposed with sulfuric acid, hydrolyzed as in the previous instances, and the purines present were determined. Again adenine and guanine, but no xanthine or hypoxanthine, were found. The fact that barium hydroxide carries down compounds of the same constituents as occur in the acetic acid precipitate from the filtrate from that precipitation was overlooked in the experiment previously described.

The filtrate from barium hydroxide precipitation still gave a strong pentose reaction. After neutralizing with acetic acid it failed to give a precipitate with normal lead acetate, but gave a heavy one on treatment with the basic salt. The filtrate from this precipitation was free from pentose nucleotide constituents. The lead salts obtained were converted into the barium salts which were finally crystallized from hot water. It was found possible to separate the material in this way into two fractions having a marked difference in solubility in water, but both giving all the reactions for pentose nucleotides and barium. The more

soluble fraction was the larger one. After removal of barium and hydrolysis with 5 per cent sulfuric acid it gave no reaction for guanine, but a heavy precipitate of adenine picrate. The filtrate from the picrate precipitation, after removal of excess picric acid by sulfuric acid and ether, gave a slight precipitate with ammoniacal silver nitrate which, after washing and dissolving in boiling nitric acid of 1.1 specific gravity, deposited on cooling characteristic crystals of hypoxanthine silver nitrate. The compound was further identified by removing the silver and crystallizing out typical whetstone-shaped crystals of hypoxanthine nitrate which gave no trace of xanthine reaction with nitric acid and caustic soda. The filtrate from hypoxanthine silver nitrate deposited a trace of red-brown precipitate on being rendered ammoniacal which, after removal of silver and evaporation of the solution of nitrate obtained, deposited a very slight residue giving a characteristic xanthine reaction.

The less soluble fraction of the barium salts was treated in the same way as the more soluble one. It contained neither guanine nor adenine, but only hypoxanthine and a trace of xanthine as purine constituents.

The filtrate from barium hydroxide precipitation of the acetic acid filtrate from the original extract contains, therefore, both adenine and hypoxanthine and a trace of xanthine in addition to conjugated phosphoric acid and pentose.

The results obtained from this experiment thus confirm the conclusion reached from the former one. For the reasons advanced in connection with that experiment coupled with the fact that, in the case just described, adenine is found with only a trace of hypoxanthine in one fraction of the barium salts obtained from the acetic acid filtrate of the extract, and hypoxanthine with practically no other base in the other fraction, it appears further that both adenine and hypoxanthine are present in mononucleotide combination.

The occurrence of the adenine compound in the acetic acid precipitate of the extract in both experiments indicates that this compound is present in the tissue in nucleoprotein combination. There is, however, no evidence that inosinic acid is similarly combined since hypoxanthine has not been found in the acetic acid precipitate.

Levene (2) has shown that adenosinphosphoric acid is best identified as the free acid obtained through the ammonium salt. It is hoped to accumulate sufficient of the more soluble fraction of the barium salt prepared as described to make identification along these lines possible.

Inosinphosphoric acid (inosinic acid) is usually identified as the barium salt. This is, according to Bennett (7), readily soluble in warm water, differing in this respect from the fraction of barium salts obtained as above described containing hypoxanthine as purine constituent. The compound obtained may be the basic salt. Haiser (16) has described such a sparingly soluble compound derived from inosinic acid by the action of excess barium hydroxide. This point also can be elucidated only on the accumulation of more material.

CONCLUSIONS.

Evidence has been advanced indicating that:

1. The β-nucleoprotein of the pancreas of the dogfish contains both adenine and guanine in pentose mononucleotide combination.

2. This β-nucleoprotein is readily broken down to its constituent mononucleotides and protein by the action of cold 1 per cent sodium hydroxide.

3. The mononucleotides thus obtained are not precipitated from either neutral or acetic acid solution. The guanine mononucleotide differs in this respect from the guanylic acid previously isolated from mammalian material.

4. The acetic acid filtrate from β-nucleoprotein precipitation contains the mononucleotides of the β-nucleoprotein in solution together with inosinic acid.

Addendum.—Since the foregoing paper was written a communication has appeared by Hammarsten[2] which bears directly on the findings recorded and has drawn the writer's attention to earlier papers by Feulgen[3] which also have an important bearing on the subject. Both these authors have found adenine as well as guanine in β-nucleoprotein prepared from the pancreas of the ox by the Hammarsten method. Feulgen, by enzymic decomposition of the β-nucleoprotein has isolated a compound of guanylic

[2] Hammarsten, E., *J. Biol. Chem.*, 1920, xliii, 243.

[3] Feulgen, R., *Z. physiol. Chem.*, 1914, xci, 165; 1919, cvii, 147.

acid with nucleic acid and Hammarsten has isolated the same, or a very similar, body by direct extraction of the dried gland with cold dilute alkali and precipitation with acid. The latter author has also found about one-third as much adenine as guanine in β-nucleoprotein by acid hydrolysis followed by precipitation of the purines as silver salts and fractionation by the method of Steudel, and, since these bases are shown to be present in about the same ratio in the conjugated nucleic acid isolated by direct extraction of the pancreas, the implication appears to be made that β-nucleoprotein may be a body of similar constitution. Both authors regard the adenine found as part of the nucleic acid (tetranucleotide) component of the conjugated nucleic acid.

It is possible that the adenine found in the acetic acid precipitate (β-nucleoprotein) from the extract of the pancreas of the dogfish is due to a conjugated nucleic acid such as has been described by these authors. The presence of such a compound in the extract would not, however, affect the conclusion which has been drawn as to the pentose nucleotide combination of the adenine found in the filtrate after precipitation by acetic acid and by barium hydroxide.

BIBLIOGRAPHY.

1. Jones, W., and Kennedy, R. P., *J. Pharm. and Exp. Therap.*, 1919, xiii, 45.
2. Levene, P. A., *J. Biol. Chem.*, 1919, xl, 415.
3. Hammarsten, O., *Z. physiol. Chem.*, 1894, xix, 19.
4. Jones, W., and Rowntree, L. G., *J. Biol. Chem.*, 1908, iv, 289.
5. Levene, P. A., and Mandel, J. A., *Biochem. Z.*, 1908, x, 221.
6. Liebig, J., *Ann. Chem.*, 1847, lxii, 317.
7. Bennett, C. B., *J. Biol. Chem.*, 1912, xi, 221.
8. Jones, W., Nucleic acids: their chemical properties and physiological conduct, Monographs on biochemistry, London, New York, Bombay, and Calcutta, 1914, 8.
9. Bang, I., *Z. physiol. Chem.*, 1898–99, xxvi, 133.
10. Berkeley, C., *J. Biol. Chem.*, 1920, xli, p. liv.
11. Grund, G., *Z. physiol. Chem.*, 1902, xxxv, 111.
12. Starkenstein, E., and Henze, M., *Z. physiol. Chem.*, 1912, lxxxii, 417.
13. Abderhalden, E., Biochemisches Handlexikon, Berlin, 1911, ii, 519.
14. Abderhalden, E., Handbuch der Biochemisches Arbeitsmethoden, Berlin, 1910, iii, pt. ii, 888.
15. Jones, W., *J. Biol. Chem.*, 1911, ix, 129, 169.
16. Haiser, F., *Monatschr. Chem.*, 1895, xvi, 190.

A CRITIQUE OF EXPERIMENTS WITH DIETS FREE FROM FAT-SOLUBLE VITAMINE.*

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(Received for publication, November 13, 1920.)

Practically all recent investigators who have fed mixtures of purified food products to small animals have either assumed or concluded from their experiments that lard is devoid of fat-soluble vitamine.[1] In fact the importance of the latter factor was discovered because of the nutritive failure of rats that were being fed on diets in which lard furnished the sole fat therein.[2,3] The results reported by numerous investigators have, however, differed in respect to the effect of such diets on young rats. While some, like ourselves, have described their animals as usually growing at a nearly, or quite, normal rate for periods of 60 to 80 days before showing signs of a nutritive deficiency in the diet, others have secured little if any growth under apparently similar conditions. All, however, agreed that such diets are defective and that the failure to maintain body weight was followed sooner or later by symptoms of disease and death unless some source of the fat-soluble vitamine was supplied. It was surprising therefore to learn from the recent paper of Daniels and Loughlin[4] that rats could be made to grow, reproduce, and rear their young ". . . .

* The expenses of this investigation were shared by the Connecticut Agricultural Experiment Station and the Carnegie Institution of Washington, Washington, D. C.

[1] Report on the present state of knowledge concerning accessory food factors, *Medical Research Comm., National Health Insurance, Special Rep. Series, No. 38*, 1919, 16.

[2] McCollum, E. V., and Davis, M., *J. Biol. Chem.*, 1913, xv, 167.

[3] Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1913, xv, 311.

[4] Daniels, A. L., and Loughlin, R., *J. Biol. Chem.*, 1920, xlii, 359.

on diets in which the only apparent source of the vitamine was the lard or cotton-seed oil." These workers concluded that " . . . . both these fats contain appreciable amounts of the fat-soluble growth stimulant. This, however, is demonstrable only when fairly large amounts are fed."

In view of these divergent results it has seemed to us worth while to subject this problem to further critical investigation so that we might learn, if possible, the reason for the existing disagreement. In our own earliest observations[3] upon rats which were fed diets consisting of casein, edestin, or ovalbumin 18, "protein-free milk" 28, starch 26 to 29, lard 25 to 28 per cent; or casein, edestin, or lactalbumin 18, "artificial protein-free milk" 29, starch 25 to 28, lard 25 to 28 per cent, the characteristic nutritive failure expressed by retardation of growth followed by decline in body weight was not recognized until the lapse of many weeks. To avoid the uncertainty of possible changes resulting from the process of preparation for the market in the case of the commercial lard fed in these early trials, the experiments were duplicated by using lard rendered at comparatively low temperatures in our own laboratory.[5] The outcome with this carefully prepared fat was no different. The characteristic nutritive failure occurred in about the usual time, and the animals were restored to health by replacing a part of the lard with butter fat. The diets in these cases of malnutrition included 28 per cent of lard, an amount equal to that supplied by Daniels and Loughlin in rations of otherwise similar character. The failures were not confined to the use of one protein; for rats living on diets supplying casein, edestin, ovalbumin, or lactalbumin (as already indicated) showed the usual period of seemingly normal growth followed by failure, frequently with manifestation of eye disease. These and similar experiences are doubtless responsible for the following conclusion reached by the Committee upon Accessory Food Factors under the Medical Research Committee of Great Britain:[4]

" . . . . for a short time the young animals are able to grow when the fat-soluble factor is deficient in their diet. The explanation of this is probably supplied by the assumption that the animal organism normally

[5] Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1915, xx, 379.

contains reserve supplies of the factor 'A' in its own body. If this hypothesis is correct it is reasonable to assume that these reserves are mobilized for use when a deficiency occurs in the diet, but as soon as they are exhausted growth is immediately inhibited. During the period of temporary growth, throughout which it has been assumed that the reserve supplies are being utilized, the animals show a normal appearance and there does not appear to be any decline in their health, but when the reserves are exhausted and the deficiency becomes felt, not only do they cease to grow but they become highly susceptible to bacterial infection."

In our own experiments just referred to, the indispensable water-soluble vitamine was furnished in the form of "protein-free milk." It has been alleged that the initial delay in the manifestation of nutritive damage was due to fat-soluble vitamine carried by this product. Subsequently we replaced the protein-free milk by an artificial mixture of inorganic salts and starch (in place of milk sugar) and supplied the water-soluble vitamine by adding dried brewery yeast. The outcome with diets made up of casein or lactalbumin 18, or meat residue 19.6, salt mixture 4, starch 47 to 52.4, lard 24 to 31 per cent, plus 0.2 to 0.6 gm. of dried brewery yeast daily, was not essentially different; the failure did not appear notably earlier than in the original experiments.[6] Separate trials showed the yeast to be devoid of fat-soluble vitamine, even enormous quantities of it failing to protect the animals on lard diets against the effects of the deficiency in fat-soluble vitamine[7] (see Chart I).

As already stated, there are in the literature of the subject several records[8] of young animals, fed on foods supposedly free from fat-soluble vitamine, which failed to grow in the way that we have described, whereas the experience of others[9] coincides with our own published results in which the nutritive decline was delayed until some considerable growth had been made. This raises the question of the complete freedom from fat-soluble vitamine of the foods used in the rations employed in different

[6] Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1920, xli, 549, Charts I and III.

[7] Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1919, xxxvii, 199.

[8] McCollum, E. V., Simmonds, N., and Pitz, W., *Am. J. Physiol.*, 1916, xli, 333. Halliburton, W. D., and Drummond, J. C., *J. Physiol.*, 1917, li, 235.

[9] Stephenson, M., and Clark, A. B., *Biochem. J.*, 1920, xiv, 502.

investigations and the methods employed for purifying their ingredients. Were there traces of fat-soluble vitamine remaining in the products employed, which will account for the initial recorded growth of young rats on diets of protein, carbohydrate, inorganic salts, and lard?

The problem of the removal of this vitamine from food products has not been satisfactorily solved. Many protocols describe

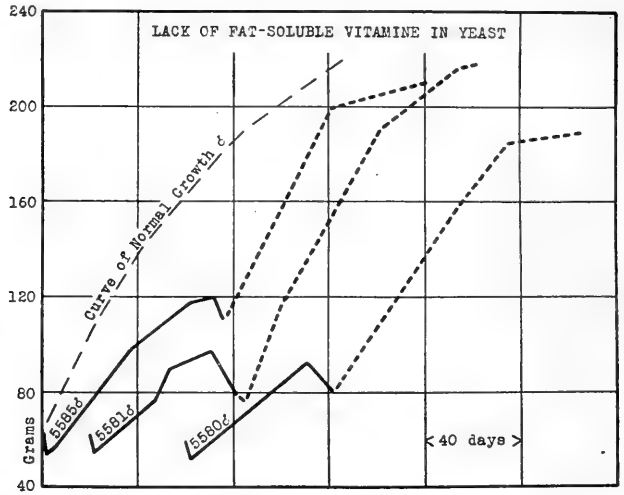

CHART I. Showing the failure of very large quantities of dried brewery yeast to furnish sufficient fat-soluble vitamine to protect rats from nutritive decline on diets otherwise adequate. Renewal of growth promptly ensued when butter fat was incorporated in the diet during the period indicated by the interrupted lines. The food mixture consisted of yeast 42.5, salt mixture 4, starch 30.5, lard 23 per cent.

the use of foods purified by extraction with various organic solvents, particularly ether. There is reason to believe that the fat-soluble vitamine is not always readily soluble in such liquids. Pure ether may be ineffective, in some cases at least, unless the food product has been previously treated with alcohol. This behavior is analogous to that of phosphatides in egg yolk, blood serum, milk, etc., which are insoluble in *pure* ether until they have been liberated from some combination with protein by

preliminary treatment with alcohol.[10] Although it has been reported[11] that ether does not extract the fat-soluble vitamine from dried leaves, we have obtained it by extraction with U. S. P. ether (which contains alcohol and water).[12] From the substances extracted by alcohol the vitamine is perhaps extractable by ether. The effect of heat on this food factor may hinge upon its preliminary liberation from other complexes.

Inasmuch as the study of problems concerned with the physiological function of the fat-soluble vitamine requires a food entirely devoid of it we have renewed the investigation from this standpoint. The protein, casein or edestin, was boiled three successive times with absolute alcohol under a reflux condenser for 1 hour, and then filtered off by suction. The commercial corn-starch used was treated in the same manner. The brewery yeast was first dried over night in an electric oven at 110°C. and then extracted in the same way.

In separate trials[13] we have assured ourselves that the yeast used as a source of water-soluble vitamine in all these tests does not lose its potency by desiccation at 110°C. followed by extraction with *absolute* alcohol. Rats have grown at a normal rate for 140 days to 336 gm. on rations in which such heated and alcohol-extracted yeast furnished the sole source of water-soluble vitamine in an otherwise adequate diet.

The curves of growth of a series of rats receiving a mixture of the extracted foods are given in Chart II. The diets consisted of extracted casein or edestin 18, extracted starch 48, salt mixture 4, lard 30 per cent, together with 0.4 gm. of extracted yeast daily.

[10] Osborne, T. B., and Campbell, G. F., *J. Am. Chem. Soc.*, 1900, xxii, 413. Osborne, T. B., and Wakeman, A. J., *J. Biol. Chem.*, 1916, xxviii, 1. van den Bergh, A. A. H., and Muller, P., *Proc. Acad. Sc. Amsterdam*, 1920, xxii, 748.

[11] McCollum, E. V., Simmonds, N., and Pitz, W., *Am. J. Physiol.*, 1916, xli, 363.

[12] Osborne, T. B., and Mendel, L. B., *Proc. Soc. Exp. Biol. and Med.*, 1918–19, xvi, 98; *J. Biol. Chem.*, 1920, xli, 549. See also Zilva, S. S., *Biochem. J.*, 1920, xiv, 494.

[13] The general methods of feeding, etc., employed in our investigations have recently been outlined by Ferry, E. L., *J. Lab. and Clin. Med.*, 1920, v, 735.

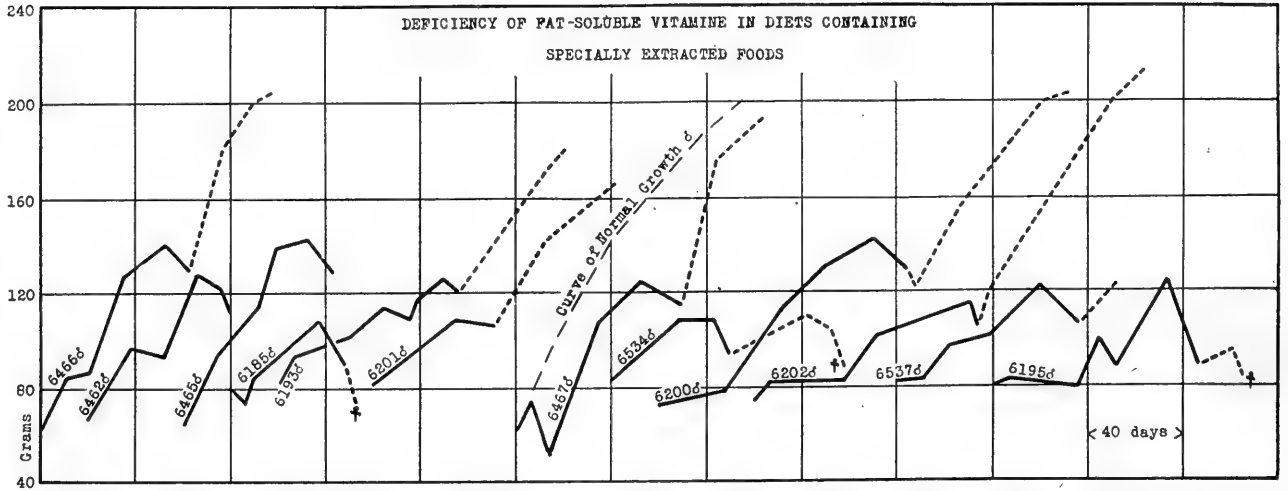

CHART II. Showing the preliminary period of growth followed by decline in body weight on diets containing specially extracted foods. During the periods represented by the interrupted lines renewal of growth was frequently secured by the addition of sources of fat-soluble vitamine to the diet. During the period represented by the uninterrupted line the food mixtures consisted of extracted casein or edestin 18, salt mixture 4, extracted starch 48, lard 30 per cent, together with 0.4 gm. of extracted yeast daily. Rats 6193, 6201, 6200, and 6202 developed the characteristic eye disease which was promptly cured after the administration of the fat-soluble vitamine.

These results should be contrasted with the records of experiments in which the foods were not specially extracted (Chart IV).

Since we have found[5] that the fat-soluble vitamine can be removed from butter fat by recrystallizing the latter from strong alcohol, commercial lard was melted and poured into absolute alcohol, heated to 60°C., cooled over night, and filtered by suction. This treatment was repeated three times, and the resulting solids were dried in a casserole over a steam bath. Experiments with this alcohol-extracted lard fed in place of the usual commercial product showed no essential differences in their outcome in so far as retardation of growth or well being were concerned.

It is a common experience that animals living on unsuitable diets tend to reduce their food intake. When the indispensable water-soluble vitamine is *incorporated* in such food the consumption of this food accessory may be greatly reduced because the animals do not eat enough of the rations containing it. A vicious circle is thus established whereby insufficient water-soluble vitamine leads to inadequate eating which in turn limits the quantity of both food and vitamine available. This criticism may be made of many investigations in which the undetermined and presumably lowered food intake occasions uncertainty regarding the supply of essential components other than the one specifically under investigation. It is especially true when the water-soluble vitamine is an integral component of the food mixture offered.

As an illustration of the difficulty of interpretation we may cite a series of unpublished experiments carried out by us several years ago with "fat-free" foods. The diets consisted of casein 18, salt mixture 4, yeast 2, and starch 76 per cent. Curves of body weight are shown in Chart III. It will be noted that on these rations supposedly devoid of fat-soluble vitamine the animals remained practically stationary in weight in contrast with what has been observed by us where lard was incorporated in the diet. All the rats scattered their food quite badly so that accurate estimates of the food intake could not be made. For this reason we are unable to conclude whether the failure to grow in the first period was due to inadequate intake of water-soluble vitamine or other essential nutrients, or was merely the expression of the *complete* lack of fat-soluble vitamine. All these animals began to grow when butter fat was added to the diet; but the food intake and consequently the intake of water-soluble

vitamine were simultaneously increased. McCollum[14] has published similar curves of complete failure to grow on a ration of casein, salts, agar-agar, and dextrin, carrying an extract of wheat embryo as a source of water-soluble vitamine, to which the criticism just applied to our own experiments may perhaps likewise be made.

The deleterious effect of a diet lacking in water-soluble (B) vitamine is far more acute in its manifestations than are the dietary failures observed by us when the fat-soluble vitamine is not supplied.[15]

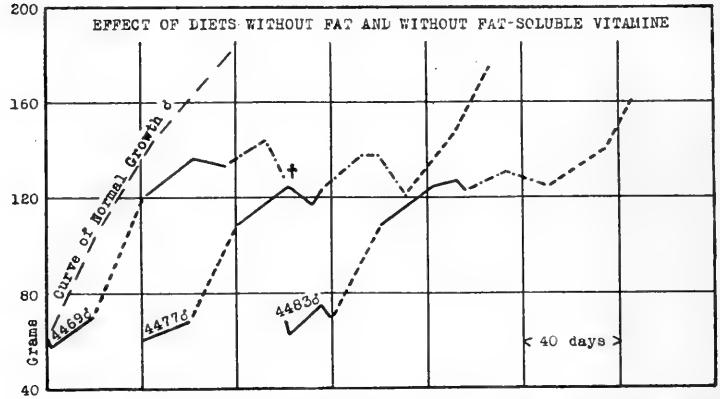

Chart III. Showing the failure to grow on fat-free foods (solid line) and on diets containing 9 per cent of lard (- - - - - -), with prompt growth whenever 9 per cent of butter fat (- - - - - -) was incorporated in the diet. The fat-free food mixture consisted of casein 18, salt mixture 4, starch 76, yeast 2 per cent.

In our newer series with alcohol-extracted foods this uncertainty has been averted by furnishing suitable daily doses of the water-soluble vitamine *apart* from the ration. Inasmuch as the animals greedily ate the separate allowance of yeast an adequate intake of this factor was assured. The outcome of the experiments with rations devoid of fat-soluble vitamine illustrated in Chart II has been a reduction of the period of undiminished

[14] McCollum, E. V., *Proc. Inst. Med.*, 1920, iii, 13, Chart I, Lot 417.

[15] Osborne, T. B., Wakeman, A. J., and Ferry, E. L., *J. Biol. Chem.*, 1919, xxxix, 35.

growth. However, even in these cases there was for some time a not negligible increment of weight. The prompt recovery of the animals tested, after their decline had begun, by the addition of butter fat, without any alteration in the dosage of water-soluble vitamine gave a clear answer to the nature of the deficiency in their previous diet. Whether the protein and starch used in our rations before they have been thoroughly extracted

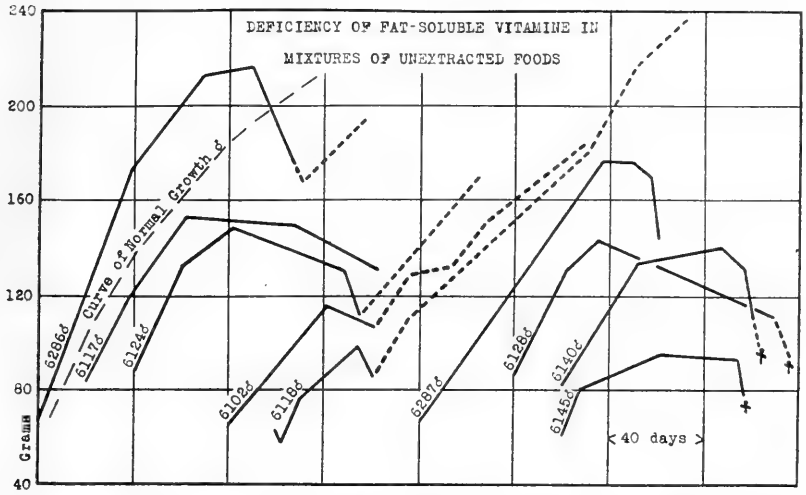

Chart IV. Showing preliminary growth followed by a decline in body weight on diets on (unextracted) foods deficient in fat-soluble vitamine, during the period indicated by the solid line. The recoveries during the periods indicated by the broken line follow the addition of butter fat as a source of fat-soluble vitamine to the diet. In the first period the food mixture consisted of casein or edestin 18, salt mixture 4, starch 48, lard 30 per cent, together with 0.4 gm. of yeast daily.

These records should be contrasted with the results of experiments in which the foods were specially extracted (Chart II).

with absolute alcohol are carriers of any noteworthy amount of fat-soluble vitamine may be judged by a comparison of the records just cited with those for a group of animals fed on precisely similar diets representing mixtures of food products which had not been extracted (Chart IV).

One gains the general impression that the extraction may

remove a small amount of the fat-soluble vitamine which if present would postpone the decline in weight somewhat longer. Steenbock and Gross[16] likewise give numerous records of a slight, though soon limited continuation of growth when a normal diet is replaced by one of highly purified food substances supposed to be free from fat-soluble vitamine. It should not be forgotten, however, that other substances which may have been removed by the extraction with alcohol may also be of importance for the well being of the animal. Convincing evidence that minute quantities of one or more lipoids are not essential has not yet been furnished.

It is interesting to note that whereas the fat-free diet used by McCollum,[14] which failed to permit growth, contained as the sole source of water-soluble vitamine the extract of only 11 gm. of wheat embryo, the precisely similar experiments of Steenbock and Gross, in which considerable initial growth was achieved on fat-free diets, contained the extract from 20 to 78 gm. of ether-extracted wheat embryo. Hence it may well be, as we have intimated, that the temporary growth on diets without any appreciable quantity of fat-soluble vitamine may be determined by the dosage of the water-soluble vitamine which the animals can secure. Indeed Steenbock and Gross have pointed out an instance

> " . . . . where presence of a subnormal amount of one vitamine was apparently brought to light by a similar situation with respect to another vitamine. When the deficiency of the water-soluble vitamine was corrected the amount of fat-soluble vitamine originally present was able to allow some further growth to result. . . . . It brings out one of the innumerable instances where a tendency to one nutritive deficiency heightens the susceptibility and results in the onset of symptoms resulting from similar or other unfavorable environmental conditions" (p. 507).

In the course of the feeding experiments it occurred to us that possibly the prolonged manipulations with alcohol render the food unpalatable. If this were true the nutritive failures might be due to undereating rather than to the lack of an essential ingredient removed by the extractions. This assumption was shown, however, to be untenable by a series of feeding trials in which the foods were treated with alcohol precisely as was the

[16] Steenbock, H., and Gross, E. G., *J. Biol. Chem.*, 1919, xl, 501.

case for the extracted foods. The products were subsequently dried to drive off the alcohol without filtration or removal of any non-volatile ingredient. Chart V shows the outcome of feeding trials with such foods. Since where butter fat was present growth was essentially normal the assumption which has found its way into the literature that heating with alcohol deteriorates the nutritive value of casein is disposed of. Where lard was the only fat present the curves of growth are not essentially unlike those

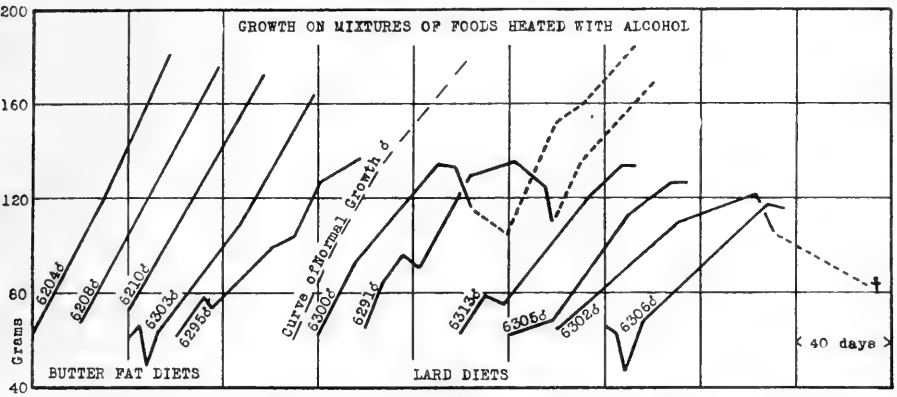

Chart V. Showing the absence of noteworthy effect from heating with alcohol in the case of mixtures of foods either adequate for growth (Rats 6204, 6208, 6210, 6303, 6295) or deficient in fat-soluble vitamine (Rats 6291, 6300, 6302, 6313, 6305, 6306). The food during the periods indicated by the solid lines consisted of casein (heated with alcohol) 18, starch (heated with alcohol) 48, salt mixture 4, butter fat 9 or 0, lard 21 or 30 per cent, with 0.4 gm. of alcohol-extracted yeast daily. During the periods indicated by the broken line butter fat was supplied as a source of fat-soluble vitamine.

for the similar experiments with unextracted foods represented in Chart IV.

In the successful feeding experiments with the lard-containing foods reported by Daniels and Loughlin, to which reference was made in our opening paragraph, it is reported that in order to reduce the content of fat-soluble vitamine to a minimum in the experimental rations " . . . . both the casein and the wheat embryo, previous to the alcohol treatment, were extracted for 48 hours with ether ('Squibb, for anæsthesia') in a Soxhlet

apparatus. The lard used was a commercial product rendered from the leaves and back fat." Must we assume for the present either that the lard from different sources varies in its content of fat-soluble vitamine or that ether alone fails to extract it from the non-fat foodstuffs?

It has been alleged that the ability of animals to grow for some time in the supposed absence of fat-soluble vitamine is due to "exceptional vitality" of the individuals or to reserve stores of the vitamine in the body.[17] Unless we are to accept some indefinite explanation of this sort, it seems necessary to conclude, in the light of our experience, that removal of the fat-soluble vitamine from even purified proteins and carbohydrates is accomplished with far greater difficulty than has been hitherto suspected. An entirely convincing crucial experiment, in which nutritive failure immediately ensues upon the administration of diets fully adequate *in every respect* except for the presence of fat-soluble vitamine, remains to be made. It is significant that older rats thrive for a longer time than do the younger ones on the same diets nearly if not entirely free from the fat-soluble vitamine. This is in contrast with the well established fact that at all periods the lack of water-soluble vitamine is speedily manifested.

[17] McCollum, E. V., and Davis, M., *J. Biol. Chem.*, 1915, xx, 641. Also Drummond, J. C., *Biochem. J.*, 1919, xiii, 81, who states that occasionally rats are encountered which "exhibit a power to grow for long periods of time although receiving a diet seriously deficient in fat-soluble A."

COMPARATIVE METABOLISM OF PROTEINS OF UNLIKE COMPOSITION.*

By WALTER G. KARR.

With the Cooperation of Edward Tolstoi.

(From the Sheffield Laboratory of Physiological Chemistry, Yale University, New Haven.)

(Received for publication, November 12, 1920.)

Proteins contain nitrogen in different groupings; *e.g.*, amino nitrogen, amide nitrogen, glyoxaline and proline, and the guanidine rest. On hydrolysis of proteins with strong mineral acids an ammonia fraction is very easily split off and represents the amide nitrogen in the molecule. This ammonia fraction runs parallel with the amount of dicarboxylic acids—aspartic and glutaminic—from the amide nitrogen of which it is probably for the most part derived (Osborne, 1909).

The proportion of these unlike types of nitrogenous groups varies in different proteins; *e.g.*, casein has a low content of amide nitrogen while the proteins of the wheat kernel are richer in this grouping of nitrogen. A sample of commercial casein containing 12.6 per cent total nitrogen, hydrolyzed for 2 hours with 20 per cent HCl, yielded 10.5 per cent of its nitrogen as ammonia nitrogen while a sample of gluten having 13.0 per cent total nitrogen gave 21.6 per cent of its nitrogen as ammonia nitrogen under the same conditions.

We know that the proportions of the end-products of nitrogenous metabolism in the urine, *e.g.* urea N, ammonia N, creatinine N, creatine N, etc., may be varied by the nature of the diet. It is not definitely ascertained, however, whether this partition

* The data in this paper are taken from the writer's dissertation presented in partial fulfillment of the requirements for the degree of Doctor of Philosophy, Yale University, 1920. A part of the expenses of the research were defrayed by a contribution from the Russell H. Chittenden Fund for Research in Physiological Chemistry.

of nitrogen can be altered by the type of distribution of nitrogen in the protein intake; *i.e.*, whether amide nitrogen has the same fate in metabolism as amino nitrogen. Therefore the following experiments on the metabolism of different diets varying only with respect to the character of the protein therein have been undertaken. Inasmuch as the character of the metabolic change may be determined by the plane of nutrition, *i.e.* whether an animal is on a low nitrogen intake barely sufficient for its needs, or on a high intake, experiments were conducted at different levels of protein intake.

Three female dogs were employed. They were kept in ordinary metabolism cages and catheterized at 24 hour intervals under conditions to preclude infection. Experimental periods lasted 4 or 5 days each. The feces were marked into periods with carmine. They were dried on the water bath with alcohol to which a few drops of acid were added. Water was given *ad libitum*. Total nitrogen was determined by the Kjeldahl method; ammonia nitrogen by the Folin aeration method; creatinine by the Folin micro method; creatine by the same procedure after treatment with 2 cc. of normal HCl in an autoclave for 20 minutes at 20 pounds pressure; phosphates by titration with uranyl acetate; hydrogen ion concentration by the Sörensen colorimetric method using sodium alizarin sulfonate as indicator for the range pH 5.0 to 6.0 and neutral red for pH 6.0 to 7.5.

The proteins fed were casein and wheat gluten. The casein[1] was a light yellow commercial preparation, finely ground, containing about 12.7 per cent of nitrogen. The gluten was a commercial variety,[2] very finely ground, containing 12 to 14 per cent of nitrogen. In addition to the protein under investigation the remaining calorific requirement was supplied by approximately equal weights of lard and sucrose to which were added 2 gm. of NaCl and 5 gm. of bone ash, the latter to give proper consistency to the feces. The diets with a few exceptions were fed in one meal soon after catheterization in the morning and were made to supply about 70 calories per kilo of body weight, but were increased in certain experiments (see Table I).

[1] Casein was kindly supplied by Lister Brothers, New York.

[2] Wheat gluten was purchased from Menley and James, New York, under the commercial name Glidine.

TABLE I.

Results of Feeding Experiment with Casein and Gluten.

(The data give daily averages of the periods.)

Experiment No.	N intake.	Food intake.	Urine.							
			Volume.	Reaction.	Total N.	NH_3 N.	$\frac{NH_3\ N}{Total\ N}$	Creatinine N.	Creatine N.	Phosphorus.
	gm.	*calories*	*cc.*	*pH*	*gm.*	*mg.*	*per cent*	*mg.*	*mg.*	*mg.*
1. Casein period, 5 days; Dog B, weight 8.4 kg.	20.2	720	500	6.1	17.3	1,140	6.6	78	*	944
2. Gluten period, 5 days; Dog B, weight 8.4 kg.	19.9	600	590	6.4	17.8	967	5.4	78	*	164
3. Gluten period, 3 days; Dog A, weight 9.7 kg.	19.9	600	470	6.4	17.3	850	5.0	95	*	140
4. Gluten period, 5 days; Dog A,† weight 10.7 kg.	13.0	900	450	6.5	11.3	640	5.7	96	2	213
5. Gluten period, 3 days; Dog C, weight 6.5 kg.	14.9	460	445	6.4	13.2	724	5.5	55	2	
6. Casein period, 5 days; Dog C, weight 6.5 kg.	6.95	440	260	6.1	6.15	406	6.6	58	16	352
7. Gluten period, 5 days; Dog C, weight 6.5 kg.	6.82	430	330	6.1	6.76	345	5.4	57	12	125
8. Casein period, 5 days; Dog A,† weight 10.2 kg.	6.32	700	220	6.1	5.21	350	6.7	100	0	375
9. Gluten period, 5 days; Dog A,† weight 10.4 kg.	6.40	670	370	6.6	5.84	352	6.0	99	3	211

* When creatine was determined on urines from dogs on a very high protein diet the combined creatinine after HCl treatment was always a few milligrams less than the preformed creatinine. The point was not further investigated except that a few normal human urines were simultaneously analyzed and did not show the phenomenon, the combined and preformed creatinine being identical.

† The dog was pregnant.

TABLE I—*Concluded.*

Experiment No.	N intake.	Food intake.	Urine. Volume.	Reaction.	Total N.	NH_3 N.	NH_3 N / Total N	Creatinine N.	Creatine N.	Phosphorus.
	gm.	*calories*	*cc.*	*pH*	*gm.*	*mg.*	*per cent*	*mg.*	*mg.*	*mg.*
10. Casein period, 5 days; Dog B, weight 8.2 kg.	6.57	600	260	6.1	5.72	380	6.6	78	5	442
11. Casein period, 5 days; Dog B, weight 8.6 kg.	2.94	610	220	6.0	2.77	195	7.1	82	5	280
12. Gluten period, 5 days; Dog B, weight 8.5 kg.	2.98	600	210	6.3	3.03	209	6.9	82	4	206
13. Casein period, 5 days; Dog C, weight 6.7 kg.	2.56	470	200	6.2	2.20	112	5.2	59	25	227
14. Gluten period, 5 days; Dog C, weight 6.5 kg.	2.57	460	200	6.3	2.32	117	5.0	59	14	164

DISCUSSION.

The urinary ammonia output is not influenced to any appreciable extent by the character or quantity of the two so widely different proteins in the diet. If the metabolism of the abundant amide nitrogen of the wheat proteins were different from that of the amino nitrogen of the casein one might anticipate that more urinary nitrogen in the form of ammonia would be excreted when the diet contained the wheat gluten. This was not the case and in general the excretion was a little lower. The average excretion on the casein diets was 6.5 per cent of the total nitrogen and 5.6 per cent on the gluten diets. The slight increase in the hydrogen ion concentration and the urinary ammonia on the casein diet might easily be explained by the greater potential acidity of the casein. The greater acidity of the casein is apparently mostly taken care of by the phosphate regulation. The creatinine output was evidently entirely of endogenous origin. The creatine found seems independent of the nature of the protein.

Additional Experiments with Yeast Protein.

Experiments similar to the preceding were conducted with yeast as the sole source of nitrogen in order to study the partition of the urinary nitrogen and to compare it with that following the ingestion of the other two proteins, and incidentally to determine the utilization of the yeast nitrogen. The literature shows rather conflicting statements as to the availability of the yeast protein in the animal body.

Völtz (1905) reported 83.6 per cent of nitrogen utilized in one experiment with a dog. Völtz and Baudrexel (1911) reported an experiment on a man in which about one-half the nitrogen was supplied by yeast; 86 per cent was utilized. Rubner (1916) giving 13.7 gm. of yeast nitrogen plus 30.9 gm. of meat nitrogen to a dog calculated that 98.4 per cent of the yeast nitrogen was utilized. Deutschland (1917) gave a dog a diet containing about one-half its nitrogen as yeast nitrogen and obtained utilizations of 83.5 and 89 per cents. Schill (1918) reports coefficients of 40 per cent in one case and 75 per cent in the other. The above data were obtained by using yeast as a part only of the nitrogen intake and obviously do not give a clear idea as to the utilization of the yeast nitrogen. Funk, Lyle, and McCaskey (1916) in four experiments on man in which yeast supplied the principal sources of nitrogen found 70 to 80 per cent utilized and decided that it "cannot very well be recommended as a sole protein source." Schill (1918) reported two experiments where yeast was the only source of nitrogen in the diet of two dogs. The intakes were low (1.75 and 1.54 gm. of nitrogen per day); the utilization was 66 and 76 per cent respectively.

The yeast used in the present investigation was a dried commercial brewery yeast.[3] It contained nitrogen 7.2, moisture 9.1, and ash 6.6 per cent. Neuberg (1915) estimated that 46 to 48 per cent of the nitrogen of yeast is protein nitrogen while Bokorny (1915) estimated it at 60 per cent. The yeast was suspended easily in water and was readily given with the other constituents of the diet each day through a stomach sound. With this exception the experiments were conducted similarly to those reported above (see Table II).

The ammonia nitrogen output compares favorably with that after feeding casein and gluten, giving further evidence that it is not influenced by the character of the protein. On certain days of the lower nitrogen intake periods the ammonia was compara-

[3] Supplied by Hinckle Brewery, Albany.

TABLE II.

Results of Feeding Experiments with Yeast.

(The data give daily averages of the periods.)

Experiment No.	Yeast.	N intake.	Food intake.	Urine.								Feces.			N balance.	N utilization.
				Volume.	Reaction.	Total N.	NH_3 N.	$\frac{NH_3\ N}{Total\ N}$	Creatinine N.	Creatine N.	Phosphorus.	Weight.	N			
	gm.	*gm.*	*calories*	*cc.*	*pH*	*gm.*	*mg.*	*per cent*	*mg.*	*mg.*	*mg.*	*gm.*	*per cent*	*gm.*	*gm.*	*per cent*
1. 5 days; Dog B, weight 8.6 kg.	90	6.52	625	560	6.5	5.27	345	6.5	84	6	972	38	4.0	1.5	−0.3	77
2. 5 days; Dog C, weight 6.4 kg.	70	5.00	460	360	6.5	3.90	240	6.2	58	30	700	32	4.0	1.3	−0.2	75
3. 5 days; Dog B, weight 8.5 kg.	40	2.90	610	345		2.29	213	9.3	81	5	510	21	3.0	0.6	0.0	79
4. 5 days; Dog C, weight 6.6 kg.	35	2.53	480	400		2.01	169	8.4	59	32	506	14	3.1	0.4	+0.1	82

tively high, but no explanation is evident until more is known concerning the nature of the nitrogenous compounds of yeast. The excretion of urinary phosphorus is high as would be expected from the high content of nucleic acid in the yeast. The nitrogen balance and percentage utilization indicate that yeast has about 80 per cent of its nitrogen fairly efficient for nutrition.

CONCLUSIONS.

As far as is indicated by the urinary nitrogenous end-products, the metabolism of two proteins, casein and wheat gluten, widely different in their chemical make-up, is essentially the same.

Despite the great diversity in amide nitrogen in the proteins compared, the relative excretion of urinary ammonia was approximately the same.

In metabolism experiments with comparable quantities of yeast as the sole source of protein, the partition of urinary nitrogen compared favorably with what was observed after feeding the other two proteins.

The utilization of the yeast nitrogen was about 80 per cent.

The writer wishes to thank Professor Lafayette B. Mendel for his suggestion of the problem and advice throughout the course of the investigation.

BIBLIOGRAPHY.

Bokorny, T., *Allg. Brau.-Hopfen Ztg.*, 1915, lv, 431.
Deutschland, A., *Biochem. Z.*, 1917, lxxviii, 358.
Funk, C., Lyle, W. G., and McCaskey, D., *J. Biol. Chem.*, 1916, xxvii, 173.
Neuberg, C., *Woch. Brau.*, 1915, xxxii, 317.
Osborne, T. B., The vegetable proteins, Monographs on Biochemistry, London, New York, Bombay, and Calcutta, 1909.
Rubner, M., *Münch. med. Woch.*, 1916, lxiii, 629.
Schill, E., *Biochem. Z.*, 1918, lxxxvii, 163.
Völtz, W., *Arch. ges. Physiol.*, 1905, cvii, 360.
Völtz, W., and Baudrexel, A., *Biochem. Z.*, 1911, xxx, 457.

RIGOR MORTIS IN SMOOTH MUSCLE AND A CHEMICAL ANALYSIS OF FIBROMYOMA TISSUE.

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(Received for publication, August 30, 1920.)

1. Experiments Demonstrating a Postoperative Rigor in Fibromyomas.

Studies of rigor mortis recorded in the literature have been made almost exclusively on skeletal muscle, and whatever conclusions there may be regarding its occurrence in smooth muscle seem to have been reached largely by inference from such studies, rather than by actual experimental observations. Wells[1] states that all forms of muscle, striped, smooth, and cardiac, undergo rigor mortis, manifested by a shortening and thickening. Saxl,[2] investigating the distribution of muscle proteins in bovine uteri, observed no difference between the analyses of tissues fresh and after 24 hours. He did not see a postmortem rigidity of these uteri. Nasse, according to this investigator, found the intestinal wall firmer several hours after death than immediately post mortem, and concluded that this is a rigor mortis contraction of the intestinal musculature. Hawk,[3] referring to Saxl's study, says there is a difference of opinion regarding the occurrence of true rigor in non-striated (smooth) muscle. Such statements in the literature are sufficiently at variance with the observed contraction and intussusception of bowel post mortem, and to similar or postoperative rigidity of uteri and fibromyomas to deserve investigation.

[1] Wells, H. G., Chemical pathology, Philadelphia, 3rd edition, 1918.

[2] Saxl, P., *Beitr. chem. Physiol. u. Path.*, 1907, ix, 1.

[3] Hawk, B. P., Practical physiological chemistry, Philadelphia, 6th edition, 1918.

Contraction of bowel segments or their intussusception has been observed post mortem, no doubt, by every pathologist, but the doughy consistency of a uterus or a fibromyoma changing to a rigid firmness soon after death or surgical removal is not so commonly appreciated. Histologically, fibromyoma tissue contains much smooth muscle, and such contractile phenomena as it may manifest are due, no doubt, to this element of its structure.

In order to study the rigidity mentioned, and, if possible, correlate it with the physical changes of skeletal muscle known as rigor mortis, sections of operatively removed uteri and fibromyomas 1 cm. square and 4 cm. long were mounted soon after

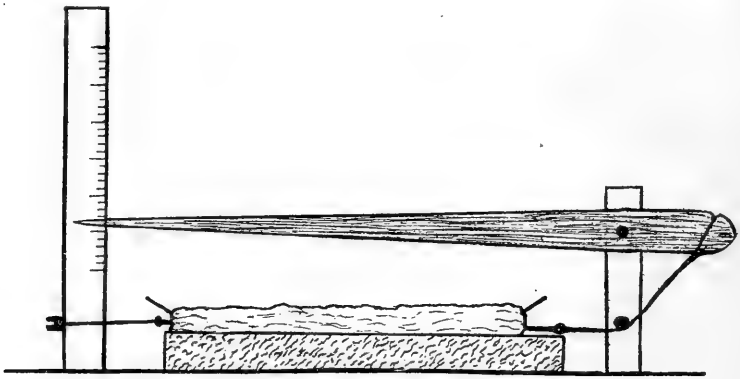

Fig. 1. Apparatus for mounting muscle.

removal, as shown in Fig. 1. The tissue preparation was placed in a moist chamber at room temperature (62–70° F.), moistened with a few drops of normal salt solution, and the contractions were observed.

As soon as the fresh tissue preparation is mounted, contraction begins and continues gradually to a maximum. In order to represent this change graphically, a curve was made by plotting along the ordinate the contraction measured on the millimeter scale of the apparatus, and along the abscissa the time in minutes. Two such curves are given in Fig. 2, one of them for uterine muscle, the other for fibromyoma tissue. After the period of contractility has passed, which may be as short as 1 hour after removal, forceful extension is not followed by shortening.

All the experiments agree in result with the graphs prepared, excepting such fibromyoma tissue as has undergone retrogressive changes known as "red degeneration." Such tissue manifests none of the contractile phenomena observed with other fibromyomas and uteri.

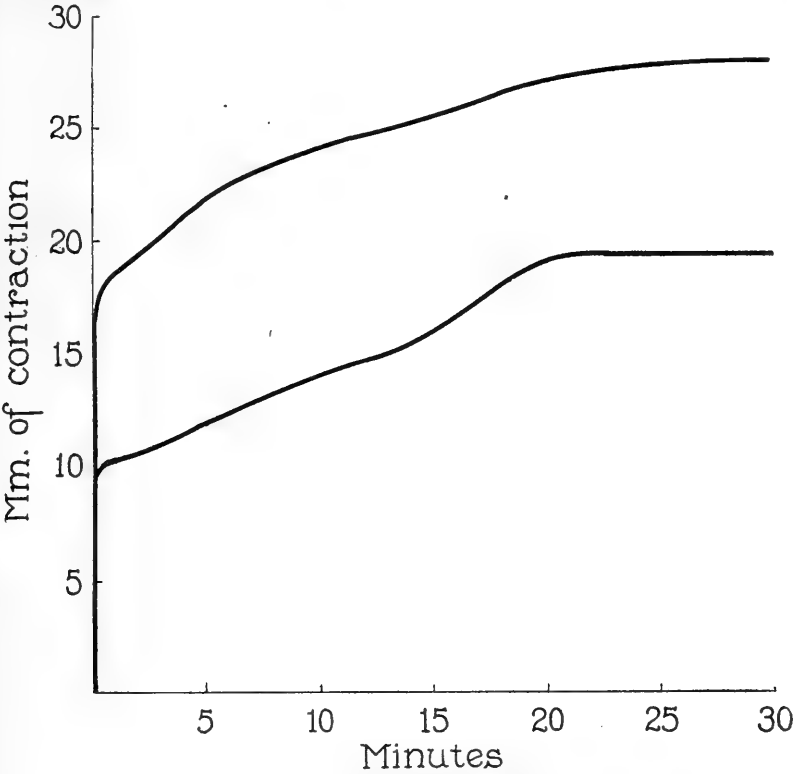

FIG. 2. Upper curve shows contraction of fibromyoma; lower curve contraction of uterus.

It is possible to say, from these observations, that a postoperative rigor occurs in smooth muscle, which is similar, at least in so far as contraction is concerned, to the postmortem rigor of striated muscle.

The heart, in postmortem examinations generally, is said to be in firm systole or to have ceased action in systolic contraction.

This statement, no doubt, is incorrect, for the postmortem rigor of heart muscle is similar to that observed with other involuntary muscle.

2. Experiments Determining a Postoperative Acidity in Fibromyomas.

Postmortem rigor and rigor induced under other conditions in skeletal muscle are known generally to be accompanied by an acidity. Lactic acid has been identified in such tissues and is believed largely to be the acid causing this change in reaction. With the exception of certain heat rigor experiments on smooth muscle by Meigs,[4] all these observations have been made on skeletal muscle.

Since a postoperative rigor can be demonstrated in fibromyoma tissue and uteri, it is possible that an acidity similar to that occurring in striated muscle may accompany this change. To determine it, standard solutions and the appropriate indicators in solution were prepared carefully according to Clark and Lubs.[5] Triple distilled "conductivity water" was used in making these solutions and in conducting the reaction experiments. The tissue reaction was determined by placing 10 gm. samples of fibromyoma and uterine tissue in 15 cc. of water, and after sufficient time (usually a minimum of 15 minutes was taken) for the reaction of the water to approach that of the tissue, 2 cc. were placed in a comparing tube, colored with the proper indicator, and compared with a series of standard solutions.

The results of the individual experiments are given in Table I.

While the contention is not made that the hydrogen ion concentration obtained by the method described represents the maximum acidity, for doubtless dilution of the tissue fluids and other factors must be considered, yet these tests demonstrate uniformly an acidity developing rapidly in fibromyoma and uterine tissue manifesting a postoperative rigor. This change in reaction is another similarity correlating the rigor of fibromyomas (non-striated muscle) with the postmortem rigor of skeletal muscle. The only exception again is the fibromyoma

[4] Meigs, E. B., *J. Biol. Chem.*, 1909, vi, p. xviii.

[5] Clark, W. M., and Lubs, H. A., *J. Bact.*, 1917, ii, 1, 191.

tissue with changes known as "red degeneration" in which neither an acidity nor a rigor developed. The substance giving the acid reaction to the tissues may be lactic acid, for several of the fluids in which the fibromyoma tissue had been contained reacted positively with the ferric chloride test for this acid.

Kondo[6] determined quantitatively the increase of lactic acid in expressed skeletal muscle fluid, and found that its formation is unusually rapid, the maximum concentration in some fluids having been reached within 30 minutes. He observed that

TABLE I.

Hrs.	1	2	3	4	5	6	7	22	23	24
	pH	*pH*	*pH*	*pH*	*pH*	*pH*	*pH*	*pH*	*pH*	*pH*
Fibromyoma				6.8	6.8	6.8		6.2		6.0
"									6.0	
"	6.4			6.4			6.2	6.2		6.2
Uterus	6.4									
Fibromyoma.*	7.0			7.2		7.2	7.2			
" †			6.6							
"			6.2	6.0		6.0			6.0	
"			6.0							

* Red fibromyoma, did not contract in moist chamber.
† " " contracted little " " "
Some fluids in these experiments reacted with the ferric chloride test for lactic acid.

lactic acid production is completely inhibited by the addition of acid (N H_2SO_4) to the muscle fluid, and is favored by the addition of sodium bicarbonate, there being an optimum concentration of this alkali in such fluids beyond which it exerts no influence. In the presence of a small quantity of alkali in the expressed muscle fluid, the formation of lactic acid soon reaches a maximum, and is inhibited at a certain hydrogen ion concentration. This concentration is not given by Kondo. His conclusion, however, is supported by the results of this study for there has been found a fairly uniform maximum (pH 6.0) which the usual tissue acidity does not exceed.

[6] Kondo, K., *Biochem. Z.*, 1912, xlv, 63.

3. Postoperative Alkali Reserve Changes in Fibromyoma Tissue.

To study further the postoperative change in the reaction of these tumors, 3 cc. of fluid were expressed from small pieces of tissue with a hand meat press. The fluids were equilibrated with air containing 5.5 per cent carbon dioxide and analyzed according to the Van Slyke and Cullen[7] method, the proper corrections for 0°C. and 760 mm. barometric pressure being made.

Two such determinations were made with fibromyoma tissue 24 hours after removal, the carbon dioxide capacities of their fluids being respectively 3.93 and 6.06 volumes per cent; the fluid of a third after 8 hours, 8.4 volumes per cent; and that of

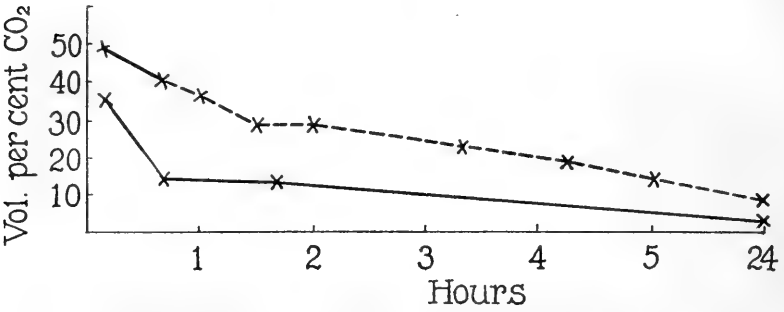

FIG. 3. Postoperative alkali reserve changes in fibromyoma tissue.

another after 48 hours, 6.56 volumes per cent. The last mentioned determination was made after the postoperative rigor had disappeared.

The rapidity with which this change in the alkali reserve occurs was determined then by testing at short intervals other fibromyomas obtained immediately after their removal. The results of two such determinations are shown in Fig. 3.

These curves and the results mentioned otherwise support those results obtained in the experiments made for determining the postoperative variation of the hydrogen ion concentration, in that they demonstrate a marked and rapid loss of the tissue alkali reserve. The curves, especially, graphically represent the speed with which this change occurs.

[7] Van Slyke, D. D., and Cullen, G. E., *J. Biol. Chem.*, 1917, xxx, 289.

4. The Reducing Carbohydrate Content of Fibromyoma Tissue.

The possibility that some carbohydrate may be the substance from which an acid or acids arise in the tissues during postoperative rigor suggested investigating the reducing sugar content of fibromyoma tissue at varying short intervals of time. To do this, the fluid of recently removed fibromyomas was expressed from small pieces of tissue with a hand meat press, a specimen as free from blood as possible being taken. Duplicate 2 cc. samples were diluted with 14 cc. of distilled water and the protein was precipitated according to the Folin and Wu[8] technique by adding 2 cc. of 10 per cent sodium tungstate solution and 2 cc. of $\frac{2}{3}$ N sulfuric acid. The precipitated proteins were filtered off, and 2 cc. of the clear filtrate used for making the sugar determinations according to the recent method of Folin and Wu[9] with the special tubes recommended. All determinations were made in duplicate on the duplicate samples of fibromyoma fluid, and the reducing power of the clear filtrate was estimated in milligrams of dextrose.

Three separate samples of fibromyoma fluid taken 24 hours after operative removal contained per cc. the equivalent of 0.2650, 0.3535, and 0.3312 mg. of dextrose. A uterus examined 24 hours after surgical removal contained three fibromyomas with values of 0.333, 0.211, and 0.198 mg. of dextrose per cc. of expressed fluid. The values for other fibromyoma tissues are charted in Fig. 4.

These curves disclose a rapid diminution of reducing carbohydrates in fibromyoma tissue, especially during the 1st hour after removal, but continuing slowly thereafter. It is not likely that these substances disappear completely, for appreciable amounts are present 24 hours after removal.

Although the experiments mentioned do not demonstrate necessarily a carbohydate origin of the tissue acidity, there is a certain parallel between the rate of acid increase, the rate of alkali reserve decrease, and the rate of reducing carbohydrate diminution, each rapidly attaining a maximum at about the same time and continuing thereafter at an approximate level.

[8] Folin, O., and Wu, H., *J. Biol. Chem.*, 1919, xxxviii, 81.
[9] Folin, O., and Wu, H., *J. Biol. Chem.*, 1920, xli, 367.

Of interest in correlating this parallelism are the comments by Haggard and Henderson[10] on asphyxial acidosis. Araki's[11] observation that partial asphyxia causes the appearance of lactic acid (lactates) in the urine has been interpreted, according to these investigators, to indicate that in the absence of adequate oxygen supply an acidosis develops. This interpretation of Araki's observations, they continue, has gained support from the

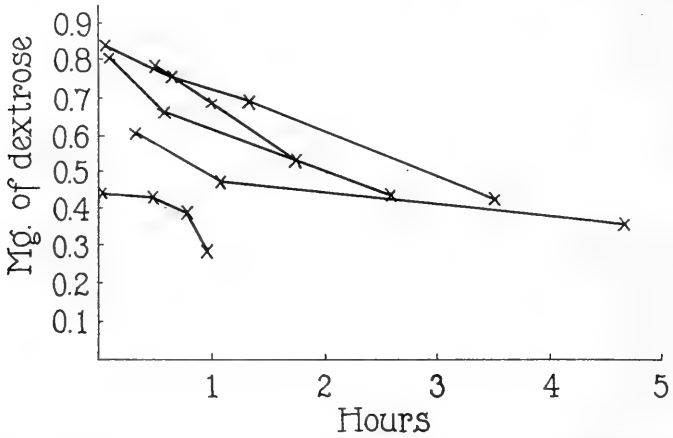

FIG. 4. Postoperative dextrose content of fibromyoma tissues per cc. of tissue fluid.

fact, now apparently well demonstrated, that the first step chemically in the production of energy from carbohydrates in the animal body is anaerobic; sugar breaking down into lactic acid, which then is oxidized to carbon dioxide, the latter reaction being secondary, more a process of elimination than one of energy production. Embden, Baldes, and Schmitz,[12] among others, have discussed the mechanism of lactic acid formation from glucose in the animal body.

[10] Haggard, H. W., and Henderson, Y., *J. Biol. Chem.*, 1920, xliii, 3.
[11] Araki, T., *Z. physiol. Chem.*, 1894, xix, 422.
[12] Embden, G., Baldes, K., and Schmitz, E., *Biochem. Z.*, 1912, xlv, 108.

5. Chemical Analysis of Fibromyoma Tissue.

As part of the study of rigor mortis in fibromyomas, a chemical analysis of this tumor tissue was made. Samples were ground finely, weighed by difference, and preserved in redistilled 95 per cent alcohol sufficient to make approximately 75 per cent concentration until the alcohol-ether extraction in a Soxhlet

TABLE II.

	per cent
Alcohol-ether residue	16.44
" extract (dried)	3.38
Total solids	19.82
Water	80.18

Alcohol-ether residue per gm. solids.

	mg.
Total protein	792.0
Protein sulfur	4.6
" phosphorus	1.5

Alcohol-ether extract.

	Extractives.	Lipins.
	mg.	*mg.*
Total inorganic (ash)	30.2	
" solids	101.3	
" sulfur	0.7	0.5
Inorganic sulfur	Trace.	
Total phosphorus	2.3	2.2
Inorganic phosphorus	1.3	
Total nitrogen	4.5	2.3

extractor was made. The tissue analysis was made according to standard methods, the sulfur and phosphorus being determined gravimetrically as barium sulfate and magnesium pyrophosphate respectively, and the nitrogen by the Kjeldahl method. The results are given in Table II.

The proportion of total solids and water in the fibromyoma tissue analyzed approximates those found in the castration granuloma of swine,[13] and in many other respects the analyses of the alcohol-ether-soluble and alcohol-ether-insoluble fractions of each correspond closely.

[13] Hirsch, E. F., and Wells, H. G., *Am. J. Med. Sc.*, 1920, clix, 356.

CONCLUSIONS.

There is a postoperative (postmortem) rigor of smooth muscle which is manifested by changes similar to those known in skeletal muscle as rigor mortis. Each variety of muscle in this change contracts, and is accompanied by a distinctly increased tissue acidity. With smooth muscle this acidity develops rapidly to a maximum, and is paralleled closely by a rapid diminution of its reducing carbohydrates, and by a decrease of its alkali reserve. There is a fairly uniform maximum (pH 6.0) tissue acidity in rigor mortis of fibromyomas that in part, at least, is due to lactic acid.

A chemical analysis of fibromyoma tissue is given.

THE CULTIVATION OF YEAST IN SOLUTIONS OF PURIFIED NUTRIENTS.

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(Received for publication, November 16, 1920.)

There has existed since the controversy between Pasteur and von Liebig (1, 2, 3) and the subsequent work of Mayer (4) and von Nägeli (5) a belief that yeast in nutrient solutions affording only ammonium salts as a source of nitrogen failed to multiply to an appreciable extent. The addition of small quantities of impurities of certain kinds, especially yeast extract, exerted a profound effect on the growth of yeast cells in such media. The nature of the substance or substances which affect the multiplication of yeast cells in so marked a degree has never been clearly stated. To define it Wildiers (6) coined the term "bios." Yeast itself was considered the best source of this growth-promoting substance. Very small amounts of "bios" were needed to insure rapid proliferation, and it was thought that the better growth secured in nutrient solutions of purified foodstuffs in which an ammonium salt served as the sole source of nitrogen when a large seeding of yeast was made, as contrasted with the results when one or very few cells were cultivated, was due to the death and autolysis of some of the cells. These dead cells contributed the hypothetical "bios" to the solution, and thus enabled the living ones to proliferate.

The proofs brought forward in support of this view have not been entirely convincing, but have been widely accepted. That the acceptance has not been universal is made evident by the numerous papers that are still being written in support of or in opposition to it. Quite recently it has been suggested that the "bios" of Wildiers is perhaps identical with the antineuritic substance concerned in the nutrition of the higher animals. Williams (7) and Bachmann (8) have devised procedures for the detection

and approximate estimation of the antineuritic substance by the use of yeast as a test organism. Their methods are based on the assumption that there exists a definite requirement by yeast of the antineuritic substance which has generally been assumed to be identical with the dietary factor water-soluble B. These authors hold the view that they have demonstrated experimentally that the multiplication of yeast cells in a solution containing pure chemical substances, all of which can be named, and the fermentative power of the yeast are quantitatively influenced by the addition of substances known to contain water-soluble B (7, 8). In a recent paper from this laboratory (9) it has been shown that extracts of certain natural foods, *e.g.* wheat germ, rolled oats, muscle tissue, etc., even when treated so as to contain so little of the antineuritic substance that its presence cannot be demonstrated experimentally with rats, still exert a profound influence on the proliferation of yeast cells. The same is true of the addition of mixtures of amino-acids and of glucose. The view that the growth of yeast can be made a quantitative method for the dietary factor water-soluble B must be regarded as questionable until supported by more convincing experimental proof.

The following experimental work was undertaken in order to throw further light on the problem of the requirement of yeast of the dietary factor water-soluble B. The problem might also be stated, because of the nature of the experimental work and the line of reasoning involved, to be a test of the identity of the antineuritic substance water-soluble B with "bios."

We were successful in securing the multiplication of yeast in fifteen successive seedings in nutrient solutions of purified chemical substances.

In order to have a synthetic solution free from any trace of water-soluble B it seemed expedient to restrict the nutrient substances as nearly as possible to those of mineral origin. More than half a century of experimentation on the nutrition of yeast furnished abundant material for references. According to Lafar (10) yeast may under proper conditions make excellent use of ammoniacal nitrogen. Enough experimental evidence in favor of at least a partial utilization of an inorganic nitrogen supply by yeast cells was found to warrant the use of ammonium sulfate as the source of nitrogen. The sucrose used as the carbohydrate supply

was prepared from the best granulated cane sugar by repeated precipitation from a concentrated water solution by absolute alcohol.

Nutrient Solution 1. 2 Per Cent Sucrose.

1 liter distilled water.
20 gm. sucrose, recrystallized.
3 " ammonium sulfate, C.P.
2 " potassium dihydrogen phosphate, C.P.
0.25 gm. calcium chloride, C.P.
0.25 " magnesium sulfate, C.P.

Nutrient Solution 2. 5 Per Cent Sucrose.

The second nutrient solution differed from the first only in that it contained 50 gm. of sucrose per liter.

The solutions were heated to boiling on 2 successive days. On the 3rd day portions of 25 cc. were measured with a sterile pipette into a number of small sterile Erlenmeyer flasks, which were then heated to boiling, stored in a cupboard, and kept free from possibilities of contamination.

Three strains of yeast, baker's yeasts "F" and "XII" and brewer's yeast "K," were obtained from the Fleishmann Laboratories. A small loopful of these pure cultures was used to seed 25 cc. of sterile nutrient solution. The flasks were then well shaken to distribute the cells and allowed to stand undisturbed in the cupboard at room temperature. There was not enough yeast to produce visible turbidity in the solutions. At the end of a week there was a very perceptible growth of yeast on the bottom of the flasks and the solutions showed considerble turbidity when shaken. 1 cc. of the well agitated suspension in Flask A was transferred to a second seeding, Flask B, which was kept for a week, when the turbidity seemed about the same as in Flask A 1 week after seeding. A third seeding was made from Flask B to Flask C. This successive seeding was continued to the fifteenth seeding with no apparent change 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 (11, 12).

The seedings in Nutrient Solution 2 were conducted in the same way as those in Nutrient Solution 1. It was, however, possible to make seedings every 5 days rather than every 7 days as in the first series. It was noted in both series that the two strains of baker's yeast, "F" and "XII," made a somewhat better growth than brewer's yeast "K."

Flask A containing 25 cc. of nutrient solution had added to it the "bios" contained in one loopful of a pure culture of yeast cells. If these cells gained no "bios" and lost none, Flask B received 1/25, Flask C received 1/25 of 1/25, or 1/650, of the original amount of "bios," and Flask J received 1/5, 667,253,614,400; *i.e.*, less than one-five trillionth of the "bios" in the one loop of yeast cells used to seed Flask A. If, as Amand (12) supposes, the original amount of "bios" decreases very rapidly, it must appear that yeast may grow without "bios" or water-soluble B, or it must be able to synthesize its own supply.

The fermentative power of the yeast used in these experiments was determined for cells taken from the fifteenth seeding. 0.5 cc. of yeast suspension was used for each test. The examination was carried out according to Durham's fermentation test with glucose solution.

	Gas. *inches*
Yeast XII after 3 days	$3\frac{1}{2}$
" K " 3 "	$3\frac{3}{4}$
" F " 3 "	$3\frac{1}{2}$
Control " 3 "	0

There can be no question about the remarkably stimulating effect of adding various substances such as amino-acids and other extractives to such purified nutrient solutions as were used in this work. Where extracts of natural foods are employed, using yeast as a test organism with a view to determining by the extent of the proliferation of the cells the content of antineuritic substance in such extracts, there is always added with the antineuritic substance so many others having a favorable influence on the growth of yeast that it seems unlikely that the test as carried out by Williams and Bachmann contains any element of specificity.

SUMMARY.

The results of our efforts to cultivate yeast in nutrient solutions containing no possible source of the antineuritic factor have been of such a nature as to make us seriously question whether yeast is dependent on a supply of the antineuritic principle for its continued multiplication. Successive seedings with very few cells have been carried sufficiently far to reduce the possible content of antineuritic substance ("bios," "water-soluble B") to vanishingly small amounts.

We have grown quantities of yeast in this purified nutrient solution sufficient to furnish from 2 to 5 gm. of dry substance. It would seem that but one of two conclusions is admissible; *i.e.*, either yeast must grow without "bios" or it must synthesize the substance to meet its own needs (as was believed by Henry (13)). Feeding experiments with yeast grown under the conditions described in this paper are contemplated and should yield results of unusual interest.

BIBLIOGRAPHY.

1. Pasteur, *Compt. rend. Acad.*, 1858, xlvii, 1011.
2. von Liebig, J., *Ann. Chem.*, 1870, cliii, 1, 137.
3. Pasteur, L., *Ann. chim. phys.*, 1872, xxv, 145.
4. Mayer, A., *Ann. Physik*, 1871, cxlii, series 1, 293.
5. von Nägeli, C., *Chem. Zentr.*, 1881, xii, 45.
6. Wildiers, E., *La Cellule*, 1901, xviii, 313.
7. Williams, R. J., *J. Biol. Chem.*, 1919, xxxviii, 465; 1920, xlii, 259.
8. Bachmann, F., *J. Biol. Chem.*, 1919, xxxix, 235.
9. Souza, G. de P., and McCollum, E. V., *J. Biol. Chem.*, 1920, xliv, 113.
10. Lafar, F., Handbuch der Technischen Mykologie, Jena, 1905–08, 155.
11. Kossowicz, A., *Chem. Zentr.*, 1903, lxxiv, pt. i, 475.
12. Amand, A., *La Cellule*, 1904, xxi, 329.
13. Henry, J., *Ann. brass. et dist.*, 1902, 129; *Woch. Brau.*, 1902, xix, 325.

THE DETERMINATION OF SODIUM IN BLOOD.*

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(Received for publication, November 26, 1920.)

A knowledge of the amounts of sodium salts in tissues and biological fluids is of considerable importance in reaching an understanding of the physiological rôle of inorganic substances. However, an adequate study of the variations in the amount of sodium in blood, for instance, under different physiological and pathological conditions would be difficult if not impossible by methods[1] now in use. The quantitative determination of sodium by existing methods requires the ashing of the material, the removal of sulfates, phosphates, iron, calcium, and magnesium, and the estimation of potassium in the weighed alkali chlorides. Such a long and tedious procedure seriously limits a study of the metabolism of sodium. Another limiting factor is the amount of material necessary for an analysis, at least 15 cc. of blood being required for the usual gravimetric method.

These considerations prompted the search for a shorter method for the determination of sodium in smaller amounts of material. It seemed essential that some insoluble compound of sodium be found which would permit its quantitative precipitation. The most promising of the very few sodium compounds having small solubility appeared to be the complex sodium cesium bismuth nitrite described by Ball (2) and used by him for sodium determinations. A method based upon the precipitation of this

* A preliminary report of this method was made at the meeting of the American Society of Biological Chemists at Cincinnati, December, 1919.

[1] Kramer (1) has recently described a method for the determination of sodium based upon the insolubility of the pyroantimonate. We have had no experience with this method, but Kramer's data indicate that it is a satisfactory procedure for the estimation of sodium in small samples of tissue.

compound has proved to be readily applicable to blood and urine and with some modification should be equally serviceable for any tissue. The results are believed to be almost as accurate as those obtained by the older procedures while the amount of material and time required are much less. As small an amount of sodium as 0.01 mg. yields a precipitate in a final volume of 2 cc. None of the ions commonly occurring with sodium interferes with the precipitation.

Certainly this method has great promise. The chief objections to it as described by Ball are the formation of a scum of bismuth subnitrate during the precipitation of the complex sodium salt, and the solubility of the latter in all solvents of the precipitating reagent. After many experiments we feel that we have overcome these difficulties.

The formation of the scum is probably due to a loss of nitrous acid and a consequent decrease of acidity of the solution. We find that if the reagent is added to the cold solution of the sodium salt and the flask immediately put in a cold room (1°C.), a scum rarely forms within 24 hours. At this temperature the precipitation of the complex nitrite is more rapid and is generally complete in 18 hours.

All the solutions used by Ball for washing the precipitate in order to remove the mother liquor dissolve so much precipitate that serious errors are introduced. He attempted to apply a solubility correction which would amount to 10 or 15 per cent of the precipitate in some of our determinations. However, none of the solutions that we have tried was so efficient as the 50 per cent acetone in removal of the excess reagent. Consequently we have retained it but have reduced the solubility of the precipitate in it to almost zero. The 50 per cent acetone is saturated with solid sodium cesium bismuth nitrite at 1°C. The excess salt is filtered off at this temperature and the solution used for washing. Its temperature probably does not rise more than 2 or 3° before the washing is complete.

The method as published by Ball was gravimetric. We have been able to show that the nitrite is susceptible of both volumetric[2] and colorimetric estimation. The nitrite may be oxidized

[2] Faber and Stoddard (3) have used the sodium method described by Ball for the analysis of potassium nitrate. They mention the titration of

to nitrate on titration with standard $KMnO_4$. 0.1 N permanganate is suitable since the precipitate from 1 mg. of sodium requires 4.35 cc. for oxidation of nitrite to nitrate.

$$9\ CsNO_2 \cdot 6\ NaNO_2 \cdot 5\ Bi(NO_2)_3 = 30\ NO_2$$
$$30\ NO_2 + 15\ O_2 = 30\ NO_3$$

1 gram-molecule (3,753.6 gm.) of the complex nitrite requires 15 gram-molecules or 480 gm. of oxygen for oxidation.

$$\frac{9\ CsNO_2 \cdot 6\ NaNO_2 \cdot 5\ Bi(NO_2)_3}{30 \times 16} = \frac{3753.6}{480} = 7.82$$

i.e., 1 mg. of O will be required for 7.82 mg. of the precipitate.
1 mg. of sodium produces 27.2 mg. of complex nitrite. Consequently $\frac{27.2}{7.82} = 3.48$ mg. of O to oxidize precipitate from 1 mg. of sodium.

$$\frac{3.48}{0.8} = 4.35\ \text{cc. } 0.1\ \text{N } KMnO_4$$

The colorimetric estimation is based on the coupling reaction of Griess (4) using napthylamine and sulfanilic acid. Advantage is taken of the fact that bismuth salts are soluble in alkaline tartrate solutions. The precipitate is dissolved in alkaline potassium tartrate, made up to a definite volume, and a suitable sample taken. The standard is 0.01 mg. of nitrite nitrogen. The colors are developed and read with a Duboscq colorimeter.

Reagents.

1. Bismuth Cesium Nitrite Solution.—Although we have attempted to improve Ball's reagent in several ways, we have been unsuccessful. It is much less stable at room temperature than at 1°C. If kept under an inert gas at 1°C. it is suitable for quantitative work for several weeks.

the precipitate with permanganate but give no data from which one may judge of its accuracy.

The authors are indebted to Mr. Faber and Mr. Stoddard for suggesting the possibilities of Ball's method for the determination of small amounts of sodium.

30 gm. of sodium-free potassium nitrite[3] are dissolved in about 60 cc. of pure water. A solution containing 3 gm. of bismuth nitrate is added. (We keep on hand a 60 per cent solution of the crystallized salt in 2 N HNO_3.) If a precipitate forms (due to excessive alkalinity of the KNO_2), dilute nitric acid is added carefully until it redissolves. A strong solution containing 1.6 gm. of $CsNO_3$ and 1 cc. of 2 N HNO_3 is added. The solution is diluted to 100 cc. and dilute nitric acid is used to remove any turbidity which may form. At this stage the reagent should be a clear orange-yellow. If sodium salts were present in any of the chemicals as impurity, the insoluble precipitate which has formed at the end of 24 hours is filtered off. The reagent is kept under illuminating gas in the cold room.

2. Acetone.—Redistilled and kept ready for use at 1°C.

3.—A 50 per cent solution of acetone saturated at 1°C. with sodium cesium bismuth nitrite.

For Volumetric Procedure.—

1. Permanganate, 0.1 N or 0.05 N.
2. Oxalic acid, 0.1 N or 0.05 N.
3. H_2SO_4, concentrated acid diluted with equal volume of water.

For Colorimetric Procedure.—

1. Alkaline tartrate. Equal volumes of KOH (10 per cent) and tartaric acid (10 per cent) are mixed.
2. Sulfanilic acid, 0.8 per cent in 5 N acetic acid.
3. α-Naphthylamine, 0.5 per cent in 5 N acetic acid.

[3] Pure potassium nitrite has been a source of considerable difficulty which we have finally overcome. The potassium salts on the market generally contain large quantities of sodium. Since the nitrite cannot be purified by recrystallization, our only recourse was to make it. We have examined various samples of carbonate and have found that both Merck's Blue Label and Eastman's are substantially free from sodium. As an emergency procedure sodium-free potassium carbonate may be made by recrystallization of the oxalate. It is dried and ignited in a platinum dish.

The pure nitrite is made by passing nitrous fumes into a 25 per cent solution of sodium-free potassium carbonate. Nitric acid (sp. gr. 1.2) is dropped from a separatory funnel into a flask containing arsenious oxide. A delivery tube carries the fumes into the carbonate. The reaction is complete when the solution in the receiving flask gives off many fine bubbles of carbon dioxide on shaking. We generally run nitrite determinations at intervals to ascertain whether the reaction is running properly.

4. Nitrite standard. Made by recrystallizing $AgNO_2$ from hot water until free from nitrate. Add NaCl equivalent to the $AgNO_2$ and filter off silver chloride. Determine nitrite nitrogen by Devarda's (5) method and dilute so that 5 cc. = 0.01 mg. N.

Preparation of the Material for Analysis.

When we started our analyses of blood and urine we thought that it would be necessary to remove the organic material by ashing. We have used both the wet and dry methods. In our hands the former has been the more successful. As many of our data were obtained on blood ashed with sulfuric and nitric acids, this procedure is given below in detail.

1 cc. of whole blood, plasma, or urine is transferred to a pointed Pyrex tube.[4] A few drops of H_2SO_4 (concentrated) and 5 cc. of HNO_3 (concentrated) are added. A low flame which keeps the liquid boiling gently is used. The digestion is continued in the usual manner until the liquid is colorless. Urine is completely oxidized in about 8 minutes but the blood generally takes $\frac{3}{4}$ hour.

As both iron salts and any appreciable amount of phosphates interfere with this method of determination of sodium, they must be removed from the blood digest. None of the samples of urine or plasma analyzed contained sufficient quantities to vitiate the analysis.

The digest of the whole blood is quantitatively transferred to a 25 cc. volumetric flask with about 20 cc. of water. 1 drop of methyl orange and 5 to 6 drops of 4 per cent bismuth nitrate are added. A strong solution of potassium carbonate (free from sodium) is added dropwise with shaking until the indicator changes color. The flask is made up to volume, mixed, and the solution transferred to a centrifuge tube. Centrifuging at moderate speed throws down the insoluble phosphates and iron salts.

[4] These tubes are reclaimed from non-protein nitrogen determinations. After a tube has been rendered unserviceable by the phosphoric acid it is heated in an oxygen-gas flame and drawn out to a point. The tubes with small tips and thin walls stand heating best. The pointed tip provides a constant stream of bubbles which promote even boiling. No boiling stones are necessary.

20 cc. of the supernatant liquid are pipetted into a 50 cc. Erlenmeyer flask. This solution is evaporated[5] on the hot plate to 2 to 3 cc. and rendered just acid with HNO_3. An excess of 0.5 cc. of 2 N HNO_3 is added and the precipitation carried out as described.

After carefully checking our method on the ashed blood, we found that a great deal of time and labor could be saved by deproteinization with trichloroacetic acid. Our data (Table I) indicate that a dilution of 1:5 or 1:10 is perfectly satisfactory.

The procedure is as follows. 5 cc. of whole blood or plasma are transferred to a 50 cc. flask containing 35 cc. of water, and 5 cc. of trichloroacetic acid (20 per cent) are added. The contents of the flask are diluted to the mark, mixed, allowed to stand about 30 minutes, and filtered through a dry paper. 10 cc. of filtrate (equivalent to 1 cc. of blood) are pipetted into a 50 cc. Erlenmeyer flask and 1 drop of concentrated nitric acid is added. The flask, closed with a trap, is heated on a piece of asbestos on a hot plate until brown fumes from the acid are evolved. It is removed, cooled, and the trap washed off with a few drops of water. Precipitation is then carried out as described below.

Precipitation.—The solution is cooled to 10–12°C. and 3 cc. of reagent are added for each milligram of sodium expected. The flask is stoppered with a two-hole rubber stopper bearing two short glass tubes bent at a right angle. One is fitted with a short rubber tube with a glass plug, the other with a Bunsen valve and plug. Illuminating gas freed from H_2S is passed into the flask for a few seconds and the plugs are replaced. The flask is put in the cold room at 1°C. A yellow crystalline precipitate begins to form in a few minutes. Precipitation is complete in 24 hours, whereas at room temperature 48 hours are required. A scum is much more likely to form before the precipitation is complete at the higher temperature.

The precipitate is rapidly filtered on a Gooch crucible which has previously been dried and weighed. Washing with the ice-cold 50 per cent acetone which is saturated with sodium

[5] We advise the use of a trap in the mouth of the flask to prevent loss by bumping. Ours is made by blowing a bulb on the closed end of a small soft glass test-tube. A hole is then blown in the side of the bulb which is then cut off from the tube.

cesium bismuth nitrite is quickly carried out. Speed during the filtration and washing is essential for good results. 10 cc. of the 50 per cent acetone are used; 2 cc. are blown from a miniature wash bottle (made from a 10 cc. graduated cylinder) into the precipitation flask. The suction is stopped and the liquid poured onto the mat. This is repeated four times. 10 cc. of pure acetone are used to complete the transfer of the precipitate to the Gooch. If the volumetric or colorimetric procedure is used, complete transference of the precipitate is not necessary.

The Gooch is dried in an air bath at 100°C. until a constant weight is obtained.

Weight of precipitate × 0.03675 = Weight of sodium in solution

We prefer to avoid the weighing necessary for a gravimetric determination. Incidentally any scum which may have formed will cause the gravimetric result to be too high but will not affect the nitrite estimation. Very frequently potassium nitrate crystallizes at 1°. This will also give erroneous gravimetric results. The precipitate is filtered as described on a Gooch crucible and estimated either volumetrically or colorimetrically.

Volumetric Procedure.—The Gooch crucible and contents are placed in a tall 200 cc. beaker. A large excess of standard permanganate (at least twice the amount necessary for oxidation) and enough water to cover the crucible are added. The precipitate is stirred loose from the crucible and asbestos. 10 cc. of 1:1 sulfuric acid are poured in while the liquid is being stirred. After a few minutes the solution is heated to 75°C., an excess of standard oxalic acid added, and the titration finished with permanganate. A blank must be run on the reagents under similar conditions.

Cc. $KMnO_4$ × normal factor × 8 = Mg. O used
Mg. O × 7.82 = Mg. precipitate
Mg. precipitate × 0.03675 = Mg. sodium

Colorimetric Procedure.—For those who prefer a colorimetric method we have established a suitable technique. The precipitate is completely transferred to a beaker and 10 cc. of the alkaline tartrate are added. Upon warming, the salt rapidly dissolves. The solution is quantitatively transferred to a 100 cc. volumetric

flask, cooled, made up to volume, and mixed. A further dilution is made so that a volume containing approximately 0.01 mg. of N can be taken for colorimetric comparison.

The standard and unknown in 100 cc. volumetric flasks are diluted to about 90 cc. 2 cc. of the sulfanilic acid and napthylamine solutions are added to each. The flasks are made up to volume, mixed, and allowed to stand 20 minutes for the full color development. There is a very wide range of proportionality of color intensity to the amount of nitrite present. The colors are very stable.

The calculation is simple.

$$\frac{20 \times 0.01}{\text{Unknown reading}} = \text{Mg. N in sample used}$$

Suppose the sample was 1 cc. of a dilution of 1:1,000 then

$$\text{Mg. N in sample} \times 1{,}000 = \text{Mg. N in precipitate}$$

$$\frac{9\ CsNO_2 \cdot 6\ NaNO_2 \cdot 5\ Bi(NO_2)_3}{30\ N} = \frac{3753.6}{420.3} = 8.93$$

$$\text{Mg. N} \times 8.93 = \text{Mg. precipitate}$$

$$\text{Mg. precipitate} \times 0.0367 = \text{Mg. sodium in sample}$$

We prefer the volumetric method to either the gravimetric or colorimetric on account of its greater speed and accuracy. Possible contamination of the precipitate with either bismuth subnitrate or potassium nitrate renders the gravimetric values doubtful. The colorimetric procedure is open to the usual errors of such methods. The red color is very bright and comparison is rather difficult.

In Table I the figures given under "Indirect" were obtained by ashing the material in a platinum dish. The ash was taken up in water, acidified with hydrochloric acid, and the sulfates were precipitated. The barium sulfate was filtered off and the filtrate made alkaline with ammonia. After the barium phosphate had precipitated, an excess of ammonium carbonate was added. The precipitate was filtered off and the filtrate evaporated in a platinum dish. The salts were carefully dried and gently ignited. The salts were dissolved in water and tested for complete removal of barium and calcium. Generally a small amount of insoluble

material was present. It was filtered off and the filtrate caught in a small weighed platinum dish. Evaporation to dryness, and ignition were carried out as previously described.

TABLE I.

Sodium in Urine and Blood.

Experiment No.	Source of sample.	Sodium per 100 cc.			Method.
		Indirect.	Direct ash.	Trichloroacetic acid filtrates.	
		mg.	*mg.*	*mg.*	
1	Urine, normal.	414	403		Volumetric.
			407		Gravimetric.
2	" "	351	349		Volumetric.
			341		"
3	" "	351	348		"
			343		"
4	Blood, beef.	284	285		Gravimetric.
			279		"
5	" "	278	268		"
			270		"
6	" "	272	263		"
			265		"
7	" "	266		265	"
8	" "	281		272	Colorimetric.
				275	"
				283	Volumetric.
9	Plasma, "	336	339	342	Colorimetric.
				343	Volumetric.
10	Blood, swine.	221	219		"
			226		"
11	" "	206	204		"
			212*		"
12	" "	217		216	"
13	" "	209	212	213	Colorimetric.
14	Plasma, "	360	358	360	"
				358	"
				352	Volumetric.

* Determination on 0.4 cc. of blood.

The mixed sodium and potassium chlorides were dried to constant weight and the potassium was determined in the usual manner as perchlorate. The sodium perchlorate was dissolved in

97 per cent alcohol containing 0.2 per cent perchloric acid. Consequently the results for potassium are possibly a little low and sodium a little high (6).

Table II illustrates the accuracy to be expected when weighed quantities of sodium cesium bismuth nitrite are titrated with

TABLE II.

Titration of Sodium Cesium Bismuth Nitrite with Permanganate.

Experiment No.	Amount of pure salt.	Amount by titration.	Error.
	mg.	*mg.*	*mg.*
1	62.6	62.0	−0.6
2	55.3	55.8	+0.5
3	89.4	88.4	−1.0
4	51.5	52.1	+0.6
5	54.1	55.1	+1.0
6	118.6	118.0	−0.6

TABLE III.

Determination of Sodium in Pure Sodium Nitrate.

Na taken.	Na found.	Time for precipitation.	Temperature.	Method.
mg.	*mg.*	*hrs.*	°C.	
1.0	0.96	44	22	Gravimetric.
1.0	1.01	65	22	Volumetric.
1.0	0.99	69	22	"
2.0	1.62	20	22	Gravimetric.
2.0	2.02	44	22	Volumetric.
2.0	1.97	44	22	"
2.0	2.03	44	22	"
2.0	2.04	44	22	Gravimetric.
2.0	1.85	7	1	Colorimetric.
2.0	1.92	15	1	"
2.0	2.04	24	1	"
3.0	3.06	44	22	Gravimetric.
3.0	2.98	24	1	Volumetric.

permanganate. The volumetric estimation of known amounts of sodium salts also throws light on the reliability of a titration procedure.

Table III gives some values obtained by the gravimetric, volumetric, and colorimetric procedures on pure sodium nitrate.

Our early work in agreement with Ball's demonstrated that at least 44 hours were necessary for the complete precipitation at room temperature. We have found 24 hours to be sufficient at 1°C. 15 hours at this temperature gave low results.

Table I shows a comparison of values obtained by the usual procedure and by our different modifications of the sodium cesium bismuth nitrite method. We think that the results are sufficiently accurate for most work but hope to increase their accuracy by a few refinements on which we are now working.

Attention should be called to the constancy of the amount of sodium in the blood of the same species. In the case of both swine and beef the maximum variation is only about 7 per cent.

BIBLIOGRAPHY.

1. Kramer, B., *J. Biol. Chem.*, 1920, xli, 263.
2. Ball, W. C., *J. Chem. Soc.*, 1910, xcvii, 1408.
3. Faber, H. B., and Stoddard, W. B., *J. Ind. and Eng. Chem.*, 1920, xii, 576.
4. Griess, P., *Ber. chem. Ges.*, 1879, xii, 426.
5. Devarda, A., *Z. anal. Chem.*, 1894, xxxiii, 113.
6. Baxter, G. P., and Kobayashi, M., *J. Am. Chem. Soc.*, 1920, xlii, 735.

A STUDY OF THE DISTRIBUTION OF IODINE BETWEEN CELLS AND COLLOID IN THE THYROID GLAND.

II. RESULTS OF STUDY OF DOG AND HUMAN THYROID GLANDS.

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(Received for publication, November 29, 1920.)

In the first paper of this series Tatum[1] described a method whereby thyroid cells may be separated from colloid material and examined chemically. Briefly the method consists in cutting frozen sections of the thyroid gland and floating these sections on Ringer's[2] solution. The colloid material immediately drops out of the acini and is presumably dissolved in the Ringer's solution. The cells may then be separated by centrifugalization, dried, weighed, and analyzed. Comparisons between the iodine content of cells so separated and the iodine content of control pieces of unsectioned whole gland may indicate the distribution of iodine between cells and colloid under different functional conditions.

In this paper I wish to report the results of a study, suggested by Dr. A. L. Tatum, of the distribution of iodine in the thyroid glands of normal and iodine-fed dogs as well as in human glands obtained from individuals subjected to operation for toxic goiter. The method earlier described by Kendall[3] was used in making the final iodine determinations.

Incidental to the determination of the iodine distribution in dog and human thyroid glands, some control experiments were performed relative to the alterability of the intracellular iodine concentration during the process of cutting. It may be argued that a portion of the iodine-containing compound diffuses from

[1] Tatum, A. L., *J. Biol. Chem.*, 1920, xlii, 47.

[2] Campbell, J. A., *Quart. J. Exp. Physiol.*, 1911, iv, 1, Formula "A."

[3] Kendall, E. C., *J. Biol. Chem.*, 1914, xix, 251.

cells as they lie suspended in Ringer's solution during the separation of the colloid material. If it is assumed that such an outward diffusion takes place, it seems reasonable to expect that the iodine compound should diffuse back into the cells if we should increase the concentration of that iodine compound in the Ringer's solution in which the cells are suspended. Several experiments like those given in Table I were undertaken to determine whether or not the iodine content of the cells could thus be increased.

TABLE I.

Effect of Floating Cells on Pure Ringer's Solution and on Ringer's Solution on Which Cells of an Iodine-Rich Gland Had Been Floated.

Animal No.	Weight of whole gland used.	Iodine in whole gland.	Weight of cell mass used.	Iodine in cell mass.	Ratio of percentage of iodine in cells to percentage of iodine in whole gland.	Remarks.
	mg.	*per cent*	*mg.*	*per cent*		
14	146.0	0.187	40.5	0.017	0.091	Cells floated on pure Ringer's solution.
			53.0	0.017	0.091	Cells floated on Ringer's solution on which previously cells of iodine-rich gland had been floated.
20	287.2	0.030	161.5	0.005	0.167	Cells floated on pure Ringer's solution.
			151.0	0.006	0.20	Cells floated on Ringer's solution on which previously cells of iodine-rich gland had been floated.

It may be seen from the data given in Table I that no increase in the iodine content of cells could be brought about by increasing the concentration of the characteristic iodine compound in the Ringer's solution.

Again the iodine concentration of the cell mass does not seem to be in the least altered whether or not the freshly centrifugalized cell mass is washed several times with iodine-free Ringer's solution.

Moreover, in the attempt to find a suspending medium more nearly related physicochemically with thyroid cells than is Ringer's solution, I have used fresh dog serum and have not found the iodine content of the cells measurably different from that of control cells suspended in Ringer's solution.

Finally autolysis does not seem to be much of a factor in the loss—if any occurs—of iodine compounds from cells as they lie in contact with Ringer's solution. Throughout the process of cutting and separating the cells, the reaction of the suspending medium should remain weakly alkaline—a reaction which has been shown to be unfavorable to autolysis.[4] Also in experiments to be reported later in which the same technique was used under slightly different conditions, every effort was made to eliminate autolysis by cutting successively small portions of the gland and floating the cells on Ringer's solution cooled by ice. The cells from the small portions cut successively were at once centrifugalized and dried. No change could be noticed in the iodine distribution.

Results of Study of Dog Glands Taken at Random.

TABLE II.

Quantitative Determination of Iodine in Whole Gland and in Cells Free from Colloid Material of Thyroid Glands of Normal Dogs.

Animal No.	Weight of whole gland used.	Iodine in whole gland.	Weight of cell mass used.	Iodine in cell mass.	Ratio of percentage of iodine in cells to percentage of iodine in whole gland.	Morphology.	
						Cells.	Colloid.
	mg.	*per cent*	*mg.*	*per cent*			
5	550.0	0.25	154.0	0.031	0.124	Flat.	Fair.
6	388.0	0.175	265.0	0.027	0.154	"	Rich.
10	480.0	0.017	342.0	0.003	0.176	Cuboidal.	Poor.
11	505.5	0.032	197.5	0.005	0.156	"	"
12*	278.7	0.006	286.5				
13	689.0	0.234	154.0	0.047	0.201	Flat.	Fair.
14	146.0	0.187	40.5	0.017	0.091	"	"
19	474.5	0.024	116.0			"	Poor.
20*	287.2	0.030	161.5	0.005	0.167		
21*	604.7	0.038	189.0	0.006	0.158		
23	458.2	0.003	390.4			Cuboidal.	Poor.
24	318.7	0.021	295.4			"	"
31	513.8	0.055	205.6	0.008	0.145	"	"
32*	235.2	0.179	103.9	0.037	0.207		
33	289.3	0.011	538.3	0.002	0.182	Flat.	Poor.

* No histological examination.

[4] Bradley, H. C., *J. Biol. Chem.*, 1915, xxii, 113. Bradley, H. C., and Taylor, J., *J. Biol. Chem.*, 1916, xxv, 261.

From the results given in Table II one sees that the ratio of iodine concentration in cells to iodine concentration in whole gland (and hence the ratio of iodine concentration in cells to colloid-iodine concentration) has a quite constant value. In some glands (Nos. 12, 19, 23, and 24), to be sure, the iodine content of the cell mass analyzed was so low that no ratio value could be obtained. But in general, despite great variations in the iodine content and morphology of the glands analyzed, the ratio values change relatively little. These findings in the dog's thyroid gland are similar to those of Tatum[1] in the beef, pig, and sheep thyroid glands. A comparison of the ratio values in different animals is given below.

Effect of Feeding Iodine.

It was thought that perhaps ratio changes could be induced by the administration of iodine or iodine compounds. Capsules containing 1 gm. of potassium iodide or 2 drops of tincture of iodine in starch were fed over varying periods of time. No more than one capsule was given in 24 hours. The results of the examination of the glands of animals so treated are given in Table III.

The ratio obtained from analyzing the cells and whole gland of the thyroid glands of animals which had received varying amounts of iodine or iodine compounds over periods of no less than 3 days is practically the same as that of the animals which had received no iodine. One gland (that of No. 16) gave an unusually high value which I cannot explain. In view of the variations in morphology and iodine content of the glands of the normal series together with the quite constant ratio exhibited by that series, it is not surprising that the ratio still remains constant despite the feeding and consequent absorption of iodine. From a consideration of the glands examined it appears that the ratio is not altered by the feeding of iodine as potassium iodide or free iodine over periods of time ranging from 3 days to 3 weeks; and yet the total iodine content of these glands undoubtedly is greatly increased during that same period.

TABLE III.

Quantitative Determination of Iodine in Whole Gland and in Cells Free from Colloid Material of Thyroid Glands of Iodine-Fed Dogs.

Animal No.	Form of iodine.	Period of feeding.			Weight of whole gland used.	Iodine in whole gland.	Weight of cell mass used.	Iodine in cell mass.	Ratio of percentage of iodine in cells to percentage of iodine in whole gland.
		Daily.	On alternate days.*	Total.					
		days	*days*	*days*	*mg.*	*per cent*	*mg.*	*per cent*	
2	KI	14	0	14	98.2	0.31	77.7	0.045	0.145
7	KI	14	7	21	147.0	0.12	113.0	0.031	0.258
8	KI	14	7	21	124.5	0.87	125.0	0.080	0.092
9	KI	14	2	16	353.5	0.251	116.0	0.040	0.159
16	KI	12	0	12	651.4	0.319	316.1	0.117	0.367
17	KI	12	0	24	646.5	0.617	302.3	0.083	0.134
	Tincture of I.	12	0						
27	" " I.	3	0	3	556.1	0.302	332.8	0.046	0.152
29	" " I.	3	0	3	195.2	0.314	158.6	0.039	0.124
30	" " I.	3	0	3	163.3	0.357	127.5	0.041	0.115

* After feeding daily.

Human Glands.

The distribution ratio of iodine between cells and colloid material was determined in thirteen human glands obtained from operative cases.[5] In Table IV are given the results of the analyses of the human glands together with the clinical diagnosis made in connection with ten of the cases.

It will be seen that most of the thyroid glands reported were clinically diagnosed as toxic goiters. Considerable variations in the ratio value occur and do not appear to be related either to the iodine content of the gland or to its morphology. The ratio value of No. 22 is inexplicably high. In the human gland series, however, as in other gland series previously reported, the ratio variations are of a much smaller magnitude than the variations in total iodine content. So here too the ratio is fairly constant despite variations in morphology and iodine content.

[5] Through the courtesy of Dr. C. B. Davis and Dr. A. D. Bevan of the Presbyterian Hospital, Chicago, and of Dr. A. J. Ochsner of Augustana Hospital, Chicago.

TABLE IV.

Quantitative Determination of Iodine in Whole Gland and in Cells Free from Colloid Material of Human Thyroid Glands.

Series No.	Weight of whole gland used.	Iodine in whole gland.	Weight of cell mass used.	Iodine in cell mass.	Ratio of percentage of iodine in cells to percentage of iodine in whole gland.	Morphology.		Diagnosis.
						Cells.	Colloid.	
	mg.	*per cent*	*mg.*	*per cent*				
22	284.7	0.081	260.8	0.049	0.605	Cuboidal.	Poor.	Colloid cystic goiter with toxic symptoms.
25*	600.2	0.274	308.6	0.105	0.383	Flat.	Rich.	Mild exophthalmic goiter.
	411.0	0.276	240.7	0.098	0.355			
35	671.8	0.286	179.2	0.051	0.178	Flat.	Rich.	Toxic thyroid.
36	439.7	0.158	219.9	0.020	0.127	Cuboidal.	Fair.	" goiter.
37†	667.5	0.092	258.4	0.027	0.293			Colloid goiter.
38	578.6	0.079	191.7	Trace.		Cuboidal.	Poor.	" " following parenchymatous hyperplasia of exophthalmic goiter.
39†	516.0	0.279	297.6	0.095	0.341			
40	469.2	0.345	225.5	0.066	0.191	Flat.	Rich.	Exophthalmic goiter.
41	559.2	0.280	187.0	0.067	0.239	"	"	
48*	549.9	0.043	312.4	0.005	0.116	Cuboidal.	Poor.	
	607.3	0.044	311.3	0.005	0.114			
49*	590.7	0.137	256.0	0.026	0.189	Cuboidal.	Fair.	Exophthalmic goiter.
			204.5	0.027	0.197			
51	731.4	0.283	294.4	0.072	0.254	Flat.	Rich.	" "
60	370.0	0.152	228.0	0.015	0.099	"	"	Cystic goiter with toxic symptoms.

* Duplicate determinations made.

† No histological examination

Comparison of Ratio Value in Different Animals.

A comparison of the ratio values of the thyroid glands of the different animals so far examined is given in Table V. The few abnormally high and unexplained ratio values are not included in the table.

From what data are available there appear to be some differences in the thyroid glands of different animals in the numerical value of the ratio of the percentage of iodine in cells to that in whole gland. The ratio value of iodine distribution for dog thyroid glands seems to be consistently lower and more constant than that for the thyroid glands of the other animals studied.

TABLE V.

A Comparison of the Value of the Ratio of the Percentage of Iodine in Cells Free from Colloid Material to the Percentage of Iodine in Whole Gland in Different Animals.

Animal.	Extremes of iodine content of whole gland.	Ratio values.		
		Extremes.	Mean.	Average.
	per cent			
Beef*.....	0.023–0.468	0.21 –0.48	0.35	0.36
Dog.....	0.011–0.870	0.091–0.258	0.175	0.154
Man.....	0.043–0.345	0.099–0.384	0.242	0.22
Pig*†.....	0.377–0.810	0.20 –0.34	0.27	0.27
Sheep*.....	0.089–0.442	0.23 –0.41	0.32	0.33

* Tatum.[1]

† Only two glands analyzed.

SUMMARY.

1. The method described by Tatum[1] was used to determine the ratio of the percentage of iodine in cells to the percentage of iodine in whole gland in the thyroid glands of normal and iodine-fed dogs as well as in human thyroid glands obtained from operative cases.

2. Evidence is presented indicating that the concentration of intracellular iodine is independent of the suspending medium, whether that is pure Ringer's solution, Ringer's solution containing iodine-rich colloid material, or homologous blood serum.

3. The ratio value was found to be relatively constant despite great variations in the morphology and iodine content of the glands examined. The ratio value for the dog's thyroid gland seems quite constant and is much lower than that of the beef or sheep.

STUDIES ON EXPERIMENTAL RICKETS.

I. THE PRODUCTION OF RACHITIS AND SIMILAR DISEASES IN THE RAT BY DEFICIENT DIETS.

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Plates 2 and 3.

(Received for publication, October 26, 1920.)

Students of the etiology of rickets have advanced the most diverse views to account for its occurrence. Heredity, dietetic factors, faulty hygienic conditions, infections of a microbial or other nature, and disturbances of the endocrine glands have all been incriminated as the causative agents.

In the conduct of nutritional experiments on the rat which have been carried on by the members of the Department of Chemical Hygiene of the Johns Hopkins University for more than a decade, it has been the custom to autopsy all animals which have died from any cause. In the course of these routine autopsies pathological conditions of the skeleton, *viz.* enlargement of the epiphyseodiaphyseal junction, fractures of the ribs, enlargement of the costochondral junctions and vertebral ends of the ribs, abnormal spinal curvatures, etc., have been observed repeatedly and have been regarded as evidences of rickets. Other problems prevented for a time any effort at intensive study of the nature of the factor or factors which operate to produce these conditions. There was never any room for doubt that the diet was the sole determining factor in the etiology of the lesions in question, for the entire colony of approximately 2,000 animals lived under identical conditions except for those related to the composition and source of their food.

Although the members of the Department of Chemical Hygiene were of the opinion that the skeletal malformations found in their rats were the result of rickets, it was not possible for them alone to arrive at definite conclusions owing to a lack of the necessary pathological experience. It so happened that the Department of Pediatrics had been engaged for a number of years in the study of rickets from the chemical and pathological standpoints and had already conducted experiments with a view to the production of rickets in animals. It seemed advisable, therefore, for the two departments to unite in a cooperative study concerning the origin and nature of the lesions which had been produced in the rat as the result of the varied and presumably insufficient diets. In this undertaking the Department of Chemical Hygiene is responsible for the planning of the diets which have produced the disease, and their administration, the care of the animals, and the interpretation of the nature of the faults in the food mixtures themselves. The Department of Pediatrics has assumed the responsibility for the determination of the exact nature of the pathological processes and has undertaken the interpretation of them in the light of human osteopathology and in so far as it is possible the correlation of the osteo- and organopathology to the dietary defects. Each department has utilized the information gained from the other so as to plan most effectually for future experiments.

The present paper, the first of a series, has for its purpose the presentation of certain faulty diets which when fed to the young rat produce several disturbances in the growth of the skeleton which have in common fractures, enlargement and distortion of the costochondral junctions, vertebral deformity, the overproduction of osteoid tissue, and irregularities in the calcification of the intercellular substance of the proliferative cartilage or absence of lime salt deposits from the matrix of this tissue. Administration of some of these diets to young rats results in the development of pathological changes in the osseous system which are identical with those found in the bones of rachitic children. Others of the dietary formulas used caused changes in the bony and cartilaginous skeleton which bear a close resemblance to those found in human rickets but can only be said to be similar and not identical with this condition. One of these diets

causes in the second generation of rats maintained upon it an extreme degree of osteoporosis with multiple fractures but with relatively slight irregularity in the calcification of the cartilage.

The study which we have undertaken is a most comprehensive one. It has already involved the feeding of several basal rations of a faulty nature supplemented by the addition of single and multiple purified food constituents. This plan originated in the Laboratory of Chemical Hygiene and has been employed for some years in that laboratory as an effective biological method for the analysis of the nature of the deficiencies of the various natural foodstuffs. More than 300 modifications of diet have been effected in producing the rachitic and the related pathological conditions in the skeleton of the rat, and in demonstrating the nature of the faults in the food mixtures. The work of making histological examinations of the rats representative of all the experimental groups has been most time-consuming and is as yet far from being completed.

The present paper is a preliminary communication. We reserve for later papers, therefore, descriptions of the diets and growth curves and the exact pathological conditions produced in the skeleton by each of the diets in question.

In order to appreciate the view we have come to hold relative to the etiology of rickets, the following diets are described and the nature of the defects is pointed out.

No. 2249.

	per cent
Whole wheat kernel	30.0
" maize "	20.0
Polished rice	12.0
Rolled oats	11.5
Peas	12.0
Navy beans	12.0
$NaHCO_3$	1.5
NaCl	1.0

This diet is faulty in that it contains decidedly less than the optimum of both the dietary factors, fat-soluble A and calcium. It is by no means free from either of these essentials. The quality of the protein while not of the best cannot be a factor of importance in inducing malnutrition on this diet.

No. 2581.

	per cent
Beef liver (steamed and dried)	20.0
Casein	10.0
NaCl	1.0
KCl	1.0
Dextrin	65.0
Butter fat	3.0

This diet contains an abundance of protein of good quality and all the inorganic elements necessary for the normal nutrition of the rat except calcium, which is present in very small amount. It is rich in fat-soluble A and contains a fairly liberal amount of water-soluble B. This diet contains a considerable excess of potential acidity. Low calcium and acidity (after catabolism) are the only known faults in this food mixture.

Nos. 2661 and 2677.

	per cent
Rolled oats	40.0
Flaxseed oil meal	8.3
NaCl	1.0
$CaCO_3$	1.5
Dextrin	49.2

This diet is satisfactory for fairly good growth in the rat when supplemented with 5 per cent of butter fat. It has but one serious fault therefore; *viz.*, lack of fat-soluble A. Its proteins are not of a very high biological value but the two seeds when supplemented as they are in this diet with NaCl and $CaCO_3$ are capable of supporting good growth.

No. 2638.

	per cent
Whole wheat kernel	30.0
" maize "	30.0
Polished rice	10.0
Rolled oats	10.0
Peas	10.0
Navy beans	10.0

This diet of cereals and legume seeds is poor in Ca, Na, and Cl ions and also poor in fat-soluble A. Its ash is somewhat acid.

No. 2733.

	per cent
Whole wheat kernel	30.0
" maize "	19.5
Polished rice	9.5
Rolled oats	8.5
Peas	8.5
Navy beans	8.5
Round steak (beef)	10.0
NaCl	1.0
$NaHCO_3$	1.5
Cod liver oil	3.0

Ration 2733, which is essentially the same as No. 2810 but with 1.5 per cent of sodium bicarbonate in place of the dextrin and 3.0 per cent of cod liver oil in place of the butter fat, enables the animals to develop in what appears from their growth curves and external appearance to be a fairly normal manner. The animals so produced have very poor skeletons, however, and the females frequently suffer complete breakdown and death when they attempt to nurse the young.

No. 2810.

	per cent
Whole wheat kernel	30.0
" maize "	14.5
Polished rice	9.5
Rolled oats	9.5
Peas	9.5
Navy beans	9.5
Round steak (beef)	10.0
NaCl	1.0
Dextrin	1.5
Butter fat	5.0

This diet of cereal grains, legume seeds, steak, sodium chloride, and butter fat is satisfactorily constituted for the nutrition of the rat except for its low calcium content. When supplemented with 1.5 per cent of calcium carbonate, growth is normal and health is maintained over a long period of adult life. Successful nutrition of five successive generations of a family of rats has been secured with this food. Without the calcium addition, notwithstanding a high content of butter fat, normal calcification in the bones does not take place.

No. 2811.

	per cent
Bolted flour	30.0
Corn-meal	20.0
Rice	12.0
Rolled oats	11.5
Peas	12.0
Navy beans	12.0
$NaHCO_3$	1.5
Dextrin	1.0

Ration 2811 contains three degerminated products, wheat flour, corn-meal, and polished rice. These are degerminated and decorticated products of the endosperm. The oats, peas, and beans represent the entire seeds. The diet is too low in calcium, fat-soluble A, and protein, and failure of nutrition on this diet is due to these factors which are enumerated in the order of their importance.

No. 2815.

	per cent
Rolled oats	40.0
Gelatin	10.0
Salts (No. 185)	3.7
Dextrin	46.3

Ration 2815 is satisfactory except in that it is very poor in fat-soluble A. The protein is of good quality and the salt mixture is of such a nature as to supplement the deficiencies in the kernel.

No. 2869.

	per cent
Whole wheat kernel	28.0
" maize "	20.0
Polished rice	9.5
Rolled oats	9.5
Peas	9.5
Navy beans	9.5
Round steak	10.0
NaCl	1.0
$CaCO_3$	3.0

This diet is too low in fat-soluble A and too high in calcium. When fed with 2 per cent of cod liver oil the above diet induces normal development of young rats.

No. 2883.

	per cent
Liver	20.0
Casein	10.0
KCl	1.0
NaCl	1.0
Dextrin	66.0
Cod liver oil	2.0

Ration 2883 is satisfactorily constituted except for an extremely low calcium content. Liver is rich in fat-soluble A but in addition the diet contains 2 per cent of cod liver oil which is extremely rich in this factor.

No. 2911.

	per cent
Wheat	30.0
Maize	20.0
Rice	11.0
Rolled oats	11.5
Peas	11.0
Navy beans	11.0
NaCl	1.0
$NaHCO_3$	1.5
Butter fat	3.0

Ration 2911 consists of cereals and legume seeds supplemented with sodium chloride, sodium bicarbonate, and butter fat. In all cases the entire seed was used except rice, which was polished. The limiting factor in this diet for the nutrition of the rat during growth is the calcium content, which is too low. This food mixture with 1.5 per cent of calcium carbonate added is a fairly satisfactory diet for the nutrition of the rat throughout life. The protein is not of the highest quality but is sufficiently good to make possible normal development. The ash of any seed is acid and when seeds are metabolized there results an excess of acid ions in the body. Sodium bicarbonate is added to neutralize this acidity.[1]

Each of the diets described produces in young rats disturbances in the growth and formation of the skeleton. Each of these

[1] In all cases our rations were fed finely ground and so mixed that the individual ingredients could not be picked out and eaten separately by the experimental animal.

diets, when fed supplemented with purified food additions to make good the deficiencies noted in the descriptions of each, is capable of inducing approximately normal nutrition and normal skeletal growth. With these factors corrected there is in no instance any evidence from the general appearance of the animals of the deformities so characteristic of those restricted to the formulas as given in this paper.

The present discussion, as we have said above, is of necessity a preliminary communication and we are not now willing to hazard any statements in regard to the factors operating to produce rickets in the child or the experimental animal. It might seem from a perusal of the diets here discussed (representative of more than 300 formulas) that the cause of these diseases might lie in a deficiency of fat-soluble A or calcium in the food, or a disturbance in the metabolism of these factors. Many years experience with feeding experiments, however, have demonstrated to us how dangerous it is to draw conclusions from apparently obvious experimental data. Any suggestions regarding the absence of a specific antirachitic substance or deficiency of either fat-soluble A and calcium as the primary agent in the production of rickets would be ill considered and might be far from the truth. At present it is only possible to say that the etiological factor is to be found in an improper dietetic regimen. The large variety of dietary formulas the administration of which results in rickets and kindred affection gives abundant evidence of the complex nature of the causes operating in the production of the disease, and we shall attempt the presentation of their analysis in later papers.

EXPLANATION OF PLATES.

h, costochondral groove; *cc*, costochondral junctions; *cv*, costovertebral junctions; *f*, fractures; *d*, shaft; *met*, metaphysis; *os*, calcified bone; *o*, osteoid tissue; *c*, epiphyseal cartilage; *m*, bone marrow; *dia*, diaphysis; *ep*, epiphysis.

PLATE 2.

FIG. 1. Rachitic rat (R) contrasted with a normal animal (N) of the same age and sex (210 and 204 days respectively). The rachitic animal weighed 100 gm., the normal animal 295 gm.

FIG. 2. Pleural surface of the thorax from a case of severe experimental rickets showing the extreme enlargement at the costochondral junctions.

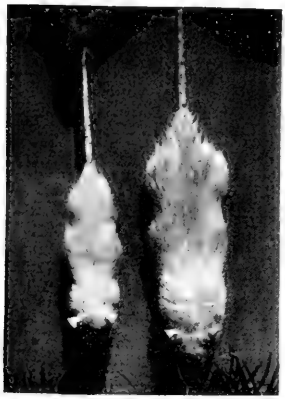
FIG. 3. An extreme case of experimental rickets. Cross-section of thorax at the level of the fifth costochondral junction. The cavity of the chest is almost completely divided into two parts by the bowed ribs and the enlargement of the costochondral junctions. Nearly the entire lungs were forced into the posterior chamber, the heart occupying the anterior chamber of the thorax. This section shows how much more marked the costochondral enlargement is on the pleural than on the external surface of the thoracic wall.

FIG. 4. Rachitic kyphoscoliosis. This picture shows also the enlargement of the ribs at the costovertebral junctions.

FIG. 5. Pleural surface of thorax from a case of experimental rickets to show multiple spontaneous fractures (healing) along the ribs.

PLATE 3.

FIG. 6. Photomicrograph of a section through the distal epiphysis and shaft of femur of a rat with experimental rachitis. The picture shows the overgrowth and irregular invasion of the epiphyseal cartilage, the presence of a metaphysis, and the overproduction of osteoid tissue. × 250.



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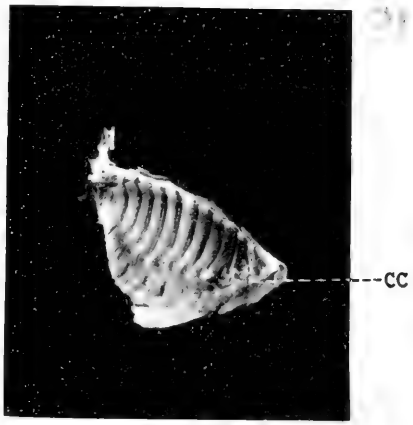

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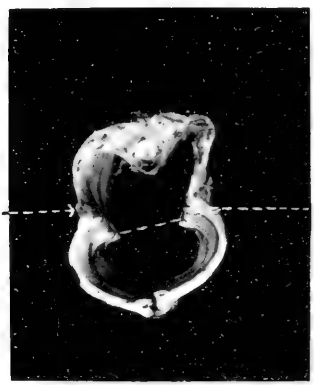

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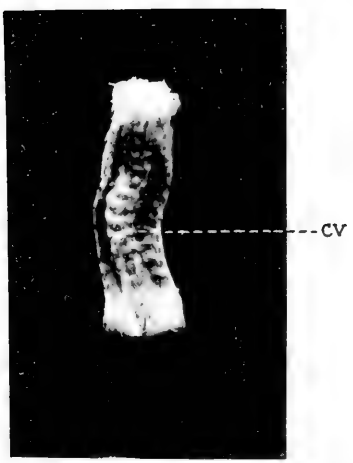

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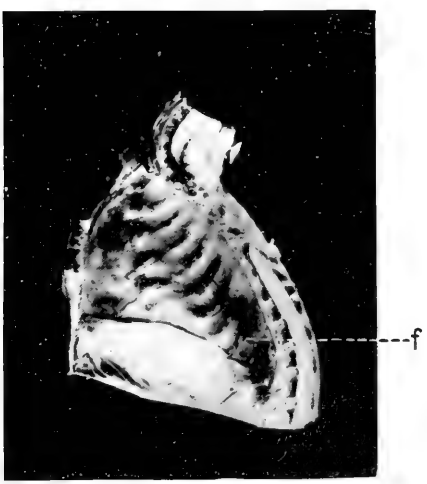

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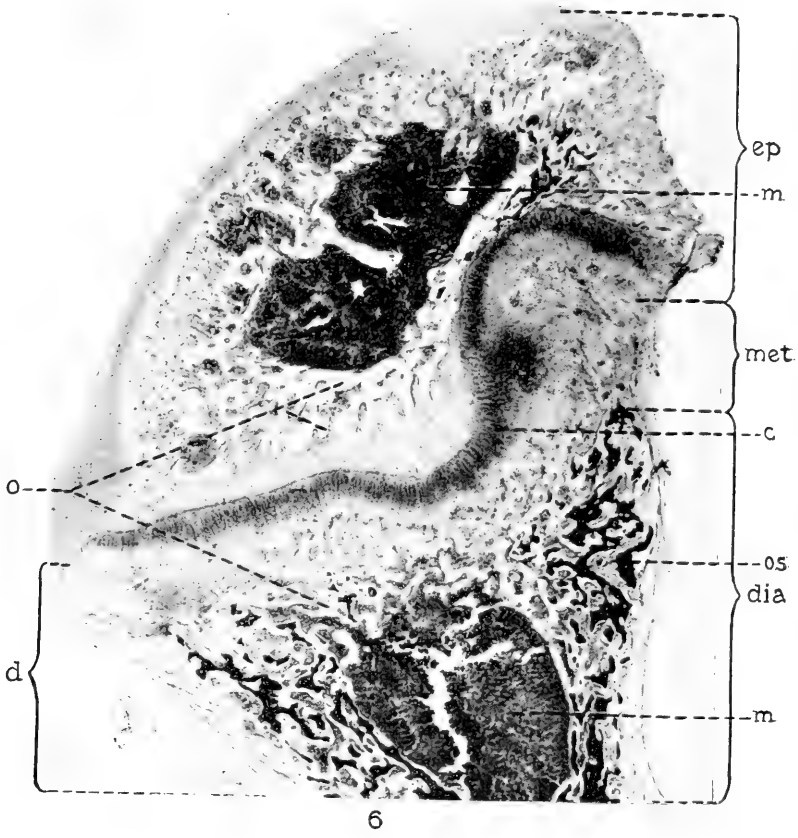
(McCollum, Simmonds, Parsons, Shipley, and Park: Experimental rickets. I.)



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(McCollum, Simmonds, Parsons, Shipley, and Park: Experimental rickets. I.)

STUDIES ON EXPERIMENTAL RICKETS.

II. THE EFFECT OF COD LIVER OIL ADMINISTERED TO RATS WITH EXPERIMENTAL RICKETS.

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Plates 4 and 5.

(Received for publication, October 26, 1920.)

For many years it has been thought as the result of clinical experience that the administration of cod liver oil in rickets exerted a favorable influence on the course of the disease. During the past 15 years studies made on the metabolism of children suffering from rickets has made it reasonably certain that the administration of cod liver oil alters the calcium balance in such a manner that calcium will be retained in the body. The natural inference from both clinical observation and metabolic investigation was that cod liver oil increased the capacity of the skeleton to take up or hold calcium. The object of the experiments reported in this paper was to furnish direct proof that cod liver oil causes calcium salts to be deposited in the bones.

We were already familiar with the observation of Schmorl that, when healing occurs in the rachitic skeleton of the human being, the initial deposition of calcium at the cartilage-shaft junction of the long bones takes place not throughout the rachitic metaphysis or at random in it but on the epiphyseal side of the metaphysis in that zone of the proliferative cartilage in which calcium deposition normally occurs and presumably would have occurred had rickets never been present. Calcium deposits in the cartilage are easy to demonstrate by histological methods and the initial deposit in healing rickets, as seen in the sections taken

longitudinally through the cartilage-shaft junctions, is situated in the lower part of the proliferative zone of cartilage. The lime salts are laid down along a line crossing the bone at right angles to its longitudinal axis and roughly paralleling the upper border of the proliferative zone, forming in the properly stained specimen a picture which cannot well be mistaken. Previous experience with the rat had taught us that by the use of faulty diets, especially certain diets deficient in the so called fat-soluble A or in both that substance and calcium, the cartilage and adjacent portions of the metaphysis of the long bones of the extremities could be rendered entirely free from calcium deposits and a condition identical with the rickets of human beings be obtained. A mode of approach to the problem at issue therefore appeared clear. If cod liver oil possessed calcium-depositing properties there seemed every reason to suppose that when added to the faulty diets of the rats whose epiphyseal cartilages and adjacent portions of the metaphyses had been rendered calcium-free, a lime salt deposit would be produced so characteristic as to have almost specific significance.

With the object of putting cod liver oil to the biological test fifteen young rats were placed on two deficient rations, Nos. 2677 and 2638. Eight animals were fed on Diet 2677 which, as will be seen from the formulas in Table I, is low in the fat-soluble A, and seven were placed on Diet 2638 which is a formula low in Ca, Na, and Cl ions, as well as in fat-soluble A. The administration of these diets it was hoped would render cartilage and the adjacent portion of the metaphysis entirely free from calcium salts.

The young rats listed as having received Diet 2638 may be said to have been on a deficient formula from their birth since the mother rats were maintained on the defective diet throughout the major part of the lactation period. Both lots of rats were maintained for from 50 to 80 days on the faulty rations.

Following the administration of the faulty diets the experimental animal ceased to gain, began to lose weight, and became extremely irritable, reacting to any stimulus with violent activity. About 40 days after the commencement of the administration of the faulty rations it was noted that those animals on Diet 2677 were beginning to develop xerophthalmia, which is characteristic for animals fed on diets deficient in fat-soluble A. When the

condition of the animals had become such as to make it evident that life could be sustained only a few days longer, they were considered ready for the test administration of cod liver oil. To the food of four of the rats on Ration 2677, cod liver oil was added in such proportion that 2 per cent replaced 2 per cent of

TABLE I.

No. and sex of rat.	Diet and principal deficiency.	Age when killed.	Deficient diet administered.	Cod liver oil added to diet.	Weight after death.	Calcium deposit in cartilage
	per cent	*days*	*days*	*days*	*gm.*	
248 ♀	Ration 2638:	75	70	5	49	+
249 ♀	Whole wheat kernel..... 30.0	75	75		66	0
250	" maize " 30.0	64	64			0
251	Polished rice..... 10.0	65	62	3	25	+
252	Rolled oats..... 10.0	67	67		31	0
253	Peas..... 10.0	67	62	5	43	+
254	Navy beans..... 10.0					
	Low in calcium, sodium, and chlorine and also poor in fat-soluble A. When cod liver oil was added to the diet 1 per cent replaced 1 per cent of maize.					
277 ♂	Ration 2677:	100	56		82	0
278 ♀	Rolled oats..... 40.0	110	41	2	64	+
298 ♀	Flaxseed oil meal..... 8.3	125	51	4	77	+
299 ♀	NaCl..... 1.0	110	68		61	0
306 ♂	$CaCO_3$..... 1.5	125	76		80	0
307 ♂	Dextrin..... 49.2	118	68	8	75	+
325 ♂	Low in the fat-soluble A. When	160	84		125	0
326 ♂	cod liver oil was added to the diet 2 per cent replaced 2 per cent of dextrin.	160	77	7	114	0

the dextrin in the ration. This modified formula was fed to the four animals for 2, 4, 7, and 8 days, respectively. To the food of the other four rats no cod liver oil was added and they were preserved as control animals. Of the seven rats on Ration 2638, three were selected as test animals. Cod liver oil was added to

the food of these three so that 1 per cent replaced 1 per cent of maize and the three rats were fed on this formula for 3, 5, and 5 days, respectively. In the case of the four rats remaining which were being fed on Ration 2638 the original diet was continued unchanged. These rats also served as control animals for this diet. As the period of the test administration of cod liver oil came to an end the test animals and their respective controls were killed with chloroform and examined. The bones of test and control animals were compared for gross differences. The ribs, the lower ends of the femora, the upper ends of the tibiæ, the lower ends of the radii and ulnæ, and the upper ends of the humeri were then split longitudinally, placed in 10 per cent formaldehyde, and saved for histological examination. After fixation had taken place the lower end of the femur of each animal was sectioned with a freezing microtome without decalcification, stained with silver nitrate according to the method of von Kossa, and counterstained with hematoxylin and eosin. Blocks of tissue from the ends of all the bones mentioned were embedded in parlodion in some instances without decalcification, in others after short decalcification in Muller's fluid, and stained according to the silver nitrate, hematoxylin, and eosin methods just mentioned.

Microscopic study of the preparations so made permits the following generalizations: When the cartilage has become free from calcium as the result of the deficient diets, the addition of cod liver oil to the food for a period of from 2 to 7 days is followed by deposition of lime salts between the cells of the proliferative zone of cartilage. The deposit of calcium salts is linear, the width of the line apparently depending on the length of time during which the animal has been fed cod liver oil. The line of deposition is at right angles to the long axis of the shaft of the bone. The granular deposit seen in sections stained with hematoxylin does not invade the protoplasm of the cell body. Stained with silver nitrate the granules are colored dark brown or black. It is difficult to say whether or not there is a coincident deposition of calcium in the osteoid tissue of the shaft but from the appearance of the section it would seem that very little lime salt is laid down in the osteoid tissue until calcification of the proliferative cartilage is complete. No gross differences between the bones of the test and control

animals were ever detected, and it was only upon microscopic examination that it was possible to note that in animals which had received no cod liver oil the epiphyseal cartilages were calcium-free. Those to whose diet the oil had been added showed a line of freshly laid down lime salt in the matrix of the proliferative zones. It will be seen from Table I that the cartilages and metaphyses of the bones of all the control animals on both diets used were successfully freed from lime salt deposits and that the administration of cod liver oil caused calcification of the matrix of the proliferative zones of cartilages of all rats fed on Diet 2638. Of the four rats which received cod liver oil after maintenance on Diet 2677 three showed calcification of the proliferative zone and one (No. 326) failed to react thus to the stimulus of the oil administration. It is difficult to assign a reason for the failure of this animal to react to the therapeutic agent but we are quite certain that the cause is to be sought with the animal rather than with the reagent itself. In a series of experiments such as those reported here there are always certain variable conditions encountered which are difficult of control. From among these might be cited variability in appetite or the presence of an absolute anorexia which is always a possibility to be reckoned when dealing with animals in poor physical condition. Again it is possible to bring an animal so near to death by prolonged feeding on a deficient diet that the organism may be without power to respond to remedial substances when they are finally received. These and other factors must be borne in mind in weighing the results of feeding experiments of this type, since the animals used must of necessity be in very poor general health before the restorative treatment is commenced.

The results of this series of preliminary experiments are twofold. They afford ocular and conclusive evidence of the specific beneficial effect of cod liver oil on rats suffering with experimental rachitis, in that some substance or substances in the oil cause calcium to be deposited in the same fashion in which deposition occurs in spontaneous healing of rachitis in man. Moreover, they prepare the way for the elaboration of a new test, which it is to be hoped may eventually prove even to be roughly quantitative, for the determination of the calcium-depositing potentiality of any substance in terms of cod liver oil units. It is

especially interesting to note in the present experiments that calcium was deposited in the cartilages following the initiation of the cod liver oil treatment in spite of the fact that in some cases the calcium intake was far below normal. In view of the fact that in human cases of rickets the blood calcium is maintained throughout the disease at approximately the normal level it is interesting to speculate concerning the source from which the calcium deposited in the proliferative cartilage is derived.

This paper is in all senses a preliminary one and experiments are now under way with a view to perfecting the biological test here reported and to determining the calcium-depositing power of certain other possible therapeutic agents.

EXPLANATION OF PLATES.

ep, epiphysis; *d*, shaft; *met*, metaphysis; *dia*, diaphysis; *m*, bone marrow; *c*, epiphyseal cartilage; *ca*, calcified intracellular substance; *o*, osteoid tissue; *os*, calcified bone.

These photomicrographs were taken of sections cut from frozen tissue previously fixed in 10 per cent formaldehyde. The bone was not decalcified and the sections were stained by von Kossa's method and counterstained with hematoxylin and eosin. Calcified tissue is seen in the photographs as in the section colored black. × 250.

Plate 4.

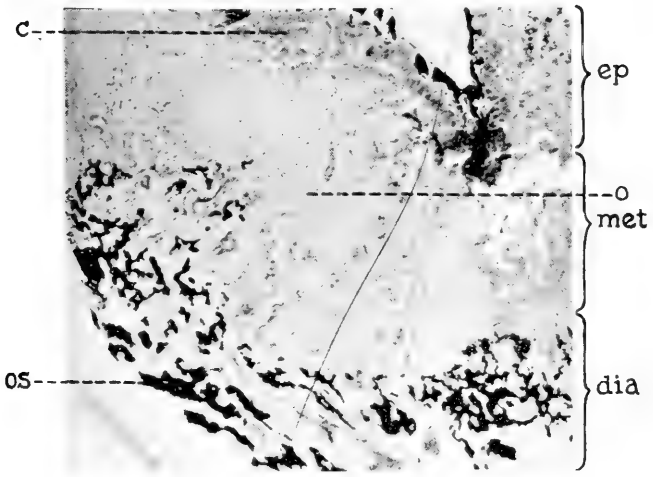
Fig. 1. Sagittal section through the lower ends of the femur of a rat fed for 84 days on Diet 2677. This picture shows besides complete absence of lime salts in the proliferative cartilage, the other cardinal microscopic signs of rickets; *viz.*, invasion of the cartilage and overproduction of osteoid tissue.

Fig. 2. Sagittal section from the distal end of the femur of a rat which was given cod liver oil after being maintained on Diet 2677. Beginning healing of experimental rickets is evidenced by the deposit of calcium (*ca*) in the proliferative zone of the epiphyseal cartilage, otherwise the bone is in the same condition as that in Fig. 1.

Plate 5.

Fig. 3. The animal from which the section shown here was made had lived on Diet 2638. The section shows marked evidences of rickets. The cartilage of the epiphysis is absolutely free from calcium salts.

Fig. 4. The rat from which this bone was taken had been given Diet 2638. *Five* days before death 1 per cent of cod liver oil was substituted for 1 per cent of maize in the ration. Note the heavy deposit of calcium (*ca*) in the epiphyseal cartilage resulting from cod liver oil administration.



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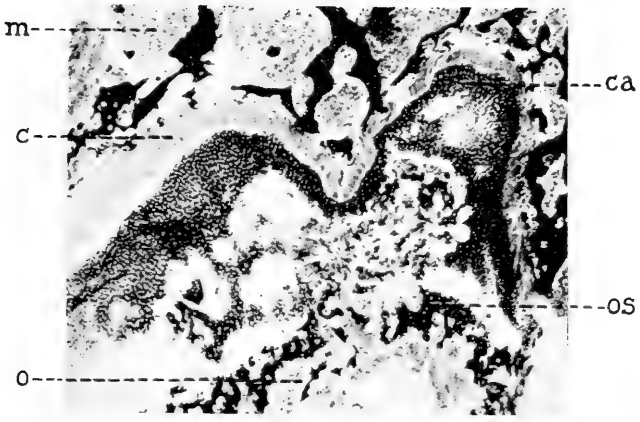

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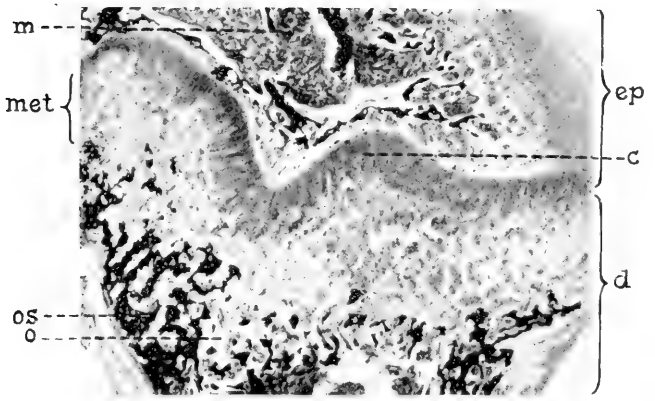
(Shipley, Park, McCollum, Simmonds, and Parsons: Experimental rickets. II.)



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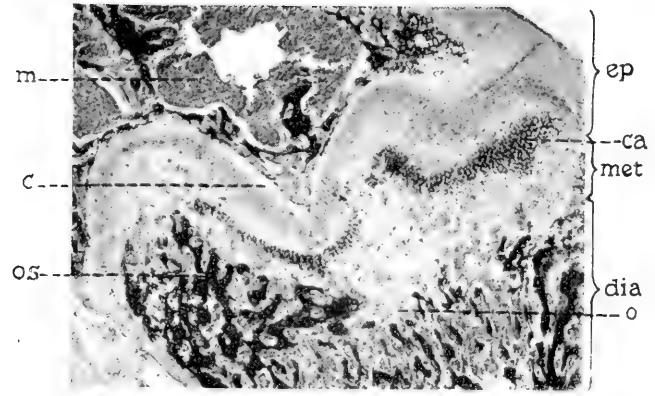

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(Shipley, Park, McCollum, Simmonds, and Parsons: Experimental rickets. II.)

THE IODOMETRIC DETERMINATION OF COPPER AND ITS USE IN SUGAR ANALYSIS.

I. EQUILIBRIA IN THE REACTION BETWEEN COPPER SULFATE AND POTASSIUM IODIDE.

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(Received for publication, November 29, 1920.)

In the course of a comparative study of several methods for the determination of sugar in blood, the results of which will be recorded elsewhere, it became desirable to investigate the reaction between copper sulfate and potassium iodide which is concerned in a number of methods for sugar determination (Bang (1), Maclean (2), Scales (3), Lehmann (4), Riegler (4), Peters (4), Kendall (5)).

In certain of these methods (Bang, Maclean, Scales) the cuprous oxide formed by the oxidation of the sugar is dissolved in acid[1] and reoxidized to cupric salt by a known amount of standard iodine—potassium iodide, the excess of iodine being then titrated with thiosulfate. The oxidation takes place in the presence of considerable excess of cupric salts which under the conditions does not appear to inhibit the oxidation, the latter going apparently to completion.

Essentially the same reaction between cupric salts and potassium iodide is the basis of the well known method for the iodometric determination of copper introduced by De Haen in 1854, the details of which have been studied especially by Gooch and Heath (6) who give the earlier literature. But in this case cupric salt is reduced to cuprous iodide in the presence of an excess of potassium iodide, with the liberation of free iodine

[1] In Bang's micro method the cuprous salt is held in solution by the presence of a large amount of potassium chloride.

equivalent to the amount of copper reduced. Under the conditions laid down by Gooch and Heath the reduction is complete, and correct results are obtained.

This latter reaction, the reduction of cupric to cuprous salt, also has been utilized in the determination of sugar, the excess of cupric copper, after filtering off the cuprous oxide, being determined in this way in methods described by Lehmann, Riegler, Peters, and others (4).

Both procedures outlined above are based upon the reversible reaction

$$Cu^{++} + 2I^- \rightleftarrows Cu^+ + I^- + \tfrac{1}{2}I_2$$

which under slightly different conditions appears to run, for practical purposes, to completion in either direction. It has long been known that this reaction is reversible and it has been shown by Bray and MacKay (7) to obey the mass law within certain limits in dilute solutions. It therefore seemed rather remarkable that conditions should exist which permit the complete removal of free iodine, one of the active factors in the reaction without perceptibly disturbing the equilibrium at *both* extremes.

In order to permit an intelligent use of the reaction in both directions we have undertaken the determination of the points of equilibria of the reaction

$$CuSO_4 + KI \rightleftarrows CuI + I_2 + K_2SO_4$$

at various initial concentrations of $CuSO_4$ and KI and at various ratios of $CuSO_4$: KI. Our results indicate the conditions under which the reaction may be utilized for the determination of either cupric or cuprous salts with ease and accuracy.

The experimental procedure was to mix the solutions of copper sulfate and potassium iodide in definite molecular ratios at widely varying concentrations. After standing at room temperature samples of the supernatant liquid (which contained the liberated iodine, unreduced cupric copper, excess potassium iodide, and a minute quantity of cuprous iodide in solution) were carefully pipetted out, and the extent of the reduction, and thus the equilibrium attained, was determined by titrating the amount of free iodine by standard thiosulfate solutions. Since according to the reaction

$$Cu^{++} + 2I^- \rightleftarrows (Cu^+) + \tfrac{1}{2}I_2$$

1 atom of iodine is liberated for each atom of copper which is reduced, one may readily calculate the amount of cuprous salt formed and this subtracted from the total copper gives the amount of cupric salt unreduced. It was clear, however, that this method of determining the equilibrium point would not work unless the equilibrium were in some way stabilized; for, on removal of iodine by titration, the equilibrium is rapidly shifted to the right by the now unbalanced cupric and iodide ions; the blue starch-iodide end-point returns very rapidly; and the results indicate much too great reduction. This was avoided simply by measuring the samples for analysis directly into an equal volume of water containing an excess of sodium hydroxide. The cupric copper which remains is precipitated as the hydroxide and removed by filtration while the iodine is converted into hypoiodite and iodide and is recovered on acidifying the filtrate.

With both cupric and cuprous salts[2] removed the reaction cannot take place, and the equilibrium attained is represented by the amount of iodine, which after acidification is titrated with thiosulfate. Under these conditions the end-point is sharp. The results are given in Table I. The details of procedure and the method of calculating the results are given below.

Preparation of $CuSO_4$-KI Mixtures.

An approximate 1.25 M solution was made of Kahlbaum's "Zur Analyse" $CuSO_4.5H_2O$. After filtering from suspended particles this was carefully standardized by titrating 20 cc. portions of a 1:10 dilution with 0.1 N thiosulfate after adding KI under the conditions described by Gooch and Heath. From the original $CuSO_4$ solution, an exactly molecular solution was made by dilution. The concentration of this solution was checked by diluting 50 cc. to 500 cc., when 20 cc. portions titrated with thiosulfate gave 20.05 cc. $\times$ 0.0997 N = 19.99 cc. 0.1 N. Potassium biiodate was used to standardize the thiosulfate. From the M $CuSO_4$, other dilutions were made as needed.

[2] The amount of cuprous iodide in solution is negligible except in the presence of high concentrations of potassium iodide. Bodländer and Storbeck (8) calculate the solubility product of $(Cu^+).(I^-)$ as 5×10^{-12} according to which value the concentration of cuprous salt in dilute solutions of potassium iodide would be less than 2.2×10^{-6} M.

TABLE I.

Equilibria of $CuSO_4 + KI$.

Experiment No.	Initial concentration of $CuSO_4$.	Initial concentration of KI.	Thiosulfate titration of 10 cc. of mixture.			At equilibrium.	
			Found at equilibrium.	Calculated for complete reduction to CuI.	Thiosulfate.	Cuprous salt.	Cupric salt.
(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)
	millimolar	*millimolar*				*per cent*	*per cent*
			$1CuSO_4$:1KI				
1	2.5	2.5	0.00	25.0	0.001 N	0.00	100.00
2	5.0	5.0	0.00	50.0	0.001 "	0.00	100.00
3	6.0	6.0	0.03	60.0	0.001 "	0.05	99.95
4	7.0	7.0	0.05	70.0	0.001 "	0.07	99.93
5	8.0	8.0	0.15	80.0	0.001 "	0.19	99.81
6	9.0	9.0	0.50	90.0	0.001 "	0.56	99.44
7	10.0	10.0	0.80	100.0	0.001 "	0.80	99.20
8	11.0	11.0	1.20	110.0	0.001 "	1.09	98.91
9	12.0	12.0	1.80	120.0	0.001 "	1.50	98.50
10	20.0	20.0	19.75	200.0	0.001 "	9.88	90.12
11	50.0	50.0	11.74	50.0	0.01 "	23.48	76.52
12	100.0	100.0		100.0	0.01 "	36.50	63.50*
13	200.0	200.0		200.0	0.01 "	40.40	59.60*
			$1CuSO_4$: 2KI				
14	2.5	5.0	0.00	25.0	0.001 N	0.00	100.00
15	3.0	6.0	0.00	30.0	0.001 "	0.00	100.00
16	4.0	8.0	0.20	40.0	0.001 "	0.50	99.50
17	5.0	10.0	0.50	50.0	0.001 "	1.00	99.00
18	6.0	12.0	1.00	60.0	0.001 "	1.67	98.33
19	7.0	14.0	0.25	7.0	0.01 "	3.57	96.43
20	8.0	16.0	0.50	8.0	0.01 "	6.25	93.75
21	9.0	18.0	0.75	9.0	0.01 "	8.33	91.67
22	10.0	20.0	1.10	10.0	0.01 "	11.00	89.00
23	11.0	22.0	1.50	11.0	0.01 "	13.64	86.36
24	12.0	24.0	2.00	12.0	0.01 "	16.67	83.33
25	13.0	26.0	2.40	13.0	0.01 "	18.46	81.54
26	14.0	28.0	2.85	14.0	0.01 "	20.36	79.64
27	15.0	30.0	3.60	15.0	0.01 "	24.00	76.00
28	16.0	32.0	4.18	16.0	0.01 "	26.13	73.87
29	17.0	34.0	4.58	17.0	0.01 "	26.94	73.06
30	18.0	36.0	5.15	18.0	0.01 "	28.61	71.39
31	19.0	38.0	5.80	19.0	0.01 "	30.53	69.47

* Determination of precipitated cupric hydroxide.

TABLE I—*Continued.*

Experiment No.	Initial concentration of $CuSO_4$.	Initial concentration of KI.	Thiosulfate titration of 10 cc. of mixture.			At equilibrium.	
			Found at equilibrium.	Calculated for complete reduction to CuI.	Thiosulfate.	Cuprous salt.	Cupric salt.
(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)
	millimolar	*millimolar*				*per cent*	*per cent*
			$1CuSO_4$:2KI				
32	20.0	40.0	6.45	20.0	0.01 N	32.25	67.75
33	30.0	60.0	12.35	30.0	0.01 "	41.17	58.83
34	40.0	80.0	19.18	40.0	0.01 "	47.95	52.05
35	50.0	100.0	2.62	5.0	0.10 "	52.40	47.60
36	100.0	200.0		10.0	0.10 "	67.25	32.75*
37	200.0	400.0		20.0	0.10 "	72.50	27.50*
38	300.0	600.0		30.0	0.10 "	75.50	24.50*
			$1CuSO_4$:3KI				
39	2.5	7.0	0.00	25.0	0.001 N	0.00	100.00
40	5.0	15.0	0.35	5.0	0.01 "	7.00	93.00
41	10.0	30.0	3.30	10.0	0.01 "	33.00	67.00
42	20.0	60.0	11.62	20.0	0.01 "	58.10	41.90
43	50.0	150.0	3.87	5.0	0.10 "	77.40	22.60
44	100.0	300.0	9.07	10.0	0.10 "	90.70	9.30
45	200.0	600.0	19.17	20.0	0.10 "	95.85	4.15
46	400.0	1,200.0	38.69	40.0	0.10 "	96.72	3.28
			$1CuSO_4$:4KI				
47	2.5	10.0	0.25	25.0	0.001 N	1.00	99.00
48	5.0	20.0	0.90	5.0	0.01 "	18.00	82.00
49	10.0	40.0	5.47	10.0	0.01 "	54.70	45.30
50	20.0	80.0	14.77	20.0	0.01 "	73.85	26.15
51	50.0	200.0	4.62	5.0	0.10 "	92.40	7.60
52	100.0	400.0	9.77	10.0	0.10 "	97.70	2.30
53	200.0	800.0	19.67	20.0	0.10 "	98.35	1.65
54	400.0	1,600.0	39.57	40.0	0.10 "	98.93	1.07
			$1CuSO_4$: 5KI				
55	2.0	10.0	0.00	20.0	0.001 N	0.00	100.00
56	3.0	15.0	0.85	30.0	0.001 "	2.83	97.17
57	4.0	20.0	3.88	40.0	0.001 "	9.70	90.30
58	5.0	25.0	10.00	50.0	0.001 "	20.00	80.00
59	6.0	30.0	18.25	60.0	0.001 "	30.42	69.58
60	8.0	40.0	33.95	80.0	0.001 "	42.44	57.56
61	10.0	50.0	5.77	10.0	0.01 "	57.70	42.30

TABLE I—*Concluded.*

Experiment No.	Initial concentration of $CuSO_4$.	Initial concentration of KI.	Thiosulfate titration of 10 cc. of mixture.			At equilibrium.	
			Found at equilibrium.	Calculated for complete reduction to CuI.	Thiosulfate.	Cuprous salt.	Cupric salt.
(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)
	millimolar	*millimolar*				*per cent*	*per cent*
			$1CuSO_4$:5KI				
62	20.0	100.0	17.00	20.0	0.01 N	85.00	15.00
63	30.0	150.0	26.72	30.0	0.01 "	89.06	10.94
64	40.0	200.0	3.70	4.0	0.10 "	92.50	7.50
65	50.0	250.0	4.80	5.0	0.10 "	96.00	4.00
66	60.0	300.0	5.78	6.0	0.10 "	96.33	3.67
67	100.0	500.0	9.77	10.0	0.10 "	97.70	2.30
68	200.0	1,000.0	19.72	20.0	0.10 "	98.60	1.40
69	300.0	1,500.0	29.56	30.0	0.10 "	98.53	1.47
70	400.0	2,000.0	39.34	40.0	0.10 "	98.35	1.65
			$1CuSO_4$:10KI				
71	1.0	10.0	0.00	10.0	0.001 N	0.00	100.00
72	2.5	25.0	6.00	25.0	0.001 "	24.00	76.00
73	3.0	30.0	12.50	30.0	0.001 "	41.67	58.33
74	4.0	40.0	23.90	40.0	0.001 "	59.75	40.25
75	5.0	50.0	3.72	5.0	0.01 "	74.40	25.60
76	8.0	80.0	7.00	8.0	0.01 "	87.50	12.50
77	10.0	100.0	9.57	10.0	0.01 "	95.70	4.30
78	20.0	200.0	19.57	20.0	0.01 "	97.85	2.15
79	50.0	500.0	4.97	5.0	0.10 "	99.40	0.60
80	100.0	1,000.0	9.62	10.0	0.10 "	96.20	3.80
81	200.0	2,000.0	17.62	20.0	0.10 "	88.10	11.90
82	300.0	3,000.0	22.12	30.0	0.10 "	73.73	26.27

A 5 M KI solution was made by dissolving in a volume of 1,000 cc. 830 gm. of a pure preparation, previously powdered and dried at 80°C. for 18 hours. The solution was filtered and from it other dilutions were made. Calibrated pipettes and burettes, the latter of 25 cc. capacity, small bore, and marked in 0.05 cc., were used throughout.

The copper sulfate and potassium iodide solutions were mixed in a uniform way in volumetric flasks. Distilled water was first added to the flasks leaving only slightly more room than needed for the solutions to be added. The $CuSO_4$ was then

added by pipette, the solution mixed, the KI measured in, and finally water added to the mark. After being stoppered and mixed by shaking, the flasks stood in a dark closet at 25–30°C. from 2 to 5 days to insure the attainment of equilibrium. During this time the precipitated CuI settles to the bottom so that it is possible to pipette off samples of the clear supernatant liquid without disturbing the precipitate. 20 cc. samples of the solutions were measured into small flasks containing exactly 20 cc. of N NaOH. After being mixed, the solutions were filtered from the precipitated cupric hydroxide, and 20 cc. of the filtrates, equivalent to 10 cc. of the $CuSO_4$-KI mixtures, were measured into small flasks and acidified with about 5 cc. of 5 N H_2SO_4. The iodine liberated was then titrated with the same thiosulfate (or dilutions made from it) which was used in standardizing the $CuSO_4$ solution (Column 4, Table I).

The use of sodium hydroxide makes necessary the application of a small correction for the amount of iodine which is removed by some impurity in the alkali, the correction being added to the amounts of thiosulfate used. The correction in these experiments amounted to 1.11 cc. of 0.01 N iodine.

At the same time other 10 cc. portions of the supernatant liquids were in most cases titrated directly with thiosulfate after adding an excess of KI. The results so obtained are equivalent to the *total* copper originally in solution and serve as an additional check upon these values. In all but a few cases the results for total copper agreed well with the calculated values.

The following example illustrates the procedure above described.

10 cc. of 1 M $CuSO_4$ were mixed with about 175 cc. of water in a 200 cc. volumetric flask, 10 cc. of 5 M KI added, and the solution was diluted to the mark. The mixture had therefore as the initial concentrations, 50 millimolar Cu and 250 millimolar KI (Experiment 65, ratio $1CuSO_4$:5KI, Table I). After standing 3 days a 20 cc. sample of the supernatant liquid was pipetted out, added to 20 cc. of N NaOH, and filtered.

	0.1 N thiosulfate. cc.
20 cc. filtrate, acidified (= 10 cc. original solution)	4.69
Correction for NaOH	0.11
(Column 4, Table I)	4.80
10 cc. original solution + excess KI = total copper (Column 5)	5.00

Since 1 atom of iodine is liberated from each atom of copper reduced, 10 cc. of 0.05 M $CuSO_4$ when fully reduced by KI should yield 10 cc. of 0.05 N or 5.0 cc. of 0.1 N iodine, which is the amount found by titration. The amount found at equilibrium (4.80 cc.) divided by the amount from total reduction (5.00) gives the proportion reduced (0.96 or 96.0 per cent) (Column 7). The remainder (4 per cent) is the proportion of cupric salt (Column 8).

The procedure of analysis and methods of calculation above described were adhered to throughout, with the following exceptions. In very dilute solutions the iodine liberated is so small in amount that all or most of it was absorbed by the impurity present in the NaOH, the correction for the blank approaching or exceeding the amount of iodine to be determined. In these cases 10 cc. of the supernatant liquid were titrated directly with 0.001 N thiosulfate without previously stabilizing the equilibrium. Fortunately at very low concentrations of copper and iodide the small amounts of iodine present can be titrated away without materially shifting the equilibrium within the brief period of making the titration, and the results of direct titration are moderately reliable as proved by a fair agreement of results by the usual procedure and by direct titrations, at the slightly higher concentrations where such comparison was possible.

Another exception to the procedure described was necessary with the higher concentrations of the series, 1 $CuSO_4$: 1 KI and 1 $CuSO_4$: 2 KI. The results first obtained with the alkaline filtrates from solutions 100 millimolar $CuSO_4$ of series 1:1 and of series 1:2 and from solutions of higher concentrations with these ratios, gave values which on being plotted on curves appeared erroneous. On inspection of the precipitate in the flasks, crystals of iodine were observed to be mixed with the cuprous iodide, which explained the low results obtained. The residual iodide, after the equilibrium was reached, was under these conditions not sufficient to hold in solution all the iodine liberated, and the titration of the iodine in solution therefore did not represent the amount formed. In such cases the points of equilibria were located by determining directly the amount of residual cupric copper. The cupric hydroxide precipitated from the supernatant liquid when the latter is run into NaOH was washed free of iodine by repeated centrifugation, decantation, and filtration,

finally dissolved in H_2SO_4, an excess of KI added, and the solution titrated with thiosulfate under the conditions under which the copper sulfate solutions are standardized. These results proved to be reliable and in agreement with those from the usual procedure in those solutions where such comparison was possible. Data were obtained in this way for the last two mixtures of ratio 1:1 and the last three mixtures of ratio 1:2 as explained in the foot-note to Table I.

DISCUSSION.

From the data given in Table I curves have been drawn which show graphically the position of the equilibria under various conditions. In Charts 1 and 2 the percentage of total copper reduced to cuprous salt (the position of equilibrium) is plotted against initial concentration of total copper for each of the molecular ratios 1 $CuSO_4$:1, :2, :3, :4, :5, and :10 KI. The initial concentration of KI for each curve is readily seen by multiplying the initial copper concentration by the KI ratio of that curve. In Chart 3 the percentage of total copper reduced is plotted against initial concentration of potassium iodide.

Each individual curve of Chart 1 shows the shift of equilibrium with change in volume for a given molecular ratio of the reacting substances. If a mixture of $CuSO_4$ and KI is made of initial concentrations as indicated and the mixture is progressively diluted the percentage distribution of total copper between precipitated cuprous iodide and cupric salt in solution is shown at any given dilution by the corresponding point on the curve for that molecular ratio. For instance, if equal volumes of 0.2 M $CuSO_4$ and 1.0 M KI are mixed, the initial concentrations being 100 millimolar $CuSO_4$ and 500 millimolar KI (1 $CuSO_4$: 5 KI), about 98 per cent of the total copper is present as cuprous iodide. If the mixture is diluted with 9 volumes of water, the equilibrium is now such that only 57 per cent of the copper exists as cuprous iodide, and on further dilution to 50 times the original volume (total copper 2 millimolar, total KI 10 millimolar) practically all the copper exists as cupric salt and only little if any iodine can be detected by starch.

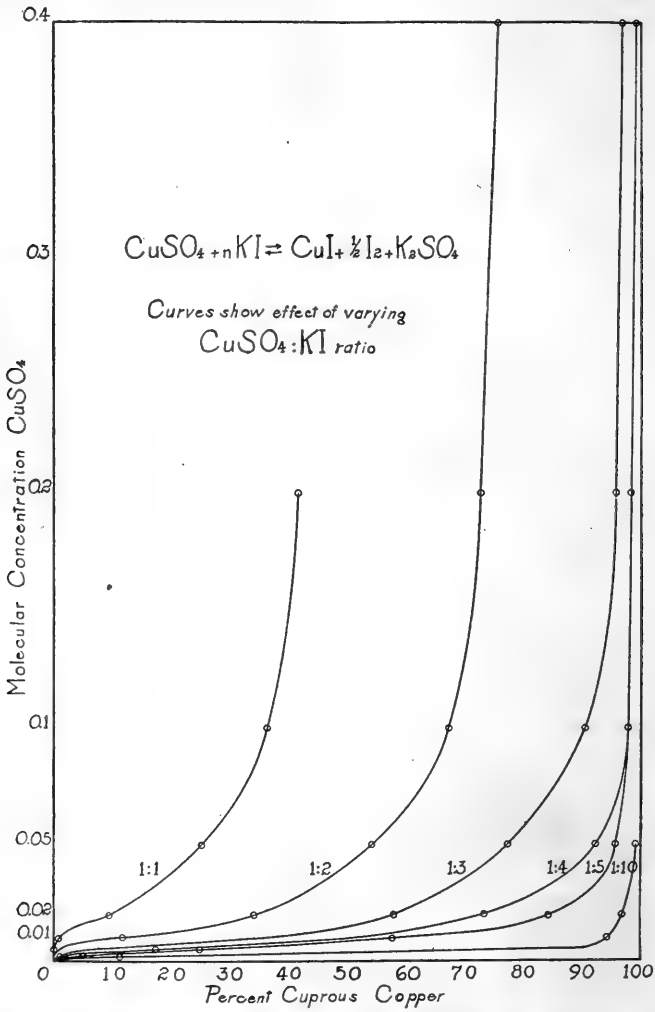

CHART 1. Curves show the *position of equilibrium* of the reaction between $CuSO_4$ and KI, expressed as percentage of total copper in the form of cuprous salt, as affected by varying the ratio of $CuSO_4$:KI, and by concentration (dilution).

The relations are illustrated by the following experiment.

Mix 2.5 cc. of M (25 per cent) $CuSO_4.5H_2O$ and 7.5 cc. of M (16.6 per cent) KI. Cuprous iodide is immediately precipitated and the solution is dark-colored from the formation of free iodine. On analysis 96 per cent of the copper would be found as cuprous iodide. This mixture had initial concentrations of 250 millimolar $CuSO_4$ and 750 millimolar KI or $1CuSO_4$:3KI. Mix the same amounts in a total volume of 1,000 cc. by adding 2.5 cc. of M $CuSO_4$ to 990 cc. of water, and to this dilute solution add 7.5 cc. of M KI. The mixture now remains clear and gives no iodine reaction with starch.[3] The initial concentrations in this case correspond to 2.5 millimolar $CuSO_4$ and 7.5 millimolar KI.[4]

It is evident that the proportion of cupric salt which is reduced to cuprous iodide is determined by the dilution and by the relative excess of iodide in solution. The predominant influence of iodide is indicated by Chart 3. These relations are implied in the mass law equation

$$(Cu^{++}) \cdot (I)^2 = \sqrt{I_2} \cdot K$$

according to which the concentration of cupric ions will vary inversely as the square of the iodide ions and directly as the square root of the free iodine. A discussion of the bearing of our data upon the validity of this equation will be deferred to a later paper, and we shall point out here only their significance for the determination of cupric and cuprous copper, for which purpose the work was undertaken.

[3] Since writing the above the work of Traube (Traube, M., *Ber. chem. Ges.*, 1884, xvii, 1064) has come to our attention. Traube established the existence of cupric iodide in solution and described experiments very similar to those given above showing the effect of dilution upon the formation and precipitation of cuprous iodide from mixtures of copper sulfate and potassium iodide. He pointed out that the mixture of 0.1 per cent KI and 0.075 per cent $CuSO_4.5H_2O$ (equivalent to final concentrations of 3 millimolar KI and 1.5 millimolar $CuSO_4$) or 0.05 per cent KI with 1.0 per cent $CuSO_4.5H_2O$ (equivalent to final concentrations of 1.5 millimolar KI and 20 millimolar $CuSO_4$) remains clear and gives no reaction with starch after 24 hours standing. Our work is thus an elaboration of facts made known 36 years ago.

[4] The same experiment may be more simply performed by diluting the first mixture to a liter, but in this case equilibrium is attained more slowly and the solution contains a small amount of free iodine for some time.

With the ratio of 1 Cu : 1 KI reduction is absent or very slight until the concentration reaches about 5 millimolar (Chart 2). With these relative amounts of cupric salt and potassium iodide and at these concentrations no cuprous salt and no free iodine are formed. Furthermore, if cuprous salt is originally

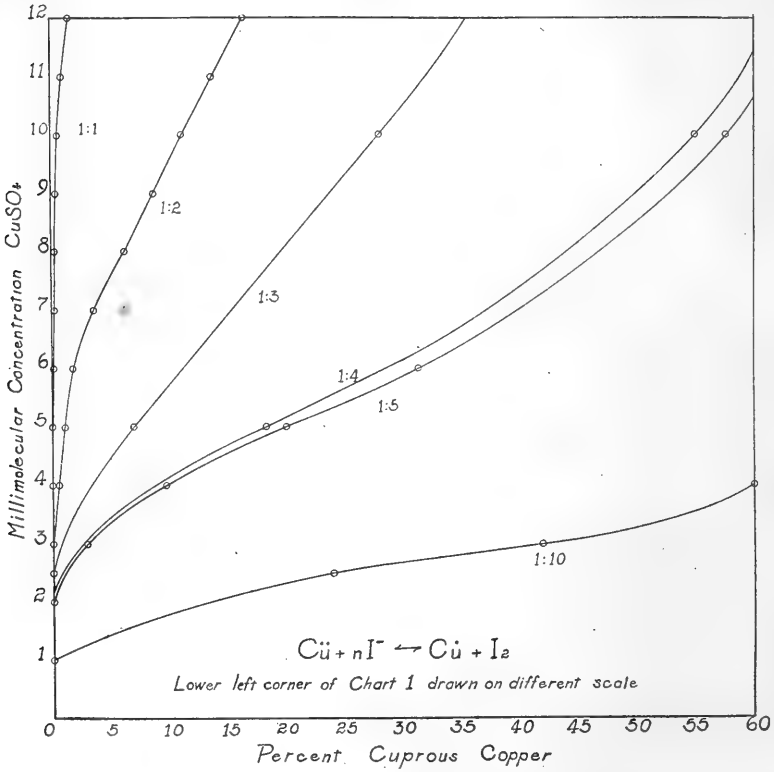

Chart 2. Curves show the same relations as in Chart 1, drawn on a larger scale to indicate the limiting concentrations for the complete oxidation of cuprous to cupric salt.

present it is quickly and completely oxidized to the cupric condition if iodine is added; and since at this concentration there is no tendency for the reaction to run to the right, and thus no cuprous salt and free iodine to form, the excess iodine may be removed by titration with a sharp end-point. The

conditions, therefore, which must be chosen for the determination of cuprous copper by this reaction are a minimum excess of KI and a final (total) concentration of less than about 3 to 5 millimolar copper. (In the following paper means are described by which this limitation is removed.) Cuprous copper will be completely oxidized to cupric salt by an excess of iodine provided the final concentration of copper and iodide do not exceed 5 millimolar each.[5]

These are the conditions which Maclean and Scales found empirically to be necessary for the reoxidation by iodine of cuprous copper formed in the oxidation of sugar. The final concentration of total copper in Maclean's titration is 2.5 millimolar and of iodide 2.9 millimolar (after the amount of iodide necessary for the reaction with the iodate has been subtracted from the original amount present) or 1:1 ratio. In the technique described by Scales the values prove to be 4.5 millimolar iodide and 2.3 millimolar total copper or 1:2 ratio. An examination of Chart 2 shows that both these conditions lie on the ordinate below the intersection of Curves 1:1 and 1:2, where no cuprous salt is present at equilibrium. At concentrations represented by points above the intersection of the respective curves, the end-points would not be sharp and the results would be incorrect. At such higher concentrations a large excess of iodine will, to be sure, oxidize more or less cuprous salt but on removal of the excess of iodine during titration with thiosulfate the equilibrium will shift to the right with the reformation of cuprous iodide and iodine and a consequent running (returning) end-point.

[5] The actual limitation is doubtless a certain value of the product $(Cu^{++}) \times (I^-)^2$. What this value is we have not determined exactly though our results indicate it to be roughly about 135, the concentrations being expressed in terms of millimolar solutions.

Ratio 10:1,	1 Cu × (10 I)²	= 100
" 5:1,	2 Cu × (10 I)²	= 200
" 4:1,	2.1Cu × (8.2I)²	= 141
" 3:1,	2.5Cu × (7.5I)²	= 140
" 2:1,	3.0Cu × (6 I)²	= 101
" 1:1,	5 Cu × (5 I)²	= 125
Average		135

For the determination of cupric copper, on the other hand, it is necessary that the cupric salt be completely reduced with the liberation of an equivalent amount of iodine, and, as shown by Chart 1, this condition depends upon the presence of a considerable excess of iodide in solution. The optimum concentration of iodide is about 0.25 to 0.5 M, in which solutions, if the ratio of

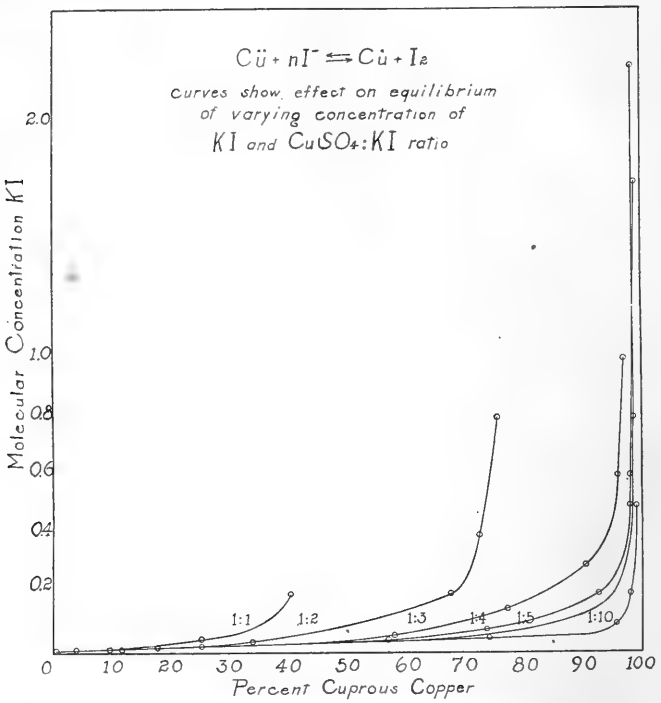

CHART 3. The curves show the predominating influence of KI in determining the equilibrium.

copper to iodide is not greater than 1:5, the copper will be almost completely converted into cuprous iodide at equilibrium, and on removal of the iodine by titration the small remainder of cupric salt is quickly reduced. Equilibrium being close to the right side of the reaction, the titration end-point is sharp. In a volume of 100 cc., 0.25 M potassium iodide (4.2 gm.) allows the correct determination of amounts of copper up to the equivalent of

1.25 gm. of $CuSO_4 . 5 H_2O$, or double this amount with 0.5 M KI. With smaller concentrations of iodide, as is apparent from Chart 1, the reduction is less complete at equilibrium, and the titration end-points are in consequence less sharp, the blue color returning as long as cupric salts are present.

It is doubtless due to the lack of such information as may be derived from Chart 1 that varying opinions have been expressed as to the value of this method for the determination of copper (cited by Gooch and Heath (6)). Those who have favored the method adopted conditions the correctness of which are confirmed by our curves. Moser (9) recommends the addition of 2 gm. of potassium iodide to 50 cc. of a solution containing about 0.6 gm. of cupric sulfate; this is equivalent to about 0.24 M iodide and at least 5 KI: 1 Cu. Gooch and Heath recommend the use of 5 gm. of potassium iodide in 100 cc. of solution containing not more than 0.3 gm. of copper. These amounts are equivalent to 0.3 M iodide and at least 6 KI: 1 Cu.

SUMMARY.

Both cupric and cuprous salts may be determined iodometrically by means of the reversible reaction

$$Cu^{++} + I^- \rightleftharpoons Cu^+ + I_2$$

The position of equilibrium has been determined for the reaction

$$CuSO_4 + N\ KI = CuI + I_2 + K_2SO_4$$

at ratios of from 1 to 10 KI : $1CuSO_4$ at various dilutions.

For the determination of cupric salts potassium iodide must be added to give a *final* concentration of about 0.25 M (4 to 5 gm. per 100 cc. of solution).

For the determination of cuprous salts the solution must be so diluted that the *final* concentration of copper and of iodide does not exceed about 5 millimolar each.

The conditions found empirically by Gooch and Heath (6) for cupric salts and by Maclean (2) and Scales (3) for cuprous salts comply with these requirements.

BIBLIOGRAPHY.

1. Bang, I., Der Blutzucker, Wiesbaden, 1913, 20.
2. Maclean, H., *J. Physiol.*, 1916, l, 168; *Biochem. J.*, 1919, xiii, 135.
3. Scales, F. M., *J. Biol. Chem.*, 1915, xxiii, 81.
4. Lehmann, K. B., *Arch. Hyg.*, 1897, xxx, 267. Riegler, E., *Z. anal. Chem.*, 1898, xxxvii, 22. Maquenne, M. L., *Bull. soc. chim.*, 1898, xix, series 3, pt. 2, 926. Citron, H., *Deutsch. med. Woch.*, 1904, xxx, 1602. Peters, A. W., *J. Am. Chem. Soc.*, 1912, xxxiv, 422, 928.
5. Kendall, E. C., *J. Am. Chem. Soc.*, 1911, xxxiii, 1947; 1912, xxxiv, 317.
6. Gooch, F. A., and Heath, F. H., *Am. J. Sc.*, 1907, xxiv, 65.
7. Bray, W. C., and MacKay, G. M. J., *J. Am. Chem. Soc.*, 1910, xxxii, 1207.
8. Bodländer, G., and Storbeck, O., *Z. anorg. Chem.*, 1902, xxxi, 469.
9. Moser, L., *Z. anal. Chem.*, 1904, xliii, 597.

THE IODOMETRIC DETERMINATION OF COPPER AND ITS USE IN SUGAR ANALYSIS.

II. METHODS FOR THE DETERMINATION OF REDUCING SUGARS IN BLOOD, URINE, MILK, AND OTHER SOLUTIONS.

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(Received for publication, November 29, 1920.)

After heating Fehling's solution or similar alkaline copper solution with a reducing sugar, the solution contains the oxidation products of the sugar, an excess of cupric salt, and suspended cuprous oxide. One may learn the amount of sugar oxidized by determining either the cuprous oxide, or, knowing the total copper, the residual cupric salt. Both forms of copper have been determined by means of iodometric titrations, as illustrated by the procedures of Lehmann (1), Riegler (2), Maquenne (3), Citron (4), and Peters (5) for residual cupric salts and of Bang (6), Scales (7), Maclean (8), and Clark (9) for cuprous salts. The principle underlying these methods and the conditions necessary in the determination of both forms of copper are more exactly defined in the preceding paper (10).

In one case the residual cupric salt is completely converted into cuprous iodide, with the liberation of an equivalent amount of iodine.

$$2\,Cu^{++} + 4\,I^- \rightarrow 2\,CuI + I_2$$

in the other case cuprous salt is completely oxidized to cupric in the presence of a known excess of iodine, with the conversion of the corresponding amount of iodine into iodide.

$$2\,Cu^{++} + 2\,I^- \leftarrow 2\,Cu^{+} + I_2$$

The iodine formed from iodide in the first case, and the excess iodine left in the second case, are determined by titration with standard thiosulfate, starch being used as indicator.

The direction in which this reversible reaction will proceed depends upon the concentration of the active substances (or ions). For the complete conversion of cupric to cuprous salt the concentration of iodide, which has much the strongest influence, must be high, the optimum being about 0.25 M.

For the complete oxidation of cuprous salts by iodine on the other hand means must be found for maintaining the concentrations of cupric and iodide ions at very low values. As shown in the preceding paper this is accomplished when the solution is diluted so that the total copper and final iodide concentrations do not exceed about 0.005 M each, the equilibrium then being for practical purposes on the cupric side of the reaction. In this way Bang, Maclean, Scales, and Clark were able to determine very small amounts of cuprous oxide with considerable accuracy, even in the presence of cupric salts and iodide; though none of these workers appears to have been aware of the limiting effect of iodide and copper concentrations.

Great dilution, however, limits the titration of cuprous copper to small amounts and curtails its general usefulness. Fortunately, other means are available for reducing the concentration of cupric ions to such a small value that even at moderate dilution and in the presence of much soluble iodide the reaction does not proceed to the right and equilibrium is maintained with the copper wholly in the cupric form. It was pointed out by Elbs (11) in 1917 that the presence of alkali oxalates more or less completely inhibits the reaction of cupric salts with soluble iodides, as well as other reactions of cupric ions. Elbs interpreted this behavior by the assumption of the formation of alkali cupric oxalate which dissociates with the formation of anions containing oxalate and copper, the cations being those of the alkali. This observation, which is easily verified, permits one to direct at will the above reversible reaction *to completion in either direction*, and at practically any concentration of copper salt. The following experiment illustrates the remarkable effect of oxalate.

To 50 cc. of approximately 0.1 M $CuSO_4$ were added 5 gm. of potassium iodide; the liberated iodine required 47.50 cc. of 0.1058 N thiosulfate or 50.23 cc. of 0.1 N. To the same solution, after titration with thiosulfate, were then added 100 cc. of 0.0969 N iodine (= 96.90 cc. of 0.1 N) and 25 cc. of

molar potassium oxalate. After a moment of shaking the cuprous iodide completely dissolved, and on again titrating with thiosulfate 44.10 cc. = 46.65 cc. of 0.1 N were required, showing that 50.25 cc. (96.90 − 46.65 cc.) of 0.1 N iodine had been used for the oxidation. The two determinations on the same sample are in full agreement, the same amount of iodine being used for oxidation as was liberated in reduction, and thus proving the reoxidation to be substantially complete.

The cupric salt is first completely reduced and precipitated as cuprous iodide in the presence of a large excess of soluble iodide, the liberated iodine being reduced to iodide on titration with thiosulfate. The subsequent addition of more free iodine shifts the equilibrium slightly toward the cupric side, but the concentration of iodide (5 gm. of KI in 200 cc. or about 0.15 M) is such that only about 15 per cent of the total copper can be in the cupric form (see Chart 3 of preceding paper). The addition of the oxalate, however, shifts the equilibrium wholly to the cupric side of the reaction and in spite of the excess of potassium iodide present the cuprous iodide is at once dissolved and oxidized by the free iodine. Equilibrium is attained almost immediately and is not affected by the removal of the excess of free iodine in the second titration with thiosulfate. As a rule the starch blue color does not reappear even on standing several days. The solution is colored blue from the complex copper salt, but the starch blue end-point is remarkably sharp and distinct, and after a single trial is never confused with the color due to the copper salt. After a short time, often before the end of the titration if the solution is cold, a precipitate of cupric oxalate or cupric potassium oxalate may form, but this in no way interferes with the titration.

The same phenomenon may be shown qualitatively as follows.

Add to a solution of copper sulfate (25 cc. of Fehling's copper solution) a few grams of potassium iodide; insoluble cuprous iodide is precipitated and the solution contains much free iodine. Add at least four molecular equivalents (5 gm.) of potassium oxalate, and shake; the cuprous iodide dissolves, the iodine disappears, and the solution assumes the blue color of cupric salts. The addition of starch indicates the presence of little or no free iodine. Or to the copper solution add first the oxalate; the addition of KI now fails to form iodine and cuprous iodide.

The only interpretation of the phenomenon would appear to be that by the formation of the complex copper oxalate the num-

ber of cupric ions is so far reduced that the product of their concentration by the square of the iodide concentration

$$(Cu^{++}) \times (I^{-})^2$$

is below the threshold value required to cause the reaction to take place to a degree sufficient for its detection by the starch test for iodine. This threshold value was roughly calculated at about 135[1] according to which the concentration of Cu^{++} ions in the above experiments (approximately 0.06 M copper solution) would be less, *probably very much less*, than 0.000006 M.[2]

Taking advantage of this effect of oxalate one has the choice of two almost equally satisfactory, convenient, and accurate iodometric methods for the determination of the amount of copper reduced by sugar. After acidifying the alkaline copper solution, either the residual cupric salt or the cuprous salt, following their reaction with iodide or iodine respectively may be titrated with standard thiosulfate. The oxidation products of the sugar do not react with iodine *in acid solution* and cause no interference in the reactions. It is unnecessary to remove the cuprous oxide in the determination of cupric salt (Maquenne (3)) as prescribed by Peters and others; nor is it necessary to isolate the cuprous oxide. Either may be accurately determined in the presence of the other. Both methods yield the same results within the error of manipulation and titration. These facts permit the application of the very delicate and accurate methods of iodometry to the determination of reducing sugar under a variety of circumstances. We have used the methods in various forms with sugar solutions, in food analysis, with urine, blood, and milk, and shall describe the procedures which we find most convenient and satisfactory. On a number of points there is room for individual choice and there are doubtless other modifications which would be equally satisfactory.

We shall describe procedures for both the "cupric titration" as we may designate the method based upon the determination of the residual cupric salt by its reaction with an excess of potas-

[1] Shaffer, P. A., and Hartmann, A. F., *J. Biol. Chem.*, 1920–21, xlv, 361, foot-note 5.

[2] The phenomenon is a simple and striking illustration of reversible reactions suitable for class experiments.

sium iodide, and the "cuprous titration" or the determination based upon the reoxidation of cuprous salt by iodine made possible by the effect of oxalate. Both methods have been applied for the determination of either large or small amounts of reducing sugar, different solutions being used for the micro titrations. The cupric titration is essentially that introduced by Lehmann and later modified by Maquenne, Riegler, Peters, and others, while the cuprous titration with the aid of oxalate has not previously been used. Because of the greater cost of the large amounts of potassium iodide required for the cupric titration, and especially the fact that the starch end-point is less easily seen in the presence of the precipitated cuprous iodide and is less permanent, we distinctly prefer the cuprous titration, but both methods are in our experience preferable on the grounds of convenience, speed, and reliability to any other volumetric or colorimetric procedures for the determination of reducing sugar with which we are familiar. The degree of accuracy depends only upon the care in manipulation, especially as to the conditions during the oxidation of sugar.

THE DETERMINATION OF LARGE AMOUNTS OF SUGAR.

With either of the reactions above mentioned, the accurate determination of copper is easily accomplished. But the determination of sugar involves also the amount of copper reduced by a given amount of sugar, and this ratio is known to be affected by the concentration of copper, the alkalinity, the duration of heating, and the extent of oxidation of sugar or reoxidation of cuprous oxide by oxygen dissolved in the solution, all of which factors must be controlled or standardized. Although we have formulated new solutions and special conditions for certain purposes it seemed desirable also to adapt the two iodometric titrations to solutions and conditions of heating now in general use. We have therefore adopted the use of Fehling's Soxhlet solutions, and the amounts and conditions of heating prescribed by Munson and Walker (12), and are thus enabled to use their tables for the conversion of copper into terms of sugar. As shown by the results given in Table I, substantially correct values are obtained by using Munson and Walker's figures. In addition we are using

a carbonate-citrate solution, containing in a single solution the iodine and oxalate required for the cuprous titration, which possesses certain advantages and which also will be described.

TABLE I.

Results of Determination of Pure Glucose Solutions. Fehling's Solutions. Cupric and Cuprous Titrations. Kahlbaum's "K" Glucose and United States Bureau of Standards Glucose, Dried at 85°C. Solutions Made by Weight.

Glucose taken.	Cuprous titration.		Cupric titration.	
	Cu reduced.	Glucose found (Munson-Walker).	Cu reduced.	Glucose found (Munson-Walker).
mg.	*mg.*	*mg.*	*mg.*	*mg.*
50	101.5	49.7		
	100.9	49.4		
100	199.0	100.3		
	199.5	100.5		
	198.4	100.0		
	198.0	99.8		
200	375.0	200.2		
	373.8	199.4		
	374.2	199.6	374.0	199.5
	373.3	199.2	373.6	199.3
	373.4	199.2	374.3	199.6
	376.5	201.0	375.5	200.4

Fehling's Solution.

Method.—Measure 25 cc. (pipette) of each of the two Fehling's Soxhlet solutions[3] into a 300 or 400 cc. flask. Add 50 cc. or less of the (approximately neutral) sugar solution containing from 20 to 200 mg. of sugar, and water (if necessary) to make a total volume of 100 cc. Cover with a small inverted beaker and heat on an asbestos mat over a flame so adjusted as to bring the solution to boiling in 4 minutes. Boil 2 minutes. Stand the flask in the sink under running water till cool (3 to 4 minutes). The subsequent treatment depends upon whether the cuprous or cupric titration is used; we prefer the former.

[3] Fehling's Soxhlet Solution 1: 34.64 gm. $CuSO_4.5\,H_2O$ in 500 cc. Solution 2: 173 gm. Rochelle salt and 50 gm. NaOH in 500 cc.

Cuprous Titration.—Add 50 cc. (accurate pipette) or 25 cc. if only little cuprous oxide is present, of the iodate-iodide solution (see below), followed by 15 to 17 cc. of 5 N H_2SO_4. The acid should be added from a cylinder or fast flowing pipette in order that the acidification of the whole solution be accomplished promptly; hypoiodite in *alkaline* solution oxidizes the organic acids present and some iodine may be thus used if acid is added very slowly. There is no danger of this error if the acid is added quickly. Shake the solution gently for a few moments until the cuprous oxide has dissolved. The solution should become clear, though often some cuprous iodide will separate. Add 20 cc. of saturated potassium oxalate and rotate the flask until the cuprous iodide is *completely* dissolved.

If the alkaline solution is taken from the tap while it is still slightly warm (about 40°) and the iodate, acid, and oxalate are added, the cuprous oxide dissolves almost immediately and the solution remains clear; when cold, cuprous iodide may first separate, and when this is dissolved after the addition of oxalate, acid potassium tartrate may crystallize, but neither affects the result. Titrate the solution with standard thiosulfate (0.1 N), adding a few cc. of starch solution toward the end, before the disappearance of the green color.

It is necessary to know accurately the amount of iodine added in the iodate solution, and the blank reduction of the Fehling's solution. Boil the Fehling's solution with 50 cc. of water instead of sugar, cool, and treat as above described. The value of the blank remains practically unchanged for long periods if the Fehling's solutions are measured separately and thus mixed only as used, and its determination need be made only occasionally.

Calculation.—From the blank titration subtract the titration of the sugar determination, the remainder representing I_2 required for the oxidation of cuprous salt. Multiply by the copper factor of the thiosulfate (1 cc. 0.1 N = 6.36 mg. Cu), and find the amount of sugar equivalent to the copper by reference to Munson-Walker tables (12)[4] or divide the amount of copper reduced by the corresponding ratio obtained from the glucose curve in Chart 1.

[4] See also Mathews, A. P., Physiological chemistry, New York, 1915, and *U. S. Dept. Agric., Bureau of Chemistry, Bull. 107*, 1912.

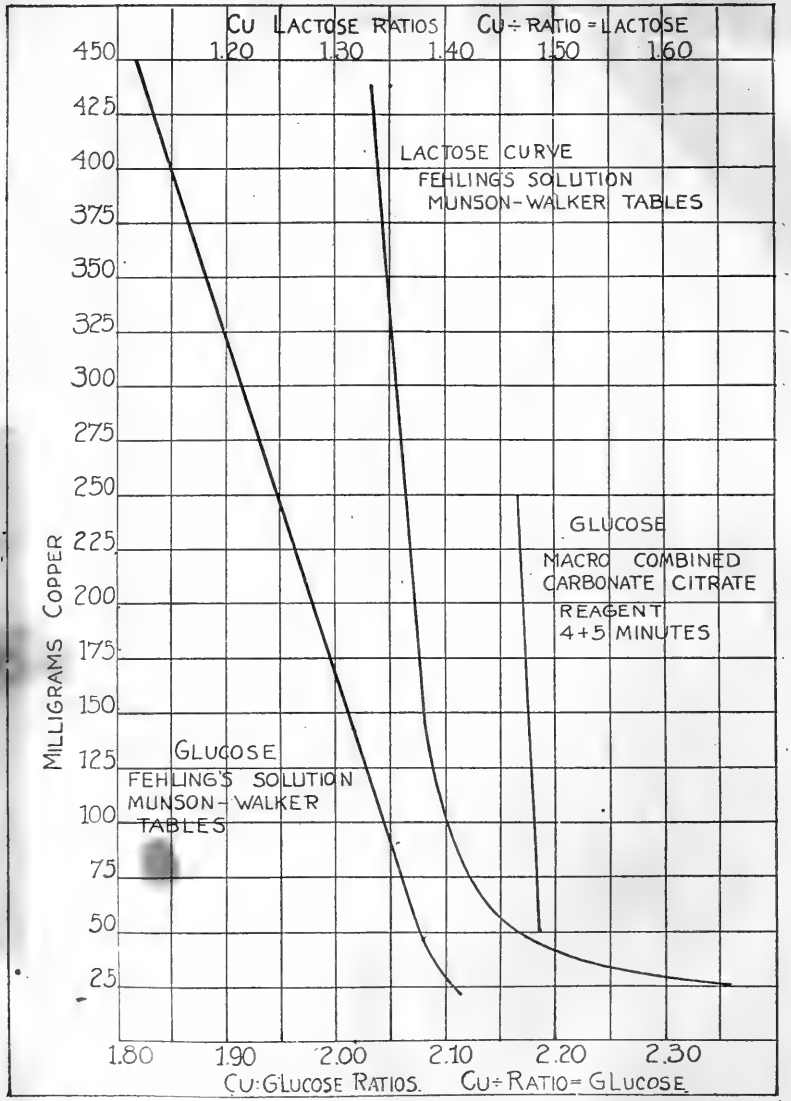

CHART 1. Copper : glucose ratios for Fehling's solution and for the macro combined reagent, and copper:lactose ratios for Fehling's solution. Divide the amount of reduced copper by its corresponding ratio as shown by the respective curves.

Composition of Iodate-Iodide Solution.—5.4 gm. of KIO_3 and 60 gm. of KI are dissolved in water to which is added a small amount of alkali (to prevent the formation of hydriodic acid and its oxidation by air), and diluted to a liter.

Cupric Titration.—To the cooled alkaline copper solution add 6 gm. of KI and 25 cc. of 5 N H_2SO_4. Titrate with standard thiosulfate, adding starch solution toward the end. The titration is subtracted from a similar blank determination on the Fehling's solution, the difference representing copper reduced by the sugar. For the cupric titration the copper solution must be measured accurately. Consult Munson-Walker tables or Chart 1 for the conversion of copper into terms of sugar.

The amount of acid added in both titrations is important. In the cuprous titration an excess sufficient to dissolve the cuprous oxide and to liberate the iodine from the iodate-iodide mixture is essential, but a *very* great excess causes the decomposition of the complex oxalate and is therefore to be avoided. An *excess* of 15 to 40 cc. of N acid over the amount required to neutralize the alkali of the Fehling's solution is satisfactory; this is equivalent to a total of 16 to 20 cc. of 5 N acid. A larger excess of acid is necessary in the cupric titration for the same reason for which it is to be avoided in the cuprous titration. The tartrate and perhaps the organic products of the sugar oxidation tend to form complex salts with cupric copper similar to the oxalate and thus to prevent its complete reduction to cuprous iodide, and these complex salts are unstable in the presence of high acidity. The cupric titration blank is somewhat lower when the solution is only slightly acidified than the value obtained on adding a large excess of acid. Sulfuric acid and not hydrochloric must be used for the neutralization.

Copper-Carbonate Solutions.

The reagent devised by Benedict (13) and widely used for the qualitative detection of sugar in urine has the advantage that, due in part to the lower alkalinity of alkali carbonate used as a substitute for the hydroxide, the reagent does not yield *precipitates* on boiling with dilute solutions of creatinine, uric acid, chloroform, and perhaps other substances which do reduce the

more alkaline Fehling's solution. A further advantage is that the reagent does not deteriorate with age. With these points in mind we have used Benedict's qualitative reagent and a number of similar copper-carbonate solutions for the iodometric determination[5] of glucose in urine and other solutions in the expectation that the normal constituents of urine would not reduce copper at the lower alkalinity. As explained later, however, this expectation is only in part realized.

By the addition to Benedict's or other similar solution of suitable amounts of potassium iodide one may prepare a single reagent of good keeping quality and in some respects satisfactory for sugar determination by "cupric titration" of the residual unreduced copper. Similarly, by adding iodide, iodate, and oxalate to such solutions one may prepare a single permanent reagent for the "cuprous titration;" and we have developed and recommend such a solution, the preparation and use of which are described below. This reagent appears to be permanent in that its blank titration does not change, and its use is rapid, convenient, and accurate as far as concerns the determination of copper, the sharpness of end-point, and agreement of duplicates. It is applicable to the determination of reducing sugar in moderately large amounts (up to 150 mg. of glucose) as an alternative reagent for Fehling's solutions. Its advantage is the convenience in having the chemicals combined in a single solution and the fact that the amount of copper reduced by a given amount of sugar is about 10 per cent greater than with Fehling's solution, and the ratio of Cu : glucose more constant. With such a combined

[5] Benedict's qualitative solution may be used for either the cuprous or cupric titration as follows: To 25 cc. of the reagent add sugar solution containing not more than 50 mg. glucose and water to make the total volume 100 cc. Boil 5 minutes and cool. For cuprous titration add 25 cc. iodate-iodide solution, acid, and oxalate, and titrate exactly as described for the use of Fehling's solution. For cupric titration add 5 gm. KI and 25 cc. 5 N H_2SO_4 and titrate. In either case subtract the titration from the corresponding blank. The amount of copper reduced by glucose with this reagent is about 10 per cent less than with Fehling's solution and about 20 per cent less than with our "combined reagent" under the above conditions of heating. (48.8 mg. glucose reduced 90 and 89.8 mg. Cu, or 1.84 Cu:1 glucose.) We have not determined the reduction with other amounts of glucose. The reduction of this reagent by normal urines (5 cc.) is equivalent to 0.05 to 0.10 per cent glucose, although no precipitate is formed.

reagent all that is necessary is to add the sugar solution, boil, cool, acidify, and titrate, and the process is accordingly very simple; but in order to combine all the necessary chemicals in a single solution potassium salts must be used which increase the cost of the reagent.[6] The use of the carbonate reagent for determination of sugar in urine and the extent of its reduction by normal constituents of urine will be treated in a later section of this paper.

Combined Carbonate-Citrate Reagent.

			gm. per liter
0.1 M		copper sulfate (crystalline 5 H_2O)	25.0
0.25 "		potassium citrate (crystalline H_2O)	81.0
0.5 "		" carbonate (anhydrous)	70.0
0.5 "		" oxalate (crystalline H_2O)	92.0
0.1 N I_2	{	" iodate	3.57
	{	" iodide	50.0

The citrate, carbonate, and oxalate are dissolved in about 600 cc. of warm water, and into this solution the copper sulfate, dissolved separately, is poured with stirring. The iodate and iodide are together dissolved in 150 to 200 cc. of warm water and added to the alkaline copper solution, which is then cooled and diluted to a liter. This solution is of course to be used only for the cuprous titration. The procedure is as follows.

50 cc. of the reagent are accurately measured into a 300 cc. Erlenmeyer flask to which are added the sugar solution containing not more than 150 mg. of glucose, and water if necessary to make the total volume 100 cc. Cover the flask with a small inverted beaker and place on an asbestos mat over a flame so adjusted as to bring the contents to boiling in 4 minutes. Boil 5 minutes.[7] Cool under running water, add 20 cc. of 5 N H_2SO_4, and

[6] We have estimated the cost of material used in this reagent for a single determination (50 cc.), at present prices, at about 6 cents. The use of sodium carbonate and citrate (in which case the oxalate cannot be incorporated in the reagent, and is added just before titration) reduces the cost by one-third.

[7] The amount of copper reduced by glucose is somewhat greater with this solution than with Fehling's solution. This is in accord with the results of Benedict who pointed out the advantage of the low alkalinity of carbonates in avoiding destruction of glucose. However, the *rate* of the reduction of copper, and presumably of the dissociation of glucose into the

shake (cautiously at first) for a few moments until the cuprous oxide and iodide are completely dissolved. If the solution is slightly warmed (40°) the precipitate dissolves quickly and the solution remains clear; otherwise crystals of cupric oxalate or the deeper blue potassium cupric oxalate may form, but their appearance does not interfere with the end-point. Titrate the solution with 0.1 N thiosulfate.[8] A blank titration on the reagent (50

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hypothetical substance actually oxidized, is slower at lower alkalinity, and the completion of the oxidation requires longer heating than at the alkalinity of Fehling's solution. For this reason it is important to adhere closely to an accepted period of boiling. If this is not done rather irregular results are obtained. Although we have selected 5 minutes of boiling, the reduction is incomplete in this time, and longer heating gives a larger amount of cuprous oxide; though longer heating also causes more reduction by the constituents of normal urine. By prolonging the period of boiling to 10 minutes the reduction is substantially complete and with glucose solutions rather more constant results are obtained, but by so doing the insensitiveness of this reagent to normal urine is largely lost and it possesses little advantage over Fehling's solution.

[8] We find the use of potassium biiodate, $KH(IO_3)_2$, convenient and accurate for the standardization of thiosulfate, and it may also be used instead of iodate in preparing the copper reagents. In the latter case the biiodate solution must be made *alkaline* before mixing with KI, and an *equivalent* amount of the biiodate is used. Molecular weight $KH(IO_3)_2 = 389.95 \times \frac{1}{12} \times \frac{1}{10} = 3.2496$ gm. for 1 liter of 0.1 N I_2 solution when reacting with an excess of KI and acid.

Pure potassium biiodate may be conveniently made as follows: 110 gm. $KClO_3$ are added to 450 cc. warm water containing 40 cc. concentrated HCl in a 2 liter Erlenmeyer flask, and placed in a well ventilated hood. 100 gm. of powdered iodine are added and the mixture is warmed slowly with occasional shaking until the reaction starts. The reaction proceeds rather violently, with the evolution of chlorine, but if not heated too far very little iodine is lost. When the reaction has subsided the solution is boiled for a few minutes and filtered boiling hot by suction. On cooling, about 150 gm. of biiodate crystallizes, which is about 90 per cent pure. This is filtered off by suction and redissolved in about three times its weight of boiling water, the solution being filtered hot. After standing over night (in the ice box) the crop of crystals is filtered by suction and dried (after removing filter paper) in the oven at 100°C. The yield is about 100 gm. or 70 per cent.

In our experience this once recrystallized salt is pure (100.0, 99.96, and 99.95 per cent by analysis; after second recrystallization 99.98 and 99.92; and after a third 99.94 and 99.98 per cent). We are indebted to Mr. M. L. Heidemann for carrying out this preparation and for the analyses, and to Dr. S. W. Clausen for calling our attention to the process which is modified from the work of Lamb, Bray, and Geldard (Lamb, A. B., Bray, W. C., and Geldard, W. J., *J. Am. Chem. Soc.*, 1920, xlii, 1636).

cc. of reagent + 50 cc. of water), after heating as above, is made, and recorded in terms of 0.1 N thiosulfate. From this value the titrations of determinations are subtracted, and the remainder multiplied by 6.36 gives the amount of copper reduced. The ratio of Cu:glucose varies somewhat with the amount of sugar but is more constant than the ratios with Fehling's solution. Chart 1 gives also a curve for this reagent showing the ratios corresponding to varying amounts of copper. The amount of copper found divided by the appropriate ratio gives the amount of sugar determined.

Carbonate-Citrate Reagent for Cupric Titration.—If it is desired to use the carbonate reagent for *either* the cupric or cuprous titrations (or if the potassium salts necessary for the "combined reagent" are not available), a solution of substantially the same reducing power may be made as follows.

	gm. per liter
0.1 M copper sulfate (crystalline)	25.0
0.25 " sodium citrate (crystalline $\frac{1}{2}(11\ H_2O)$)	90.0
0.5 " " carbonate (anhydrous)	50.0

This solution is to be used as above described for the combined reagent, with the following difference. For *cupric* titration add, after cooling, 6 gm. of KI and 25 cc. of 5 N H_2SO_4, titrate with 0.1 N thiosulfate, and subtract the titration from the similarly determined cupric blank for the reagent. For *cuprous* titration, add, after cooling, 25 cc. of the iodate-iodide solution (p. 373), 20 cc. of 5 N H_2SO_4, and 20 cc. saturated potassium oxalate, warm the solution to about 40°, and when the cuprous oxide is all dissolved titrate with thiosulfate and subtract from the blank for the reagent with 25 cc. of the iodate solution.

THE DETERMINATION OF SMALL AMOUNTS OF SUGAR.

For some purposes, such as blood analysis, it is essential to be able to determine a few milligrams or tenths of milligrams of sugar, and in many other circumstances the determination of such small amounts is desirable and convenient. For such conditions the delicate iodometric procedures are admirably suited. We use in such cases a copper-carbonate solution of the following composition.[9]

[9] The composition of this solution as regards copper, tartaric acid, and carbonate is nearly the same as that used by Folin and Wu, and appears to be optimum for copper reduction by dilute sugar solutions. The in

Micro Carbonate-Tartaric Acid Reagent.

Final concentration.		gm. per liter
0.02 M	copper sulfate (crystalline)	5.0
0.05 "	tartaric acid	7.5
0.4 "	sodium carbonate (anhydrous)	40.0
0.02 N I_2	potassium iodide	10.0
	" iodate	0.7
0.1 M	" oxalate	18.4

Dissolve the carbonate in about 400 cc. of warm water, and into this pour with stirring the copper sulfate and tartaric acid dissolved in about 150 cc. of water. Dissolve the iodate, iodide, and oxalate in about 250 cc. of water, rinse into the alkaline copper solution, cool, and dilute to a liter. The above formula is used only for the cuprous titration, but by omitting the iodate, iodide, and oxalate the solution may be used for either method, suitable additions being made previous to titration.

Molecular equivalent amounts of either Rochelle salt (14 gm.) or sodium citrate (16 gm.) may be substituted for the tartaric acid, but in either case the resulting solutions have a lower reducing power, about 10 and 20 per cent less, respectively, than the reagent with tartaric acid, and when such solutions are used it is suggested that a table of standards be prepared by determining 0.25, 0.5, 1, and 2 mg. quantities of pure glucose. When plotted the results should give a straight line, or nearly so, and from the curve intermediate values are obtained by interpolation.

The amount of copper reduced by a given amount of glucose depends not only upon the composition of the solution but upon the conditions of heating and the total volume of the solution. A small amount of glucose is oxidized by the oxygen dissolved in the solution and some reoxidation of cuprous oxide takes place especially during the heating. Somewhat greater reduction is

sion of iodate and iodide in this solution as in the combined macro reagent, ollows the suggestion of Maclean. Their presence in the alkaline solution s no effect during the oxidation of the sugar, but has the great advantage, 99.9 the procedure described by Scales for instance, that the iodine formed and idification at once oxidizes the cuprous salt as rapidly as the latter Heid med by solution of cuprous oxide and thus avoids all danger of its Dr. tion by air. It will be noted that in using Fehling's solution as above from ibed the iodate solution is added before the mixture is acidified, and he reason just stated.

obtained by heating in an atmosphere of illuminating gas, but the extent of oxidation of sugar and cuprous oxide by air is practically constant as proved by agreement of duplicate determinations under the same conditions, and, by being included in the standard determinations on which the tables are based, does not introduce error. The standard values must, however, be determined under definite conditions as to volume of liquid, duration and manner of heating, and as to size and shape of vessel containing the solution.

The following series of determinations show the effect of varying the conditions of heating, especially the effect of exposure to air during and after the heating.

5 cc. of the reagent and 5 cc. of solution containing 0.5 or 1.0 mg. of glucose were boiled for 2 minutes in large test-tubes over a microburner. These results show that the greatest error is due to oxidation of sugar or of cuprous oxide *during the heating*, and that when the tubes are covered so as to minimize convection currents the amount of reduction is reasonably constant and the error small. When cold the reoxidation by air is slow.

	Copper reduced.	
	1.0 mg. glucose.	0.5 mg. glucose.
	mg.	*mg.*
Test-tube covered.		
Titrated at once when cold	1.97 2.02 2.05 2.00	1.01 1.04 0.98 0.96
Titrated 20 min. after cooling		1.01 1.04
After cooling, aerated 2 min		0.95
Before boiling, added 5 cc. water, total volume 15 cc.	1.95 1.95	0.82
Heated reagent to boiling, then added glucose	2.02 2.00	0.98 1.01
Not covered during boiling.		
Aerated *during* boiling	1.71	0.78 0.59

TABLE II.

Amounts of Glucose Corresponding to Copper. Micro Reagent. Cuprous Titration. 5 Cc. Reagent + 5 Cc. Sugar Solution.

Cu reduced.	Microburner 2½ min.		Water bath 15 min.		0.005 N iodine required to oxidize cuprous oxide.	Glucose in blood.*	
	Glucose.	Glucose in blood.*	Glucose.	Glucose in blood.*		Microburner 2½ min.	Water bath 15 min.
(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)
mg.	*mg.*	*per cent*	*mg.*	*per cent*	*cc.*	*per cent*	*per cent*
0.1	0.07	0.014	0.07	0.014	0.5	0.020	0.020
0.2	0.133	0.026	0.13	0.026	1.0	0.040	0.040
0.3	0.186	0.037	0.18	0.037	1.5	0.056	0.056
0.4	0.235	0.047	0.24	0.047	2.0	0.067	0.067
0.5	0.278	0.055	0.29	0.058	2.5	0.082	0.083
0.6	0.326	0.065	0.33	0.066	3.0	0.100	0.096
0.7	0.374	0.075	0.37	0.074	3.5	0.114	0.109
0.8	0.415	0.083	0.41	0.082	4.0	0.130	0.122
0.9	0.465	0.093	0.45	0.090	4.5	0.145	0.135
1.0	0.515	0.103	0.50	0.100	5.0	0.160	0.148
1.1	0.564	0.113	0.54	0.108	5.5	0.175	0.162
1.2	0.614	0.123	0.58	0.116	6.0	0.189	0.176
1.3	0.664	0.133	0.62	0.124	6.5	0.202	0.188
1.4	0.711	0.142	0.66	0.132	7.0	0.217	0.200
1.5	0.758	0.151	0.70	0.140	7.5	0.232	0.214
1.6	0.804	0.161	0.75	0.150	8.0	0.245	0.227
1.7	0.852	0.170	0.79	0.158	8.5	0.259	0.243
1.8	0.90	0.180	0.83	0.166	9.0	0.274	0.255
1.9	0.945	0.189	0.88	0.176	9.5	0.287	0.268
2.0	0.99	0.198	0.91	0.182	10.0	0.300	0.280
2.2	1.077	0.215	1.00	0.200	10.5	0.315	0.292
2.4	1.17	0.234	1.08	0.216	11.0	0.332	0.305
2.6	1.255	0.251	1.17	0.234	11.5	0.349	0.317
2.8	1.34	0.268	1.25	0.250	12.0	0.365	0.329
3.0	1.428	0.285	1.33	0.266	12.5	0.380	0.342
3.2	1.515	0.303	1.42	0.284	13.0	0.396	0.356
3.4	1.61	0.322	1.49	0.298	13.5	0.413	0.369
3.6	1.714	0.343	1.56	0.312	14.0	0.430	0.383
3.8	1.815	0.363	1.63	0.326	14.5	0.449	0.397
4.0	1.914	0.383	1.71	0.342	15.0	0.457	0.410
4.2	2.02	0.404	1.80	0.360			
4.4	2.12	0.424	1.89	0.378			
4.6	2.24	0.448	1.98	0.396			
4.8			2.06	0.412			

* Figures in Columns 3, 5, 7, and 8 are used only when 5 cc. of 1:10 blood filtrate, equivalent to 0.5 cc. of blood, are taken for the determination. They apply also for urine containing small amounts of sugar, when 5 cc. of 1:10 diluted urine or urine filtrate are taken. For urine containing more than 0.4 per cent sugar, which must be diluted 1:50, 1:100, or 1:200, and 5 cc. of the dilution are used, the values in these columns are to be multiplied by 5, 10, or 20, respectively.

TABLE III.

Amounts of Glucose Corresponding to Copper. Micro Reagent. Cuprous Titration.

(1) 2 cc. reagent + 2 cc. sugar solution heated 10 min. in boiling water bath.			(2) 10 cc. reagent + 10 cc. sugar solution heated 20 min. in boiling water bath.		
Copper reduced.	Glucose.	Glucose in blood.	Copper reduced.	Glucose.	Glucose in blood.
mg.	*mg.*	*per cent*	*mg.*	*mg.*	*per cent*
0.1	0.083	0.042	0.4	0.38	0.038
0.2	0.124	0.062	0.6	0.47	0.047
0.3	0.165	0.083	0.8	0.52	0.052
0.4	0.206	0.103	1.0	0.62	0.062
0.5	0.247	0.124	1.5	0.84	0.084
0.6	0.288	0.144	2.0	1.03	0.103
0.7	0.329	0.165	2.5	1.25	0.125
0.8	0.370	0.185	3.0	1.45	0.145
0.9	0.410	0.205	3.5	1.66	0.166
1.0	0.452	0.226	4.0	1.87	0.187
1.1	0.493	0.247	4.5	2.07	0.207
1.2	0.534	0.267	5.0	2.27	0.227
1.3	0.575	0.288	5.5	2.48	0.248
1.4	0.614	0.307	6.0	2.69	0.269
1.5	0.656	0.328	6.5	2.90	0.290
1.6	0.698	0.349	7.0	3.10	0.310
1.7	0.739	0.370	7.5	3.30	0.333
1.8	0.780	0.390	8.0	3.50	0.350
1.9	0.821	0.411	9.0	3.92	0.392
2.0	0.862	0.431	10.0	4.32	0.432

We have determined the degree of reduction with the tartaric acid solution with varying amounts of glucose under the following conditions, the heating being carried out in all cases in Pyrex test-tubes, 20 × 200 mm., covered with small inverted beakers or bottle caps.

2 cc. reagent + 2 " sugar solution } ..... 10 min. in boiling water bath.

5 " reagent + 5 " sugar solution } ..... 15 " " " " " "
..... 2½ " boiling over microburner.

10 " reagent + 10 " sugar solution } ..... 20 " in boiling water bath.

From a number of series of careful determinations on solutions of United States Bureau of Standards glucose, the standard values given in Tables II and III have been calculated. We

find it most convenient to use 5 cc. of the reagent for a determination and for this volume Table II contains detailed data for the two methods of heating, and for the cuprous titration.

Procedure.—Measure 5 cc. of the reagent into a large test-tube, and add an equal volume of the sugar solution containing not more than 2.0 mg. of glucose. Cover the tube and either boil for 2½ minutes over a small flame or place in a boiling water bath for 15 minutes. Cool for several minutes under the tap. If using the combined reagent containing oxalate and iodate, add 5 cc. of N H_2SO_4 and after about 1 minute titrate with 0.005 N thiosulfate. A blank titration on the reagent is determined after heating with an equal volume of water. The value of the blank does not change during at least several months and therefore need be determined only occasionally. The difference between the blank and the titration of a determination represents reduced copper, and the corresponding amount of sugar is learned by inspection of the table (1 cc. 0.005 N thiosulfate = 0.318 mg. copper). When 10 or 2 cc. of the reagent are used a proportionate amount of acid is used for acidification, and the corresponding table is used for the calculation.

The following results of a series of determinations of copper reduced by known amounts of pure glucose, using 10 cc. of reagent (not containing oxalate) + 10 cc. of sugar solution, and heated 20 minutes in a boiling water bath, show the close agreement of the cupric and cuprous titrations and are illustrations of the data from which the standard tables and curves are derived.

Glucose taken.	Copper reduced.	
	Cuprous titration.	Cupric titration.
mg.	*mg.*	*mg.*
0.4	0.47	0.46
0.8	1.40	1.40
1.2	2.34	2.34
1.6	3.32	3.38
2.0	4.30	4.24
3.0	6.88	6.92
4.0	9.42	9.50
5.0	11.61	11.68

The Determination of Sugar in Milk, Urine, and Blood.

Milk.

Precipitate the protein and fat by tungstic acid as proposed by Folin and Wu (14) for blood. 10 cc. of milk and 80 cc. of water are measured into a 200 cc. flask. Add 5 cc. of 10 per cent sodium tungstate and 5 cc. of $\frac{2}{3}$ N H_2SO_4. Shake well and pour through a folded filter. The filtrate should be perfectly clear. 25 or 50 cc. of the filtrate, equivalent to 2.5 or 5.0 cc. of milk, are added to 25 cc. of each of the Fehling's Soxhlet solutions (25 cc. of water are also added if 25 cc. of filtrate are used), and the determination is carried out as above described, using either the cuprous or the cupric titration, the former being somewhat preferable. Consult Munson-Walker tables (15) for the conversion of copper into terms of lactose. Instead of the Munson-Walker tables the lactose curve of Chart 1 may be conveniently used. This and the glucose curve have been prepared from their tables and give the ratio of copper: sugar with varying amounts of copper. The amount of copper found divided by the corresponding ratio gives the amount of sugar.

Urine.

The Fehling's Soxhlet solutions may be used as above described also for the determination of sugar in urine, using 5 cc. of urine + 45 cc. of water, though the results so obtained are too high because of the reduction of copper by constituents other than sugar. Normal urine, of specific gravity 1.020 and giving a negative test with Benedict's qualitative reagent, gives a reduction corresponding to 0.18 to 0.25 per cent of glucose, though no precipitation of cuprous oxide occurs. This normal reduction which is only slightly decreased, if at all, after fermentation is equivalent to a plus error of about 2 to 4 gm. of glucose in a 24 hour urine. With urines containing much glucose, this error, although considerable, is in many cases not important. The procedure is convenient in that no preliminary dilution of the urine is necessary unless it contains over 5 per cent of sugar. But for urines containing only little sugar, or when greater accuracy is desired, the *direct* use of Fehling's solution is not to be recommended.

With the carbonate reagents the error due to reduction by normal constituents is less, but still considerable. With 5 or 10 cc. of urine, diluted to 50 cc., with 50 cc. of the combined carbonate-citrate reagent (p. 375), a series of ten different normal urines, giving negative qualitative tests with Benedict's reagent, gave results equivalent to from 0.07 to 0.14 per cent of glucose, or about half the values obtained with Fehling's solutions. With the micro carbonate reagent, 5 cc. of normal urines previously diluted 1:10 (0.5 cc. of urine) give about the same values. In neither case is the apparent amount of sugar in such normal urines very materially decreased by fermentation, which shows that the reduction with the carbonate reagents also is due in great part to substances other than glucose. Except for urines containing very small amounts of glucose, when the percentage error is larger, and when the preliminary precipitation to be described later should be used if more accurate results are desired, the error caused by the normal reduction with the carbonate reagents may perhaps be disregarded. When the amount of sugar present is such that preliminary dilution of the urine is necessary, the concentration of creatinine and other interfering substances is thereby reduced to a point at which they no longer cause reduction and the small error due to their presence quite disappears. When used as stated below, neither of the carbonate reagents is reduced by such amounts of protein, acetone, acetoacetic acid, or chloroform as may occur in urine; nor do these substances or the normal constituents of urine interfere with the iodometric titrations.

With the above statement of limitations, the application to urine of the methods here described may be summarized. From a qualitative test judge whether the amount of sugar present is large or small, and use one of three alternative methods. Because of its simplicity and quickness we commonly use the micro method with the "combined reagent" and the cuprous titration.

Fehling's Solution.—Use 5 cc. urine + 45 cc. water with 25 cc. each of Fehling's Soxhlet solutions as described on page 371. Applicable with urines containing up to 5 per cent sugar; if known to be very high, use 2 cc. or less urine.

Macro Carbonate-Citrate Reagent.—Use 5 cc. urine + 45 cc. water with 50 cc. of reagent as described on page 375. Applicable up to 2 per cent sugar; if strong qualitative test, use 1 or 2 cc. urine, or 5 cc. previously diluted 1:2 or 1:5.

Micro Reagent.—Dilute the urine 1:20, 1:50, or 1:100, as indicated by the qualitative test, and use 5 cc. with 5 cc. of reagent as described on page 382.

If the qualitative test is only faintly positive the preliminary treatment described below should be followed.

The Reduction of Normal Urine and the Determination of Small Amounts of Sugar in Urine.

With urines containing only a few tenths of a per cent of sugar, no one of the existing methods gives, according to our experience, even approximately correct results without preliminary removal of the interfering substances. Of the methods used for such removal, those based on precipitation by mercuric salts appear to be the most satisfactory and have been most often used. While such methods perhaps are an outgrowth of the older urea method of Liebig, the first to use mercuric salts preliminary to sugar determination appears to have been Johnson (16) who in 1887 used $HgCl_2$ to remove creatinine previous to the determination of sugar in urine by the picric acid colorimetric method which he says had been introduced in 1883 by George Johnson. Patein and Dufau (17) in 1902 described the preparation and use of a solution of acid mercuric nitrate for the treatment of urine and other solutions preliminary to sugar determination. Their formula has been repeatedly used (18) and is probably the origin of the solution recently described by Benedict and Osterberg (19). The procedure of Patein and Dufau, improved by the substitution of sodium bicarbonate for sodium hydroxide, proposed by Benedict, is an almost ideal preparation for glucose determination. It removes nearly all interfering substances, and does not precipitate, adsorb, or destroy glucose. It does, however, leave some mercuric salt in the filtrate, and as pointed out by Patein and Dufau this must be removed before treatment with alkaline copper solution. It is not clear why this should be necessary for the cuprous iodometric titration, which we expected would measure the total reduction, both copper and mercury, but the presence of mercury salts does cause slightly low results.[10]

[10] With pure glucose solutions, the filtrates contain only the merest trace of mercury and correct results are obtained without subsequent treatment with sulfide and copper, while with glucose added to urine the results are low unless the mercury, present in much greater amount, is removed.

The mercury is removed from the acidified filtrate by Denigès (18), Schöndorff (20), and Benedict and Osterberg (19) by the use of Zn or Zn-Cu. This plan is not permissible for the iodometric methods because of the reduction of nitrate to nitrite, the latter liberating iodine from KI on later acidification. We remove the mercury from the filtrate by a small excess of $(NH_4)_2S$ or Na_2S, the excess of sulfide being in turn removed by $CuSO_4$, the excess of which remaining in the filtrate is with the cuprous titration not objectionable. Treated in this way, the details being presented below, urines yield filtrates which behave altogether like sugar solutions, and may be analyzed with as great accuracy. Added glucose is recovered quantitatively.

The filtrates from normal urines contain according to our method surprisingly little reducing substance, commonly the equivalent of about 0.02 or 0.03 per cent of glucose in the urine. Very little if any of this reducing substance is as a rule fermented by yeast, which indicates that it is not glucose; and from this fact it follows that at least many normal urines actually contain practically no glucose. We shall consider in a later paper the extent of the variations in the amount of the reducing substance of the urine of normal subjects; but it may be noted here that our results are similar to but by no means identical with those recently reported by Benedict, Osterberg, and Neuwirth (21) who found the reduction of normal urine to be equivalent to from 0.05 to 0.10 per cent or more of glucose, *about one-third of which was fermentable by yeast.* The results by our technique indicate somewhat smaller amounts of reducing substance, and that *little if any of it is, as a rule, fermented by yeast.*

The solutions used for the mercury precipitation and subsequent treatment are: the Patein-Dufau solution of acid mercuric nitrate, prepared in the manner recently stated by Benedict and Osterberg (19); a strong solution of Na_2S or $(NH_4)_2S$, free from sulfites;[11] and a strong solution of $CuSO_4$. The precipitation is

[11] Sulfites (or thiosulfate), if added with the sulfide, are not removed by the later treatment and on acidifying are oxidized by and use up iodine, giving too high results. On adding an excess of $ZnSO_4$ to a few cc. of the sulfide solution, the filtrate when acidified and when starch is added should give a blue color with the first or second drop of iodine. A satisfactory solution of Na_2S may be made by passing washed H_2S from a generator into NaOH.

TABLE IV.

Reduction of Normal Urine, and Normal Urine with Added Glucose.

Normal urines.	Final dilution of filtrate.	Blank minus titration.	Copper reduced.	Glucose found in urine.		Average.	After fermentation.
		cc. 0.005 NI	*mg.*	*mg.*	*per cent*	*per cent*	*per cent*
I	1:4	1.60	0.508	0.28	0.022		
	1:4	1.35	0.43	0.25	0.020		
	1:4	1.40	0.445	0.254	0.020	0.021	0.018
Same + 0.04 per cent glucose.	1.4	4.5	1.43	0.73	0.058		
	1:4	4.6	1.46	0.74	0.059		
	1:4	5.1	1.62	0.814	0.065		
	1:4	5.2	1.65	0.828	0.066	0.062	0.019
II	1:2	3.2	1.02	0.52	0.021		
	1:5	1.2	0.38	0.23	0.023		
	1:5	1.2	0.38	0.23	0.023	0.022	
III	1:5	0.45	0.14	0.09	0.009		
		0.40	0.13	0.09	0.009	0.009	0.012
Same + 0.10 per cent glucose.	1:5	7.3	2.32	1.14	0.114		
	1:5	7.2	2.29	1.12	0.112	0.113	0.012
IV (decomposed).	1:5	0.2	0.06				
Same + 0.100 per cent glucose.	1:5	6.5	2.07				
	1:5	6.6	2.10				
			Average 2.08				
			Minus 0.06				
			2.02	1.00	0.100		
V	1:2	4.7	1.50	0.75	0.030		0.036
Same by Benedict method.					0.046		
VI	1:2				0.035		0.037
VII (after eating apples).	1:5	5.2	1.65	0.83	0.083		0.084

carried out substantially as described by Benedict and Osterberg, by the use of equal volumes of urine and mercuric nitrate (25 cc.) and by the addition of solid $NaHCO_3$ to slight alkaline reaction to litmus paper. A drop of caprylic alcohol lessens frothing and introduces no error. If the urine is known to contain sugar it may be diluted with a known amount of water before precipitation; even "normal" urines may be diluted so that the filtrate is 1:5 instead of 1:2 and so allow more rapid filtration. The filtrate, 15 cc. or more, is faintly acidified by a few drops of concentrated H_2SO_4, a few drops of Na_2S (excess), and, after shaking, a few drops (excess) of $CuSO_4$ are added. The sulfides of mercury and copper are filtered off, leaving a water-clear filtrate which should contain a very small amount of copper. If acid, it is quite permanent and keeps indefinitely without change in its reducing power, but if slightly alkaline a faint precipitate of cuprous hydroxide may appear after a day or so. Unless the filtrate is to be analyzed the same day, it should be made faintly acid, if not already so. 2, 5, or 10 cc. of this filtrate are heated with an equal volume of the combined micro reagent as already described, using the cuprous titration (p. 382). The results in Table IV illustrate the amount of copper reduced by filtrates of normal urines and show the degree of accuracy in the determination of added glucose by the method here described.

Blood.

For the preparation of the blood filtrates we employ the Folin-Wu (14) tungstic acid precipitation, since such filtrates are used also for the determination of other constituents. But coagulation of protein by heat and colloidal iron is also satisfactory.

To a measured quantity of oxalate blood (2 to 15 cc., 2 or even 1 cc. being sufficient for the sugar determination) add seven volumes of water and allow to lake. Add one volume of 10 per cent sodium tungstate and one volume of $\frac{2}{3}$ N H_2SO_4. Stopper and shake; filter after 5 minutes. 2, 5, or 10 cc. of this filtrate, equivalent to one-tenth the volume of blood, are used with an equivalent volume of the micro reagent as described on page 382.

These directions are applicable for all bloods containing not more than 0.4 per cent of glucose; for higher concentrations, the

blood filtrates are diluted or a smaller amount (and water) is taken for the determination.

A comparison of the results by this and other methods will be reported by one of us in a later paper. It may be stated, however, that results by our method are in general substantially the same as by the methods of Folin and Wu, Maclean (8), and usually by the Myers-Bailey modification of the Lewis-Benedict method. All these methods in our hands not infrequently yield results which are much lower than the last procedure described by Benedict (22), and higher than the method described by Shaffer (23). According to our experience the technique here described is more reliable as well as more convenient than any of the other methods above mentioned.

SUMMARY.

1. The reversible reaction

$$Cu^{++} + I^- \rightleftarrows Cu^+ + I_2$$

is applied to the determination of the mixture of cuprous and residual cupric copper resulting from the action of reducing sugar upon alkaline copper solutions. The reaction may be caused to take place to completion *in either direction*, thus enabling one to determine by iodometric titration either the cupric or the cuprous copper in a mixture of the two forms.

2. The details of methods are given for both cupric and cuprous titrations, the latter being generally preferred. The use of several reagents for the determination of glucose by means of the cupric and cuprous titrations is described.

3. The methods are applied to the determination of reducing sugar in milk, blood, and urine, as well as in other solutions.

BIBLIOGRAPHY.

1. Lehmann, K. B., *Arch. Hyg.*, 1897, xxx, 267.
2. Riegler, E., *Z. anal. Chem.*, 1898, xxxvii, 22.
3. Maquenne, L., *Bull. soc. chim.*, 1898, xix, series 3, pt. 2, 926; abstracted in *J. Chem. Soc.*, 1899, lxxvi, pt. 2, 529.
4. Citron, H., *Deutsch. med. Woch.*, 1904, xxx, 1602.
5. Peters, A. W., *J. Am. Chem. Soc.*, 1912, xxxiv, 422, 928.

6. Bang, I., Der Blutzucker, Wiesbaden, 1913; *Biochem. Z.*, 1918, lxxxvii, 248.
7. Scales, F. M., *J. Biol. Chem.*, 1915, xxiii, 81.
8. Maclean, H., *J. Physiol.*, 1916, l, 168; *Biochem. J.*, 1919, xiii, 135.
9. Clark, W. B., *J. Am. Chem. Soc.*, 1918, xl, 1759.
10. Shaffer, P. A., and Hartmann, A. F., *J. Biol. Chem.*, 1920–21, xlv, 349.
11. Elbs, K., *Z. Electrochem.*, 1917, xxiii, 147.
12. Munson, L. S., and Walker, P. H., *J. Am. Chem. Soc.*, 1906, xxviii, 663.
13. Benedict, S. R., *J. Biol. Chem.*, 1908, v, 485.
14. Folin, O., and Wu, H., *J. Biol. Chem.*, 1919, xxxviii, 81.
15. Walker, P. H., *J. Am. Chem. Soc.*, 1912, xxxiv, 202.
16. Johnson, G. S., *Proc. Roy. Soc. London*, 1887, xlii, 365.
17. Patein, G., and Dufau, E., *J. pharm. et chim.*, 1902, xv, 221; abstracted in *Chem. Centr.*, 1902, lxxiii, 954.
18. Denigès, G., *Ber. internat. Kongr. angew. Chem.*, 1903, iv, 130, cited in Neuberg, C., *Biochem. Z.*, 1910, xxiv, 426.
19. Benedict, S. R., and Osterberg, E., *J. Biol. Chem.*, 1918, xxxiv, 195.
20. Schöndorff, B., *Arch. ges. Physiol.*, 1908, cxxi, 572.
21. Benedict, S. R., Osterberg, E., and Neuwirth, I., *J. Biol. Chem.*, 1918, xxxiv, 217.
22. Benedict, S. R., *J. Biol. Chem.*, 1918, xxxiv, 203.
23. Shaffer, P. A., *J. Biol. Chem.*, 1914, xix, 285.

THE REMOVAL OF AMMONIA FROM URINE PREPARATORY TO THE DETERMINATION OF UREA.

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(Received for publication, November 27, 1920.)

In the determinatian of urea by the urease method of Marshall (1) as modified by Van Slyke and Cullen (2) the ammonia determination can be eliminated by preliminary removal of ammonia by the permutit method of Folin and Bell (3). It is evident that such a simple and rapid process should be very desirable in place of the present complete ammonia determination required whenever urea is determined.

The more satisfactory and less expensive urease solution of Folin and Youngburg (4) may also be used instead of the acetone-insoluble preparation of Van Slyke and Cullen. The latter does not go into solution well and in the course of a day is largely precipitated, forming a muddy viscous mixture-solution which is rather undesirable to use, especially in aeration.

Method for Removal of Ammonia.—Dilute 5 cc. of the urine to 50 cc. (10 to 50 if very dilute urine) and mix well. Place 3 to 4 gm. of dry permutit in a wide bottom flask, preferably a 200 cc. or 250 cc. volumetric, and add 20 to 25 cc. of the diluted urine. Agitate for 5 minutes. Allow to settle 15 to 30 seconds and then pour through a thin filter paper.[1] If there is no permutit "dust" the urine may be decanted without filtering. The ammonia is completely removed.

Determination of Urea.—The Van Slyke and Cullen method is slightly modified to use the alcoholic urease solution and the filtrate from the permutit-urine mixture. To 5 cc. of the filtrate

[1] Filter paper must not contain any appreciable amount of ammonia. It was found during the work reported here that some filter papers contained as much as 1 to 2 mg. of ammonia N.

2 cc. of the alcoholic urease solution of Folin and Youngburg are added, together with 2 drops of a buffer solution, which contains Na_2HPO_4 and NaH_2PO_4, each in molecular concentration (142 and 120 gm. respectively per liter). 15 minutes are allowed for decomposition of the urea by the enzyme. The remainder of the determination is carried through as described by Van Slyke and Cullen.

TABLE I.

Effect of Increased Amounts of Ammonia.

Urine A.

	mg. per cc.
Total N	8.43
Urea N	7.26 (86.10 per cent of total N).
Ammonia N	0.41 (4.86 " " " " N).

To 10 cc. portions of the urine were added different amounts of ammonia N $[(NH_4)_2SO_4]$ and the volume was made up to 50 cc. according to the usual dilution in urea determination. After agitating with permutit in the proportion of 6 gm. to 30 cc. of the diluted urine, the total N was determined on a 50 cc. portion and urea on a 5 cc. portion.

Sample No.	Ammonia N added.	Total N obtained.	Urea N obtained.	
	mg.	*mg. per cc.*	*mg. per cc.*	*per cent**
1	0	8.11	7.22	85.69
2	5	7.95	7.17	85.05
3	10	8.02	7.14	84.70
4	15	8.09	7.17	85.05
5	20	8.02	7.21	85.36
6	25	7.95	7.36	87.35
7	30	8.01	7.34	87.02
8	35	7.97	7.34	87.02
9	40	7.99	7.20	85.36
10	45	8.06	7.28	86.36
11	45 + 250 mg. NaCl.	8.12	7.34	87.02

* Total N = 8.43 mg. per cc.

The data from the experiments described in Tables I and II show that urea is not absorbed or retained in any way by permutit, also that the urinary salts even in somewhat more than the maximum amounts found in urines (undiluted) are without effect.

In Table I there have been added increasing amounts of ammonia N up to 45 mg. per 10 cc. of urine. This amount is more than has been found in urines. The total N and urea N

determinations, however, show that all the ammonia N was removed by the permutit.

In Table II ammonia N was added so that the urine contained 13.35 per cent of its N in the form of ammonia. Increasing amounts of NaCl were added, also a mixture of ordinary urinary

TABLE II.

Effect of Increased Amounts of Salts.

Urine B.

	mg. per cc.
Total N	9.86
Ammonia N	1.0
NaCl	19.4

Ammonia N [$(NH_4)_2SO_4$] and other salts were added to the urine and dilution was made as shown below. The urine was then agitated for 5 min. with permutit and filtered and the total N determined on 10 cc. of the filtrate (representing 4 cc. of the undiluted urine).

Sample No.	Urine taken.	NH_3 N added.	NaCl added.	Water.	Total N calculated to undiluted urine.	Ammonia N removed.	
	cc.	*mg.*	*mg.*	*cc.*	*mg. per cc.*	*mg. per cc.*	*per cent of total N**
1	20	7.3†	200	50	8.89	1.335	13.05
2	20	7.3	400	50	8.85	1.375	13.45
3	20	7.3	800	50	8.79	1.435	14.03
4	20	7.3	1,600	50	8.94	1.285	12.47
5	20	7.3	3,200	50	8.82	1.405	13.64
6	20	7.3	6,400	50	8.79	1.435	14.03
7	20	7.3	8,000	50	8.89	1.335	13.05
8	20	7.3	1,600 + 540 $NaH_2PO_4.4H_2O$ + 500 $Na_2SO_4.10H_2O$.	50	8.80	1.425	14.03

* Total N = 9.86 mg. 0.365 mg. per cc. added = 10.225 per cent.

† Through a miscalculation this amount of N makes the total ammonia N 13.35 per cent instead of 10 per cent as intended. 7.3 mg. in 20 cc. = 0.365 mg. per cc.

salts in amounts exceeding those found normally or pathologically. The results show that the ammonia was removed in all cases.

Urines A and B were normal urines of acid reaction. Total N was determined by the simplified macro-Kjeldahl method of Folin and Wright (5). Urea was determined by the Van Slyke

and Cullen method except that alcoholic urease solution was used. Ammonia was determined by the permutit colorimetric method (3) and chlorides by the usual Volhard method.

The removal of ammonia by permutit may of course be employed equally well in any of the methods for urea determination. The writer has employed it especially for the determination of urea by direct Nesslerization (4). It is particularly desirable in that case because no aeration is employed.

BIBLIOGRAPHY.

1. Marshall, E. K., Jr., *J. Biol. Chem.*, 1913, xiv, 283.
2. Van Slyke, D. D., and Cullen, G. E., *J. Biol. Chem.*, 1914, xix, 211.
3. Folin, O., and Bell, R. D., *J. Biol. Chem.*, 1917, xxix, 329.
4. Folin, O., and Youngburg, G. E., *J. Biol. Chem.*, 1919, xxxviii, 111.
5. Folin, O., and Wright, L. E., *J. Biol. Chem.*, 1919, xxxviii, 461.

CHEMICAL STUDY OF SEVERAL MARINE MOLLUSKS OF THE PACIFIC COAST.*

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(Received for publication, December 20, 1920.)

The marine invertebrate forms of animal life have not often been the subject of chemical investigation. Lacking, as a class, the greater freedom of movement and the more highly organized nervous and circulatory systems which the possession of a bony skeleton allows, these forms, in general, are more sluggish in their metabolic activities, less responsive to chemical influences, and less attractive subjects of chemical study.

Among the mollusks, the oyster has been the subject of considerable scientific investigation in regard to its composition, food value, and commercial propagation. The clam and *Abalone* have in recent years, particularly on the Pacific Coast, risen to a position of considerable economic importance, and regulations limiting the size of the animals collected for commercial use and the season of the year during which they may be taken are now in force.

The following investigations deal specifically with five distinct species of marine mollusks which occur in considerable abundance along the coast of California. These are *Abalone*, Pismo clam, *Cryptochiton*, *Ischnochiton*, and owl limpet.

1. Abalone.—Abalone belongs to the family of marine snails, the Haliotidæ. It appears to be exclusively herbivorous in its habits, feeding chiefly on kelp and sea lettuce. The animals can often be obtained at low tide, as they occur on the rocks at depths varying from 1 to 30 meters below mean sea level. When full

* This paper is a part of a thesis to the Department of Chemistry of Stanford University in partial fulfillment of the requirement for the degree of Doctor of Philosophy.

grown they weigh from 0.5 to 2 kilos. For commercial purposes *Abalone* on this coast is usually obtained by Japanese divers, who, equipped with diving suits, work from boats at some distance off-shore at depths up to 30 meters.

2. Pismo Clam, Tivella stultorum.—This bivalved mollusk occurs along the ocean shore imbedded in mud and sand at depths of from 1 to 4 feet. Its length is from 1 to 4 inches, depending on the age of the animal and the environment in which it grows. Clams, as all other lamellibranchs or bivalves, feed chiefly on diatoms carried by the brackish tidal currents.

3. Chitons, Coat-of-Mail Shells.—The chitons are an ancient family, biologically speaking, ranking among the most primitive of the molluscan forms. The shell is composed of eight separate but overlapping plates, forming a dorsal shield, and so giving the specimen the form of a boat embedded in a leathery girdle of skin. Underneath these plates are found the organs of the animal essential for its life and below these a muscular foot. Two chitons obtainable in quantity on the Pacific Coast were studied. They are:

Cryptochiton stelleri, the giant chiton.

Ischnochiton conspicuus, the *Ischnochiton.*

Cryptochiton is the largest of the family of chitons, and is readily obtained at low tide clinging loosely to rocks or lying in tide pools. It feeds on the marine vegetation of that zone, and frequently upon sand with attached microscopic particles. Its weight is from 0.5 to 1.5 kilos.

Ischnochiton is much smaller in size, weighing from 30 to 50 gm.

4. Owl Limpet, Lottia gigantea.—This form is small in size, the oval shell averaging from 5 to 6 cm. in length. It feeds chiefly on diatoms and seaweed.

The material for this investigation was secured in abundance at various points along the coast of California. The *Abalones* were obtained chiefly in Monterey Bay, near Carmel, Point Lobos, Point Sur, and the bay around La Jolla in southern California. At times when unfavorable weather made shore collecting impossible, specimens were secured from Japanese divers. *Cryptochitons* were gathered at low tide from the rocks or tide pools of Half Moon Bay and near the shore at Carmel

Beach. The Pismo clams were obtained from Pismo Beach, where the beaches are plowed at certain seasons for a large commercial yield. Special care was taken to obtain fresh, uninjured material. The tissues and organs studied were the muscle, liver, reproductive system, and blood under normal, and in some cases under fasting conditions. Material not used in a fresh condition was preserved with alcohol.

The Chemical Investigation.

Digestive Enzymes in Stomach and Intestines.

The first investigations on this subject on various land and marine mollusks were carried on by Bernard (1). He found that the acid fluid in the intestines of *Loligo*, *Limax*, and *Ostrea* digests starches and fats. The same fact was established later by Yung (2) on the stomach juice of *Helix*, a common land mollusk. Biedermann and Moritz (3) found that during fasting periods the fluid of the stomach and intestines of snails is free from sugar, but contains a strong starch-splitting enzyme and is also able to invert cane-sugar. At the same time they discovered the presence of cytase in the secretion of the liver which had passed into the intestine, while the extract of the liver itself was inactive. The experiments of Biedermann and Moritz were confirmed by Müller (4) in 1900. Fredericq (5) showed that a proteolytic enzyme is present in the intestinal fluid of *Arion rufus* which acts in weakly alkaline but not in acid solution, while in *Mytilus* this fluid is active in acid solution as well. De Bellesme (6) established further the presence of a tryptic enzyme in the secretion of the *Octopus* liver. Examining the intestinal contents of *Aeolis*, Krukenberg (7) found also a peptic enzyme. Since then Abderhalden and Heise (8) have shown that proteolytic enzymes are present in the alimentary canal of many invertebrates.

Sellier (9) reported a proteolytic enzyme and Bernard a lipase in the gastric secretion of a few marine invertebrates. Mendel and Bradley (10) found in their study of *Sycotypus* that digestion in this mollusk is affected by the secretion of two different kinds of glands: the salivary glands, containing a proteolytic enzyme with amphoteric action in the cold, resembling trypsin; and the liver and hepatopancreas glands, elaborating enzymes which hydrolyze carbohydrates and fats.

In the present investigation tests were carried out on glycerol extracts for the following enzymes employing the usual methods: amylase, cytase, emulsin, glycogenase, lactase, lipase, maltase, pepsin, sucrase, trypsin, urease, uricase.

It was found that a temperature of 37°C. was satisfactory for the reactions involved. The results are presented in Table I.

TABLE I.

Enzymes of the Digestive Tract.

	Abalone.	Pismo clam.	*Cryptochiton.*	*Ischnochiton.*	Owl limpet.
Amylase	+	+	+	+	+
Cytase	−	−	−	−	−
Emulsin	+	+	+	+	+
Glycogenase	+	+	+	+	+
Lactase	+	+	+	+	+
Lipase	+	+	+	+	+
Maltase	+	+	+	+	+
Pepsin	+	+	+	+	+
Sucrase	+	+	+	+	+
Urease	+	+	+	+	+
Uricase	−	−	−	−	−

The Muscle.

The most important contractile tissue of *Abalone*, the chitons, and the limpet, in point of size, is the large pedal muscle, occupying the open end of the shell and when extended projecting some distance beyond it. This tissue makes up approximately half the body mass exclusive of the shell in the last two forms and considerably more than half in *Abalone*.

Among invertebrates very few investigations of muscle tissue have dealt with molluscan forms. The most favored animal in this respect seems to be *Octopus*. Other marine animals studied regarding muscle extractives are *Mytilus edulis*, *Pecten opercularis*, *Venus mercenaria*, *Sepia*, *Sycotypus*, *Fulgur*, *Ostrea*, and *Haliotis*.

Glycocoll was found in the adductor muscle of *Pecten irradians* by Chittenden (11) in quantities from 0.39 to 0.71 per cent. Tyrosine and leucine, decomposition products of proteins, were reported by Dohrn (12) in crab muscle, further by Krukenberg (13) in the muscle of the lobster, but the occurrence of free tyrosine in any quantity in the muscle of living mollusks is by no means established.

Of the inorganic constituents, special importance has been attached to iron as one of the essential constituents of the invertebrate muscle. Schneider (14) made a special study on iron in muscle, liver, and mantle of mollusks. Giunti (15) and later Dubois (16) determined the quantity

of copper present in lower animals. A complete inorganic analysis has been carried out by Henze (17) on *Octopus* and by Meigs (18) on *Venus mercenaria*, a clam occurring on the eastern coast of this country.

Methods of Analysis.

Muscle tissue of *Abalone*, Pismo clam, *Cryptochiton*, *Ischnochiton*, and owl limpet was examined for the following components: water, total solids, ash, alcohol-soluble portion, ether-soluble portion, proteins, purines, creatine, creatinine, uric acid, glycogen, inorganic constituents, and enzymes, employing in the preliminary treatment in the main the methods of Koch and Carr (19). The alcoholic extract was examined for total solids, ash, total nitrogen, proteose nitrogen, urea nitrogen, ammonia, total sulfur, total phosphorus, inorganic sulfur, inorganic phosphorus, creatine, creatinine, uric acid, and reducing sugars. The analysis of the ether-soluble portion included total solids, total nitrogen, total sulfur, and total phosphorus, while that on the alcohol- and ether-insoluble portion included total nitrogen, protein, total sulfur, total phosphorus, phospho-protein phosphorus, uric acid, creatine, creatinine, and reducing sugars.

Ammonia was determined by Folin's method, based upon the absorption of free ammonia in acid and the titration of the excess of acid. Urea was estimated by Marshall's urease method (20), and later checked by the method of Van Slyke and Cullen (21). Creatinine and creatine were determined by the methods of Folin and uric acid by that of Benedict and Hitchcock.

The results of these analyses are given in Table II.

The only positive tests for enzymes in muscle extracted with glycerol were an amylase and glycogenase in *Cryptochiton*, and a urease in the Pismo clam.

DISCUSSION.

The water content of the muscle tissue is higher in gastropods and lamellibranchs than in mollusks not very closely related to them, as Table III indicates.

No records are available regarding the content of fat in the muscle of mollusks. Apparently it may vary widely among different species. *Ischnochiton* carries larger amounts of fat than

TABLE II.

Constituents.	*Abalone.*	Pismo clam.	*Crypto-chiton.*	*Ischno-chiton.*	Owl limpet.
100 gm. of fresh muscle tissue.					
	per cent	*per cent*	*per cent*	*per cent*	*per cent*
Water	70.90	76.40	75.10	70.50	73.80
Total solids	29.10	23.60	24.90	29.50	26.20
Ash	3.42	5.10	3.52	3.88	7.54
Alcohol extractives, F1 + F2	8.59	8.04	4.01	12.53	7.34
Lipin fraction, F1	1.56	0.99	1.00	5.84	0.30
Water-soluble fraction, F2	7.03	7.05	4.05	6.69	7.04
Alcohol- and water-insoluble fraction, F3	20.5	15.57	20.93	16.94	18.83
Ash of F1 + F2	1.22	1.20	1.30	1.48	1.74
" " F3	2.20	3.90	2.22	2.40	5.80
Total N	3.61	1.61	3.55	2.70	3.19
Protein	23.00	10.26	22.60	17.27	20.30
Purine N	0.06	0.056	0.94	0.083	Not determined.
Creatine and creatinine	0.01	Trace.	Trace.	Trace.	Trace.
Uric acid	None.	None.	None.	None.	None.
100 gm. of F1 + F2 + F3.					
Alcoholic extracts, F1 + F2.	29.50	34.10	16.10	42.50	28.00
Alcohol-insoluble residue, F3	70.50	65.90	83.90	57.50	72.00
100 gm. of F1 + F2.					
Total solids, F1	17.90	12.30	25.00	46.00	4.10
" " F2	82.10	87.70	75.00	54.00	95.90
Ash of F1 + F2	17.35	16.90	13.20	22.10	24.70
" " F3	11.00	25.00	10.50	14.10	30.70
100 gm. of F1.					
Total N	6.46	5.20	0.40	1.45	15.0
Amino-acid N.	0.227	0.270	0.390	0.127	1.020
Total sulfur	1.03	1.00	1.50	0.51	3.67
" phosphorus	1.08	1.83	1.68	0.48	1.05
Lipin sugar	None.	None.	None.	None.	None.

TABLE II—*Concluded.*

Constituents.	*Abalone.*	Pismo clam.	*Crypto-chiton.*	*Ischno-chiton.*	Owl limpet.
		100 gm. of F2.			
	per cent	*per cent*	*per cent*	*per cent*	*per cent*
Total N	6.40	8.64	16.2	6.75	6.16
Amino-acid N	3.07	3.60	5.51	0.99	2.75
Proteose N	0.34	0.34	1.08	0.45	0.41
Urea N	0.16	0.06	None.	0.12	0.04
Urea	0.35	0.14	"	0.30	0.09
Ammonia	0.35	0.35	0.89	0.37	0.36
Total sulfur	6.55	5.65	7.2	7.17	6.23
Inorganic sulfur	0.085	0.1	1.0	0.28	0.17
Total phosphorus	0.49	0.53	1.41	0.41	0.19
Inorganic phosphorus	0.043	0.175	0.360	0.037	0.082
Creatine and creatinine	Trace.	None.	None.	None.	None.
Uric acid	None.	"	"	"	"
Reducing sugars on hydrolysis	14.4	27.2	9.67	9.58	5.26
		100 gm. of F3.			
Total N	13.23	10.81	14.73	14.06	12.80
Protein (× 6.37)	82.80	67.70	92.20	88.01	80.13
Total phosphorus	0.42	0.53	0.19	0.40	0.52
Phospho-protein phosphorus	0.47	0.78	0.64	0.47	0.46
Total sulfur	0.69	0.86	0.97	0.58	1.03
Creatine and creatinine	Trace.	None.	None.	None.	None.
Uric acid	None.	"	"	"	"
Reducing sugars after hydrolysis	10.40	19.24	0.25	4.10	0.64
		Inorganic constituents per 100 gm. of tissue.			
SiO_2	1.24	0.66	1.57	2.31	2.34
Fe_2O_3	1.35	1.56	1.21	1.20	1.72
CaO	0.31	0.85	0.47	1.05	0.46
MgO	0.29	0.43	0.42	0.40	0.42

Abalone and the bivalved clam. The lowest is the owl limpet, which anatomically is more closely related to *Abalone* than to *Cryptochiton* or *Ischnochiton*. The muscle of *Abalone*, limpet, and *Cryptochiton* contains relatively large amounts of combined nitrogen, which is an approximate measure of the total protein. The

muscle of *Ischnochiton* and Pismo clam, in the order named, runs notably lower in these constituents.

Determinations for total nitrogen were extended to the sexes in the case of *Abalone*, *Cryptochiton*, and *Ischnochiton*, but the results show no substantial difference between them.

TABLE III.

Animal.	Water.	Total solids.	Ash.	Reference.
	per cent	*per cent*	*per cent*	
Octopus (muscle)	77.5	22.70		Henze (17).
Mytilus edulus (total animal)	82.20	17.80		Voit (22).
Ostrea (" ")	80.50	19.50		Balland (23).
Astacus fluviatilis (" ")	77.11	22.89	9.061	von Bezold (24).
Venus mercenaria (" ")	74.44	25.56	1.46	Meigs (18).
Haliotis (muscle)	70.90	29.50	3.42	Albrecht.
Tivella stultorum (muscle)	76.40	23.60	5.10	"
Cryptochiton (")	75.10	24.90	3.52	"
Ischnochiton (")	70.50	29.50	3.88	"
Lottia gigantea (")	75.80	26.20	7.54	"

TABLE IV.

Animal.	Purine N.	Reference.
	per cent	
Octopus	0.0456	Henze (17).
Vertebrates	0.055–0.071	" (17).
Abalone	0.06	Albrecht.
Pismo clam	0.056	"
Cryptochiton	0.094	"
Ischnochiton	0.083	"

The purine nitrogen content of the muscle of these mollusks is small, yet relatively large as compared with the corresponding tissue in *Octopus* and the vertebrates, as Table IV indicates.

Uric acid was not present in the muscle tissue of the species examined, and in fact it has never been reported in the muscle of mollusks. Creatine and creatinine were found in appreciable quantities only in *Abalone* muscle. The absence of creatinine in the muscle of the chitons, limpet, and clam was confirmed by the addition and accurate *redetermination* of known amounts of

creatinine. This discovery of creatine and creatinine in *Abalone* muscle is of particular interest, as it is the first time these two substances, which are among the most important metabolic products in the vertebrates, have been found in the muscle tissue of mollusks.

In *Abalone*, Pismo clam, and *Ischnochiton*, the ammonia content is almost the same. The large amount of lipins in *Ischnochiton* is of especial interest. It is approximately four times the amount found in *Abalone*, six times that in *Cryptochiton* and the Pismo clam, and nineteen times that in the owl limpet. Mammalian muscle contains about 2 to 3 per cent of lipins. The Pismo clam, not especially high in its glycogen content, leads in reducing sugars. No results on other marine animals, besides those examined in the course of this investigation, are available for comparison.

The large amount of iron is worthy of note. Former analyses carried out by Henze on *Octopus* and by Meigs on the clam show no iron or only traces. In the mollusks examined by the author it is almost the same in amount but notably high in the various forms.

Calcium which is present in all mammalian and invertebrate tissue was also determined, the largest amount being found in *Ischnochiton* while *Cryptochiton*, anatomically closely related, carried only a very small quantity. The other closely related forms, *Abalone* and owl limpet, contain almost the same amount of calcium. Magnesium does not vary much in the mollusks examined. Potassium and sodium were not determined.

Very few enzymes are present in the muscle of mollusks. Careful estimations gave as a rule negative results. This is astonishing at first, since, having found large amounts of glycogen and fat in the muscle tissue, we might expect to find the corresponding enzymes. An article by Bradley and Kellersberger (25) leads, however, to the general conclusion that tissues rich in glycogen are poor in diastatic enzymes, and, conversely, those poor in glycogen are rich in diastatic enzymes. This is practically what was found in this investigation.

It is possible that in an animal, the whole metabolism of which is on so low a level, an amount of enzyme too small to be detected is still sufficient to hydrolyze the glycogen stored in the pedal

muscle. The same condition seems to hold in the case of lipase, the fat-splitting enzyme, as Bradley has shown for higher animals. Some of the most active fat-producing tissues are poorer in lipase than many which never contain or produce more than a small percentage of fat. Experiments carried out with a glycerol extract of muscle of *Abalone*, Pismo clam, *Cryptochiton*, *Ischnochiton*, and owl limpet gave striking examples which support this general view. An exception was noticed in *Cryptochiton* muscle on diastase, and in Pismo clam on urease.

SUMMARY.

The above investigation has dealt with the chemical composition of five species of marine mollusks occurring in abundance along the coast of California. These are *Abalone*, Pismo clam, giant chiton, *Ischnochiton*, and owl limpet, of which the first two are of rapidly growing importance as food for human consumption.

1. The digestive juices of the stomach and intestines of each of these forms have a distinct acid reaction and are notably rich in enzymes. A catalase, glycogenase, lactase, lipase, maltase, protease, invertase, urease, emulsin, and an amylase gave pronounced reactions. A cellulose-digesting enzyme was not found in any case.

2. A complete analysis of the muscle tissue was made. Particularly notable among the results on this tissue are the relatively high protein content of *Abalone*, 23 per cent; the low protein content of the Pismo clam, 10.26 per cent; the large amount of ash in all the forms; and especially in the limpet 7.54 per cent and the Pismo clam 5.1 per cent as against an average of approximately 1 per cent in the common mammalian muscle tissues used as food and 1 to 1.5 per cent in the sea fishes; the presence of determinable amounts of urea in *Abalone*, Pismo clam, *Ischnochiton*, and limpet; the large amounts of reducing sugars yielded on hydrolysis of the alcoholic extract from 5.26 to 27.2 per cent of the residue after removal of the alcohol; and finally the occurrence of creatinine and creatine in *Abalone*, here reported for the first time in molluscan tissue. Enzymes were found only in three cases: an amylase and glycogenase in *Cryptochiton*, and a urease in the Pismo clam.

BIBLIOGRAPHY.

1. Bernard, C., Leçons de physiologie expérimentale, 1855, i, 70, 101–103; 1856, ii, 489 ff.
2. Yung, E., *Acad. Roy. Bruxelles*, 1888, xlix, 1–116.
3. Biedermann, W., and Moritz, P., *Arch. ges. Physiol.*, 1898–99, lxxiii, 219
4. Müller, E., *Arch. ges. Physiol.*, 1900–01, lxxxiii, 619.
5. Fredericq, L., *Arch. zool. exp.*, 1878, vii, 578.
6. de Bellesme, J., *Compt. rend. Acad.*, 1879, lxxxviii, 428.
7. Krukenberg, C. F. W., *Unters. Physiol. Inst. Heidelberg*, 1878, ii, 402.
8. Abderhalden, E., and Heise, R., *Z. physiol. Chem.*, 1909, lxii, 136.
9. Sellier, J., *Compt. rend. Soc. biol.*, 1909, lxvii, 237.
10. Mendel, L. B., and Bradley, H. C., *Am. J. Physiol.*, 1905, xiii, 23–27.
11. Chittenden, N. H., *Ann. Chem.*, 1875, clxxviii, 266.
12. Dohrn, A., Inaugural dissertation, Berlin, 1861.
13. Krukenberg, C. F. W., Vergleichend-physiologische Studien an den Küsten der Andria: Experimentelle Untersuchungen, 1880–81, i, series 1, 32; iv, 54–56; 1882, ii, series 2, 143–147. Krukenberg, C. F. W., and Wagner, H., *Z. Biol.*, 1885, xxi, 25.
14. Schneider, R., *Abhandl. Kais. Akad. Berlin*, 1888.
15. Giunti, M., *Gazz. chim. ital.*, 1879, ix, 546–555.
16. Dubois, R., *Compt. rend. Soc. biol.*, 1900, lii, 392.
17. Henze, M., *Z. physiol. Chem.*, 1904–05, xliii, 477.
18. Meigs, E. B., *J. Biol. Chem.*, 1915, xxii, 493–496.
19. Koch, W., and Carr, E. P., *J. Am. Chem. Soc.*, 1909, xxxi, 1341.
20. Marshall, E. K., Jr., *J. Biol. Chem.*, 1913, xiv, 283.
21. Van Slyke, D. D., and Cullen, G. E., *J. Biol. Chem.*, 1914, xix, 211.
22. Voit, E., *Sitzungsb. Ges. Morphol. u. Physiol. München*, 1892, vii, 159.
23. Balland, *Compt. rend. Acad.*, 1898, cxxvi, 1728.
24. von Bezold, A., *Z. wissen. Zool.*, 1857, viii, 517.
25. Bradley, H. C., and Kellersberger, E., *J. Biol. Chem.*, 1912–13, xiii, 419.

HEMICELLULOSE OF APPLE WOOD.*

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(Received for publication, December 21, 1920.)

This paper covers the preliminary chemical phase of an investigation upon conditions of composition associated with fruitfulness in the apple and other fruit trees. Roberts[1] has recently presented the general economic aspects of the problem, placing special emphasis upon the relation of the amount of spur growth to fruiting, with a discussion of some external conditions which modify it. The present article deals with the composition of apple spurs and adjacent wood, with special reference to the hemicellulose fraction.

Butler, Smith, and Curry[2] have published data on the proximate composition of different aged branches, trunk, and roots of the apple tree at successive stages, from spring dormancy to leaf fall. These investigators concluded that storage carbohydrates in their material were in the form of sucrose and starch. The most abundant constituent of 1 year branches, aside from crude fiber, was starch. At the time of bud swelling the branches contained over 30.0 per cent of this constituent. At this time also the nitrogen content was greatest in the 1 year branches, amounting to about 1.0 per cent, equivalent to over 6.0 per cent of protein. It should be noted that in the investigation here referred to starch was determined by the method of acid hydrolysis[3] and

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The writers are indebted to J. A. Anderson and S. N. Epstein for some of the data presented.

[1] Roberts, R. H., *Wisconsin Agric. Exp. Station, Bull. 317*, 1920.

[2] Butler, O. R., Smith, T. O., and Curry, B. E., *New Hampshire Agric. Exp. Station, Techn. Bull. 13*, 1917.

[3] Correspondence with Dr. O. R. Butler.

therefore includes the hemicellulose compounds, as defined by Schulze.[4] Hooker[5] has made similar analyses of apple spurs, including determinations of the true starch, at successive dates throughout the year. He concludes that fruit bud differentiation occurs as a result of conditions leading to high starch and low nitrogen content.

Analytical.

In the work here reported samples were taken from the trees soon after the dew had evaporated, in an attempt to avoid metabolic changes which might follow the onset of rapid photosynthesis in the leaves. The samples were collected in covered glass jars, weighed as soon as they could be taken to the laboratory, and dried to constant weight at 80°C. The dried material was ground in a drug mill and reduced to a fine powder in a Dreef[6] mill, in which condition it passed through a 100 mesh sieve. Fig. 1 shows the origin of the new growth (*N*), spur (*S*), and base wood (*BB*) samples. It represents the first stage of development of fruiting branches examined.

Moisture was determined by drying 2.0 gm. samples to constant weight at 110°C. *Ether extract* was determined in the residue by extracting for 12 to 14 hours with anhydrous, alcohol-free ether in the Caldwell type of apparatus for continuous extraction. *Sugars* were extracted by boiling the fat-free residue with 100 cc. of 90 per cent alcohol for 1 hour under a reflux condenser. About 0.3 gm. of $CaCO_3$ was added previously, to prevent inversion of disaccharides by acids. After filtering and washing the residue with hot alcohol the extract was evaporated at 60°C., taken up with water, filtered again, and washed to a volume of 100 cc. The reducing sugars in this solution were determined as glucose by the Munson-Walker method.[7] Total sugars were determined as glucose by the Defren-O'Sullivan method,[8] after

[4] Schulze, B., *Landw. Jahrb.*, 1894, xxiii, 1.

[5] Hooker, H. D., Jr., *Univ. Missouri Agric. Exp. Station, Research Bull. 40*, 1920.

[6] Wiley, H. W., Principles and practice of agricultural analysis, Easton, 2nd edition, 1914, iii, 12.

[7] Munson, L. S., and Walker, P. H., *J. Am. Chem. Soc.*, 1906, xxviii, 665.

[8] Defren, G., *J. Am. Chem. Soc.*, 1896, xviii, 751.

boiling for 45 minutes with HCl (1: 10), neutralizing, and diluting to 500 cc.

Starch and *dextrin* were determined by digesting the residue from the extraction of sugars with saliva, after gelatinizing the starch by treatment with boiling water. The resulting sugars and dextrins were washed out, hydrolyzed with acid, and determined by the Defren-O'Sullivan method. *Acid-hydrolyzable* material was determined by boiling the residue from the starch

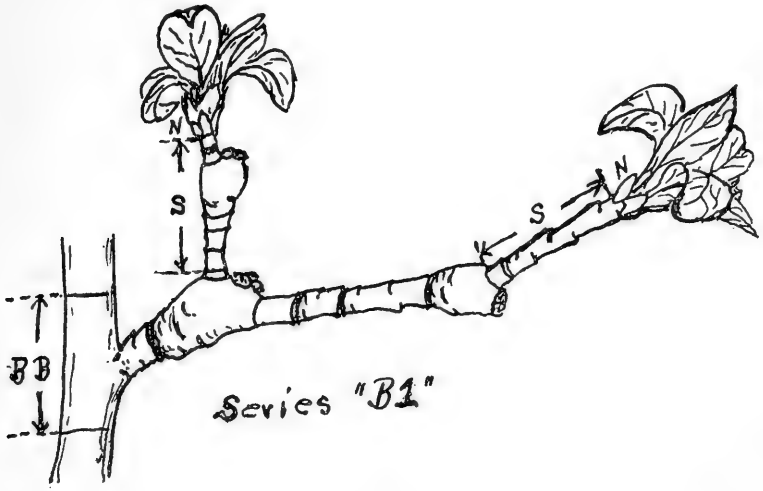

FIG. 1. Tissue taken in sampling apple wood of blossom-bearing branches. *BB*, base wood; *S*, spur; *N*, new growth of spurs.

determination with dilute HCl (1: 10) for 3 hours under a reflux condenser. After filtering and washing, the extract was neutralized and analyzed for glucose by the Defren-O'Sullivan method. *Lignin* was determined by extraction with 72 per cent H_2SO_4, after extraction of the tissue with benzene and alcohol, according to the method of Dore.[9] *Cellulose* was determined by successive chlorinations, after extraction with benzene and alcohol, also according to Dore.[9] *Protein* was calculated by multiplying the total nitrogen by 6.25, the factor commonly used in this connection. The nitrogen was determined by the Gunning-Kjeldahl

[9] Dore, W. H., *J. Ind. and Eng. Chem.*, 1920, xii, 477.

method,[10] with the added use of $CuSO_4$ in the digestion. *Ash* was determined by charring and extracting with water, subjecting the residue to complete incineration, and finally combining the extract, evaporating, and igniting at low heat.

The materials upon which the determinations were made in the case of base wood were composites of samplings on May 3, 12, 28, and June 8 and 24, 1918. In the case of spurs the data are averages of the composition of the samples taken on the above successive dates. The data for new growth of spurs are from one sample taken July 7. All the analytical results are assembled in Table I.

TABLE I.

Composition of Apple Wood in Fruiting Branches, Calculated on the Dry Matter.

	Ether extract.	Reducing sugars.	Other sugars.	Starch and dextrin.	Acid-hydrolyzable material.	Lignin.	Cellulose.	Protein.	Ash.
	per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
New growth of spurs.	1.62	0.94	5.89	4.87	21.75				
Spurs.....	1.40	4.13	3.77	3.27	27.90			5.36	
Base wood.....	1.76	1.50	2.07	3.50	28.70	18.60	36.50	2.37	3.90

Examination of the analytical data shows that while the acid-hydrolyzable material is very abundant in both spurs and base wood, the true starch and dextrins form only a small percentage of the total weight. The results from a similar analysis of plum wood, in which dextrins were extracted with water before digesting the starch, showed that the percentages of these two constituents were about equal in that material. In the case of the complete analysis of base wood the data form a total of 98.90 per cent.

In view of the probable abundance of pentosans in the wood, determinations of these were made by the method of conversion to furfurol and precipitation with phloroglucin, as modified by Kröber.[11] The result for the base wood was 19.0 per cent. In

[10] Gunning, J. W., *Z. anal. Chem.*, 1889, xxviii, 188.

[11] Kröber, E., *J. Landw.*, 1901, xlviii, 357.

the case of the products recovered from acid hydrolysis of the base wood, after these had been subjected to fermentation with ordinary yeast to remove the hexoses, the result was 10.1 per cent, based also upon the original wood. From these relations it appeared probable that in the acid-hydrolyzable fraction of the wood we were dealing primarily with a mixture of hexose and pentose sugars.

Examination of the Products from Acid Hydrolysis.

This examination is confined to the base wood. A determination of mannan by the method of Schorger[12] gave negative results.

For the preparation of a quantity of the products of hydrolysis 100 gm. of wood were extracted successively with ether and 90 per cent alcohol, and digested free from starch with saliva. 1 liter of dilute H_2SO_4 (2.5 per cent by volume) was added and the mixture was boiled for 2 hours under a reflux condenser. After filtering and washing, the filtrate was digested with $BaCO_3$ until neutral. The $BaSO_4$ thus produced was filtered off and the solution was made faintly acid with H_3PO_4. On concentrating under a pressure of about 130 mm. at 45–50°C. a thick syrup remained.

This syrupy product was extracted for some time with about 200 cc. of boiling 99 per cent alcohol. The extract was filtered and concentrated *in vacuo* as before. It was then taken up with a little water and decolorized by boiling with norite. After filtering off the norite, the solution was concentrated as before and dried at 44° under a pressure of 127 mm. The final product was 9.9 gm. of a very viscous, pale brown solid.

The residue from extraction with 99 per cent alcohol was subjected to a similar extraction with 95 per cent alcohol, but the amount of material extracted was very small. The product was therefore combined with the previous fraction, and its amount is included therein.

The residue from the alcoholic extractions was taken up in about 35 cc. of cold water. A small amount of pale brown residue was filtered off and the soluble material was recovered and dried as in the case of the alcohol-soluble fraction. There were

[12] Schorger, A. W., *J. Ind. and Eng. Chem.*, 1917, ix, 748.

recovered 2.4 gm. of a solid which was paler and more brittle than that previously recovered.

The specific rotatory power, reducing power, and pentose content of these two fractions of the hydrolysis products were determined. The galactose content of the alcohol-soluble fraction was also determined, through oxidation to mucic acid by the procedure of Dore.[9] All these results appear in Table II.

TABLE II.

Properties of Products from Acid Hydrolysis of Apple Wood.

	Specific rotation.	Reducing power, in equivalents of glucose.	Pentose content.	Galactose content.
	degrees	*per cent*	*per cent*	*per cent*
Alcohol-soluble fraction	26.1	70.0	58.6	0.98
Water-soluble "	27.0	25.1	29.2	

Special attention was given to identifying the sugars in the alcohol-soluble fraction of the hydrolytic products. Tests of the osazones prepared in the usual way with phenylhydrazine hydrochloride showed a high degree of solubility in boiling water, indicating a high percentage of pentose sugars. Fractional preparations of the osazones taken after periods of 5, 10, and 20 minutes in the water bath, when compared under the microscope with osazones freshly prepared from glucose, galactose, xylose, and arabinose, showed a preponderance of either glucose or fructose in the first fraction, while the pentoses predominated in the later fractions. A few crystals which appeared to be galactosazone were observed in the last fraction. Both the Seliwanoff test for ketoses and the osazone test by means of methylphenylhydrazine, as directed by Neuberg,[13] were negative, thus establishing the absence of fructose.

As with the wood itself, a test for mannose as hydrazone by the use of phenylhydrazine gave a negative result. By the absence of other sugars which give the glucosazone test, the presence of glucose as the chief hexose sugar in our preparation is made certain. The absence of arabinose was proved by nega-

[13] Neuberg, C., *Ber. chem. Ges.*, 1902, xxxv, 960.

tive results from the test with diphenylhydrazine according to Neuberg.[14] It is apparent, therefore, that the pentose of our preparation is xylose.

While it is not possible to separate sharply a mixture of sugars of this sort, it is possible to use the analytical data in a comparative way by which the approximate proportions may be ascertained. On the basis of the pentose determination of Table II, and as a result of the identification of sugars, it appears that the alcohol-soluble fraction resulting from hydrolysis consisted of about 58 per cent of xylose. Allowing 1 per cent for galactose, on the basis of the mucic acid test, there would remain 41 per cent of glucose. The specific rotation of a mixture of *l*-xylose, *d*-glucose, and *d*-galactose in these proportions should approximate 33°, according to Tollens' data.[15] It will be noted that the determined rotation is considerably less than this. Furthermore, the determined reducing power is much less than the above simple mixture of sugars should possess. This discrepancy invalidates calculation of the percentages of sugars by simultaneous equations, as given by Browne.[16] It appears, therefore, that compounds other than the sugars identified are present, or the latter are not entirely free, or both conditions may exist. The results are suggestive of glucosidic di- and trisaccharides analogous to those described by Browne.[17] The water-soluble fraction recovered from hydrolysis appears to be a product, or products, of partial hydrolysis.

Preliminary tests indicate that it may be possible to extract a gum-like substance from apple wood which will correspond in composition to the alcohol-soluble fraction of the products of hydrolysis. Somewhat similar polysaccharide materials are mentioned by Tollens[18] and by Czapek.[19]

[14] Neuberg, C., *Ber. chem. Ges.*, 1900, xxxiii, 2248.

[15] Tollens, B., Kurzes Handbuch der Kohlenhydrate, Leipsic, 3rd edition, 1914, 128, 175, 286.

[16] Browne, C. A., A handbook of sugar analysis, New York, 1912, 477, 489.

[17] Browne,[16] pp. 643, 731.

[18] Tollens,[15] pp. 474, 563.

[19] Czapek, F., Biochemie der Pflanzen, Jena, 2nd edition, 1913, i, 654, 685.

Work in progress shows that the acid-hydrolyzable material of apple wood in the spurs varies in a manner which indicates that it may function as an important reserve carbohydrate material. If substantiated, this relation will throw unusual importance upon the so called hemicellulose fraction of wood, the function of which has hitherto been obscure.

We purpose following the composition of this material as it is deposited in the branches and drawn upon for subsequent growth. It will also be desirable to compare it with corresponding material in trees of other species.

SUMMARY.

Analysis of apple wood from fruiting branches shows a high content of the acid-hydrolyzable material commonly designated as hemicellulose. The alcohol-soluble fraction resulting from partial hydrolysis of this material has been found to contain large amounts of xylose and glucose, with a little galactose. It is suggested that this hydrolyzable material forms a reserve source of carbohydrate in the metabolism of the apple tree.

THE ESTIMATION OF BILE ACIDS IN BILE.

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(Received for publication, October 16, 1920.)

In a recent paper Foster and Hooper (1) have described a rapid method for the estimation of bile acids in dog bile. The method depends on the fact that the two bile acids usually found in dog bile, *viz.* taurocholic and taurocholeic, on hydrolysis with alkali give taurine, which yields its nitrogen quantitatively in 4 minutes when shaken with HNO_2. Taurocholic and taurocholeic acids do not react with HNO_2 before hydrolysis. The proteins in bile are precipitated by hot alcohol which at the same time holds the bile salts in solution. A portion of the filtrate is evaporated to dryness and then hydrolyzed with 8 per cent NaOH for 6 hours. The taurine thus set free can be estimated from its yield of amino nitrogen. Since bile normally contains a small amount of free amino nitrogen, an estimation to allow for this is carried out on a separate portion of evaporated filtrate. Other nitrogenous constituents present in bile do not yield amino nitrogen with the exception of urea. But as the amount of urea present in bile—32 mg. per 100 cc. (2)—is small and since only about 3 per cent of its nitrogen is liberated in 3 minutes, this factor can be neglected.

While this method is applicable to bile acids which on hydrolysis yield only taurine it does not differentiate between these acids and those having glycocoll in the molecule. Since bile from most animals, especially man, contains acids of both the glycocoll and the taurine series, we have concerned ourselves with the problem of devising a method which will differentiate between the two series of bile acids.

Strecker (3) found that hydrolysis of glycocholic acid by means of alkali gave cholic acid and glycocoll and we have confirmed

his observation by quantitative experiment. A solution containing about 500 mg. of twice crystallized glycocholic acid per 25 cc. yielded 0.0141 gm. of nitrogen by the Kjeldahl method and 0.0142 gm. of nitrogen (corrected for the abnormal value given by glycocoll) when hydrolyzed for 6 hours with 8 per cent NaOH and the amino nitrogen was estimated. Glycocoll, like taurine, is not decomposed by heating with alkali in the concentration and time necessary to hydrolyze glycocholic acid. A solution containing 65 mg. of glycocoll per 25 cc. yielded 11.9 mg. of amino nitrogen and the same value was obtained on adding NaOH to a concentration of 8 per cent and heating at 100°C. for 10 hours. Hydrolysis of a mixture of bile acids of the taurine and glycocoll series and subsequent estimation of the amino nitrogen will give a value which is too high since glycocoll yields by Van Slyke's method 103 per cent of the theoretical nitrogen (4). If, however, the total amino nitrogen and the taurine nitrogen are known the usual correction for the high value given by glycocoll can be made.

In a mixture of pure bile acids taurine can be estimated from its sulfur content. This method has been utilized for its estimation in bile. It is seemingly open to objections since protein-free bile is known to contain sulfur compounds other than taurocholic and taurocholeic acids. Hammarsten (5) states that he has found ethereal sulfates in human and shark bile while von Bergmann (6) could not detect it in dog bile. Estimations of total sulfates which were carried out by us on the specimens of bile reported in this paper did not give a value greater than 1 mg. of $BaSO_4$ per 10 cc. of bile, which is within the experimental error of the method. It is probable that small quantities of taurine, cystine, and jecorin occur in bile but their concentrations are not known. Estimations of phosphorus in protein-free bile showed that the phospholipoid nitrogen is less than 0.1 per cent of the nitrogen present as bile acids and since jecorin is probably only a small fraction of the concentration of phospholipoid nitrogen, no error in the estimation of bile acids on the basis of the total sulfur will result by neglecting this factor. The amount of cystine and taurine cannot be large since the free amino nitrogen present in unhydrolyzed bile is only about 5 per cent of the nitrogen present as bile salts. Undoubtedly a part of this free amino

nitrogen results from heating the protein-free bile. To an alcoholic solution containing 200 mg. of twice recrystallized glycocholic acid sufficient NaOH was added to give a neutral reaction and the solution was evaporated in the usual manner. Estimation of the free amino nitrogen gave a value of 0.12 mg. or 2 per cent of the total bile acid nitrogen. This is not due to the splitting of the glycocholic acid by HNO_2 since shaking for 8 minutes gave a practically identical value; *viz.*, 0.14 mg. Other amino-acids besides taurine and glycocoll are probably also found in bile and contribute to the free amino nitrogen. If in bile containing acids of both the taurine and the glycocoll series we assume that the free amino nitrogen is made up of taurine and glycocoll in the ratio in which they occur in bile combined with cholic acid, the resultant error in the estimation of the taurine series of bile acids on the basis of the total sulfur will be less than the error (6 per cent) found by Foster and Hooper on adding known amounts of taurocholic acid to bile. The method employed by us with biles containing either an excess of glycocholic acid or nearly equal quantities of acids of the taurine and glycocoll series is to subtract the free amino nitrogen from the total obtained after hydrolysis, thus neglecting the sulfur present as taurine. If the bile contains predominantly acids of the taurine series the free amino nitrogen is assumed to consist wholly of taurine and a correction for the sulfur is made.

Croftan (7) has pointed out that the protein precipitate may contain a large proportion of the bile acids and Bang (8) states that taurocholic acid may be precipitated by means of protein solutions. To overcome this factor, Foster and Hooper filter off the protein precipitate after bringing the alcoholic solution to the desired volume and then take an aliquot of the filtrate for the estimation of bile acids. This procedure is based on the assumption that taurocholic acid is uniformly distributed throughout the two phases. We have estimated the error which would result if the amount of taurocholic acid adhering to the protein precipitate after several washings with alcohol is neglected. The proteins from 100 cc. of ox bile were precipitated by 10 volumes of alcohol, filtered, and washed a number of times with alcohol. The sulfur content of the precipitate was 2 mg. and the nitrogen 11 mg. Since the sulfur content of mucin is about 13 per cent

of the nitrogen (9), the amount of taurocholic acid in the precipitate from 10 cc. of bile is less than the experimental error of the method.

Our procedure for the estimation of taurine and glycocoll nitrogen combined as bile acids is as follows: To 10 cc. of bile (more may be necessary if the bile is dilute) 8.5 volumes of alcohol are added, the mixture is heated almost to the boiling point, filtered through a dry filter paper, and repeatedly washed with small quantities of alcohol until the volume of the filtrate, when cold, has been brought to 100 cc. Two 20 cc. portions are evaporated to dryness in porcelain dishes and the remainder is evaporated in a nickle crucible. The residue in the crucible is fused with a mixture of Na_2CO_3 and Na_2O_2, the sulfates are precipitated with $BaCl_2$, and weighed as $BaSO_4$. The residue from one of the 20 cc. portions is dissolved in water, brought to a volume of 10 cc., and 2 cc. portions are used for the estimation of the amino nitrogen. The other is washed into a 10 cc. volumetric flask with 8 per cent NaOH, placed in a water bath, and heated at 100°C. for 6 to 8 hours. The amino nitrogen is estimated in 2 cc. of the hydrolysate. The difference between the amino nitrogen in the hydrolyzed and unhydrolyzed bile gives the taurine and glycocoll nitrogen of the bile acids. The taurine nitrogen is calculated from the figures obtained in the estimation of the sulfur. If the bile acids consist predominantly of the taurine series the free amino nitrogen is assumed to consist wholly of taurine and a correction applied to the total sulfur value. The difference between the amino nitrogen due to bile acids and the taurine nitrogen, less 3 per cent, gives the glycocoll nitrogen.

To test the accuracy of this method a known quantity of glycocholic acid, dissolved by addition of NaOH, was added to a specimen of ox bile, Sample B, the bile acids content of which was estimated on a separate portion, Sample A. The following results, per 10 cc. of bile, were obtained:

	Sample A. *mg.*	Sample B. *mg.*
Amino nitrogen after hydrolysis	13.4	15.4
" " before "	0.3	0.4
Nitrogen of the bile acids	13.1	15.0
Taurine nitrogen calculated from the sulfur value (0.0103 S)	4.5	4.5

	Sample A. *mg.*	Sample B. *mg.*
Glycocoll nitrogen (uncorrected)	8.6	10.5
" " (corrected)	8.4	10.2
Nitrogen recovered from bile acid added		1.8
Total nitrogen of the glycocholic acid (66.7 mg.) added		2.0
Free amino nitrogen in bile acid added		0.1
Nitrogen combined as glycocholic acid		1.9
Glycocholic acid recovered, *per cent*		95

The accuracy of the method, based on duplicate estimations, depends to a large extent on the concentration of bile salts. Duplicate sulfur estimations checked within 1 mg. of $BaSO_4$. Since the aliquot (0.6) taken is large the resultant error is usually less than 1 per cent. The chief error lies in the estimation of the amino nitrogen since the aliquot (0.04) taken for analysis is small. Duplicate estimations agree within 3 per cent. Other factors, such as the presence of amino-acids besides taurine and glycocoll, sulfur compounds other than taurine, splitting of bile acids during evaporation of the alcoholic filtrate, etc., probably do not contribute an error greater than 3 per cent. This gives a maximum error of about 6 per cent which probably represents the accuracy of the method.

The analyses reported in Table I were made with the aid of this method. With the exception of the biles from the dog and human fistulas, the specimens were mixed samples obtained from the slaughter house. In ox bile we note that the predominance of acids of the taurine and the glycocoll series, respectively, varies in different specimens. This had been previously noted by Marshall (10) who examined a large number of specimens of ox bile. Pig bile contains chiefly acids of the glycocoll series while bile from the sheep and the dog contains only those of the taurine group. In human bile we note a slight excess of glycocholic acid over the acids of the taurine series. These results are essentially in accord with the analyses reported by other workers using more laborious methods (11).

TABLE I.

Bile.*	Amino N after hydrolysis.	Amino N before hydrolysis.	N in bile acids.	Sulfur.	Taurine N.	Glycocoll N.
	gm.	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*
Ox 1	0.0182	0.0006	0.0176	0.0162	0.0071	0.0102
" 2	0.0172		0.0172	0.0270	0.0118	0.0052
Pig 1	0.0187	0.0003	0.0184	0.0089	0.0039	0.0141
" 2	0.0202	0.0001	0.0201	0.0076	0.0032	0.0164
" 3	0.0172	0.0008	0.0164	0.0092	0.0040	0.0120
Sheep 1	0.0216	0.0033	0.0183	0.0488	0.0181	None.†
" 2	0.0247	0.0026	0.0221	0.0526	0.0204	" †
Dog 1	0.0023	0.0004	0.0019	0.0057	0.0021	" †
" 2	0.0024	0.0004	0.0020	0.0058	0.0021	" †
Human 1	0.0017	0.0001	0.0016	0.0019	0.0008	0.0008
" 2	0.0013	0.0001	0.0012	0.0013	0.0006	0.0006
" 3	0.0018	0.0003	0.0015	0.0016	0.0007	0.0008‡
" 4	0.0086	0.0009	0.0077	0.0067	0.0029	0.0047§

* All figures are per 10 cc. of bile.

† Calculations are based on the assumption that the free NH_2 is due wholly to taurine.

‡ Slight decomposition.

§ Obtained on autopsy.

SUMMARY.

A method for the estimation of the nitrogen of bile acids of the taurine and the glycocoll series in bile is described. It is based on the fact that on hydrolysis with NaOH the bile acids are split, setting free taurine and glycocoll which react quantitatively with HNO_2. The taurine nitrogen can be estimated from the total sulfur content of protein-free bile. The difference between the amino nitrogen resulting from the splitting of the bile acids, and the taurine nitrogen, less 3 per cent, gives the glycocoll nitrogen.

BIBLIOGRAPHY.

1. Foster, M. G., and Hooper, C. W., *J. Biol. Chem.*, 1919, xxxviii, 355.
2. Marshall, E. K., Jr., and Davis, D. M., *J. Biol. Chem.*, 1914, xviii, 60.
3. Strecker, A., *Ann. Chem.*, 1848, lxv, 130.
4. Van Slyke, D. D., *J. Biol. Chem.*, 1911, ix, 185.
5. Hammarsten, O., *Ergebn. Physiol.*, 1905, iv, 12.
6. von Bergmann, G., *Beitr. chem. Physiol. u. Path.*, 1904, iv, 192.

7. Croftan, A. C., *Am. J. Med. Sc.*, 1902, cxxiii, 150.
8. Bang, I., *Beitr. chem. Physiol. u. Path.*, 1906, vii, 148.
9. Hammarsten, O., and Mandel, J. A., A textbook of physiological chemistry, New York, 1914, 169.
10. Marshall, J., *Z. physiol. Chem.*, 1887, xi, 233.
11. Gundelach, C., and Strecker, A., *Ann. Chem.*, 1847, lxii, 205. Bensch, A., *Ann. Chem.*, 1848, lxv, 194. Strecker, A., *Ann. Chem.*, 1849, lxx, 149. Jolin, S., *Z. physiol. Chem.*, 1888, xii, 512; 1889, xiii, 205. Örum, H. P. T., *Skand. Arch. Physiol.*, 1904, xvi, 273.

THE EFFECT OF ALKALI ON THE EFFICIENCY OF THE WATER-SOLUBLE VITAMINE B.*

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(Received for publication, December 22, 1920.)

While there is convincing evidence that the growth-promoting capacity of the water-soluble vitamine B is little, if at all, affected by heating with relatively strong acids, even for many hours, comparatively little is to be found in the literature respecting the effect of alkalies on this vitamine. Thus McCollum and Simmonds,[1] Drummond,[2] and Voegtlin and Lake[3] agree that heating with more or less dilute alkalies destroys the water-soluble vitamine B, while on the other hand Daniels and McClurg[4] and Whipple[5] state that under the conditions of their experiments it was not destroyed.

Without entering into a critical discussion of these conflicting views until our knowledge of this subject is more complete we wish to record some observations which we have made on a concentrated preparation of this vitamine obtained from brewery yeast according to the method of Osborne and Wakeman.[6]

The preparation used for these experiments sufficed in daily doses of 12 mg. to promote the prompt recovery of young rats which had failed on a diet adequate in respect to every factor

* The expenses of this investigation were shared by the Connecticut Agricultural Experiment Station and the Carnegie Institution of Washington, Washington, D. C.

[1] McCollum, E. V., and Simmonds, N., *J. Biol. Chem.*, 1918, xxxiii, 55.

[2] Drummond, J. C., *Biochem. J.*, 1916–17, xi, 255.

[3] Voegtlin, C., and Lake, G. C., *Am. J. Physiol.*, 1918–19, xlvii, 558; *J. Pharmacol.*, 1918, xi, 167.

[4] Daniels, A. L., and McClurg, N. I., *J. Biol. Chem.*, 1919, xxxvii, 201.

[5] Whipple, B. K., *J. Biol. Chem.*, 1920, xliv, 175.

[6] Osborne, T. B., and Wakeman, A. J., *J. Biol. Chem.*, 1919, xl, 383.

except the water-soluble vitamine B (see Chart I, Rats 5917 and 5924).

An aqueous solution of 1 gm. of this preparation required 0.0530 gm. of NaOH to make its reaction neutral to litmus. Accordingly 5 gm. were dissolved in 67.4 cc. of water and 32.6 cc. of a solution of NaOH containing 0.6650 gm. were added. This sufficed to neutralize the acidity of the preparation and leave an excess equivalent to a 0.1 N solution of the alkali. After

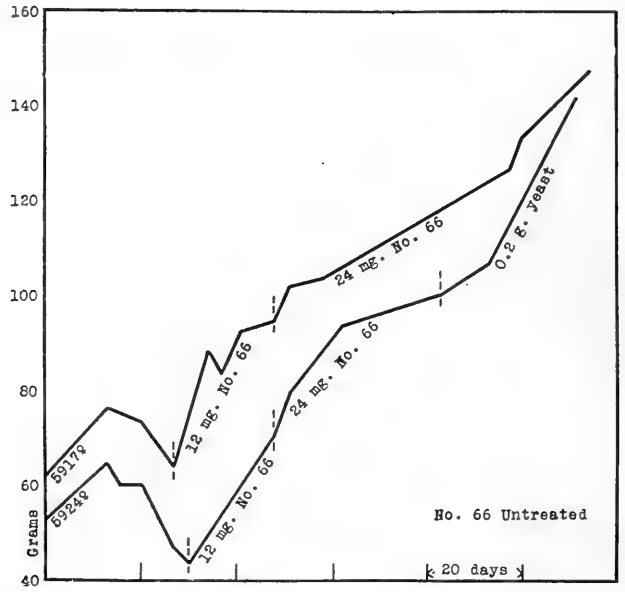

Chart I.

this alkaline solution had stood for ½ hour at a temperature of 20°, 22 cc. of 0.1 N HCl were added to 20 cc., thus making the reaction faintly acid to litmus. After standing over night at 0°, the solution was mixed with 5.67 gm. of starch, evaporated on the steam bath, and made into tablets, each containing 15 mg. of the vitamine preparation, No. 88.

After the main alkaline solution of the vitamine preparation had stood at about 20° for 18 hours, 22 cc. of 0.1 N HCl were added to another portion of 20 cc. and the slightly acid solution was evaporated on starch and made into tablets as above described, No. 90.

A third lot of tablets was made in exactly the same manner after the solution had stood at room temperature for 90 hours, No. 91.

A fourth portion of 20 cc., after standing at room temperature for 18 hours, was heated in a bath of boiling water to 90°, kept

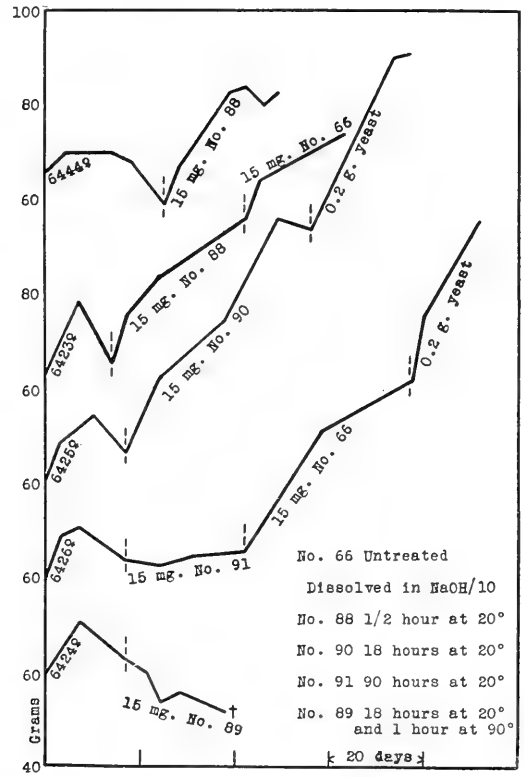

Chart II.

immersed therein for 1 hour longer, 22 cc. of 0.1 N HCl were added, and tablets made as before, No. 89.

Chart I shows the efficiency of the untreated yeast fraction, No. 66, in promoting the recovery and growth of young rats

declining on a diet[7] free from water-soluble vitamine B (Rats 5917 and 5924). That this efficiency was not appreciably impaired by being dissolved in 0.1 N NaOH solution during ½ hour at 20° is shown by Chart II (Rats 6444 and 6423) nor by even 18 hours at 20° (Rat 6425). That, however, it was seriously affected after 90 hours at 20° is shown by the failure of Rat 6426 to recover when supplied with 15 mg. daily of the fraction thus treated, and its prompt gain in weight when the alkali-treated vitamine was replaced by a like quantity of the untreated preparation. That heating in 0.1 N NaOH solution, on the other hand, quickly destroys the activity of this preparation is shown by Rat 6424.

These experiments confirm the observations of those investigators who have reported the destruction of the water-soluble vitamine B when heated with alkalies. It thus appears that in attempting to concentrate, or isolate, the water-soluble vitamine B dilute alkaline solutions can be used without materially affecting its activity provided a low temperature and a short time of exposure to the alkali are employed.

[7] The diet consisted of meat residue 20, salt mixture 4, starch 52, butter fat 9, and lard 15 per cent. The preparation of the meat residue is described by Osborne, Wakeman, and Ferry (Osborne, T. B., Wakeman, A. J., and Ferry, E. L., *J. Biol. Chem.*, 1919, xxxix, 35). The composition of the salt mixture is given by Osborne and Mendel (Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1919, xxxvii, 572).

A METHOD FOR THE DETERMINATION OF CHLORINE IN SOLID TISSUES.

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(Received for publication, December 29, 1920.)

In the course of some investigations in inorganic metabolism it became necessary to determine the total chlorine in small samples of various tissues. While several rapid and satisfactory methods have been devised for blood, milk, and other liquids, these are not applicable to solid tissues. The size of the samples available made necessary the use of a method capable of determining less than 5 mg. There is no difficulty in determining this amount in pure solution with sufficient accuracy by the titrations of Bang (1913) or McLean and Van Slyke (1915), and the problem was simply one of removing the organic matter. For this purpose the wet ashing process of Neumann (1900, 1902–03) seemed the most suitable and we have adapted this method to our needs.

Neumann's method consists in ashing the tissues with a mixture of dilute nitric and sulfuric acids and distilling the hydrochloric acid thus formed into a standard silver nitrate solution. The excess silver is titrated by Volhard's method after removing all traces of nitrous acid. All rubber must be avoided in the apparatus since it is attacked by the hydrochloric acid in the hot gases, causing low results. Plimmer (1904) called attention to the formation of hydrocyanic acid as a source of error in this method and recommended boiling the acid silver solution containing the precipitate for $\frac{1}{2}$ hour before titrating. With this modification, which Neumann (1904–05) later admitted to be necessary, Plimmer secured accurate results on proteins.

To adapt this method to our needs, we have modified the apparatus to deal with small samples of tissue, and have substituted

the more sensitive titration of McLean and Van Slyke for that of Volhard. We have also shortened the time by using a more vigorous digestion mixture.

Neumann used for the digestion a mixture of equal parts of water, concentrated sulfuric acid, and concentrated nitric acid. He found that if more concentrated acid was used, some free chlorine was formed which was not absorbed in the silver solution. We use for our digestion concentrated sulfuric acid and persulfuric acid. This gives extremely rapid digestion but forms a large proportion of free chlorine. Accordingly we do not absorb the gases in the silver solution but in an alkaline sulfite solution. The sulfite is not added as such but is formed in the alkali by absorption of sulfur dioxide from the digestion gases. Plimmer (1904) found that no cyanides were formed by ashing proteins with sulfuric acid alone or in combination with potassium permanganate or manganese dioxide. We have never been able to detect cyanides in our distillates.

Apparatus.

Our apparatus is arranged as shown in Fig. 1. We use suction rather than the pressure of the boiling digestion mixture to carry the gases through the absorption tubes. The tube *A* carries at one end a small bulb *B* perforated with a number of small holes and is provided at the other end with a glass stopper *C*. The tube *A* is passed through the T-tube *D* and attached to it by a piece of rubber tubing. A bent side arm terminating in the bulb *E* is attached just below the glass stopper *C*. A second absorption tube *F* is attached to the side arm of the T-tube *D* and the outlet of this second tube is connected to the suction. The digestion is carried out in the hard glass tube *G*. The internal diameter of *G* is about 1 mm. greater than the external diameter of the bulb *E*. When suction is applied, air enters the annular space between *E* and *G* and carries the gases through the absorption tubes. This space is so narrow and the speed of the entering gases so great, that none of the digestion gases can escape. The apparatus as described presupposes a halogen-free atmosphere. The entering air can be washed, if necessary, by modifying the apparatus as in Fig. 2. *G* and *E* are, as in Fig. 1, the digestion

tube and the bulb. The washed air enters the side arm of the T-tube *H*. The speed of the entering air prevents any gases passing *E* and attacking the rubber stopper. It is necessary to provide a joint in the side arm to allow the replacement of the stopper and rubber tube.

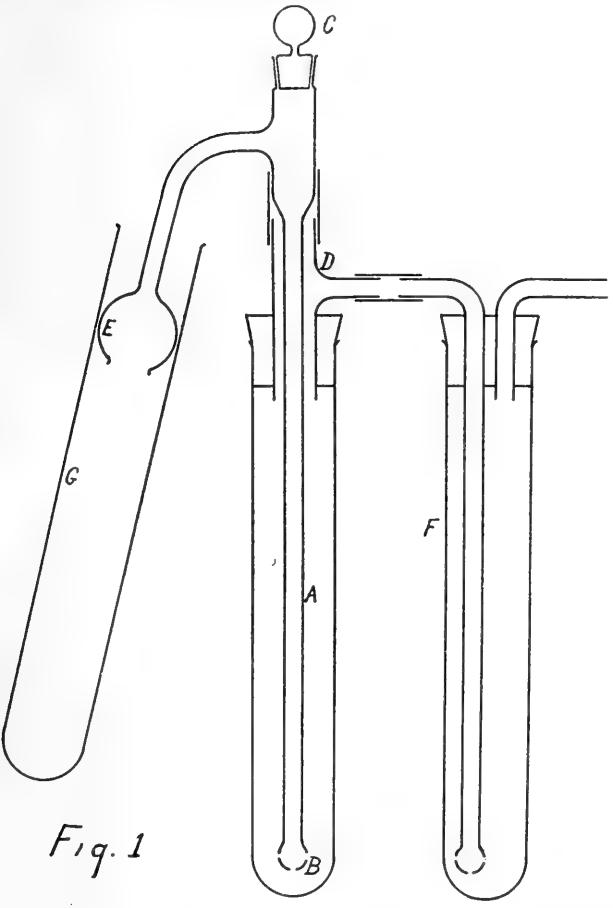

Fig. 1

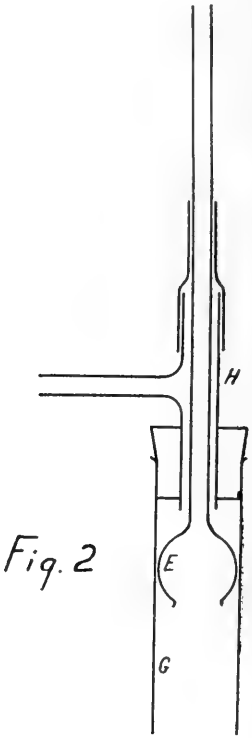

Fig. 2

It can be seen that the gases come in contact with nothing but glass until they have passed through the liquid in the first absorption tube when they are cool and nearly free from hydrochloric acid and chlorine. We have found that only 1 or 2 per cent of the total chlorine escapes absorption in the first tube.

Technique of the Determination.

The actual determination is carried out as follows. The weighed sample of tissue is placed in the digestion tube *G* with a minute drop of mercury as a catalyzer, and two or three pieces of quartz to prevent bumping. (Small pieces of broken silica dishes are much superior to pebbles for this purpose on account of their sharp edges. Broken silica ware may be bought quite cheaply from dealers.) 2 gm. of solid, chlorine-free sodium carbonate (monohydrate) for each gram of tissue sample are placed in the first absorption tube and about 10 cc. of distilled water added. In the second absorption tube are placed about 1 gm. of carbonate and 10 cc. of water. A few drops of caprylic alcohol to prevent foaming are added to each tube and the apparatus is set up as shown. A rather rapid current of air is started and 2 cc. of concentrated sulfuric acid for each gram of tissue are added to the digestion tube above the bulb *E*. The acid is mixed with any liquid in the tube by gentle shaking and the tube is then gently heated until the large pieces of tissue have dissolved. If the heating is too rapid at first, explosions of steam from the interior of the tissue may force gases past the bulb *E*. The tube *G* is inclined so that water condensing on the bulb will not drop directly into the hot acid. The heat is then increased and the acid boiled vigorously for about 5 minutes. The tube is allowed to cool for a minute or two, the bulb is removed, and 2 gm. of ammonium or potassium persulfate and a fresh piece of quartz are added, the bulb being replaced as quickly as possible. The heating is resumed and if, after boiling a few minutes, the acid is not decolorized, another gram of persulfate is added in the same manner. The digestion is continued, adding more persulfate if necessary, until the acid is perfectly clear and colorless.

When the digestion has been completed, the air current is stopped and all the connecting tubes are washed into the two absorption tubes by removing the various joints and the stopper *C*.

The liquid in these tubes is then transferred quantitatively to a 150 cc. Erlenmeyer flask, a few pieces of quartz and a few drops of methyl orange are added, and the mouth of the flask is closed by a bulb having a small hole in its side and a short open stem hanging inside the flask. Dilute (30 per cent) sulfuric acid is

added through the side hole in this trap by means of a pipette until the solution is slightly acid. The solution is boiled for a few minutes, keeping faintly acid to methyl orange by adding a few drops of acid from time to time. An excess of acid is to be avoided as it decreases the sensitiveness of the end-point of the silver titration. When no more sulfur dioxide or carbon dioxide comes off, 5 cc. of standard silver nitrate solution (see below) are added and the solution is evaporated to about 15 cc. The liquid is then transferred to a 25 cc. volumetric flask, cooled, made up to volume, and centrifuged. The silver chloride is well coagulated by the boiling and is easily thrown down in the centrifuge. We prefer to centrifuge rather than filter on account of the difficulty in securing filter paper free from traces of halogens. 20 cc. of the clear liquid are taken for the titration.

Titration.

The solutions used for the titration are those described by Van Slyke and Donleavy (1919) except that the picric acid is omitted from the silver solution. These solutions are made up as follows.

Silver Solution.—5.812 gm. of pure fused silver nitrate, and 250 cc. of nitric acid (specific gravity 1.42) are made up to 1 liter with distilled water.

Potassium Iodide Solution.—2.4 gm. of potassium iodide are dissolved in 1 liter of distilled water, titrated against the silver solution in the manner described by Van Slyke and Donleavy, and diluted so that 12.65 cc. equal 5 cc. of the silver solution.

Indicator Solution.—2.5 gm. of soluble starch are dissolved in about 500 cc. of distilled water, 446 gm. of crystalline sodium citrate ($Na_3C_6H_5O_7.5\frac{1}{2}H_2O$) and 20 gm. of sodium nitrite are added and dissolved by heat. The solution is filtered through cotton, cooled, and made up to 1 liter. It should be noted that according to U. S. P. IX, sodium citrate contains only 2 molecules of water, while according to U. S. P. VIII, it contains the usual $5\frac{1}{2}$ molecules. If the drier preparation is used, proportionately less should be taken. The use of citrate free from chlorides appreciably sharpens the end-point.

The calculation is simple:

$$\frac{1015 - (100 \times \text{cc. KI})}{\text{weight tissue in gm.}} = \text{mg. NaCl per 100 gm. tissue, or}$$

$$\frac{1015 - (100 \times \text{cc. KI})}{1.65\ (\text{weight tissue in gm.})} = \text{mg. Cl per 100 gm. tissue}$$

The color of the methyl orange which is used during the evaporation is entirely destroyed by the nitrous acid formed on adding the indicator solution and does not interfere with the end-point of the titration. The iodide solution should be frequently compared with the silver. If they are found not to agree, the comparison should be repeated using a new indicator solution, since substances are occasionally formed in the indicator on standing which cause the blue color to appear before all the silver has been precipitated. The solutions should be compared whenever a new indicator solution is made up.

Reagents.

All the reagents, including the distilled water, must be frequently tested to insure the absence of halogens, and it is advisable to run blanks often.

Good grades of sulfuric and nitric acids are usually sufficiently free from halogens and we have never had any samples of persulfate which were not pure. Potassium persulfate may be recrystallized easily from water. The ammonium salt is decomposed by hot water but may be converted into the potassium compound by adding a potassium salt to its solution, since the potassium salt is much less soluble.

The purity of the alkali is most important since large amounts of it are used. We have been unable to buy or prepare sodium hydroxide free from halogens. Even when prepared from metallic sodium it contains too much chlorine. However, the preparation of chlorine-free carbonate is quite simple.

400 gm. of $Na_2CO_3.10H_2O$ or an equivalent amount of a drier preparation are dissolved in water and made up to 500 cc. Alcohol is added in small quantities with shaking until the mixture just begins to separate into two layers. About 100 cc. of alcohol are required. The upper alcoholic layer should be as small as possible. The mixture is seeded with about a gram of solid

carbonate and cooled under the tap to below 18°, with constant shaking to prevent the crystals from adhering to the sides of the vessel as they form. About 250 cc. more of alcohol are added and the mixture is again cooled until no more carbonate separates. The crystals are sucked dry on a Buchner funnel, and washed with about 200 cc. of alcohol in small portions. When nearly dry, the carbonate is made up to 500 cc. with distilled water and recrystallized in exactly the same manner as before. This second crystallization and washing should be done with alcohol free from chlorides, which may readily be obtained by distilling over a little carbonate. The purified salt is best preserved as the monohydrate.

If the original preparation is nearly free from chlorides, the first crystallization may be omitted. In all cases the final product should be again tested for chlorides just before using.

If desired, sodium citrate for the indicator solution may be recrystallized in a similar manner.

RESULTS.

Table I gives some results obtained on blood by this method and by Foster's (1917) modification of McLean and Van Slyke's

TABLE I.

NaCl Content of Blood and Plasma, per 100 Cc.

No.	Tissue.	Van Slyke-Foster.	Digestion method.
		mg.	*mg.*
1	Defibrinated blood	491	495
2	Whole blood, normal	494	504
3	" " "	494	506
4	" " "	506	511
5	" " "	438	433
6	" " "	471	468
7	" " "	425	427
8	" " "	475	478
9	Plasma, normal	644	641
10	" "	638	635
11	" "	631	631
12	" "	700	692
13	" "	596	591
14	" "	594	605

method. The original method of McLean and Van Slyke could not be used on account of the lack of Merck's blood charcoal. 1 cc. of blood was used in all cases.

TABLE II.

NaCl Content of Human Rectus Muscle, per 100 Gm.

No.	Diagnosis.	Weight of sample.	NaCl
		gm.	*mg.*
1	Normal	4.067	84
		5.200	82
2	Unknown	3.55	108
		3.98	107
		3.275	107
3	Streptococcus septicemia	3.45	97
		4.508	94
4	Tuberculosis	3.108	169
		2.585	168
5	Pulmonary tuberculosis	4.337	158
		4.378	157
6	Tuberculosis; pneumonia	2.903	124
		3.137	125
7	Diphtheria; empyema	3.855	103
		3.675	106
8	Empyema; surgical shock	4.921	119
		3.407	117
9	Miliary tuberculosis	2.941	149
		2.997	147

We have used this method successfully on many different tissues including various abdominal organs, bone, brain, skin, and muscle. Certain tissues may require slight modifications of the digestion. It is often necessary to add more acid during the digestion of tissues containing much inorganic matter such as bone. Tissues containing much fat are among the most resistant,

and it is best to prolong the digestion with acid alone, before adding the persulfate.

Table II shows the agreement of duplicates obtained on human muscle.

BIBLIOGRAPHY.

Bang, I., *Biochem. Z.*, 1913, xlix, 31.
Foster, G. L., *J. Biol. Chem.*, 1917, xxxi, 483.
McLean, F. C., and Van Slyke, D. D., *J. Am. Chem. Soc.*, 1915, xxxvii, 1128; *J. Biol. Chem.*, 1915, xxi, 361.
Neumann, A., *Arch. Physiol.*, 1900, 159; *Z. physiol. Chem.*, 1902–03, xxxvii, 115; 1904–05, xliii, 36.
Plimmer, R. H. A., *J. Physiol.*, 1904, xxxi, 65.
Van Slyke, D. D., and Donleavy, J., *J. Biol. Chem.*, 1919, xxxvii, 551.

THE DETERMINATION OF CHLORIDES IN TRICHLORO-ACETIC ACID FILTRATES FROM WHOLE BLOOD AND PLASMA.

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(Received for publication, December 1, 1920.)

There are at present at least three methods commonly in use for the determination of chlorides in blood. They are the Van Slyke and Donleavy,[1] Foster,[2] and Rappleye[3] methods. The first two are adaptations of the original McLean and Van Slyke[4] starch-iodide titration, merely employing different procedures for the removal of blood proteins, while the latter method utilizes the Volhard titration and another modification for precipitation of blood proteins. It would seem unnecessary to add an additional method to these apparently satisfactory ones but certain considerations seem to the writer to justify the introduction of the present modification of the original McLean-Van Slyke titration.

The method of Foster gives reliable figures and is very satisfactory for a large proportion of blood chloride determinations but frequently there are bloods, especially pathological, from the filtrates of which it is impossible to flock out the silver chloride completely. This happens in spite of large additions of magnesium sulfate and careful fusing of the *m*-phosphoric acid before the precipitation of blood proteins. This is a very serious drawback as a satisfactory remedy (heating) would make the method complicated. Two bloods which presented this difficulty with Foster's method gave no trouble with the method to be described.

While comparing the method of Van Slyke and Donleavy with that of Foster it was found that the latter gave results much lower than the former. This was rather surprising and it was not

[1] Van Slyke, D. D., and Donleavy, J. J., *J. Biol. Chem.*, 1919, xxxvii, 551.

[2] Foster, G. L., *J. Biol. Chem.*, 1917, xxxi, 483.

[3] Rappleye, W. C., *J. Biol. Chem.*, 1918, xxxv, 509.

[4] McLean, F. C., and Van Slyke, D. D., *J. Am. Chem. Soc.*, 1915, xxxvii, 1128.

until the silver solutions for both methods had been again carefully prepared and blanks and known chloride solutions had been determined simultaneously with the bloods that the former figures were considered reliable. Invariably the method of Van Slyke and Donleavy gave correct figures on known solutions of chloride in water and high figures on plasma. These figures were further confirmed by comparing with the method of McLean and Van Slyke[5] and also with the digestion method of Bell and Doisy[6] which through the kindness of Professor Bell the writer was permitted to use.

A probable explanation for the high figures by the method of Van Slyke and Donleavy is that some silver is carried down with the blood proteins during their simultaneous precipitation with the chlorides. It might be mentioned that plasmas used for comparison were carefully obtained to insure freedom from hemolysis although it is doubtful whether a small amount of hemolysis would introduce an appreciable error (see Table I). The method of Rappleye was not compared with that of Bell and Doisy but it would seem to be open to the same error as that of Van Slyke and Donleavy.

This left the problem of finding some blood protein precipitant which would satisfactorily remove blood proteins and give a filtrate from which silver chloride could easily be precipitated. Picric acid with nitric acid used by Austin and Van Slyke[7] for whole blood is open to the same difficulty as *m*-phosphoric acid. The same is also true of nitric acid with magnesium sulfate. Tungstic acid filtrates, even after removing the excess tungstate with barium hydroxide, while being well suited for the precipitation of silver chloride, gave slightly high results (about 2 per cent). Heat with acetic acid as used by McLean and Van Slyke in their original method is rather laborious and necessitates Merck's blood charcoal. Trichloroacetic acid, a very efficient protein precipitant and one introduced by Greenwald[8] for precipitation of blood proteins, was tried and found to be very satisfactory in the determination of blood chlorides.

[5] McLean, F. C., and Van Slyke, D.D., *J. Biol. Chem.*, 1915, xxi, 361.
[6] Bell, R. D., and Doisy, E. A., *J. Biol. Chem.*, 1920–21, xlv, 427.
[7] Austin, J. H., and Van Slyke, D. D., *J. Biol. Chem.*, 1920, xli, 345.
[8] Greenwald, I., *J. Biol. Chem.*, 1915, xxi, 61.

TABLE I.

Sodium Chloride per 100 Cc. of Blood.

	Bell and Doisy.	Trichloracetic acid filtrate.	Van Slyke and Donleavy.	McLean and Van Slyke.	Foster.	von Korányi.
	mg.	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*
Plasma, normal		612	649	618	618	
" "		608	641	609	615	
" *		607	645	633		
" normal	636	640	665		640	
	641	640			641	
" "			635	606		
" " †	643	646	688			
	647					
" "	640	645	676			
	645	646				
" diabetic‡	618	628	649			
		628				
" normal	631		661			
	632		662			
" "	629		665			
	630					
" "	609		638			
	611					
" nephritic	595	608				
	609	608				
" normal (diluted)	460	465				
	465	466				
" diabetic	601	607				
	607	608				
Whole blood, normal	466	460				
		462				
Plasma, same blood		629				
		630				
Whole blood, diabetic	487	483				
	491	484				
" "	499	500				
	502	501				
Ascitic fluid		679				676
Plasma, nephritic		588				583
Ascitic fluid, nephritic		623				625

* Mixture of diabetic plasmas. Hemolyzed. Considerable lipemia.

† Very slightly hemolyzed.

‡ Considerably hemolyzed. Plasma had stood 24 hours at 27°C. in an open tube.

Trichloroacetic acid would on first thought seem to be a rather dangerous reagent to use in connection with the determination of blood chlorides since it contains chlorine. Moreover this chlorine will react with silver nitrate in solutions of trichloroacetic acid which have stood for some time. A fresh solution will give no opalescence with silver nitrate, providing it is prepared from chloride-free crystals. At the end of 24 hours, if silver nitrate is added, a slight opalescence will appear but not enough to make an error outside the limit of error of the method. The fact that the solution of trichloroacetic acid even at the end of 24 hours will introduce no appreciable error into the results gives a wide margin of time for a determination.[9]

Trichloroacetic acid filtrates are adapted for the determination of chlorides in plasma, whole blood, or other body fluids. Moreover, the trichloroacetic acid solution is very easily made up and requires no previous preparation as does *m*-phosphoric acid. The filtrates are identical with those in which Bell and Doisy[10] determine phosphates and are also adapted for the determination of non-protein nitrogen according to Folin and Wu[11] (the nitrogen values are slightly higher than in tungstic acid filtrates) so that these determinations may be done simultaneously with the chlorides.

While the starch-iodide titration is superior to the Volhard titration for chlorides in blood, one may get into serious difficulty by not understanding thoroughly the various factors which deter-

[9] A 25 per cent solution of trichloroacetic acid, containing 10 cc. of concentrated nitric acid per 100 cc., which had stood for 2 weeks exposed to ordinary daylight and which gave a strong opalescence with silver nitrate, when used on a solution of sodium chloride in water gave a result only 1.5 per cent too high.

A 20 per cent solution of trichloroacetic acid, which had stood in ordinary daylight for 19 days, when used on a plasma and compared with a freshly prepared solution of trichloroacetic acid on the same plasma gave 632 and 631 mg. of NaCl as compared with 620 and 623 mg. of NaCl for the fresh solution.

A trichloroacetic acid blood filtrate which was determined immediately and after standing 5 days gave the values 609 and 608 respectively, the slightly lower figure on standing being within the limit of error of the titration.

[10] Bell, R. D., and Doisy, E. A., *J. Biol. Chem.*, 1920, xliv, 55.

[11] Folin, O., and Wu, H., *J. Biol. Chem.*, 1919, xxxviii, 81.

mine its sensitiveness. As McLean and Van Slyke have shown in their first paper, the final acidity is the most important factor. The blue color is developed best in slightly acid solution. Too much acid abolishes the blue color and leaves only the brown color of the iodine liberated by the nitrous acid. If the solution is not acid enough no color at all will be formed because it is necessary for nitrous acid to be present to liberate hydrogen iodide from the potassium iodide. (This is a valuable criterion to use in determining roughly whether the proper amount of citrate solution has been added, for when a blue color is formed after the addition of each drop of potassium iodide during the titration it is an indication that the optimum conditions are approximated, although this should be checked up with an end-point determination as will be described.)

While using Foster's method it was noticed that the end-point was much sharper and bluer in color than for the other methods. Upon investigation of the cause it was found that the *m*-phosphoric acid present seemed to increase the sensitiveness of the end-point. Further investigation showed that *o*-phosphoric acid or its sodium or potassium salts have the same effect. This is not entirely due to the buffer action of the phosphate for in a titration which was too acid (with nitric acid), due to too little citrate solution having been added, the addition of a small amount of phosphoric acid, notwithstanding that this made the solution more acid, improved the end-point. The addition of phosphoric acid to the citrate solution also greatly increases its flexibility. A variation greater than 0.25 cc. of the McLean-Van Slyke citrate solution will appreciably affect the end-point while a similar variation of the same solution containing a proper amount of phosphoric acid will hardly be noticeable. The most curious fact about the addition of phosphoric acid is that it will make the color of the end-point a deeper blue, even though the optimum amount of citrate solution has been added.

Another important factor influencing the sensitiveness of the end-point is the amount and character of the starch used. Increasing the amount of starch in a titration makes the end-point more intense and therefore easier to distinguish. The starch should be soluble but this can be carried too far as has evidently been done in the soluble starches obtainable on the market at

present. They give an end-point which is not a pure blue, indicating that some dextrin is present. This difficulty may be overcome by boiling ordinary corn-starch 4 or 5 hours and then filtering through cotton. This gives a soluble starch which shows an intense blue color in the presence of iodine. The writer feels that it is more satisfactory to keep the starch solution separate from the citrate solution.

The presence of other salts during the titration makes the end-point less sharp. Too much nitrate, magnesium sulfate, or sodium chloride are examples. While it is not imperative it is very desirable for this reason to use sodium citrate, sodium nitrite, and phosphoric acid free from chlorides.

The final volume for titration bears an inverse ratio to the intensity and sharpness of the end-point. This makes it desirable to perform the titration in as small a volume as possible.

Finally as pointed out by Van Slyke and Donleavy the blue color is not produced by the starch until a considerable but definite excess of potassium iodide solution has been added. They give this amount as 0.15 cc. but it may vary, even under optimum conditions, with the character of the starch and other factors discussed above. It is therefore very important to determine the amount of this excess for every new buffer solution, silver nitrate solution, or starch solution.

Bearing in mind the above factors it has been the purpose of the writer to make the titration as sensitive and flexible as possible. The final volume for titration has been considerably reduced, the starch solution has been made from corn-starch and kept separate, phosphoric acid has been added to the citrate solution, and finally the potassium iodide solution has been made slightly stronger.

Preparation of Solutions.

1. Trichloroacetic Acid 20 Per Cent.—A simple procedure for making this solution when needed is as follows: Counterpoise a small beaker or flask on ordinary laboratory scales. Weigh directly into the beaker or flask enough trichloroacetic acid crystals to give a 20 per cent solution of the volume desired. Then add the proper amount of distilled water with a pipette or small cylinder. Great accuracy is not required in making up this solution.

2. Starch Solution.—Place 5 gm. of corn-starch or potato starch in a liter beaker containing about 400 cc. of distilled water. Heat the mixture to

boiling with stirring to prevent scorching. After it once begins to boil the stirring is no longer necessary. Continue the boiling gently for 5 hours keeping the volume of the fluid at about 400 cc. by adding distilled water from time to time. Then pour the solution into a liter flask and make to 1,000 cc. when cool. Filter through a good thickness of cotton and the solution is then ready for use. The solution is quite opalescent but gradually settles out forming a clear supernatant portion. It is not necessary to wait for this before using. (The solution can be cleared by filtering through filter paper but it soon fills the pores and the filtration is extremely slow.) Add about 10 drops of xylene or toluene and thoroughly mix with the solution. This will prevent the formation of moulds or growth of bacteria. Such a solution prepared by the writer has kept perfectly for 5 months with contant use.[12]

3. Buffer Solution.—Place in a liter flask 446 gm. of sodium citrate ($5\frac{1}{2}$ mols H_2O) and 50 cc. of 80 per cent phosphoric acid syrup. Make the whole up to a volume of about 800 cc. with distilled water and dissolve with the aid of boiling. Pour this solution quantitatively into a liter volumetric flask and allow to cool. To about 100 cc. of distilled water add 20 gm. of sodium nitrite. This will dissolve without the aid of heat. When it is dissolved, pour into the cooled citrate-phosphate solution, mix, and make to volume. This solution may then be filtered, if necessary, and it is ready for use.[12]

4. Silver Nitrate Solution.—

Silver nitrate..... 11.624 gm.
Nitric acid (sp. gr. 1.42)..... 300 cc.
Make to 1,000 cc. with distilled water.

1 cc. of this solution is equivalent to 4 mg. of NaCl.

5. Potassium Iodide Solution.—1 cc. of this solution is equivalent to 0.8333 mg. of NaCl.

Dissolve 6.0 gm. of potassium iodide in 2,000 cc. of distilled water. Standardize this solution against the silver nitrate solution as follows: To 10 cc. of distilled water, accurately measured, add 2 cc. of silver nitrate solution from an Ostwald pipette accurately standardized for blow-out delivery. After mixing, pipette 10 cc. of this solution into a 50 cc. Erlenmeyer flask and then add 2.5 cc. of the buffer solution and 1.0 cc. of the starch solution. (Mohr pipettes may be used for the buffer and starch solutions.) Titrate with the potassium iodide solution to the first permanent blue color. It should take about 8.02 to 8.06 cc. as discussed under "End-point." Make the necessary dilution of the potassium iodide solution and then carefully standardize again both for end-point (see "End-point") and strength.

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[12] This solution may be used in the titration of either Foster's method or the method of Bell and Doisy by using double the quantity required for the titration described in this paper.

End-Point.

The end-point should be a pure, permanent blue. The permanency of the end-point may be determined by shaking gently for 30 seconds to see if it fades. I have found it desirable to run the titration not to the first permanent blue but 1 drop past this point to a deeper blue. If the potassium iodide solution is standardized for such an end-point the procedure is unquestionable. All subsequent titrations should be run to the same depth of color. When about 0.2 cc. from the end-point, if 5 or 6 drops of potassium iodide solution are added at once a false end-point will appear and fade in about 15 to 45 seconds. This is due to the presence of chlorides in the indicator solutions. It is therefore best to approach the end-point a drop at a time. This will insure a definite and permanent end-point upon the addition of 1 drop (0.02 cc.) of potassium iodide and no confusion will result from fading and necessary addition of more potassium iodide until the permanent color is obtained.

The optimum conditions for the end-point for any particular set of solutions used may easily be determined. Place 5 cc. of a solution containing 850 to 900 mg. of NaCl per 100 cc. in a 50 cc. volumetric flask, add 5 cc. of 20 per cent trichloroacetic acid, make to the mark with distilled water, and mix. Accurately measure four 10 cc. portions into four 15 cc. centrifuge tubes. Add 2 cc. of the silver nitrate solution to each tube with an Ostwald pipette, insert a clean, dry stopper, vigorously shake the tube for a moment to flock out the silver chloride, and centrifuge. Accurately pipette 10 cc. of the supernatant fluid in each tube into 50 cc. Erlenmeyer flasks. Add 2.0, 2.5, 3.0, and 3.5 cc. portions of the buffer solution respectively to the four flasks. Then add 1.0 cc. of the starch solution to each flask and titrate[13] each to the desired permanent blue color. As all the silver nitrate will have been removed by the sodium chloride, the amount of potassium iodide solution added will represent the amount necessary to add

[13] It is best to use a small (5 cc.) burette for the titration as it permits of greater accuracy. The Folin sugar burette (supplied by Emil Greiner Company, New York) is very well adapted as it is of 5 cc. capacity and graduated in 0.02 cc. The tip may be drawn out slightly in the flame to make the drops smaller. It is possible to do this so that 1 drop will be 0.02 cc.

in excess of the neutral point in order to obtain the blue color. Ordinarily the flask containing only 2 cc. of buffer solution will require 0.06 to 0.08 cc. The others will require 0.02 to 0.05 cc. The best color will usually be given by the flask containing 2.5 cc. of buffer solution, and I have found that the end-point is quite intense when 0.04 cc. of the potassium iodide solution has been added. However, as pointed out this will vary with the solutions. When this procedure has been completed the potassium iodide solution can then be accurately diluted so that $8.0x$ cc. will be required for a blank determination where x cc. is the amount required for an optimum end-point.

Procedure for Blood Chloride Determination.

1. Preparation of Blood Filtrate.—Carefully measure (an automatic burette is convenient) 40 cc. of distilled water[14] into a clean, dry flask or tube. Then run 5 cc. of plasma, whole blood, or other fluid to be determined into the water from a pipette graduated to contain 5 cc. (The determination may be done with as little as 2 cc. of plasma with proportional quantities of water and trichloroacetic acid.) Blow out the last drop and then rinse out the pipette twice by sucking up the mixture of water and blood, and finally blowing out the pipette. Accurately add 5 cc. of the trichloroacetic solution, stopper, and shake at intervals for 10 to 15 minutes. Filter the mixture through a dry chloride-free filter[15] into a dry tube or flask. The filtrate is then ready for the chloride or other determinations.

[14] It is impossible to obtain C.P. trichloroacetic acid on the market at present. The U.S.P. grades give a filtrate from which silver chloride will not completely precipitate. This trouble is not experienced with pure trichloroacetic acid. The difficulty is easily remedied by the use of 5 cc. of 95 per cent ethyl alcohol (or ethyl alcohol denatured with methyl alcohol; methyl alcohol alone is not so satisfactory) in the precipitating mixture. This may best be done by adding 100 cc. of alcohol to 700 cc. of distilled water and using 40 cc. of this mixture for diluting the blood. The filtrates are suitable for the other determinations as well as chloride.

[15] The use of chloride-free filter paper should be emphasized. Two chloride determinations on a blood were made using the method of Van Slyke and Donleavy with Alpha paper (chloride-free) and a good grade of Whatman paper. The filtrate from the Whatman paper gave a result 1.2 per cent higher than that from the Alpha paper. Of course this danger is perhaps not so great when solutions containing no silver nitrate are filtered.

2. Chloride Determination.[16]—Measure 10 cc. of the filtrate into a 15 cc. centrifuge tube, add 2 cc. of the silver nitrate solution as has been described, stopper, shake vigorously, and then centrifuge. (The silver chloride, especially in whole blood determinations, will be in very fine suspension appearing to be colloidal but after vigorous shaking and centrifuging a perfectly clear supernatant solution is obtained.) Pipette 10 cc. of the clear supernatant solution into a 50 cc. Erlenmeyer flask and add the proper amount of buffer solution (as indicated by the end-point determination) and 1.0 cc. of starch solution. Titrate to the desired permanent blue color with the potassium iodide solution.[13]

3. Calculation.— $8.0x$ − cc. KI = gm. of NaCl per liter of blood, where x represents the cc. of potassium iodide solution necessary to give the end-point.

It is evident that the highest blood chloride that may be determined is 800 mg. of NaCl per 100 cc. of blood. In a large number of determinations on pathological bloods the writer has never encountered such a high blood chloride. If such a blood should occur, however, the trichloroacetic acid filtrate may be diluted with an equal volume of distilled water and the determination made in the way described. The result is then multiplied by two.

RESULTS.

Table I gives a comparison of figures obtained by the method described with those of Bell and Doisy, Van Slyke and Donleavy, McLean and Van Slyke, and the digestion method of von Korányi.[17] All plasmas, unless otherwise stated, which were used for comparison with the method of Van Slyke and Donleavy were perfectly free from hemolysis. Merck's blood charcoal was used in the McLean-Van Slyke determinations. With each determination a known chloride solution as well as a blank was determined. These

[16] This determination may also be made by placing 10 cc. of filtrate in a 25 cc. flask, adding 5 cc. of a silver nitrate solution containing per liter 5.812 gm. of $AgNO_3$ and 250 cc. of HNO_3 (sp. gr. 1.42), making to mark, centrifuging, and titrating 20 cc. of the clear supernatant solution with N/73.1 KI after the addition of 5 cc. of buffer solution and 2 cc. of starch solution. Calculation: 10. xx − cc. KI = gm. of NaCl per liter of blood, where xx is the amount necessary for the development of the end-point.

[17] von Korányi, A., *Z. klin. Med.*, 1897, xxxiii, 1.

figures are not included in the table but in all cases the variation from theoretical was less than 1 per cent.

Table II gives a series of nine complete determinations on the same plasma in comparison with the digestion method of von Korányi. The purpose of this table is to show approximately the

TABLE II.

Comparison of Nine Complete Analyses of a Plasma with That of a von Korányi Digestion Determination.

Trichloroacetic acid filtrates.	von Korányi. 10 cc. plasma used.
mg.	*mg.*
582	583
579	
584	
583	
582	
583	
584	
583	
585	
Average..... 583	

degree of accuracy of the method. With careful work errors should be less than 1 per cent. The titration is capable of even greater accuracy as the variations very probably occur in the precipitation of the blood proteins.

CONCLUSION.

1. The determination of blood chlorides by several different methods is discussed.

2. It is pointed out that the simultaneous precipitation of blood proteins and chlorides is open to error.

3. A modification of the McLean-Van Slyke starch-citrate-nitrite solution is introduced.

4. The use of trichloroacetic acid filtrates for determination of chlorides is described.

I wish to thank Professor Otto Folin and Professor Richard D. Bell for helpful suggestions and encouragement during my work. I am also indebted to Mr. J. C. Whitehorn who kindly made the chloride determinations according to the method of von Korányi.

A SYSTEM OF BLOOD ANALYSIS.*

SUPPLEMENT II.

SIMPLIFIED METHOD FOR THE DETERMINATION OF CHLORIDES IN BLOOD OR PLASMA.

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(Received for publication, December 29, 1920.)

The method of preparing protein-free filtrates by the use of tungstic acid, as developed and used by Folin, has proved so highly satisfactory in the determination of a number of blood constituents, that it has seemed desirable to apply it to the determination of chlorides.

The procedure developed for this purpose is based upon the principle of the Volhard method;[1] namely, the precipitation of silver chloride from a known amount of silver nitrate and titration of the excess silver nitrate by means of sulfocyanate, using ferric ammonium alum as an indicator.

A number of preliminary experiments were conducted to ascertain the most convenient conditions which would give a good end-point. Efforts to obtain increased accuracy by greatly increased dilution of reagents were disappointing. In order to insure a sharp end-point, the volume of fluid at the end of titration must be kept small, which cannot be done when very dilute solutions are used. As a result of dilution the end-point tends to be yellowish, rather than red, and cannot be accurately perceived, at least by the writer's eye, except in strong daylight. This difficulty in the use of very dilute solutions is somewhat increased by the presence of oxalate, because of the lemon-yellow color of

* By agreement between Mr. Whitehorn and myself, this paper is published as Supplement II of the "System of blood analysis" devised by Folin and Wu.—*Professor Otto Folin.*

[1] Volhard, J., Ueber eine neue methode der maassanalytischen Bestimmung des Silbers, *J. prakt. Chem.*, 1874, ix, 217.

iron oxalate. It was presumably for this reason that Rappleye's method for plasma chlorides[2] required the use of citrated rather than oxalated plasma. The personal element in the reading of such faint end-points is perhaps rather large, for Myers and Short have very recently published fairly accurate results obtained on picric acid filtrates by a modified Volhard-Arnold method in which they used ammonium thiocyanate of only M/117 strength.[3] The writer, however, has found it more satisfactory to use a thiocyanate solution of M/35.5 strength, of which 0.03 cc. gives, under the conditions specified, an unmistakable end-point.

For reasons which will appear below, it was necessary to avoid the customary procedure of filtration or centrifugalization of the silver precipitate. This simplification has been accomplished without loss of accuracy by the liberal use of nitric acid and ferric alum. Accurate results were obtained on known chloride solutions, with or without the addition of small amounts of tungstic acid.

The following method was thereupon developed.

I. Preparation of Protein-Free Filtrates.—The filtrate is prepared by the use of the same reagents as have been described in detail by Folin and Wu[4] for the determination of non-protein nitrogen, urea, uric acid, creatinine, creatine, and sugar. Because even slight variations in the chlorides are significant, great accuracy is necessary. The writer customarily uses volumetric flasks in order to insure an accurate 1:10 dilution. The method is applicable, without alteration, to either whole blood or plasma. An amount of filtrate equivalent to 1 cc. of blood or plasma is needed. Less may be used but with proportionate loss of accuracy.

II. Determination of Chloride Content of Filtrate.—(*a*) *Reagents Required.*—1. Silver nitrate solution (M/35.46).

2. Potassium (or ammonium) sulfocyanate (M/35.46).

3. Powdered ferric ammonium sulfate ($FeNH_4(SO_4)_2$).

4. Concentrated nitric acid (HNO_3 of specific gravity 1.42).

[2] Rappleye, W. C., A simple application of the Volhard principle for blood plasma chlorides, *J. Biol. Chem.*, 1918, xxxv, 509.

[3] Myers, V. C., and Short, J. J., The estimation of chlorides in blood, *J. Biol. Chem.*, 1920, xliv, 47.

[4] Folin, O., and Wu, H., A system of blood analysis, *J. Biol. Chem.*, 1919, xxxviii, 81.

(*b*) *Procedure.*—Pipette 10 cc. of the protein-free filtrate into a porcelain dish. Add with a pipette 5 cc. of the standard silver nitrate solution and stir thoroughly. Add about 5 cc. of concentrated nitric acid, mix, and let stand for 5 minutes, to permit the flocking out of the silver chloride. Then add with a spatula an abundant amount of ferric ammonium sulfate (about 0.3 gm.) and titrate the excess of silver nitrate with the standard sulfocyanate solution until the definite salmon-red (*not yellow*) color of the ferric sulfocyanate persists in spite of stirring for at least 15 seconds.

(*c*) *Calculation.*—

5.00 − titer (in cc.) = mg. of Cl per cc. of blood (or plasma)

Since each cc. of thiocyanate solution used is equivalent to 1 cc. of silver nitrate solution, the difference between the volume of silver nitrate solution taken and the excess determined by the titration, that is 5−titer, represents the volume which reacted with chloride at the ratio of 1 cc. to 1 mg. of Cl. And the 10 cc. of filtrate taken represents 1 cc. of blood (or plasma).

To convert Cl figures into NaCl figures divide by 0.606. The same result may be more easily obtained by the following rule: To obtain mg. NaCl per 100 cc., divide mg. Cl per liter by 6, and subtract 0.001 of the result. Conversely, to obtain mg. Cl per liter, add to mg. NaCl per 100 cc. 0.001 of itself and multiply by 6.

The examples in Table I illustrate the principles involved in the calculation.

TABLE I.

Specimen.	Titer.	Cl per cc.	NaCl per 100 cc.	
			By simple rule.	By long calculation.
	cc.	*mg.*	*mg.*	*mg.*
Filtrate A.....	0.75	(5 − 0.75) = 4.25	$\frac{1}{6}$(4,250) − 7 = 701	701
" B.....	2.25	(5 − 2.25) = 2.75	$\frac{1}{6}$(2,750) − 5 = 453	453
" C.....	1.35	(5 − 1.35) = 3.65	$\frac{1}{6}$(3,650) − 6 = 602	602

(*d*) *Preparation of Reagents.*—Dissolve 4.791 gm. of C.P. silver nitrate in distilled water. Transfer this solution to a liter volumetric flask and make up to the mark with distilled water. Mix

thoroughly and preserve in a brown bottle. 1 cc. = 1 mg. Cl. (It is to be noted that the silver nitrate and nitric acid are not added to the protein-free filtrate simultaneously. To do so may result in the mechanical enclosure of silver nitrate solution within the curds, and a consequent error in the positive direction.)

Because sulfocyanates are hygroscopic, the standard solution should be prepared volumetrically. As an approximation about 3 gm. of KCNS or 2.5 gm. of NH_4CNS should be dissolved in a liter of water. By titration under the conditions specified under "Procedure" and by proper dilution prepare a standard such that 5 cc. are equivalent to 5 cc. of the silver nitrate solution.

The solid ferric alum is used rather than a solution, in order to insure a very high concentration in the mixture to be titrated. It is powdered in order to facilitate its solution.

Remarks.

1. Essentially the same procedure and reagents may be used in determining urine chlorides, except for the silver nitrate solution, which should be of M/7.092 strength. When 5 cc. of urine and 5 cc. of this strong silver solution are used in urine chloride determination the calculation becomes $5 - \frac{\text{titer}}{5} = \text{mg. Cl per cc.}$ of urine.

2. When a determination of both urea and chlorides is desired on a small sample, as may sometimes occur in cases where nephritis is suspected, one may pipette 2 cc. of the plasma into a 25 cc. flask, dilute to about 20 cc. with water, add 2 cc. each of the tungstate and acid solutions, make up to the mark with water, and shake. This will give sufficient filtrate for both determinations, 5 cc. for urea by Folin's distillation method and 10 cc. for the chlorides by the method described. In either case the figure obtained must be multiplied by $\frac{5}{4}$, since the filtrate has been diluted 2 : 25 instead of 2 : 20.

3. The glassware should be checked to within at least 0.5 per cent.

4. Reagents must be halogen-free. Some samples of nitric acid contain much chloride. None of the samples of tungstate tested has contained chloride. To guard against the possibility

of contamination, however, all samples should be tested as follows before using: Mix one volume of 10 per cent sodium tungstate solution with two volumes of concentrated, chloride-free nitric acid, and filter into a test-tube containing silver nitrate solution. Turbidity indicates contamination with halogen.

5. At Dr. Folin's suggestion, purification of tungstates containing added chlorides has been accomplished by recrystallization with alcohol. Sodium tungstate containing 0.3 per cent NaCl was so nearly purified by one recrystallization that the above mentioned silver test gave only a faint opalescence, scarcely perceptible even by transmitted light—not enough to produce an appreciable error. After a second recrystallization no chloride whatever could be detected. The following procedure was used: To a cooled 50 per cent solution of the contaminated tungstate, prepared with the aid of heat, add slowly an equal volume of 95 per cent ethyl alcohol and let stand for 10 minutes. Pour the suspension of crystals on a Buchner funnel, wash twice with alcohol, and dry.

6. The writer's attention has very recently been called to the method published by Rieger for chloride estimation on tungstic acid filtrates.[5] Certain features of this article deserve comment.

(*a*) The method therein described retains the centrifugalization or filtration procedure, which at times causes erroneously high results on tungstic acid filtrates, whether or not there is sufficient tungstate present to give a precipitate on the addition of an equal volume of concentrated nitric acid. Incidentally, the absence of such a precipitate does not indicate the absence of tungstate, as Rieger has stated, for solutions containing as much as 25 mg. of sodium tungstate per 100 cc. may not give a precipitate with nitric acid except when heated.

(*b*) The article mentioned contains a method for the "purification of sodium tungstate;" *i.e.*, for the preparation of chloride-free tungstate. It is very difficult to believe that the method so designated really accomplishes its purpose, since it calls for the use of 7 cc. of 40 per cent sodium hydroxide solution for each 10 gm. of sodium tungstate taken. Preparations of sodium hydroxide always contain large amounts of chloride. On the other

[5] Rieger, J. B., The estimation of chlorides in whole blood, *J. Lab. and Clin. Med.*, 1920–21, vi, 44.

hand, none of the samples of sodium tungstate tested by the writer has contained perceptible amounts of chloride. It has therefore not been necessary to purify. Certainly "purification" should not be attempted by the use of sodium hydroxide.

(*c*) Instead of precipitating the plasma proteins before dilution it is preferable to dilute the plasma with distilled water before adding the acid, in order to insure an even distribution of chlorides between solution and precipitate.

(*d*) It is not necessary to wait an hour for protein precipitation. 5 minutes are sufficient. Time of standing before filtering off the precipitated protein causes no appreciable difference in the chloride determination, as shown by the following results on the same plasma: Stood for 5 minutes before filtering, 3.64 mg. Cl per cc.; 15 minutes, 3.68; 30 minutes, 3.68; 1 hour, 3.64; and $3\frac{1}{4}$ hours, 3.70.

Soundness of the Method.

The soundness of the method of chloride determination described obviously depends upon the answers to two questions:

I. Does the titration figure indicate accurately the excess of silver nitrate?

II. Does the amount of silver precipitation so found indicate accurately the chlorides of the plasma?

I. The first of these questions deserves careful attention. Many chemists will quite properly be very skeptical on this point. Sutton states[6] "In cases where chlorine is precipitated by excess of silver, and the excess has to be found by thiocyanate, experience has proved that it is absolutely necessary to filter off the chloride and titrate the filtrate and washings." Rosanoff and Hill[7] have shown that the error is due to the reaction of silver chloride with sulfocyanate. Their figures would indicate that silver chloride, when shaken in an equimolar water solution of ammonium sulfocyanate, reacts so rapidly as to precipitate 43 per cent of the sulfocyanate in 2 minutes. This occurs because

[6] Sutton, F., Systematic handbook of volumetric analysis, Philadelphia, 10th edition, 1911, 145.

[7] Rosanoff, M. A., and Hill, A. E., A necessary modification of Volhard's method for the determination of chlorides, *J. Am. Chem. Soc.*, 1907, xxix, 269.

silver chloride is more soluble than silver sulfocyanate. Harvey[8] has shown, however, that both ferric alum and nitric acid retard this reaction. By using nitric acid in a concentration of 5 per cent, he obtained practically identical results on known chloride solutions and on urines whether or not he filtered off the silver chloride. His results, indeed, indicate a slightly greater accuracy without filtration, but the difference is so small as to be entirely without significance (*e.g.* 0.7052 without filtration and 0.7069 per cent NaCl with filtration on a known 0.7039 per cent NaCl solution).

The following experiments were designed and carried out to test the accuracy of the titration in the presence of silver chloride. Since the purpose was to discover if the presence of silver chloride influenced the accuracy of the titration, all factors such as tungstate which might modify or conceal such an influence, had to be excluded. Therefore a pure solution of sodium chloride was substituted for the blood filtrate. A 0.1 N solution was prepared by dissolving in distilled water 0.5846 gm. of recrystallized, thoroughly dried sodium chloride, and making up to 100 cc. in an accurately calibrated flask. Then 1 cc. of this solution and 9 cc. of water were used instead of 10 cc. of blood filtrate, but all the subsequent details were followed as specified under the heading "Procedure." Eleven determinations were made in this manner. The chlorine content of 1 cc. of 0.1 M NaCl solution, as determined by these experiments, was: 3.55; 3.52; 3.56; 3.56; 3.54; 3.53; 3.56; 3.55; 3.56; 3.55; and 3.54 mg. The average was 3.547, compared to a theoretical value of 3.546 mg. The highest and lowest deviations were 0.7 and −0.3 per cent. The presence of silver chloride during the titration had therefore produced no error.

Similarly the chlorine content of 4 cc. of 0.1 M NaCl solution, as determined in the same manner (which of course necessitated the use of 3 cc. of M/7.092 $AgNO_3$ solution instead of 5 cc. of M/35.46) in three such experiments, was found to be 14.28, 14.23, and 14.30 mg., compared to the theoretical value of 14.2 mg. Here also the presence of chloride produced no error in titration.

As a final crucial test, another determination was made on 1 cc.

[8] Harvey, S. C., The quantitative determination of the chlorids in the urine, *Arch. Int. Med.*, 1910, vi, 12.

of 0.1 M NaCl solution, but with this difference, that the mixture containing silver chloride was centrifugalized and an aliquot portion of the water-clear supernatant liquid taken for titration. The whole mixture measured 15 cc. and the titration of 10 cc. of the supernatant liquid required 0.97 cc. of M/35.46 KCNS solution. Since $5 - \frac{3}{2}(0.97) = 3.55$, it is evident that the method of titration gives the same result whether or not the silver chloride is removed.

The essential part of the procedure is the use of nitric acid, and the principle is a physical one—the flocking out of the silver chloride, with the consequent reduction of the surface exposed for reaction with the sulfocyanate. It is for this purpose that so large an amount of nitric acid is used, approximately 25 per cent of the volume of the mixture in which the titration is carried out.

Much higher concentrations of nitric acid should not be used, as the sulfocyanate is decomposed by them quite rapidly. To determine if such decomposition played an important part in the disappearance of the end-point, a small drop (0.03 cc.) of the M/35.46 KCNS solution was added to each of two dishes, one containing 10 cc. of 30 per cent nitric acid, and the other 10 cc. of 15 per cent nitric acid, and each containing about 0.3 gm. of ferric alum. The red color persisted in the first mixture for 15 minutes, in the second it was still present at the end of 15 hours. It is evident, therefore, that under the conditions which may occur in using this method, the decomposition of sulfocyanate by nitric acid is not rapid enough to affect the accuracy of the titration.

In accordance with the geometrical principle that volumes vary as the cubes of a dimension, whereas surfaces vary as the squares, the surface of the silver chloride, when flocked out, is so small that the reaction with ferric sulfocyanate, although probably still going on, is negligible. (The color due to 0.03 cc. of the M/35.46 KCNS solution persisted for 19 hours in the presence of such curds of silver chloride.) But the small amount of silver chloride which in spite of the nitric acid remains in fine suspension, has a larger aggregate surface and therefore reacts much more rapidly with the sulfocyanate.

Herein lies the explanation of the phenomena observed when KCNS is added to the mixture containing silver nitrate, silver

chloride, ferric alum, and nitric acid. The red color which appears with each drop disappears in about 3 seconds, because the ferric sulfocyanate reacts with the solution of $AgNO_3$. But when all the silver present as $AgNO_3$ has been precipitated, the red color which appears when the next drop is added persists about 30 seconds (from 10 seconds to 1 minute, depending upon how much of the drop was really excess, and upon the thoroughness of the flocking out of AgCl). Its disappearance is due to the reaction between the sulfocyanate and the very finest of the suspended particles of silver chloride. If another drop is added, the color will persist several minutes, for the sulfocyanate must then react with silver chloride particles of larger size and smaller aggregate surface. If still another drop is added the color will persist from 15 minutes to an hour or more (even for 19 hours),

TABLE II.

Centrifuge time.	Persistence of color ($Fe(CNS)_3$).			
	End-point.	Excess drops of KCNS solution.		
		First.	Second.	Third.
min.				
None.	40 sec.	4 min.	15 min.	More than 1 hr.
1	90 "	24 "	More than 1 hr.	
6	75 "	23 "	" " 1 "	
40	30 min.	More than 1 hr.		

for the fine suspension of silver chloride has all been used up, and the aggregate surface left for reaction is very small.

The influence of the silver chloride particles of different sizes can be demonstrated by fractional centrifugalization, as in the experiments given in Table II.

In addition to the action of nitric acid in flocking out the chloride, the abundance of ferric alum used also retards the reaction between silver chloride and sulfocyanate by reducing the ionization of the latter. This also deepens the end-point color by preventing the ionization of the red salt, $Fe(CNS)_3$, into yellow Fe^{+++} ions and colorless CNS^- ions.

II. There remains the second question: "Does the amount of silver precipitation, as found by titration of the excess, indicate accurately the chlorides of the plasma?"

Mixtures of tungstic acid and chloride bring down more silver than can be accounted for by the chloride alone. This additional precipitation of silver has been found by centrifugalization and titration of the supernatant fluid. Errors as large as 4 per cent were obtained in this way. Similar errors sometimes occurred when centrifugalization was introduced into the chloride determination of tungstic acid filtrates. The amount of error which may so occur is not always proportional to the amount of tungstic acid present, and so appears to be more probably dependent upon physical than upon chemical reactions.

This possibility of error is, however, entirely avoided in the method described, by carrying on the titration in the presence of the precipitate, when all the silver which has not been truly precipitated by chloride is available for titration. Determinations of the same 0.1 M NaCl solution gave practically identical results whether determined directly or after the addition of equal volumes of 10 per cent sodium tungstate solution and $\frac{2}{3}$ N sulfuric acid; for example, with tungstate, 3.53, 3.54, and 3.54 mg. of Cl per cc., and without tungstate, 3.547 as the average of eleven determinations.

The evidence given above has been presented in order to demonstrate that the method is free from error at the two points which seemed, *a priori*, the most probable sources; namely, the presence of tungstic acid at the time silver chloride is precipitated, and the presence of silver chloride at the time of titration.

Checks.

The final test of the accuracy of the method consists of course in its comparison with methods of known accuracy. The check determinations are given in Table III.

These check determinations indicate the essential accuracy of the method described. In order to determine the limit of error, seventeen duplicate determinations, involving nine separate precipitations with tungstic acid, were made on the same plasma, with the following results: 3.56; 3.51; 3.52; 3.54; 3.50; 3.55; 3.57; 3.54; 3.48; 3.49; 3.52; 3.50; 3.52; 3.53; 3.56; 3.54; 3.55 mg. of Cl per cc. The digestion method of von Korányi showed 3.53 mg. of Cl per cc. The average by the method described is 3.528. The

TABLE III.

Specimen.	Method described.		NaCl checked by method of
	Cl per cc.	NaCl	
	mg.	*per cent*	*per cent*
			Foster.*
Whole blood. M.	2.88	0.473	0.481
	2.88	0.473	0.479
Plasma. M.	3.69	0.608	0.619
	3.71	0.612	0.617
Whole blood. Wh.	3.06	0.503	0.504
Plasma. Wh.	3.84	0.633	0.635
			McLean-Van Slyke.†
Plasma I.	3.76	0.619	0.633‡
" II.	3.78	0.623	0.618‡
" III.	3.75	0.618	0.609‡
" IV.	3.74	0.617	0.606‡
			von Korányi.§
" V.	3.68	0.606	0.603
" VI.	3.80	0.626	0.619
" VII.	3.52	0.580	0.584
" VIII.	3.80	0.626	0.631
" IX.	3.52	0.582	0.582
Whole blood I.	3.24	0.535	0.542
Ascitic fluid I.	4.10	0.676	0.677
" " II.	3.78	0.623	0.625
			Bell and Doisy.‖
Plasma X.	3.75	0.619	0.614‡
" XI.	3.38	0.558	0.567‡
" XII.	3.64	0.600	0.596‡
" XIII.	3.70	0.610	0.605‡
" XIV (diluted).	2.84	0.467	0.465‡
Plasma XV.	3.86	0.637	0.641‡
Average		0.5885	0.5896

* Foster, G. L., A modification of the McLean-Van Slyke method for the determination of chlorides in blood, *J. Biol. Chem.*, 1917, xxxi, 483. (Allowance for end-point has been made, as introduced by Van Slyke and Donleavy (Van Slyke, D. D., and Donleavy, J. J., *J. Biol. Chem.*, 1919, xxxvii, 551).

† McLean, F. C., and Van Slyke, D. D., A method for the determination of chlorides in small amounts of body fluids, *J. Biol. Chem.*, 1915, xxi, 361.

‡ I am indebted to Mr. L. M. Smith for these determinations.

§ von Korányi, A., Physiologische und klinische Untersuchungen über den osmotischen Druck thierischer Flüssigkeiten, *Z. klin. Med.*, 1897, xxxiii, 1.

‖ Bell, R. D., and Doisy, E. A., *J. Biol. Chem.*, 1920–21, xlv, 427.

greatest deviations are −1.3 and +1.2 per cent. The limit of error with careful technique must therefore be less than 1.5 per cent.

SUMMARY.

1. A simple and rapid method is described for the determination of blood and plasma chlorides.
2. The same reagents as are used for the determination of urine chlorides are employed.
3. The method is especially adapted to the system of blood analysis developed by Folin and Wu.
4. The limit of error is less than 1.5 per cent.

The writer is indebted to Professor Otto Folin for encouragement in this work, and to Mr. L. M. Smith for assistance in checking determinations.

THE DETERMINATION OF CHLORIDES IN BLOOD PLASMA.

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(Received for publication, December 6, 1920.)

Van Slyke and Donleavy (1919) have published a method for the determination of chlorides in blood plasma, in which both proteins and Cl are precipitated together by a solution containing picric acid, nitric acid, and standard silver nitrate. The excess silver was titrated in the filtrate by the iodometric method of McLean and Van Slyke (1915). The results in a series of normal human plasmas were identical with those obtained by the method of McLean and Van Slyke, which involved two successive precipitations and filtrations; one of the proteins, the other of the silver chloride. It seemed, therefore, that the Van Slyke-Donleavy method, which involved only one precipitation and filtration, constituted a desirable simplification in the technique for determining plasma chlorides, although it was found still necessary to use the double precipitation for chlorides in whole blood (Austin and Van Slyke, 1920).

We have in the meantime tested the single precipitation method on plasmas from pathological cases by comparing the results with those of the double precipitation method used by Austin and Van Slyke on whole blood, and with those of the total chlorine determination by the Carius method (Table I). In the double precipitation method the preliminary precipitation of the proteins was performed as described by Austin and Van Slyke, except that for 3 cc. of plasma only 10 cc. of picric acid solution are used, instead of the 30 cc. used for 3 cc. of whole blood. The larger amount of picric acid is not needed for plasma, and if employed may result later, when nitric is added, in an undesirable separation of picric acid crystals. The Carius determination was carried out as described by Austin and Van Slyke.

TABLE I.

No.	Appearance of oxalate plasma.	Condition of donor.	Chloride, calculated as NaCl, per liter of plasma.					
			Carius method.		Double precipitation method.		Single precipitation method.	
			Separate determinations.	Average.	Separate determinations.	Average.	Separate determinations.	Average.
			gm.	gm.	gm.	gm.	gm.	gm.
1	Clear.	Normal.	6.02	6.08	6.08	6.08	6.07	6.08
			5.97		6.08		6.08	
			6.16					
			6.15					
2	"	"	6.23	6.27	6.24	6.24	6.32	6.31
			6.31		6.24		6.30	
3	"	"	6.09	6.11			6.07	6.05
			6.13				6.02	
4	"	"	6.06	6.07	5.98	6.03	6.18	6.13
			6.06		6.07		6.08	
			6.08					
5	Deep amber.	Nephritic.	5.29	5.30	5.29	5.30	5.48	5.49
			5.31		5.31		5.49	
6	" "	Cardiac.	5.87	5.92	5.86	5.86	6.05	6.04
			5.97		5.86		6.03	
			5.87					
			5.97					
7	Amber.	Nephritic.	5.87	5.90	5.95	5.95	6.09	6.14
			5.91		5.95		6.19	
			5.92					
8	Creamy opaque.	"			6.02	5.98	6.07	6.08
					5.94		6.08	
9	Clear.	Osteomyelitis.	5.76	5.73	5.72	5.70	5.88	5.87
			5.76		5.68		5.85	
			5.76					
			5.64					
10	"	Arteriosclerosis.	6.06	6.07	5.98	6.01	6.08	6.13
			6.06		6.03		6.18	
			6.08					

CONCLUSIONS.

We have encountered plasma specimens from hospital patients in which the single precipitation gave results indicating a higher chloride content than that obtained when a preliminary removal of the proteins was performed. When such disagreement occurred, the results by the Carius method confirmed those by the double precipitation method of Austin and Van Slyke.

Since the factors which interfere with the single precipitation method in pathological plasmas are not known, it is desirable in all plasmas, both normal and pathological, to remove the proteins by a preliminary precipitation, as in the Austin-Van Slyke method for whole blood, before the chlorides are precipitated with standard silver nitrate.

BIBLIOGRAPHY.

Austin, J. H., and Van Slyke, D. D., *J. Biol. Chem.*, 1920, xli, 345.

McLean, F. C., and Van Slyke, D. D., *J. Am. Chem. Soc.*, 1915, xxxvii, 1128; *J. Biol. Chem.*, 1915, xxi, 361.

Van Slyke, D. D., and Donleavy, J. J., *J. Biol. Chem.*, 1919, xxxvii, 551.

AN IMPROVED APPARATUS FOR USE IN FOLIN AND WU'S METHOD FOR THE ESTIMATION OF UREA IN BLOOD.

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(Received for publication, December 21, 1920.)

In carrying out many determinations of urea in blood by the method of Folin and Wu[1] we encountered much difficulty in preventing frothing while distilling over the ammonia. Many of the usual antifoaming mixtures were tried with little success.

The apparatus described below was devised to overcome this difficulty and has been found to prevent entirely any liquid being carried over as froth into the receiving tube.

A glass tube with a bulb in the center (a 25 cc. pipette) is bent as shown in Fig. 1. A number of small holes are blown in the side, and a constriction is made at the end, of the tube from which distillation proceeds.

The large bubbles of foam, in passing through the numerous small holes, are broken up and the bulb in the tube further safeguards any possibility of liquid being carried over into the receiver.

This apparatus, somewhat enlarged, makes a perfect substitute for the distilling bulb used in the ordinary Kjeldahl determination of nitrogen.

[1]Folin, O., and Wu, H., *J. Biol. Chem.*, 1919, xxxviii, 81.

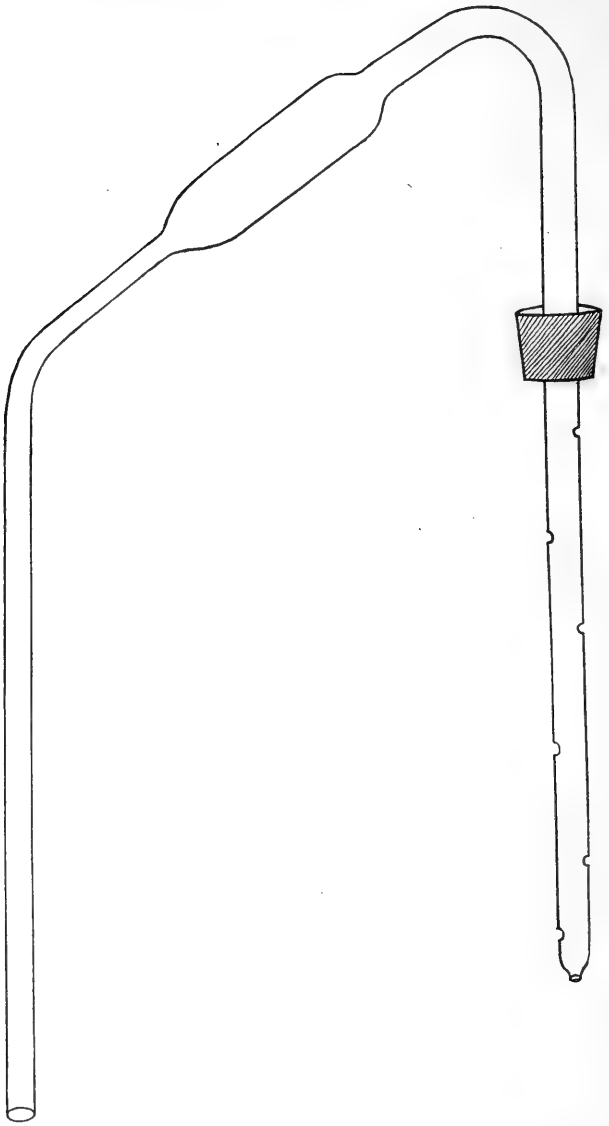

FIG. 1. Apparatus for use in urea determinations by the method of Folin and Wu.

THE CHEMICAL STRUCTURE OF CHONDRIDIN.*

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(Received for publication, December 31, 1920.)

Hebting,[1] working in Hofmeister's laboratory, has obtained on hydrolysis of chondroitin sulfuric acid a crystalline product which he named chondridin. Hebting recognized that the substance was related to chondrosin, but found that it differed from the latter in its elementary composition and its capacity to crystallize.

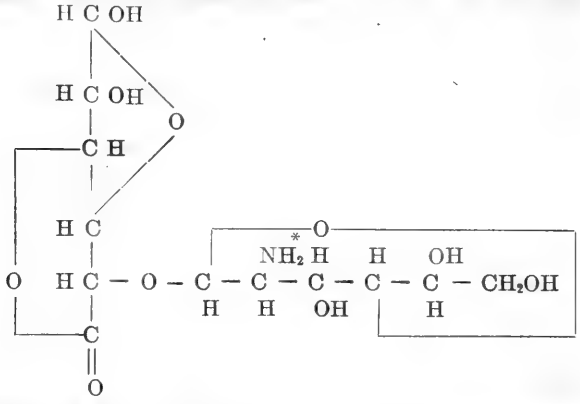
The structural representation of chondroitin sulfuric acid as formulated by Schmiedeberg, and later by Levene and La Forge, admits in the organic radical of the molecule the presence of only one derivative of chondrosin; namely, of its acetyl derivative. It therefore seemed probable that chondridin was not a primary decomposition product, but one formed from chondrosin through manipulation. This hypothesis was borne out by the present investigation. Hebting has found that the composition of chondridin differed from chondrosin by the presence in the molecule of a surplus of hydrogen and oxygen equivalent to 1 molecule of water.

A simple explanation of this difference may be given by assuming that chondridin consists of unchanged chondrosin containing a molecule of crystal water. On the other hand this assumption was scarcely adequate to explain the great difference in the capacity for crystallization of the two substances. More adequate seemed the assumption that chondridin was a lactone of chondrosin, crystallizing with crystal water. The substance

* I regret that the article of Schmiedeberg (Schmiedeberg, O., *Arch. exp. Path. u. Pharmakol.*, 1920, lxxxvii, 47) had escaped my attention until after this work was prepared for publication. The present communication incidentally contains an answer to his criticism on my theory of the place of linkage of chondrosamine and of glucuronic acid. Other criticisms of Professor Schmiedeberg have been met in previous publications. —P. A. L.

[1] Hebting, J., *Biochem. Z.*, 1914, lxiii, 353.

analyzed by Hebting apparently contained 2 molecules of crystal water. The samples analyzed by us seemed to contain $2\frac{1}{2}$ molecules. The graphic representation of the anhydrous substance would then be as follows:



* Allocation of NH_2 arbitrary.

The elementary composition of this substance containing $2\frac{1}{2}$ molecules of crystal water is $C_{12}H_{19}NO_{10} + 2\frac{1}{2}\ H_2O$.

The experimental evidence to be reported in this communication seems to support the second hypothesis.

The lactone structure of the substance was suggested by the method of preparation and was confirmed by the result of the titration of the aqueous solution with alkali. When to a cold solution of the substance 0.1 N alkali is added rapidly, the solution reacts neutral after the addition of 1 drop of the alkali (alizarin being used as indicator). When to the aqueous solution of the substance the alkali was added in excess, and when the alkaline solution was titrated back after standing 18 hours, it was found that 0.1000 gm. of the substance neutralized 2.55 cc. of 0.1 N alkali. On this basis the molecular weight is calculated to 382, while the theory for $C_{12}H_{19}NO_{10} + 2\frac{1}{2}\ H_2O$ is 392. Thus, of the two assumptions that of the lactone structure of the substance seems the more acceptable.

The substance reduces Fehling's solution, has its primary amino group unsubstituted, and yields a quantity of furfurol which approximates the one required by theory for chondrosin.

The air-dry substance on drying under diminished pressure at the temperature of water vapor loses $1\frac{1}{2}$ molecules of crystal

water. If the lactone structure of the substance is correct one has to accept that under the given conditions of drying the resulting substance still retains 1 molecule of crystal water.

EXPERIMENTAL PART.

The mode of preparation of the substance differed in its details from that of Hebting. The analytical data published by this author for chondridin seemed to agree with those required by theory for chondrosin oxalate. At the outset of the work it was planned to test this possibility. Chondrosin chlorohydrate was prepared following the conditions employed in the earlier work by Levene and La Forge. The chondrosin chlorohydrate obtained in this manner was freed from hydrochloric acid. To the aqueous solution of chondrosin a slight excess over one equivalent of oxalic acid was added and to the solution alcohol was added to opalescence. On standing there was no evidence of crystallization. However, when the solution was allowed to stand on a boiling water bath for an hour prior to the addition of alcohol, crystallization did take place. Alcohol was added to marked opalescence and the solution was allowed to remain on the water bath until it clarified. On scratching along the walls of the beaker a crystalline deposit soon began to form. The substance was recrystallized by dissolving in hot water, adding to the aqueous solution 99.5 per cent alcohol to opalescence, and boiling the solution until it clarified. After three or four recrystallizations the substance contained only traces of mineral impurities.

In later experiments the preparation of the substance was somewhat simplified; namely, no attempt was made to isolate the chondrosin hydrochloride before the digestion with oxalic acid. The procedure was as follows. Portions of 50.0 gm. of the barium salt of chondroitin sulfuric acid were hydrolyzed by heating for 1 hour on the water bath in a solution of 150 cc. of 20 per cent hydrochloric acid. From the product of reaction barium and hydrochloric acid were removed and the solution was concentrated to a small volume under diminished pressure. The subsequent treatment was as above described.

Properties of the Substance.—The lactone is a white crystalline powder. It does not melt, but contracts and turns dark at 200°C. The analysis of the substance was as follows:

1. 0.1176 gm. of the substance (No. 43) dried to constant weight at the temperature of water vapor and under diminished pressure lost 0.0078 gm.

2. 0.1130 gm. of another sample (No. 81) under the same conditions lost 0.0075 gm.

	Calculated for $C_{12}H_{21}NO_{11} + 1\frac{1}{2}\ H_2O$. *per cent*	Found. *per cent*
H_2O	7.20	6.63

1. 0.1698 gm. of the above substance gave on combustion 0.1626 gm. of CO_2 and 0.0580 gm. of H_2O.

0.1867 gm. of the substance used for Kjeldahl nitrogen estimation required for neutralization 5.3 cc. of 0.1 N acid.

For the amino estimation 0.050 gm. of the substance was dissolved in 5 cc. of water.

2 cc. of the solution in the Van Slyke micro apparatus gave 1.36 cc. of nitrogen gas at $T = 20°C.$ and $P = 764$ mm.

2. 0.1055 gm. of the substance gave 0.1564 gm. of CO_2 and 0.0568 gm. of H_2O.

	Calculated for $C_{12}H_{19}NO_{10} + H_2O$. *per cent*	Found. No. 1. *per cent*	Found. No. 2. *per cent*
C	40.54	40.43	40.11
H	5.96	5.98	5.92
N	3.94		3.97
$NH_2\ N$	3.94		3.89

The rotation of the air-dry substance was as follows:

$$[\alpha]_D^{20} = \frac{+ 0.97 \times 100}{1.6 \times 1} = + 60.6°$$

Titration of the Substance with Alkali.—0.1000 gm. of the substance was dissolved in 25 cc. of water and titrated with 0.1 N sodium hydroxide. Alizarin was used as indicator. After the addition of the first drop the solution reacted neutral. 0.1000 gm. of the substance was dissolved in 25 cc. of water. 15 cc. of 0.1 N alkali were added and the solution was allowed to stand over night. It required 12.45 cc. of 0.1 N acid to titrate the solution to neutral.

	Calculated for $C_{12}H_{19}NO_{10} + 2\frac{1}{2}\ H_2O$.	Found.
Molecular weight	382.17	392

Furfurol Estimation.—0.2000 gm. of the substance was distilled in the usual way with hydrochloric acid having a specific gravity of 1.06. The yield of the phloroglucide was 0.0248, which corresponds to 0.00744 gm. of glucuronic acid.

	Calculated for $C_{12}H_{19}NO_{10} + 2\frac{1}{2}\ H_2O$. *per cent*	Found. *per cent*
Glucuronic acid	50.77	37.2

Taking into consideration the limit of error of the method the result is not unsatisfactory.

SULFOCYANATE CONTENT OF THE SALIVA AND URINE IN PELLAGRA.

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(Received for publication, December 27, 1920.)

I. The Sulfocyanate Content of the Saliva.

In previous work on the saliva of pellagra patients[1] at the United States Pellagra Hospital, Spartanburg, South Carolina, it was found that the sulfocyanate reaction with ferric chloride and dilute hydrochloric acid was much less marked for the salivas of the pellagra patients than for normal people.

Subsequent to the general survey of the saliva and the testing of the saliva with ferric chloride, Sullivan and Jones, using the very accurate Rupp-Schied[2] method, improved by Thiel[3] made quantitative estimations of the sulfocyanate content of the saliva of seven patients who were somewhat improved as regards general physical condition and extent of erythema and dermatitis. The sulfocyanate content of the saliva of these seven cases estimated as KSCN varied from 8 to 25.8 parts per million with an average of 18.6 parts per million. These quantitative findings were utilized by Sullivan and Jones as a basis for the conclusion[4] that the sulfocyanate content of patients, even in a convalescing stage, was less than normal.

In 1919 the study of the sulfocyanate content of the saliva of pellagra patients was again taken up. The type of case admitted to the hospital in 1919, however, was somewhat different from those of 1917. In 1917 the patients admitted were rather marked

[1] Sullivan, M. X., and Jones, K. K., *Public Health Rep., U. S. P. H.*, 1919, xxxiv, 1068.

[2] Rupp, E., and Schied, A., *Ber. chem. Ges.*, 1902, xxxv, 2191.

[3] Thiel, A., *Ber. chem. Ges.*, 1902, xxxv, 2766.

[4] Sullivan and Jones,[1] p. 1076, foot-note 5.

cases of pellagra on the whole, while in 1919 the cases were of a milder type and in better physical condition. The difference in the two sets of patients held for the saliva. Thus in 1917, out of 40 cases tested in the hospital, the saliva of 32 gave no perceptible pink or red color with ferric chloride with or without the addition of hydrochloric acid, while in 1919, of 66 cases so tested, 47 were faintly or strongly positive. Thus, crude as the ferric chloride test admittedly is, it showed difference between the salivas of the 1917 patients and of the 1919 patients.

Two quantitative methods had been tested in the laboratory. Munk's[5] gravimetric method and the Rupp and Schied[2] method improved by Thiel.[3] In the Munk method, the sulfocyanate is precipitated by silver nitrate in the presence of dilute nitric acid and the silver precipitate melted with sodium carbonate and potassium nitrate to convert the sulfur of the sulfocyanate to sulfate. From the sulfur in the sulfate precipitated by barium chloride the sulfocyanate is calculated. The Rupp and Schied method improved by Thiel is an iodometric method.

The method is based on the fact that sulfocyanate in the presence of excess of bicarbonate decolorizes large amounts of iodine solution as illustrated by the following equation.

$$KSCN + 4\ I_2 + 4\ H_2O = H_2SO_4 + 6\ HI + KI + CNI$$

The process is completed in the course of 4 hours at ordinary temperature. Then with careful acidification with hydrochloric acid[3] hydriodic acid is liberated and the cyanogen iodide decomposed.

$$HI + CNI + HCN = I_2$$

The entire process can be expressed as follows:

$$KSCN + 3\ I_2 + 4\ H_2O = H_2SO_4 + 5\ HI + KI + HCN$$

1 molecule of sulfocyanate is thus equivalent to 6 atoms of iodine as iodide.

The Rupp-Schied-Thiel method, which was found very satisfactory by Gies and his collaborators[6] in their extensive study of sulfocyanate in the mammalian body, was preferred by us to the Munk method. For the saliva work done at the hospital the procedure is given below.

After thoroughly rinsing the mouth with water the saliva was collected under the stimulus of chewing white paraffine wax. Obviously, it was not possible to get 24 hour samples of saliva so a sample collected for a definite length of time, in most cases 30 minutes, was used for comparison. This sample was collected between 10 and 11 a.m. or approximately 1½ hours before the noon meal. The samples collected shortly after the pa-

[5] Munk, I., *Virchows Arch. ges. Physiol.*, 1877, lxix, 350.

[6] Gies, W. J., and Kahn, M., *Dental Cosmos*, 1913, lv, 40; Gies, W. J., Lieb, C. C., and Kahn, M., *Dental Cosmos*, 1914, lvi, 175.

tient's entrance to the hospital and shortly before discharge were compared. The saliva was expectorated into a beaker, while the subject directed his entire attention to the collection of saliva.

The specimens thus obtained were carefully measured, ranging in volume from 10 to 100 cc., and transferred to suitably sized Erlenmeyer flasks. The mucin was precipitated by treatment with 1 per cent acetic acid and moderate heating on a hot plate; and the coagulated matter, as well as any other solid material present, was removed by filtering through folded filters. It was discovered that salivas vary widely in the ease with which this precipitation is effected and in some cases filtration was rendered difficult through plugging of the filter with colloidal matter. The residue and filters were thoroughly washed with warm water to remove any traces of $-CNS$ that might be retained thereon; and the combined filtrates and washings were transferred to 200 cc. volumetric flasks and made up to volume. In later work it was found advisable to add a little 95 per cent alcohol to these preparations before making up to volume. The addition of a small amount of alcohol in no way interfered with the subsequent determinations and prevented the development of microorganisms, in case the material was to stand any length of time before analysis, and aided in preventing the development of colloidal precipitates when treated with silver nitrate in the next stage of the process.

Of the 200 cc. preparations, definite quantities, in most cases 75 cc., were measured with a pipette into moderate sized beakers for duplicate determinations. The material was acidified with dilute HNO_3 and the $-CNS$ precipitated as the silver salt by addition of an excess of $AgNO_3$. Owing to the already great dilution, it was found more satisfactory to add the $AgNO_3$ in the form of a concentrated solution, approximately 25 per cent, drop by drop, accompanied by thorough agitation with a glass rod, until a slight excess was reached, instead of using the dilute 3 per cent solution generally employed with other types of material. To facilitate sedimentation a small amount of infusorial earth (previously calcined, washed in HNO_3, and dried) was added, thoroughly stirred in, and the solution placed upon a boiling water bath for about 10 minutes. Filtration was effected by means of suction through an ordinary filter paper on a platinum cone. The filtrate should be perfectly clear. If not, and this frequently occurred, it was passed through the filter again after the addition of another small amount of infusorial earth, the process being repeated until an absolutely clear filtrate was obtained. At this stage in the process some salivas gave a good deal of trouble on account of the formation of colloidal precipitates after the addition of $AgNO_3$, making them difficult or practically impossible to filter. However, with few exceptions, it was found that repeated passing through the filter and liberal use of infusorial earth would result in complete separation of the material. The contents of the beaker were thoroughly washed out into the filter, using a rubber "policeman," and the precipitate on the filter was likewise thoroughly washed with 1 per cent HNO_3.

The filter and precipitate were then transferred to a wide necked Erlenmeyer flask of 1 liter capacity, 3 gm. of $NaHCO_3$, 3 gm. of KI, and a little water added, and the whole was stirred until the filter paper was completely disintegrated. In practice it was found more convenient to employ solutions of KI and $NaHCO_3$ and add volumes of each sufficient to provide the equivalent of 3 gm. in each case. The KI is added to convert any chlorides present to iodides and thus prevent reaction with the standard iodine solution added later; 3 gm. were found more than sufficient for the quantities encountered in this work.

To the mixture was now added, quantitatively, standard iodine solution until the brown color persisted after several minutes gentle agitation. A standardized iodine solution of 0.05 N strength was used and 10 cc. of this, measured out with a calibrated pipette, were found to be sufficient to provide excess in any of the material worked with. The advantage of adding a uniform quantity, eliminating difficult burette readings, is obvious. The flask was now stoppered tightly with a paraffined cork and set away in a dark place at room temperature for a period of 4 or 5 hours, which is sufficient time for completion of the reaction at ordinary temperature.

At the expiration of this time the mixture was acidified with 10 per cent HCl, using caution to avoid too rapid evolution of CO_2 and hence risk loss of volatile iodine; a few cc. of dilute starch paste (about 3 per cent) were added; and the excess of iodine was titrated with a standard 0.05 N sodium thiosulfate solution. The difference between the amount of 0.05 N iodine originally added and the excess thus determined represents the iodine which has reacted with the −CNS. Since, as shown previously, 1 molecule of −CNS corresponds to 6 atoms of iodine under such conditions, 1 cc. of 0.05 N iodine corresponds to 0.81015 mg. of −CNS, expressed as KCNS, or 1 cc. of 0.1 N iodine corresponds to 1.6203 mg. of KCNS. Likewise the number of cc. of iodine reacted multiplied by the factor 0.81015 for 0.05 N or 1.6203 for 0.1 N will give the quantity of −CNS expressed in mg. of KCNS.

It was found that this method, carried out as just described, gave uniformly accurate results even with the small quantities of material sometimes available for analysis and with the small concentration of −CNS in many samples, provided proper precautions were taken to insure absolutely correct standardization of solutions and to correct for marked changes in temperature or other conditions. Determinations made upon a pure solution of KCNS of known strength comparable to the quantity of sulfocyanate calculated as KSCN in the saliva, gave results with an error of less than 1 per cent.

Though it has not been proved that the sulfocyanate exists in the body as a potassium salt, in our work it is always computed

as KSCN. The results with the saliva of the Pellagra Hospital patients are given in Tables I and II. In Table I the results

TABLE I.

Sulfocyanate Content of Saliva Collected in 30 Min.

Status of pellagra at time of testing.					
Active.		Convalescent.		Normal.	
Case No.	K S C N	Case No.	K S C N	Case No.	K S C N
1919	*mg.*		*mg.*		*mg.*
579	0.88	555	1.41	1	1.82
586	3.53	556	1.55	2	1.95
10*	0.50	557	0.69	3	2.43
11	1.05	563	2.20	4	1.35
12	1.67	567	4.36	5	1.37
15	1.01	572	1.24		
16	2.67	577	1.36		
19	0.49	582	3.70		
20	0.63	586	8.18		
21	1.90	11	4.79		
22	0.61	12	4.36		
23	1.07	15	1.35		
24	0.89	16	3.66		
26	1.61	19	1.25		
28	1.80	20	0.90		
29	1.28	21	2.13		
30	1.36	22	1.61		
31	1.89	23	1.47		
32	2.21	24	2.24		
33	0.39	26	2.56		
34	1.04	28	1.08		
		29	1.28		
		30	5.14		
		31	3.32		
		32	3.57		
		33	0.93		
Average ..	1.36		2.55		1.78

*A new series of case numbers was started July 1.

from all patients tested are given. In Table II only those cases where the saliva was tested within a few days after admission and also within a few days of their discharge are presented.

With the exception of Case 28, which gives a lower value for the sulfocyanate content of the saliva at discharge than at entrance, and Case 29 with the same sulfocyanate content at entrance and at discharge, the sulfocyanate content of the saliva is lower at entrance than at discharge from the hospital.

Table II shows that the increase of the sulfocyanate is not proportional to the increase in volume of saliva. Thus in three

TABLE II.

Comparison of K S C N in Saliva at Admission and at Discharge.

Case No.	First test.			Last test.		
	Volume of saliva.	K S C N in sample.	K S C N per 100 cc. saliva.	Volume of saliva.	K S C N in sample.	K S C N per 100 cc. saliva.
	cc.	*mg.*	*mg.*	*cc.*	*mg.*	*mg.*
586	35	3.53	10.09	66	8.18	12.40
11	54	1.05	1.94	95	4.79	5.04
12	36	1.67	4.64	54	4.36	8.07
15	50	1.01	2.02	50	1.35	2.70
16	84	2.67	3.18	87	3.66	4.21
19	49	0.49	1.00	55	1.25	2.27
20	40	0.63	1.58	59	0.90	1.53
21	60	1.90	3.17	38	2.13	5.61
22	48	0.61	1.27	62	1.61	2.60
23	71	1.07	1.51	70	1.47	2.10
24	55	0.89	1.62	49	2.24	4.57
26	48	1.61	3.35	55	2.56	4.65
28	60	1.80	3.00	47	1.08	2.30
29	25	1.28	5.12	51	1.28	2.51
30	24	1.36	5.67	65	5.14	7.91
31	21	1.89	9.00	39	3.32	8.53
32	53	2.21	4.17	78	3.57	4.58
33	45	0.39	0.87	39	0.93	2.38
Average.....	47.7	1.45	3.51	58.8	2.77	4.66

cases (Nos. 15, 16, and 23) the volume is the same while the KSCN content is increased. In three other cases (Nos. 21, 24, and 33) the volume is decreased while the KSCN is increased. For Case 28 there is at discharge a decrease both in volume of the saliva collected in 30 minutes and in the sulfocyanate content thereof. Case 29 has an increase in volume at discharge with no variation in the sulfocyanate content. In ten cases there is an

increase in both the volume and the KSCN content of the saliva of the patients about to be discharged as compared with the saliva at entrance, but the increase in the KSCN content is proportionately very much greater. Thus the average volume of these ten cases (Nos. 586, 11, 12, 19, 20, 22, 26, 30, 31, and 32) is 40.8 cc. at entrance and 62.8 cc. at discharge, while the corresponding content of KSCN is 1.51 and 3.57 mg. The same conclusion holds for the saliva as a whole; namely, that the sulfocyanate is increased at discharge to a much greater degree than the volume.

Calculated on the basis of 100 cc. of saliva, the sulfocyanate is higher at discharge than at entrance in fifteen of the eighteen cases given in Table II. For Case 20 the sulfocyanate calculated to 100 cc. is slightly lower at discharge than at entrance, while for Cases 28 and 29 it is distinctly lower. Still, as a rule, the percentage of sulfocyanate in the saliva is higher at discharge than at entrance to the hospital.

The sulfocyanate content of the saliva as given in the literature is rather variable, due, perhaps, to different methods. Munk[5] using his own method found the saliva of man to contain 0.014 per cent of NaSCN (0.0167 per cent KSCN); Bruylants[7] found a trace to 0.0698 gm. of HSCN per liter, with an average for forty-five cases of 0.0374 gm. of HSCN (or the equivalent of 0.0062 per cent of KSCN). Krüger[8] using Munk's method found 0.0072 per cent of KSCN in the saliva; Mayer[9] using the Rupp-Schied-Thiel method found 0.0003 per cent of KSCN in the saliva; while Gies and Kahn,[6] using the same method, found 12.8 mg. of KSCN in 500 cc. of saliva.

The only reference to the saliva in pellagra that we have found is that by Mense,[10] who tested the saliva of pellagrins by means of a pad of test paper presumably impregnated with starch and iodic acid, which permits judging of the content in KSCN by the formation of a blue color following the release of iodine. Of twenty-nine pellagrins or suspects selected, seven showed absence

[7] Bruylants, J., *Bull. Acad. méd., Belgique*, 1888, ii, series iv, 21.

[8] Krüger, F., *Z. Biol.*, 1899, xxxvii, 6.

[9] Mayer, in Oppenheimer, C., Handbuch der Biochemie des Menschen und der Tiere, Berlin, 1908, iii, pt. i, 33.

[10] Mense, C., *Arch. Schiffs. u. Tropenhyg.*, 1913, xvii, 788.

of KSCN; eight a very feeble reaction (about 0.001 per cent); seven a feeble reaction (0.003 per cent); and only six a normal reaction (0.01 per cent).

As the excretion of sulfocyanate seems to be somewhat of an individual question, we believe the best control on the pellagra patient is the same patient recovered to such a degree that all the signs and symptoms characteristic of pellagra have disappeared. Accordingly stress is laid on the difference in the sulfocyanate of the saliva as found at entrance and at discharge. Of the eighteen cases of Table II, the saliva of sixteen increased in sulfocyanate during their stay in the hospital.

TABLE III.

Standard Diet for 1919.

Diet 1.	Diet 2.
Soda biscuits.	Soda biscuits.
Corn bread.	Corn bread.
Rice.	Rice.
Tomatoes.	Irish potatoes.
Orange or evaporated apples.	Grits.
Grits.	Gravy.
Mush.	Syrup.
Gravy.	Corn-meal mush.
Cheese.	Milk.
Veal, beef, chicken, or fish.	
Milk.	

In actual average figures, the KSCN content of the saliva shortly after admission of the patient was 1.45 mg. in the sample collected, with a percentage of 0.00351, while at discharge it was 2.77 mg. and 0.00466 per cent. The sulfocyanate content of the saliva was, as a rule, increased at discharge despite the fact that, barring the possible difference of utilization, the diet at entrance and shortly thereafter was more varied and potentially could yield more HSCN than the latter diet.

At entrance the diet given the patients contained cereals, vegetables, meat or fish, and milk. In a short time the meat and fish were eliminated, and as much milk as the patient would take, usually 1,000 to 1,800 cc. a day, was given. Examples of the diets used are given in Table III. No measurement of quantity

was made aside from the measurement of the milk. Diet 1 is the diet used for a short time after entrance; Diet 2 the diet used for most of the time the patient was in the hospital until discharge.

II. The Sulfocyanate Content of the Urine.

As the sulfocyanate of the saliva was found in general to increase as the patients passed from the pellagrous stage to the convalescent stage, attention was paid to the sulfocyanate content of the urine. The patients available for the urine study were rather mild cases of pellagra. The diet employed during the last tests just before discharge was Diet 2 of 1919, which was less varied than Diet 1 and relied upon milk as the main source of nitrogen. Still of the fourteen cases given in Table IV, eleven gave a higher content of KSCN in the 24 hour urine at discharge than at entrance. In two cases (Nos. 26 and 32), the increase was slight. In three cases (Nos. 28, 30, and 31), there was a reduction in sulfocyanate at discharge as compared with entrance. The average of the fourteen urines studied comparatively, however, is higher for the absolute amount of KSCN at discharge than at entrance.

Of the fourteen cases given in Table IV, the volume of the urine is increased at discharge in twelve cases; in two (Nos. 15 and 28) it is decreased. As the volume of the urine of the patients at discharge, when they showed no signs or symptoms, which in the judgment of the medical staff indicate pellagra, is on the whole decidedly increased, the percentage of sulfocyanate in the urine, that is the quantity in 100 cc. of urine, is as a rule less at discharge than at entrance. Thus in ten cases (Nos. 16, 19, 20, 21, 26, 29, 30, 31, 32, and 33), the percentage of sulfocyanate in the urine is reduced while in only four cases (Nos. 15, 23, 24, and 28) is it increased. It may be emphasized, however, that the absolute amount of sulfocyanate excreted in the 24 hour urine, is, as a rule, increased at discharge.

In Table V are given data to show that, as a rule, the same order obtains with both saliva and urine with both collected within the same 24 hours; that is, that the milligrams of KSCN in the saliva and in the 24 hour urine are, as a rule, greater at discharge than at entrance.

It may be noted, however, that of the eleven cases given in Table V, the sulfocyanate content of the saliva is reduced at discharge in two cases (Nos. 28 and 29), and that the sulfocyanate content of the urine is reduced at discharge in two cases (Nos. 28 and 30). However, the fact obtains that in general for both saliva and urine, when taken within the same 24 hours, the sulfocyanate content is increased, as a rule, at discharge—a corroboration of the data given for the saliva and the urine in Tables II and IV, respectively.

TABLE IV.

K S C N of the 24 Hr. Urine.

Case No.	Active pellagra.			Convalescent.		
	Volume.	K S C N in urine.	K S C N in 100 cc.	Volume.	K S C N in urine.	K S C N in 100 cc.
	cc.	*mg.*	*mg.*	*cc.*	*mg.*	*mg.*
15	1,220	34.1	2.80	1,120	43.3	3.87
16	680	50.0	7.35	1,650	94.2	5.71
19	1,230	60.9	4.95	1,955	67.3	3.44
20	1,325	79.6	6.00	2,240	85.3	3.81
21	640	51.8	8.09	1,400	68.6	4.90
23	530	26.1	4.92	1,115	76.4	6.85
24	1,470	15.7	1.07	1,690	58.2	3.44
26	1,530	52.9	3.46	1,960	55.1	2.81
28	2,965	78.2	2.64	1,770	47.9	2.71
29	455	45.8	10.07	1,390	62.0	4.46
30	1,000	61.8	6.18	1,600	43.3	2.71
31	1,420	52.2	3.68	1,830	44.2	2.42
32	930	93.5	10.05	1,260	96.6	7.67
33	550	49.7	9.04	1,230	54.1	4.40
Average.....	1,139	53.7	5.74	1,586	64.0	4.23

Fenwick[11] believes that sulfocyanate as a product of protein metabolism affords indirect evidence of the extent of the nitrogenous metabolism of the organism and that its amount diminishes under conditions in which the activity of the nutritive function is diminished.

According to Grober,[12] sulfocyanate excretion is very slight in the case of cachetic and sick people.

[11] Fenwick, S., The saliva as a test for functional disorders of the liver, London, 1889.

[12] Grober, J. A., *Deutsch. Arch. klin. Med.*, 1901, lxix, 243.

The physiological properties of sulfocyanate and references thereto are summarized by Abderhalden.[13] The urine of healthy men contains on the average 0.0476 gm. of HSCN (0.0796 gm. of KSCN), somewhat less for women. According to Willanen[14] the administration to rabbits of glycocoll, creatinine, creatine, and adenine, which by oxidation or splitting yield HCN, causes increased urinary excretion of sulfocyanate. Lang[15] found that administration of acetonitrile, propionitrile, butyronitrile, capronitrile, and

TABLE V.

KSCN in Saliva and in 24 Hr. Urine at Admission and at Discharge.

Case No.	Saliva.		Urine.	
	Entrance.	Discharge.	Entrance.	Discharge.
	mg.	*mg.*	*mg.*	*mg.*
16	2.67	3.66	50.0	94.2
19*	0.69	1.25	60.9	67.3
21	1.90	2.13	51.8	68.6
23	1.07	1.47	26.1	76.4
24	0.89	2.24	15.7	58.2
26	1.61	2.56	52.9	55.1
28	1.80	1.08	78.2	47.9
29*	1.43	1.28	45.8	62.0
30	1.36	5.14	61.8	43.3
32	2.21	3.57	93.5	96.6
33	0.39	0.93	49.7	54.1
Average....	1.46	2.30	53.3	65.8

* The salivas at entrance for Cases 19 and 29 in this table were collected a day or two later than those of Table II—at the time the 24 hr. urine was being collected. So the sulfocyanate figures in the two tables do not agree for these two cases.

HCN increased urinary sulfocyanate. Gies and collaborators[6] in their study of sulfocyanate in relation to dental caries added much to the knowledge of the origin of sulfocyanate. They conclude from experiments with dogs that the addition to the diet of simple substances containing or yielding sulfide radicals do not markedly affect the sulfocyanate excretion. On the other hand, they

[13] Abderhalden, E., Biochemisches Handlexicon, Berlin, 1911, iv, 945.
[14] Willanen, K., *Biochem. Z.*, 1906, i, 129.
[15] Lang, S., *Arch. exp. Path. u. Pharmakol.*, 1894, xxxiv, 247.

found increased excretion of sulfocyanate after administration of acetonitrile (CH_3CN), and mandelic acid nitrile ($C_6H_5CHOHCN$), which contain the CN radical; and after administration of the amino-acids, alanine, glycocoll, and leucine, the first two of which Plimmer[16] found would by suitable oxidation yield hydrocyanic acid.

Sulfocyanate arises in the body from the union of $-SH$ and $-CN$ radicals both of which can be obtained from protein by various laboratory methods that involve chemical processes similar to those which obtain in the animal body. The production of $-SCN$ compounds appears to be a process of detoxification—a defensive measure similar to the detoxification of indole by the liver into indican normally found in the urine.

The analytical data given by Gies, Lieb, and Kahn on the sulfocyanate content of the tissues of dogs did not give positive support to the theory that sulfocyanate results from the metabolism of protein as such. They say

> "The production of sulfocyanate, as indicated by the excretion, was perceptibly decreased during fasting, but was increased very slightly if at all during overfeeding with protein. If, however, the production of sulfocyanate is estimated from the data for its distribution, the figures indicate that it was not materially affected by either fasting or overfeeding."

They suggest that sulfocyanate may result normally wholly from endogenous protein changes that are not materially affected by the amount of ingested protein. They regard the liver as the main organ of production of the sulfocyanate.

Dezani[17] has somewhat different views. Thus, in a series of articles, dealing with the genesis of thiocyanic acid in animals he declares Lang's hypothesis of a quantitative transformation of aliphatic nitriles into HCNS is inadmissible since six-sevenths to nine-tenths of the nitrile is decomposed in some other way. He did not find an increased urinary excretion of sulfocyanate on administering guanine, uric acid, glycine, aspartic acid, alanine, asparagine, or glycerol. In the dog, and in man as well as in rabbit, ingestion of cauliflower resulted in the elimination of

[16] Plimmer, R. H. A., *J. Physiol.*, 1904, xxxi, 65.

[17] Dezani, S., *Arch. farm. sper.*, 1917, xxiii, 245; 1917, xxiv, 113, 193; 1918, xxv, 83, 278; 1918, xxvi, 257; 1919, xxvii, 134; 1919, xxviii, 23.

HSCN in the urine. The thiocyanogenic substance belongs to the group of so called extractives. It is not a protein, or an amino-acid precipitable by phosphotungstic acid, or an organic sulfide. It does not occur in meat extract.

During a prolonged fast, a dog continued to eliminate HSCN in the urine to the extent of about one-third of that of a similar period preceding the fast. No relation was observed between the amount of total urinary nitrogen and of the HSCN eliminated. The elimination of urinary HSCN, however, depends on the nature of the food. In changing from a food poor in N to one rich in N the urinary HSCN excreted by the dog doubled in 24 hours. In the final paper of the series, Dezani concludes that the HSCN normally excreted by animals (guinea pig, rabbit, dog, man) is purely exogenous. Its origin is traced to the plant food consumed directly by herbivorous and indirectly by carnivorous animals.

That the excretion of sulfocyanate in the urine is somewhat independent of the total protein metabolized is shown by our urinary data in Table VI. Thus with low total urinary nitrogen in the tests made shortly after entrance to the hospital the nitrogen of the urinary KSCN is 0.1352 per cent of the total nitrogen while in the tests made shortly before discharge the nitrogen of the urinary KSCN is 0.0891 per cent of the total nitrogen. In short, though the sulfocyanate of the urine is, as a rule, increased at discharge from the hospital as compared with excretion at entrance, the increase is not in proportion to the increase of total nitrogen of the urine.

As pointed out by Gies the normal yield of sulfocyanate is, then, not dependent altogether on the total protein metabolized but on the production or evolution of CN from parts of protein; in short the production of sulfocyanate depends to a great extent on the nature of the protein and the amino-acid constituents thereof, and the capacity of the system to synthesize the sulfocyanate. As judged by the work of Willanen and of Gies, a protein yielding glycocoll, for example, should, barring lack of sulfur compounds in the food and barring insufficiency of the liver, give a greater yield of sulfocyanate in the urine.

A modifying factor in the urinary KSCN data obtained shortly after the patients' entrance to the hospital and again

TABLE VI.

Urinary KSCN.

Compared with total nitrogen, urea nitrogen, and ammonia nitrogen.

Case No.	Shortly after entrance.					Shortly before discharge.				
	Total N.	Urea N.	NH_3 N.	KSCN	KSCN (N) in percentage of total N.	Total N.	Urea N.	NH_3 N.	KSCN	KSCN (N) in percentage of total N.
	gm.	*gm. per cent of total N*	*gm. per cent of total N*	*mg.*		*gm.*	*gm. per cent of total N*	*gm. per cent of total N*	*mg.*	
16	10.595	8.798 83.0	0.532 5.0	50.0	0.0680	11.286	6.999 62.0	0.293 2.6	94.2	0.1203
15	8.604		0.265 3.1	34.1	0.0571	9.818	8.495 86.5	0.219 2.2	43.3	0.0636
19	8.659		0.321 3.7	60.9	0.1014	8.516	6.822 80.0	0.246 2.9	67.3	0.1139
20	5.854	3.089 52.8	0.922 15.8	79.6	0.1960	9.762	7.948 81.4	0.578 5.9	85.3	0.1260
21	5.538	4.150 74.9	0.254 4.6	51.8	0.1349	9.262	6.976 75.3	0.268 2.9	68.6	0.1068
23	1.700	1.009 59.3	0.113 6.6	26.1	0.2214	9.294	6.619 71.0	0.188 2.0	76.4	0.1185
26	5.673	4.341 76.5	0.236 4.2	52.9	0.1344	10.450	7.957 76.1	0.850 8.1	55.1	0.0760
28	7.430	5.560 74.8	0.205 2.8	78.2	0.1517	13.293	10.938 80.4	0.397 2.9	47.9	0.0520
29	6.579	2.992 45.5	1.181 17.8	45.8	0.1004	10.064	7.250 72.0	0.421 4.2	62.0	0.0888
30	4.760	3.667 74.9	0.270 5.7	61.8	0.1872	8.928	6.797 76.1	0.694 7.8	43.3	0.0699
31	5.098	2.198 43.1	0.738 14.5	52.2	0.1476	10.438	7.793 74.7	0.551 5.3	44.2	0.0611
32	9.906	5.957 60.1	1.673 16.9	93.5	0.1360	14.941	13.126 87.8	0.368 2.5	96.6	0.0932
33	5.885	4.240 72.1	0.365 6.2	49.7	0.1218	11.454	8.179 71.4	0.526 4.6	54.1	0.0681
Average ..	6.637	4.182 65.2	0.544 8.22	56.7	0.1352	10.577	8.146 76.5	0.431 4.1	64.5	0.0891

shortly before discharge lies in the change in diet. In the first tests the diet contained meat, which gives glycocoll on digestion; in the last tests milk was the main source of protein and milk contains little glycocoll. Despite the change in the diet in the two periods mentioned the absolute amount of sulfocyanate in the saliva and urine is, as a rule, greater during convalescence and shortly before the patients' discharge than during the active stage of the disease. The difference in favor of the convalescent stage would undoubtedly have been greater if the wider variety of diet used at entrance had been maintained throughout. As judged by work with a group of patients in 1917 the utilization of protein by pellagra patients tends to be somewhat subnormal.[18] The difference in the KSCN of the two periods must be due then to an increased assimilation and to an increase in detoxifying processes[19] or the sulfocyanogenetic powers of the organism as a whole.

In 1917 with marked cases of pellagra the difference in the sulfocyanate content of the saliva of the same patient in the active stage of the disease and in the convalescent stage was very marked. In 1919 with milder cases of pellagra the differences in the KSCN of saliva and of urine in the two periods were still noticeable.

The variations in sulfocyanate excretion between the active stage of the disease and the highly convalescent stage is associated with the difference in general condition. In the active stage of the disease when the patients are in a more or less cachetic condition the KSCN formation is less, as it is in other cachetic conditions as found by Grober. At discharge, when the patients have a greater feeling of well being and better assimilation, a higher protein metabolism, and presumably a greater detoxifying power of the system as a whole, the KSCN formation is increased. As the diet in the convalescent stage was if anything less ample than that of the active stage, the increase of sulfocyanate in the convalescent stage offers little support for the theory of its purely exogenous origin, but rather lends support to the view of Gies,

[18] Sullivan, M. X., Stanton, R. E., and Dawson, P. R., Metabolism in pellagra; a study of the urine, unpublished data.

[19] In the active pellagrous period one of us (S.) has found for several patients tested that the blood possessed a lower oxidative power as tested by the conversion of phenolphthalin to phenolphthalein.

Lieb, and Kahn that it is of endogenous origin. Thus the work herein outlined shows that the increased production of sulfocyanate is not closely bound up with total nitrogen but is more probably a product of some endogenous activity perhaps of the synthetic, detoxifying activity of the liver, coupled with a greater assimilation of sulfocyanogenetic complexes.

CONCLUSIONS.

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 of entrance, however, is not proportional to the increase of the total nitrogen of the urine.

I. THE CARBON DIOXIDE ABSORPTION CURVE AND CARBON DIOXIDE TENSION OF THE BLOOD OF NORMAL RESTING INDIVIDUALS.

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(Received for publication, December 2, 1920.)

INTRODUCTION.

In 1919 Haggard and Henderson (1) outlined a method for the direct determination of the carbon dioxide tension of arterial and venous blood. They also demonstrated the applicability of the method to the study of abnormal physiological conditions involving disturbances of the respiratory system and the acid-base equilibrium in the blood. Stadie's (2) demonstration of the practicability of arterial puncture as a safe procedure suggested the application of Haggard and Henderson's method to the study of problems of human physiology and pathology. The method consisted of the simultaneous determination of the carbon dioxide absorption curve of the blood and the carbon dioxide content of the arterial and venous blood. By interpolating the arterial and venous values on the absorption curve the carbon dioxide tension and hydrogen ion concentration of the blood as they existed in the body were estimated. This method, with such modifications as were necessary to render it applicable to work with human subjects, has been employed by us in the study of a series of normal and pathological subjects.

In brief the procedure followed has been to obtain specimens of arterial and venous blood simultaneously, protecting them from contact with the air and consequent loss of CO_2 by means of albolene. Just before and just after the arterial puncture specimens of alveolar air were obtained by the Haldane and Priestley method (3). The carbon dioxide and oxygen content of both

arterial and venous specimens and the oxygen capacity of the blood were determined at once. Specimens of the blood (usually venous) were then exposed to various mixtures of CO_2 and air at 37.5°C. The carbon dioxide content of each specimen was measured and the absorption curve obtained from the results. The values for the CO_2 content of the arterial and venous blood and the CO_2 tension of the alveolar air were then placed on the curve by interpolation. In many cases we determined also the CO_2 capacity of the venous plasma.

This work was practically completed when Means (4) presented before The Association of American Physicians the results obtained by the application to fifteen normal and pathological subjects of a method for determining arterial carbon dioxide tension similar in all essential respects to our own. Means confined his efforts entirely to a determination of the absorption curve and the arterial CO_2 tension. To this we have added several other factors, especially the alveolar CO_2 tension. This has led us to draw different conclusions with regard to certain points. Dr. Means has kindly permitted us to see his paper in manuscript form so that we are able to call attention to these differences at this time.

Before this method could be applied to the study of pathological subjects it seemed necessary to ascertain the limits of variation of the various factors involved, in normal persons. As the subjects of this investigation three adult males were employed: J. P. (Case 1) and D. P. B. (Case 3), two of the authors of the paper; and W. S. M. (Case 2), Dr. William S. McCann, whose assistance in this respect and in other parts of the work we gratefully acknowledge. All three subjects had successfully passed examinations for the Medical Corps of the United States Army and on subsequent examinations had shown no signs of any existing pathological condition or abnormality that could in any way render their use as normal controls open to question. It will appear in the course of this investigation that some of our results fall definitely outside what has hitherto been considered as the range of normal variation. These deviations may be due to faults in the theory or methods employed by us or by previous observers. It may be that the amount of normal control material hitherto available has been too small to cover the entire range of

normal variation. It is possible that subsequent developments may prove that the deviations are indications in our subjects of pathological conditions at present undemonstrable by other methods.

The use of only three normal subjects offers an entirely inadequate amount of control material. The scope and nature of the investigation rendered it difficult to make a more extensive study. Of course it would have been possible to make use of selected patients in the hospital and thus enlarge the number of individuals in this group. The use of patients with pathological conditions as normal controls is, however, open to criticism in any investigation in which the influence of all factors attending these pathological conditions is not definitely established. The limits of variation of the carbon dioxide absorption curve as established by Straub and Meier (5) from a study of 56 hospital patients whose clinical condition, they say, gave no reason to suspect any pathological change of the blood, are very different from the limits of variation found by all other observers in known normal individuals.

If it were necessary to rely entirely on the results of our own observations for the delimitation of normal variations, certainly our material is inadequate. There is, however, a considerable mass of experimental data in the literature which we have employed freely for comparison and corroboration.

Details of Method as Applied to Both Normal and Pathological Subjects.

The experiments on patients were usually performed in the afternoon, about 2 hours after the midday meal. In some cases they were begun in the morning, 4 hours or more after breakfast. No attempt to establish standard dietary conditions was made. Most of the patients were unable to get out of bed. In the few cases in which the condition of the subject permitted him to be up and about, a preliminary rest period of 30 minutes was given. During this time the patient remained in the reclining position. The experiments on normal persons were usually performed very shortly after the midday meal and were preceded by a 30 minute rest period.

Specimens of alveolar air were obtained both before and after the arterial puncture. The arterial puncture was done under novocaine anesthesia. The blood was withdrawn under albolene into a Luer syringe and then placed under albolene in a test-tube from which specimens were removed for analysis within a few minutes.

The venous blood was collected as soon as possible after the arterial puncture. To prevent venous stasis no tourniquet was applied and the subject was directed to keep his arm relaxed. The blood was collected under albolene, without any contact with the air.

Coagulation of the blood was prevented by the addition of a minimal amount of dry, neutral potassium oxalate.

As soon as possible after the blood had been obtained from the subject under investigation, 3 cc. portions were placed in cylindrical separatory funnels of about 300 cc. capacity. These were filled with a mixture of CO_2 and air. The mixture was made up in a cylinder of about 1,200 cc. capacity and passed through the separatory funnel in an interrupted stream in order to wash the funnel out completely. Simultaneous analysis of the air in the cylinder and that in the separatory funnel showed differences of 0.2 per cent or less, indicating that the method was sufficiently accurate for our purposes. The mixture in the cylinder was analyzed for carbon dioxide each time. The distal opening of the separatory funnel was tightly stoppered while the gas was still flowing through it, and the pressure equalized by water level in the cylinder. The proximal stop-cock was now shut off and the separatory funnel rotated in a water bath at 37.5°C. for 20 minutes. As it was rotated with its long axis in the horizontal position a maximum blood surface was exposed to the gas mixture. At the end of the 20 minute period rotation was discontinued and the separatory funnel was placed in the vertical position long enough to allow the blood to collect in the narrowest portion. The neck was now withdrawn from the bath and carefully dried. The stopper was then removed and duplicate specimens were withdrawn by two observers as rapidly as possible in Ostwald pipettes calibrated to deliver .1 cc. between two points.

The carbon dioxide content of both specimens was determined simultaneously in two Van Slyke pipettes. The technique was the same as that described by Van Slyke (6) except for the fact that 20 per cent tartaric acid was substituted for N sulfuric. The coagulum produced by tartaric acid in whole blood is not so firm as that produced by sulfuric and consequently does not clog the pipette or render it difficult to clean. The blood was introduced into the cup of the pipette beneath a drop of dilute, carbon dioxide-free ammonia water to prevent the escape of CO_2. A blank determination of CO_2 in the reagents was made with each experiment and subtracted from the observed reading. This correction was rendered as small as possible by the use of carbon dioxide-free water and ammonia water. It was usually about 0.01 cc. On one or two occasions it rose to 0.02 or 0.025 cc.

Calculation and Extent of Error.

Owing to the fact that the gas mixture in the separatory funnel was equilibrated with atmospheric pressure at room temperature only, and not at 37.5°C., a correction had to be made for the altered CO_2 tension caused by the change in temperature, and for the vapor pressure. This should intro-

duce no error, as the concentration of carbon dioxide in solution is dependent directly on the carbon dioxide tension of the atmosphere with which it is in contact. The error that may occur from incomplete washing out of the separatory funnel is, as we have stated, less than 0.2 per cent, or 1.5 mm., and must lie always in the same direction. A further slight error is introduced by the loss or gain of CO_2 by the blood in coming to equilibrium with the air in the separatory funnel. This must be very small and can be more or less accurately estimated. The maximum amount of CO_2 change from this source was only 0.2 to 0.3 cc. per cc. of blood. With 3 cc. of blood in a 300 cc. separatory funnel this again might produce a change of 1.5 to 2.0 mm. in the CO_2 tension of the gas mixture. In point of fact this change must have been very much smaller in most instances. The error thus produced will vary in direction and extent according to the original carbon dioxide content of the blood. Roughly we may say that at carbon dioxide tensions below that of the blood employed the apparent CO_2 tension cannot be more than 1.5 mm. above or below the actual and most usually be much less, because the two main sources of error tend to compensate one another. At carbon dioxide tensions above that of the blood the apparent CO_2 tension may be as much as 3 mm. too high.

Duplicate determinations with the Van Slyke pipette should differ by not more than 1 volume per cent. Occasionally such agreement was not obtained. Sometimes this was directly referable to errors in technique; in some instances no such errors were discovered. The faulty agreement in these cases may have been occasioned by some loss of CO_2 due to a delay in the transfer of the blood from the separatory funnel to the pipette. On the whole we believe that this was a minimal source of error. The blood was withdrawn from the funnels with the least possible loss of time. Although we have made no direct experiments to determine the rate of loss of CO_2 under these conditions the fact that duplicate samples check so accurately in most instances indicates that such loss must be very slight.

A certain error is introduced by the volumetric measurement of small amounts of blood. The pipettes were carefully calibrated to deliver 1 cc. of distilled water between two marks with an accuracy of ± 0.002 cc. The error entailed in measuring a viscous solution like blood must vary with the physical characteristics of the individual specimen under investigation and cannot be estimated, but can never be entirely negligible. It must lie always in the same direction and tend to make the apparent volume of carbon dioxide too low.

Van Slyke (6) showed that the addition of potassium oxalate to plasma did not affect its carbon dioxide-combining capacity. De Corral (7) found that anticoagulant concentrations of potassium oxalate produced no recognizable effect on the hydrogen ion concentration of whole blood. In this case it should also be without effect on the carbon dioxide-combining capacity of whole blood. Joffe and Poulton (8), in point of fact, could demonstrate no change in the level of the absorption curve of fully oxygenated blood as a result of the addition of oxalate. This seems hardly possible, however, in view of the extreme lability and sensitiveness of the

ionic balance between cells and plasma. We have attempted to determine the presence and extent of the changes produced by the addition of potassium oxalate to whole blood.

Blood was drawn into a tube containing 1 mg. of hirudin for each 2.5 cc. of blood. 2 cc. portions of this blood were placed in two separatory funnels. To the blood in one of these were added 40 mg. of crystalline, neutral potassium oxalate. Both funnels were then filled with the same air-CO_2 mixture from a large spirometer and rotated at room temperature for 15 minutes. At the end of this time both specimens of blood were analyzed for CO_2 by the Van Slyke method. Other samples of blood were defibrinated and the effect of adding oxalate to defibrinated blood was also determined. The results appear in Table I.

TABLE I.

Effect of Potassium Oxalate in a Concentration of 2 Per Cent on the Carbon Dioxide-Combining Capacity of Blood.

	CO_2 content of blood after exposure to the same air-CO_2 mixture.		
	Without oxalate.	With oxalate.	Difference.
	cc.	cc.	cc.
Hirudinized blood.	0.685	0.686	+0.001
	0.696	0.734	+0.038
	0.696	0.728	+0.032
	0.559	0.620	+0.061
	0.597	0.695	+0.098
	0.569	0.600	+0.031
	0.554	0.618	+0.064
Defibrinated blood.	0.648	0.677	+0.029
	0.635	0.688	+0.053
Average difference			0.045

The addition of potassium oxalate in 2 per cent concentration produces an average increase of about 4 volumes per cent in the CO_2-combining capacity of the whole blood. This change occurs whether the oxalate is added to hirudinized or defibrinated blood. The increase was not constant in magnitude in all the observations. However, in this concentration oxalate produces visible physical changes in blood. The blood appears a lighter and brighter red. Whether this is due to hemolysis or not was not determined. The variations in our results may be somewhat dependent on the speed with which the oxalate was dissolved.

The concentration of oxalate employed in this experiment was far in excess of that used for the purposes of preventing coagulation. Such a concentration was chosen for the preliminary observations in order to

insure the detection of any change that might occur. When it was found that this change was constant in direction, the experiment was repeated with only 10 mg. of oxalate to each 2 cc. of blood (see Table II). In this concentration (0.5 per cent) which is well above that used to prevent coagulation, no visible physical changes in the blood were detected. The addition of oxalate increases the carbon dioxide-combining power of blood only about 1 volume per cent.

Christiansen, Douglas, and Haldane (9) found that if blood was allowed to stand its CO_2 capacity gradually diminished as if it became more acid. This change occurred more rapidly at body temperature than at the temperature of the room. For this reason we have exposed a fresh sample of blood to each gas mixture. In this way no sample remained at 37.5°C. more than 20 minutes. However, the multiplicity of procedures, involved

TABLE II.

Effect of Potassium Oxalate in a Concentration of 0.5 Per Cent on the Carbon Dioxide-Combining Capacity of Blood.

	CO_2 content of blood after exposure to the same air-CO_2 mixture.		
	Without oxalate.	With oxalate.	Difference.
	cc.	cc.	cc.
Defibrinated blood.	0.599	0.622	+0.023
	0.612	0.632	+0.020
	0.624	0.628	+0.004
	0.748	0.755	+0.007
	0.745	0.742	−0.003
	0.787	0.810	+0.023
	0.797	0.793	−0.004
	0.785	0.808	+0.023
Average difference			+0.0116

in simultaneous studies of so many factors, prolonged the total time of the experiments more than we should have wished. When only the absorption curve was determined, the duration of the procedure was within the limits of safety; in the complete experiments this was not always the case.

The order of procedure in the laboratory was:

I. The simultaneous determination of

1. The carbon dioxide content of arterial blood (in duplicate).
2. The carbon dioxide content of venous blood (in duplicate).
3. The oxygen content of arterial blood.

II. The determination of the oxygen content of venous blood. (In the later experiments four Van Slyke pipettes were used and the arterial and venous bloods were analyzed for oxygen at the same time, one observer manipulating both oxygen pipettes and extracting one specimen while the other was being laked.)

III. Determination of the oxygen capacity of the blood.

IV. Determination of the absorption curve. (Unfortunately the size of the water bath permitted the introduction of only one separatory funnel at a time.)

TABLE III.

Effect of Time on the CO_2-Combining Capacity of Blood.

	Time after withdrawal of blood.	CO_2 content of blood.	Change of CO_2 content.
	min.	*cc.*	*cc.*
1. Hirudinized blood.	0	0.597	0.000
	120	0.569	−0.028
	165	0.554	−0.043
2. Defibrinated blood.	0	0.648	0.000
	60	0.635	−0.013
3. Defibrinated blood.	10	0.787	0.000
	65	0.797	+0.010
	170	0.785	−0.002
4. Oxalated blood.	30	0.720	0.000
	60	0.705	−0.015
	190	0.711	−0.009
	210	0.720	0.000
5. Oxalated blood.	5	0.790	0.000
	30	0.782	−0.008
	150	0.758	−0.032
	210	0.750	−0.040
6. Oxalated blood.	5	0.724	0.000
	60	0.723	−0.001
	125	0.703	−0.021
	180	0.676	−0.048
7. Defibrinated blood with 0.5 per cent oxalate.	10	0.810	0.000
	65	0.793	−0.013
	170	0.808	−0.002

In spite of the continuous work of three persons the total elapsed time of an experiment sometimes exceeded 3 hours. As skill and team work improved, this was steadily reduced until in the last observation we were able to complete the whole procedure within 2 hours from the time that the blood was withdrawn. It seemed advisable to find out the magnitude of the change which occurred in the blood on standing in the laboratory at

room temperature for this length of time. Blood was therefore withdrawn into oxalate and samples were removed at intervals, exposed to an air-CO_2 mixture, and the CO_2 content was measured. The results appear in Table III. Although not very consistent they indicate that the element of time may be a rather serious source of error. If blood is kept in an ordinary tube at room temperature, no change in the carbon dioxide-combining capacity can be demonstrated until the 2nd hour. At the end of $2\frac{1}{2}$ hours a loss of about 2 per cent is found. After 3 hours this has increased to about 3 per cent. The fact that this loss occurred in some specimens, but not in others, suggests that it may be hastened by extraneous factors.

If all these errors should fall in the same direction one might conceivably obtain an absorption curve that was apparently 3.5 volumes per cent lower than the actual curve. The mean variation, however, is much smaller than this and cannot be considerably greater than the error of the actual measurement with the Van Slyke pipette. The general tendency is to make the curve low. The influence of these errors, in any case, cannot be very great as we are using the curves for the most part as a basis for comparative studies only.

Method of Representation.

In all charts the abscissa represents the carbon dioxide tension with which the blood is in equilibrium, expressed in mm. of mercury at 37.5°C., corrections having been made for the barometer and vapor tension. The ordinate represents the carbon dioxide content of whole blood, both physically dissolved and chemically combined. As the physically dissolved CO_2 is a direct linear function of the carbon dioxide tension, there is nothing to be gained by subtracting this from the total as is done by Haggard and Henderson (1).

For the calculation of the diagonals expressing pH, Henderson's formula has been used, modified to show the pH directly instead of his C_H7. The use of the negative logarithm, introduced by Sörensen, has become general and is convenient. The further multiplication of forms of expression can only tend to produce confusion and misunderstanding. The Henderson scale agrees fairly closely with that of Hasselbalch (10). That the calculation of the hydrogen ion concentration of the blood from the H_2CO_3: $B$$HCO_3$ ratio rests on a sound theoretical basis was first shown by L. J. Henderson (11). Hasselbalch (10) later demonstrated the accuracy of the method by practical experiments. Both Henderson and Hasselbalch dealt with completely oxygenated blood. Parsons (12) has recently questioned Hasselbalch's results on the ground that the application of the H_2CO_3:$B$$HCO_3$ ratio as a measure of hydrogen ion concentration is possible only in the case of homogeneous solutions such as plasma. Whole blood is a mixture consisting of two phases, cells and plasma. In a comparison of the hydrogen ion concentration measured electrometrically with that calculated by means of the H_2CO_3:$B$$HCO_3$ ratio in whole blood, Parsons found differences of as much as 0.09 in the pH. His values for pH are also

consistently higher than those of Hasselbalch. The difference of scale implied in the latter is of no importance if we use our figures only for comparative purposes. Until the point at issue is definitely settled it seems better to continue the use of the Hasselbalch pH scale with the realization that, if Parsons is correct, it should be rotated to the right 0.03 to 0.04 points. The discrepancy of 0.09 between calculated and observed values of pH found by Parsons indicates a considerable error in the method of calculation. This is, however, the maximum error. The average error, as we have calculated his results, is only about 0.03, if allowance is made for the difference in scale noted above. If we recognize the possibility of the presence of an error of this magnitude in our method of calculation we may use the values here obtained for purposes of comparison where the observed changes are large and consistent and occur in completely oxygenated blood. Work of Parsons (12) and L. J. Henderson (13) suggests that the application of the same scale to partially or completely reduced blood is not entirely proper because of the nature of hemoglobin. This will have little or no effect as regards the absorption curve figures, as these are determined on completely oxygenated blood. It may have considerable effect on the interpretation of the carbon dioxide content values of arterial and venous blood; but this will be discussed later. In any event each diagonal line must represent the locus of points on which the H_2CO_3:$BHCO_3$ ratio is identical.

The carbon dioxide capacity of the venous plasma is reduced to terms of alveolar carbon dioxide with which it should correspond if the normal relation found by Van Slyke obtained. The formula employed is

$$(\text{Mg. } CO_2 \text{ in chemical combination in 1 cc. plasma}) \times 35 = x$$

The Effect of Oxygen Unsaturation on the Carbon Dioxide Absorption Curve.

One difficulty at once presents itself. The absorption curve is obtained from completely oxygenated blood, while both the arterial and venous blood are partially reduced. Christiansen, Douglas, and Haldane (9), Hasselbalch (10), Parsons (12), and L. J. Henderson (13) have all shown that reduced blood has a greater power of combining with CO_2 than has oxygenated blood. All these observers employed defibrinated blood. Haggard and Henderson (14) reported recently that they had been unable to demonstrate this effect of oxygen on the absorption curve of oxalated blood. On the other hand Joffe and Poulton (8) found no difference in this respect between defibrinated and oxalated blood. The findings of the two observers are flatly contradictory. Collateral evidence, however, strongly favors the view of Joffe

and Poulton. The effect of oxygen unsaturation on the absorption of carbon dioxide in defibrinated blood is unquestionable. If oxygen unsaturation is without effect on the absorption curve of oxalated blood, the absorption curves of defibrinated and of oxalated venous blood should be quite different. Venous blood, as drawn, may be as much as 50 per cent unsaturated with oxygen. If this blood is defibrinated and then completely oxygenated, the usual procedure in the determination of the absorption curve, the change from partial reduction to complete oxygenation should make the absorption curve low in comparison with that of oxalated blood.

That this is not the case and that the carbon dioxide-combining capacity of oxalated blood is increased by oxygen unsaturation is shown by the following experiment.

50 cc. of blood were withdrawn from an arm vein of J. P. under albolene, without contact with the air. 25 cc. of this blood were defibrinated under albolene, while to the remainder was added a small amount of neutral potassium oxalate.

The oxygen content of both portions was then determined. The oxalated blood contained 7.1 volumes per cent of oxygen, representing an oxygen saturation of 33 per cent; the defibrinated portion contained 6.65 volumes per cent of oxygen, an oxygen saturation of 31 per cent.

Samples of each portion were then exposed for equal lengths of time to an air-CO_2 mixture of 38 mm. of CO_2 tension, at 37.5°C. Analysis at the end of this period gave 42.3 volumes per cent of CO_2 in the oxalated blood and 42.0 volumes per cent in the defibrinated. Both showed an oxygen saturation of 98.4 per cent.

Another sample of the oxalated blood was, at the same time, exposed to a mixture of CO_2 and nitrogen of 38 mm. of CO_2 tension. Analysis showed that this contained 47.5 volumes per cent of CO_2 and an oxygen saturation of only 21 per cent.

As a result of a reduction of 77.4 per cent in the oxygen saturation the carbon dioxide-combining power of the oxalated blood was increased 5.2 volumes per cent. This agreed with the value obtained by calculation from our empirical formula (see below), which was 5.66 volumes per cent.

That oxalated blood is suitable for the determination of the absorption curve is indicated by the experiments on the effect of oxalate detailed above (see "Methods"). Van Slyke and Cullen (15) have also shown that if arterial and venous bloods, oxalated, are exposed to the same mixture of CO_2 and air they are found to have the same carbon dioxide-combining power. If, then, oxygen does affect the carbon dioxide-combining power of blood in the

body, the addition of oxalate to the blood does not interfere with the reaction. As we shall point out later, unless such a reaction occurs in the body, certain respiratory phenomena become quite incomprehensible. At present the weight of evidence favors those who claim that oxygen influences the absorption of carbon dioxide in the blood. In this case arterial and venous carbon dioxide tensions must be corrected for oxygen unsaturation in order to approximate conditions that exist in the body.

On theoretical grounds Parsons (12) and L. J. Henderson (11) have concluded that the effect of oxygen on the CO_2-combining power of blood must depend on the amount of hemoglobin in the blood and the ratio of reduced hemoglobin to oxyhemoglobin. Christiansen, Douglas, and Haldane (9) and Joffe and Poulton (8) have presented experimental data that conform to this theory. It should be possible, then, to find an empirical mathematical expression for the effect of oxygen unsaturation on the level of the carbon dioxide absorption curve, the accuracy of which will depend on the accuracy of the data at our disposal. The simplest equation that will express the fact that the effect of oxygen unsaturation on the curve is dependent on the amount of reduced hemoglobin is

$$K \times \mathrm{Hb} = D$$

where K = a constant

Hb = the oxygen unsaturation of the blood expressed in volume per cent of oxygen

D = the change of level of the absorption curve as a result of the effect of oxygen unsaturation, expressed in volume per cent of carbon dioxide

Of course this formula cannot express the reaction accurately. K cannot be a simple constant because the curves of reduced and oxygenated blood meet at the origin. K must, therefore, have a value of zero at 0 mm. of CO_2 tension and must increase as the CO_2 tension rises. The value of K may, however, be sufficiently constant at CO_2 tensions that exist in the body to permit the use of such a simple equation.

As experimental data we have the carbon dioxide absorption curves of the blood of J. S. H. (Christiansen, Douglas, and Haldane) saturated with oxygen and 98 per cent reduced and the curves of the blood of J. (Joffe and Poulton) completely oxy-

genated and 90 per cent reduced. The hemoglobin values given for J. S. H. and J. are 100 and 101 per cent respectively, according to the Haldane scale. To these data we have applied the above equation and calculated values of K. The results are

TABLE IV.

Effect of Oxygen on the Carbon Dioxide Absorption Curve of Whole Blood, from Equation $K \times Hb = D$.

Subject.	CO_2 tension.	Observed value of D.	Value of K.
	mm. Hg		
J. S. H. (reduced blood 2 per cent saturated with oxygen. Hemoglobin 100 per cent.)	0	0	0
	10	5.5	0.303
	20	5.5	0.303
	30	5.6	0.309
	40	6.0	0.331
	50	6.2	0.342
	60	6.2	0.342
	70	6.6	0.364
	80	6.7	0.370
	90	7.0	0.368
J. (reduced blood 10 per cent saturated with oxygen. Hemoglobin 101 per cent.)	0	0	0
	10	4.5	0.268
	20	5.3	0.315
	30	5.6	0.333
	40	5.5	0.327
	50	5.5	0.327
	60	5.9	0.351
	70	6.5	0.386
	80	7.4	0.440
	90	8.0	0.476
Average values from 30 to 70 mm. inclusive.....		J. S. H.	0.337
		J.	0.345

shown in Table IV. It will be seen that the average values of K between 30 and 70 mm. of CO_2 tension are practically identical, averaging 0.34, and that the maximum deviation from this mean at these tensions is only about 10 per cent. This is within the

error of the method employed and a constant value of K can therefore be assumed for our purposes. We have applied this equation to our results in order to correct the arterial and venous CO_2 tensions observed for the effect of oxygen unsaturation. In practice a curve is drawn at the distance D above the curve of completely oxygenated blood and the corrected point is placed where the carbon dioxide content of the blood intersects this curve.

In the charts of experiments and in the tables of this paper the arterial and venous points are shown both in relation to the absorption curve of completely oxygenated blood and also after corrections have been made for oxygen unsaturation. The quantitation of the effect of oxygen unsaturation is, of course, based on insufficient data. If, however, the data of Haldane and of Joffe are correct and the value of K does vary by only 10 per cent, the maximum error in such a correction cannot be more than one-tenth of the variation produced by the change from the completely oxygenated to the completely reduced state. For a hemoglobin of 100 per cent the error would be 0.64 volume per cent, well within the limit of error of the analytical methods we have employed. Even a considerable error in the equation would make no distinguishable difference in the results.

Level of the Absorption Curve of Normal Resting Subjects.

Christiansen, Douglas, and Haldane (9), in 1914, studied the carbon dioxide absorption curve of two normal individuals (J. S. H. and C. G. D.) at rest, and during and after exercise. They also made a few observations on five other normal persons at rest. At about the same time Morawitz and Walker (16) made some observations on the carbon dioxide-combining power of whole blood, by an entirely different technique, and obtained almost identical results. Hasselbalch (10), in 1917, determined the curve of the normal subject, K. A. H. In 1918 Straub and Meier (5) published the results of a study of the absorption curves of 56 hospital patients in whom, they state, there was no reason to suspect any pathological change of the blood. Liljestrand and Lindhard (17), Krogh and Liljestrand (18), Joffe and Poulton (8), Parsons (12), Davies, Haldane, and Kennaway (19),

and Means, Bock, and Woodwell (4) have also given normal absorption curves.

In Fig. A are presented the limits of variation of the carbon dioxide absorption curves of normal resting individuals which we have been able to find in the literature, and those of three normal persons which we have determined. Some of these represent only single observations. Others are the results of repeated studies. Altogether observations on twenty-one normal persons appear, besides Straub's 56 hospital patients.

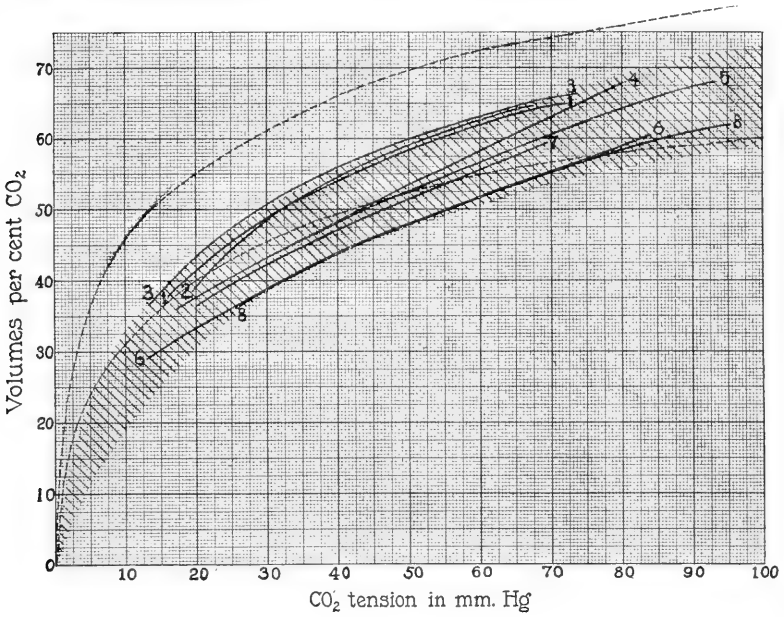

FIG. A. Absorption curves of three normal subjects.

1. J. P., Feb. 27, 1920.
2. " Mar. 5, 1920.
3. " " 12, 1920.
4. W. S. M., Feb. 13, 1920.
5. " May 21, 1920.
6. D. P. B., Mar. 26, 1920.
7. " " 29, 1920.
8. " May 14, 1920.

Shaded area shows the range of variation of the absorption curves of seventeen normal individuals, obtained from the literature. The area enclosed by broken lines represents the range of variation exhibited by Straub and Meier's 56 cases.

The methods employed are almost as numerous as the authors. Nevertheless, with the exception of Straub's highest figures, the agreement in the results of different observers is remarkably close. The normality of Straub's cases may reasonably be questioned and the data which he presents are not sufficient to permit analysis of his results. The simple statement that the clinical condition of these patients gave no reason to suspect any pathological change of the blood is not a sufficient characterization to warrant the assumption that they are normal subjects. As yet the study of the carbon dioxide absorption curve of whole blood has been too limited to permit one to say that pathological changes occur in certain restricted conditions, only. It seems better, then, to omit Straub's figures in discussing the limits of variation of the absorption curve in normal subjects. If this is done, it will be seen that the limits of variation of the eighteen cases reported by other observers are the same as those found in our three normal subjects. For the present, these must be considered the limits of range of the carbon dioxide absorption curve of normal resting subjects. It may be that further studies will reveal greater variations or that more careful investigation will prove that the extreme curves are due to some pathological or abnormal physiological condition not yet discovered.

The carbon dioxide content of blood is composed of two parts: (1) That portion which is present in simple solution and as H_2CO_3; (2) that portion which is present in chemical combination as bicarbonate. The amount of CO_2 in solution at any given temperature is dependent entirely on the tension of carbon dioxide to which the blood has been exposed, and can be calculated directly from this and the solubility coefficient. It has been shown by Bohr (20), L. J. Henderson (11), and more recently by Parsons (12) that practically all the chemically combined CO_2 must exist as bicarbonates. Henderson has also shown that the concentration of bicarbonates may be used as a measure of the alkali of the blood not bound by acids other than carbonic, without any significant error. If this is so, the alkali reserve of the blood must determine the height of the absorption curve.

Inspection of the curves discloses the fact that they vary considerably in shape and slope. In most cases these variations are minimal within the limits of CO_2 tension that are believed to

obtain in the arterial blood. If the curves of patients with severe anemia are omitted, the remainder will be found, with few exceptions, to maintain the same relative height within the limits 30 to 60 mm. In order to compare the heights of different absorption curves, it is necessary to study them at the same level. The ideal level from a theoretical standpoint is the one that conforms as nearly as possible to the conditions supposed to exist in the body; that is, where the absorption curve crosses the pH 7.35 line. In the lowest curves, however, this point lies at an extremely low carbon dioxide tension, occasionally below those tensions employed in the experimental work. The use of the point of intersection with the pH 7.35 line would, in these instances, demand extrapolation with the introduction of a considerable error. We have, therefore, chosen the point at which the absorption curve crosses the 40 mm. line. This point has been chosen because it is the generally accepted figure for average, normal alveolar CO_2 tension and because it has been rather generally employed by other investigators in the determination of the CO_2 capacity of whole blood and plasma. Except in certain pathological conditions which we shall discuss later, the differences in the results of the two methods (estimation of height at the 40 mm. line and at the pH 7.35 line) are insignificant. The height of the curve at either of these points may be considered to represent the alkali of the blood available for combination with CO_2 under conditions that exist in the body. This is expressed in terms of total carbon dioxide instead of carbon dioxide present as bicarbonate. The latter may be derived by subtracting 2.7 volumes per cent from the value given. The results obtained by others and by us, from both normal and pathological subjects, appear in Table V.

From these figures the height of the normal absorption curve at 40 mm. of CO_2 tension may be said to lie between 43 and 56 volumes per cent, with an average of about 50. The limits of the whole number of normal curves are practically the same as those found in our three normal subjects, if Straub's figures are omitted.

Christiansen, Douglas, and Haldane (9) found that, although the absorption curves of different individuals differed considerably in level, the absorption curve of any given individual was constant and quite characteristic in both height and shape.

TABLE V.

Height of the Absorption Curve at 40 Mm. CO_2 Tension.

Subject and condition.	CO_2
	vol. per cent
Christiansen, Douglas, and Haldane: 6 normal subjects; limits of variation	46.6 51.2
Morawitz and Walker: 1 normal subject; limits of variation	51.7 54.2
Hasselbalch: 1 normal subject, K. A. H	50.0
Krogh, Liljestrand, and Lindhard: 5 normal subjects; limits of variation	44.0 48.3
Joffe and Poulton: 1 normal subject, J	45.1
Parsons: 1 normal subject, T. R. P., Oct., 1917	51.9
July, 1918	46.7
Davies, Haldane, and Kennaway: 1 normal subject, J. B. S. H.	49.4
Means, Bock, and Woodwell: 2 normal subjects, M. N. W	51.1
J. H. M	50.9
Straub and Meier:* 56 hospital cases with presumably normal blood; limits of variation	49.5 65.6
J. P., normal subject, Feb. 27, 1920	53.9
Mar. 5, 1920	54.5
" 12, 1920	55.9
W. S. M., normal subject, Feb. 13, 1920	48.3
May 21, 1920	48.2
D. P. B., normal subject, Mar. 26, 1920	43.3
" 29, 1920	47.0
May 14, 1920	43.6

Summary.

	No. of cases.	CO_2 at 40 mm.		
		Maximum.	Minimum.	Average.
		vol. per cent	*vol. per cent*	*vol. per cent*
Other observers*	18	54.2	44.0	49.3
Subjects of this series	3	55.9	43.3	49.2
All normal subjects	21	55.9	43.3	49.3

* Straub and Meier's figures are omitted from this summary for reasons given in the text.

Our results seem to confirm this statement. The total variation in the height of the curve of J. P. (Case 1), determined three times in a period of 6 weeks, was only 2 volumes per cent. Two curves of W. S. M. (Case 2), obtained over 3 months apart, show even closer agreement. In the case of D. P. B. (Case 3), two curves at an interval of 6 weeks are almost identical. A third, taken 3 days after the first, lies about 4 volumes per cent higher. Christiansen, Douglas, and Haldane showed that exercise reduced the level of the curve. The variations that were found may have been due to a failure to establish proper resting metabolic conditions. The two curves of Case 3 that agree most exactly were obtained under standard conditions. The very close agreement of these curves derived from a considerable number of observations, scattered over a period of 2 months or more, can hardly be a matter of coincidence. It is also additional evidence that, for purposes of comparative study at least, the errors of the method are of no great importance.

The meaning of the differences in the height of the absorption curves of different individuals is not clear. That it is paralleled by no corresponding difference in the carbon dioxide tension of the alveolar air has been stated by Christiansen, Douglas, and Haldane. By a strange chance it happens that the absorption curves of Cases 1 and 3 are very different and lie respectively at the extreme upper and lower limits of the normal range. Both persons have been studied by a variety of respiratory methods and they have been found to differ in many respects. Some of these differences and their possible bearing on the height of the absorption curve will be pointed out later.

Carbon Dioxide Tension and the Hydrogen Ion Concentration of the Arterial Blood of Three Normal Subjects.

The carbon dioxide tension of the arterial blood of three normal persons (see Table VI, Columns 4 and 5; and Charts 1, 2, and 3[1]) was found to be 36 to 50.5 mm. of Hg uncorrected for oxygen unsaturation, 34.5 to 49 mm. after correction. This variation

[1] To facilitate the reference to charts and tables, the same numbering has been retained throughout tables, charts, and protocols. Thus Chart 1 refers to Case 1. Charts of general characteristics are lettered.

TABLE VI.

Results of the Study of Three Normal Subjects.

Subject.	Date.	Alveolar CO_2 tension.	Alveolar reaction.	Arterial CO_2 content.	Arterial CO_2 tension.		Arterial reaction.		Venous CO_2 content.	Venous CO_2 tension.		Venous reaction.		Difference between arterial and alveolar CO_2 tension.	Difference between venous and arterial CO_2 content.	Difference between venous and arterial CO_2 tension.	Difference between venous and arterial reaction.
					Uncorrected.	Corrected.	Uncorrected.	Corrected.		Uncorrected.	Corrected.	Uncorrected.	Corrected.				
		(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)	(11)	(12)	(13)	(14)	(15)	(16)
	1920	*mm. Hg*	*pH*	*vol. per cent*	*mm. Hg*	*mm. Hg*	*pH*	*pH*	*vol. per cent*	*mm Hg*	*mm. Hg*	*pH*	*pH*	*mm. Hg*	*vol. per cent*	*mm. Hg*	*pH*
1. J. P.	Feb. 27								57.4	47.5		7.33					
	Mar. 5								64.3	65.5		7.23					
	" 12	38.7	7.42	54.1	36.0	34.5	7.43	7.45	62.0	56.3		7.28		−4.2	7.9		
	May 19	36.0	7.42	54.1	39.0	38.7	7.40	7.40	55.6	42.0	39.5	7.37	7.40	2.7	1.5	0.8	0.00
2. W. S. M.	Feb. 13								64.1	72.0		7.19					
	May 21	35.9	7.35	50.9	47.0	45.0	7.28	7.30	59.4	67.5	52.5	7.18	7.30	9.1	8.5	7.5	0.00
3. D. P. B.	Mar. 26	38.5	7.29	45.5	45.0		7.25		53.4	65.0		7.15			7.9		
	" 29	37.6	7.33						57.5	65.0		7.18					
	May 14	37.4	7.30	47.9	50.5	49.0	7.22	7.23	56.2	72.0	58.5	7.12	7.22	11.6	8.3	9.5	0.01
Maximum		38.7	7.42	54.1	50.5	49.0	7.43	7.45	64.3	72.0	58.5	7.37	7.40	11.6	8.5	9.5	0.01
Minimum		35.9	7.29	45.5	36.0	34.5	7.22	7.23	53.4	42.0	39.5	7.12	7.22	(0.0)	1.5	0.8	0.00

did not bear a direct relation to the height of the corresponding absorption curves. The consequence is that our results indicate a considerable range of variation in the pH of normal arterial blood (see Table VI, Columns 6 and 7); from 7.22 to 7.43 uncorrected for oxygen unsaturation, 7.23 to 7.45 after correction. This is somewhat larger than the variation found by Michaelis (21) by direct electrometric titration in the venous blood of twelve normal resting persons, which was 7.28 to 7.43. As we shall show later the pH of venous blood is practically the same as that of arterial blood so that Michaelis' figures and ours are quite comparable. His average pH is 7.35, ours is 7.32, a very fair agreement. There are, as yet, no direct determinations of the pH of human arterial blood to be found in the literature. Most observers have relied, instead, on the values obtained from venous blood equilibrated with alveolar air.

Parsons' (12) objections to the use of the ratio of free to combined carbon dioxide as a measure of the hydrogen ion concentration of whole blood have been discussed above. If these objections are valid our figures cannot be interpreted as absolute values, but they must be very close to the true values. The differences of pH in the three individuals, moreover, are too great to be accounted for by errors in the method of calculation. Parsons considers the maximum error in calculation to be about 0.09; Hasselbalch (10) found the maximum error no greater than that of the method for determining CO_2, which he places at a difference of 0.04 in the pH. This may be increased somewhat by the error in the oxygen correction formula. A greater error, however, results if this correction is not made. Certainly it expresses the change in pH as in carbon dioxide qualitatively and, as we have shown, deals with a value of such small magnitude that the error must be minimal, probably not greater than 0.02 in pH. On the whole the maximum error should never exceed 0.06 and the average error must be nearer 0.02 to 0.03.

It is quite difficult to believe that the differences in pH found in J. P. (Case 1) and D. P. B. (Case 3) are due to errors in method, because repeated determinations revealed the same differences. Values of 7.22 and 7.25 were found in Case 3 at an interval of 6 weeks. In Case 1 in two experiments more than 2 months apart, arterial CO_2 contents were found to be identical. Unfortunately

on the second occasion the determination of the absorption curve failed. However, Case 1's absorption curve had been sufficiently well established by three other observations to warrant the assumption that the arterial CO_2 tension and the hydrogen ion concentration were the same on both occasions. In order to represent this second experiment graphically (see Chart 1) we have employed an absorption curve made up from the three previous experiments. Under these conditions the arterial pH of Case 1 on these two occasions was 7.40 and 7.45.

There is a variation in the pH of normal resting individuals of about 0.2, a little more than has been generally assumed. There seems to be a tendency for each individual to maintain his pH at a constant level. This is characteristic of the individual. We have other evidence that this must be so in the fact that the level of the carbon dioxide absorption curve and the level of the alveolar CO_2 tension, both determinants of the hydrogen ion concentration of the blood, are both constant in any given individual, although subject to a certain variation in different individuals.

If this is so one cannot talk in absolute terms of compensated or uncompensated acidosis or carbon dioxide retention from a single observation of the arterial pH under pathological or abnormal physiological conditions. If Case 1 normally maintains a pH above 7.4 in his arterial blood a pH of 7.30 would, in his case, indicate a retention of carbon dioxide or an uncompensated acidosis. But the same pH (7.30) would be normal for Case 2 and would mean an uncompensated alkalosis in the blood of Case 3. It is necessary to establish the normal pH level of an individual before one can accept as proof of uncompensated acidosis or alkalosis any values except such as lie beyond the limits of normal variation.

Relation of Alveolar and Arterial Carbon Dioxide Tension.

Hasselbalch (10) has shown that if the blood of an individual is exposed at body temperature to a mixture of CO_2 and air of the same CO_2 tension as that of the alveolar air of the subject, the pH of the blood will be found to vary only slightly about a mean of 7.35. This must mean that, in general, the alveolar carbon dioxide tension varies with the height of the absorption curve.

On the other hand, Christiansen, Douglas, and Haldane (9) make the statement that the alveolar CO_2 tension does not vary with the height of the absorption curve.

Table VII gives the calculated pH of blood equilibrated with an air-CO_2 mixture of the same CO_2 tension as that of the alveolar

TABLE VII.

pH of Blood Exposed to an Air-CO_2 Mixture of the Same CO_2 Tension as That of the Alveolar Air of the Individual from Whom Blood Was Removed.

(Values obtained by calculation from the absorption curve.)

Observer.	Subject.	Alveolar CO_2 tension.	Reaction.
		mm. Hg	*pH*
Christiansen, Douglas, and Haldane.	J. S. H.	40.8	7.35
	C. G. D.	38.5	7.35
	J. C.	35.0	7.35
	J. G. P.	40.2	7.35
Krogh and Lindhard.	A. K.		7.34
	J. L.		7.30
Means, Bock, and Woodwell.	J. H. M.	40.6	7.37
	M. N. W.	37.5	7.35
	1920		
The authors.	J. P.		
	Mar. 12	38.7	7.42
	May 19	36.0	7.42
	W. S. M.		
	May 21	35.9	7.35
	D. P. B.		
	Mar. 26	38.5	7.29
	" 29	37.6	7.33
	May 14	37.4	7.30
Average			7.35
Maximum			7.42
Minimum			7.29

air in the three normal persons of our series; four normal persons given by Christiansen, Douglas, and Haldane; A. K. and J. L. from data presented by Krogh and Liljestrand (18) and Krogh and Lindhard (22); and J. H. M. and M. N. W. from Means, Bock, and Woodwell (4). In the case of the subjects A. K. and

J. L. the absorption curves were taken from recently published work, while the figures for alveolar CO_2 tension employed were calculated from several series of observations reported in earlier studies. In all the other cases including our own, absorption curves and alveolar carbon dioxide were determined at the same time. We have calculated the pH by taking the point where the absorption curve of the individual crosses the line representing his alveolar CO_2 tension.

The average normal pH under these conditions is, as Hasselbalch found, 7.35. In spite of the statement of Christiansen, Douglas, and Haldane that they could find no exact relation between the height of the absorption curve and the alveolar carbon dioxide tension, their figures show a remarkable agreement. The difference between our conclusions and those of Christiansen, Douglas, and Haldane may be partially explained by unavoidable differences in the construction of the curves. Hasselbalch's calculations of the "alveolar pH" of J. S. H., C. G. D., and K. A. H. differ from ours by 0.01 and 0.02. This is about the error that should be expected in the construction of the most probable curve from a series of points that has an inherent error as great as that of methods for determining carbon dioxide in blood. The failure of Christiansen, Douglas, and Haldane to recognize the relation between the alveolar carbon dioxide tension and the height of the absorption curve may have been due also to the fact that this relation is not a linear function. They say that although the alveolar CO_2 of J. C. is 5 to 6 mm. lower than that of J. S. H. the corresponding absorption curves are almost indistinguishable. As we have reconstructed the curves from their data, that of J. C. appears to be 2.5 to 3.0 mm. lower than that of J. S. H. Owing to the slope of the pH lines and the dissociation curves, the difference in CO_2 tension between the points of intersection of these curves with the pH 7.35 line is about 6 mm.

The results of our experiments are not quite so satisfactory. Case 1 gives a pH of 7.42, while Case 3's is 7.29 to 7.33, with an average of 7.30. The fact that repeated observations gave identical results suggests that these relations are individual characteristics of the subjects under investigation. If we can accept the composite data for J. L. it is interesting to note that both his

alveolar CO_2 tension and absorption curve agree with those of Case 3, giving in both cases a low pH of only 7.30. In both, the absorption curves are so low that an alveolar CO_2 tension of 30 to 31 mm. would be required to produce a pH of 7.35. This is below the figures usually accepted for the lower limits of the normal range of alveolar CO_2 tension. Arguing in the same way, Case 1 would require an alveolar CO_2 tension of 45 mm. to bring his pH to the 7.35 line. Such an alveolar tension has never been observed in this subject although he has been studied for 5 years.

When the alveolar CO_2 tension is compared with that of the arterial blood (see Table VI, Column 12), the close agreement which has been generally assumed does not appear. From Charts 1, 2, and 3 it is found that, in Case 1, Experiment 1, the alveolar CO_2 tension is 4 mm. higher than that of the arterial blood; in the other two cases the alveolar tension is 6 to 11 mm. lower than that of the arterial blood.

The difference found in Case 1 can hardly be accepted at its face value. One might consider the findings in this case an indication that Krogh (22) and his followers were right in their statement that the values obtained by the Haldane method were too high. But every other observation here reported contradicts this. It is not easy to believe that there was an error in the collection or analysis of the alveolar samples. The subject is experienced in respiratory methods. Duplicate specimens checked perfectly. Not only is this true in this experiment, but in the experiment 2 months later values obtained for both alveolar CO_2 tension and arterial CO_2 content were almost identical with those of the earlier experiment. Finally the alveolar CO_2 was determined in conjunction with a respiratory experiment in which the dead space was calculated by the Haldane (3) formula. With the alveolar CO_2 as determined, a dead space of 130 cc. was obtained. If the alveolar CO_2 tension were assumed to be only 1.5 mm. below the arterial tension, the dead space would have to be as small as 75 cc. and the alveolar tension only 33 mm. Determinations of the dead space by Pearce's (23) method on the same subject gave a value of 130 cc. and his alveolar carbon dioxide tension has never been found as low as 35 mm. An alveolar CO_2 tension of 33 mm. would also, if placed on the dissociation

curve, give a pH of 7.47. The fault is, therefore, probably not in the determination of the alveolar CO_2 tension.

If the absorption curve were only 1.5 volumes per cent lower, the alveolar and arterial tensions would coincide. As we have pointed out, there is sufficient error in the method of determining the absorption curve to make it impossible to draw any conclusions from such small differences. The curve obtained on March 12 is perceptibly higher than three others obtained from the blood of Case 1 on other dates. If the average of all the curves of Case 1 is employed it is found that, in the experiments of March 12 and May 19, the alveolar CO_2 tension is respectively the same as and 3 mm. lower than the arterial tension. In Chart 1 such an average absorption curve has been used (Curve 2) in representing the experiment of May 19. For the experiment of March 12 (Curve 1), the absorption curve as actually determined on that day has been used. Curve 2, we believe, probably represents more nearly the actual relation between alveolar and arterial CO_2 tension in both experiments. In this case the two are, for practical purposes, identical.

The remaining cases are, however, not at all in keeping with the accepted theory which regards the alveolar CO_2 tension as a measure of the arterial tension. Krogh and Krogh (24) found that the alveolar CO_2 tension of rabbits was about 0.1 to 0.2 per cent lower than that of the arterial blood. Our experiments show a difference of 0.8 to 1.5 per cent in normal persons and, as we shall show, a much greater difference in some pathological conditions. Against the assumption that the alveolar CO_2 is in error the same arguments which were proposed in Case 1 may be advanced. In order to make the alveolar and arterial tensions agree by correcting the absorption curve the latter would have to be placed at least 2.5 volumes per cent higher. But in both these subjects repeated determinations of the absorption curve agreed perfectly. It is improbable that a systematic error would occur in four determinations on two subjects and fail altogether to appear in three experiments on a third. The difference between arterial and alveolar CO_2 tension in these cases seems to be a real one.

CO_2 Tension and Hydrogen Ion Concentration of Venous Blood and the Difference between Arterial and Venous Blood.

Results obtained from the analysis of venous blood are inherently less reliable than those obtained from arterial blood because the carbon dioxide and oxygen tension of the venous blood are subject to the influence of local conditions. The influence of venous stasis on both CO_2 content and CO_2-combining capacity has been brought out by Morawitz and Walker (16), as has the effect of exercise. Although it is possible to avoid artificial stasis by omitting the use of a tourniquet, it is impossible to control completely local changes in the blood flow or in the muscular activity of the arm. The fact that no simultaneous studies of arterial and venous blood from either normal or pathological subjects have given consistently satisfactory respiratory quotients is an indication that such factors are active even under the most carefully controlled conditions. Harrop's (25) blood respiratory quotients range from 1.81 to 0.62 in normal persons, 1.76 to 0.25 in cardiac subjects. Our own vary from 1.62 to 0.34. Arterial blood is not subject to the influence of local circulatory conditions and it is, therefore, probable that the chief source of error lies in the venous blood. It is obvious that, unless satisfactory quotients are obtained, the results cannot be considered to represent conditions that exist in the general circulation. Nor is it proper to accept as satisfactory isolated experiments in which satisfactory quotients are obtained. The method must be capable of returning such quotients with a certain degree of regularity in order to permit its use for the determination of the blood flow, venous CO_2 tension, and other similar characteristics in individual experiments. It may be possible, however, to draw general conclusions from the study of a considerable number of experiments.

In Table VI, Columns 9, 10, 11, 12, and 15, and Charts 1, 2, and 3 are given the CO_2 tension and the hydrogen ion concentration of the venous blood of the three normal subjects of our series with the difference in CO_2 tension between the arterial and venous blood. The CO_2 tension of venous blood was found to vary between 42 and 72 mm. uncorrected for oxygen unsaturation, 39.5 to 58.5 mm. after correction, with an average of 50.2

mm. The corresponding values for pH were 7.37 to 7.12 uncorrected, 7.40 to 7.22 corrected, with an average of 7.31. The difference between arterial and venous CO_2 tension was from 0.8 to 9.5 mm. corrected.

The range of variation of all these factors is very considerable. Especially noticeable is the very high venous carbon dioxide tension found in Cases 2 and 3. The first attempt to ascertain the venous carbon dioxide tension was made by Christiansen, Douglas, and Haldane (9) in 1914. Since then many other workers have proposed methods. All these methods are indirect and all depend on the same principle, the use of the lungs as a tonometer. The subject rebreathes a certain amount of air or some other gas mixture for a length of time less than that consumed by a single complete circulation of the blood. During this time the rebreathed air is supposed to attain equilibrium with the venous blood in the pulmonary circulation. From the CO_2 tension of the rebreathed mixture the CO_2 tension of the venous blood is calculated after a correction has been made for oxygen unsaturation. In order to find the arterial CO_2 tension the method of Haldane has been most commonly employed. By the use of these or similar methods all observers have found a difference between the arterial and venous carbon dioxide tension of from 4 to 7 mm. In some studies on the subjects J. P. (Case 1) and D. P. B. (Case 3) made with the Haldane method for arterial CO_2 tension and the Henderson (26) method for venous CO_2 tension we found the difference between the two to be from 2.7 to 7.0 mm. uncorrected for oxygen unsaturation, 1.6 to 4.2 mm. after correction. (The method of correction proposed by Christiansen, Douglas, and Haldane was used. In this 40 per cent of the total difference in CO_2 tension is subtracted as the effect of oxygen unsaturation.) The results obtained by our direct method and those obtained by the older, indirect methods are entirely incompatible. The new method gives more variable, but on the whole higher values.

It may be objected that it is not proper to criticize a method that aims to measure the carbon dioxide tension of the mixed venous blood in the pulmonary circulation on the basis of data obtained from the analysis of venous blood taken from an extremity. It may well be questioned whether the oxygen unsaturation

of the mixed venous blood of Cases 2 and 3 was as great as that found in the arm veins. The oxygen unsaturation in Case 3, Experiment 2, was 67.8 per cent and that of Case 2 was 76.7 per cent. (Lundsgaard (27), Stadie (2), and Harrop (25) found no normal subjects with a venous oxygen unsaturation of more than 40 per cent.) But this cannot explain the whole difference between the results of the direct and indirect methods. The marked oxygen unsaturation will be at least partially compensated by a change in the carbon dioxide-combining capacity of

TABLE VIII.

Difference between Arterial and Venous CO_2 Tension.

(Calculated from ten normal cases presented by Harrop.)

No.	Arterial CO_2 content.	Arterial O_2 saturation.	Venous CO_2 content.	Venous O_2 saturation.	Difference between arterial and venous CO_2 content.	Difference between arterial and venous CO_2 tension.	O_2 capacity.
	vol. per cent	*per cent*	*vol. per cent*	*per cent*	*vol. per cent*	*mm. Hg*	*vol. per cent*
1	51.8	97.5	57.2	74.3	5.4	8.8	23.7
2	54.7	100.0	56.7	84.9	2.0	2.1	17.2
3	52.9	94.3	55.9	64.4	3.0	6.0	16.3
4	46.5	96.3	51.7	67.1	5.2	8.0	20.6
5	44.8	95.1	48.3	80.8	3.5	6.5	18.7
6	49.7	96.0	54.6	61.6	4.9	6.3	20.6
7	48.1	96.3	52.2	75.0	4.1	7.8	14.4
8	50.6	97.6	58.7	64.0	8.1	13.3	24.7
9	53.3	94.4	60.4	73.5	7.1	14.5	18.9
10	44.6	99.0	50.9	61.8	6.3	8.5	21.2
Average					5.0	8.3	
Maximum					8.1	14.5	
Minimum					2.0	2.1	

the blood. Furthermore, it has already been shown that the air obtained by the Haldane method is not necessarily in equilibrium with the arterial blood, but may be as much as 9.5 mm. of CO_2 tension too low. In this case the values for the venous CO_2 tension obtained by the Henderson method must also be too low, because the two have been shown to differ by only 4 to 7 mm.

Three cases are hardly enough from which to come to very definite conclusions, but it is possible, for general purposes, at least, to use Harrop's figures. Harrop presents the carbon dioxide

content and oxygen unsaturation of both arterial and venous blood from ten normal persons. He gives no absorption curves so that it is impossible to estimate actual arterial and venous tensions from his data. We can, however, deduce with little error the difference between arterial and venous tension by assuming an average slope of 4 volumes per cent of CO_2 for each 10 mm. change in CO_2 tension at tensions that exist in the body. (The variations observed between 30 and 80 mm. are 5.75 to 3.53 volumes per cent per 10 mm., the curves gradually approaching the horizontal as the tension increases.) Assuming such an average slope and employing our formula for correction for oxygen unsaturation we arrive at the results shown in Table VIII. The difference between arterial and venous CO_2 content is from 2.0 to 8.1 volumes per cent, with an average of 5.0 volumes per cent. The difference in tension produced by such a difference in content is 5.0 to 20.0 mm. of Hg uncorrected for oxygen unsaturation, or 2.1 to 14.5 mm. after correction, with an average of 8.4 mm. These values are of essentially the same magnitude as those we obtained.

DISCUSSION.

It has been shown that the alveolar CO_2 tension varies with the height of the absorption curve in such a way that if blood is brought into equilibrium at 37.5°C. with an air-CO_2 mixture of the same CO_2 tension as that of the alveolar air of the subject from whom the blood was obtained, the hydrogen ion concentration of the blood so treated will be found to vary between pH 7.40 and 7.30, with an average of 7.35. At the same time the hydrogen ion concentration of the arterial blood shows a variation twice as great, from pH 7.45 to 7.23. The alveolar and arterial tensions are not identical.

As has been pointed out (28), the alveolar CO_2 tension can be considered from two points of view: (1) As a measure of the arterial CO_2 tension; (2) as an indication of the functional condition of the respiratory mechanism. As a measure of the arterial CO_2 tension it seems to be of less value than it has been commonly considered. But this detracts nothing from its value as an indication of the functional state of the respiratory mechanism. It still remains the effective respiratory air. The real

significance of the fact that the CO_2 tension of this "effective air" varies with the height of the absorption curve may be better appreciated if it is stated in terms of effective ventilation. In this sense it is the portion of the respired air which effects the exchange of gases between the blood and the outside air. From the standpoint of carbon dioxide it is the portion of the expired air that effects the necessary CO_2 elimination. A knowledge of the carbon dioxide output and the alveolar carbon dioxide tension is all that is necessary, therefore, to permit the calculation of the effective ventilation by the formula

$$\frac{\text{Cc. } CO_2 \text{ expired per minute} \times 100}{\text{Per cent } CO_2 \text{ in alveolar air}} = \text{Effective minute volume}^2$$

As the cc. CO_2 expired per minute under resting conditions is comparatively constant, one can say with sufficient accuracy for our purposes that the effective ventilation varies inversely as the alveolar CO_2 per cent. But as the latter varies with the height of the absorption curve, it follows that the effective ventilation must vary inversely as the height of the absorption curve.

This can have but one result: it must tend to maintain the hydrogen ion concentration of the blood at a constant level. Why, then, do we find the variations in arterial pH so great? Because of the slope of the absorption curve small changes in the carbon dioxide content of the blood produce relatively large changes in the carbon dioxide tension. The carbon dioxide content of the blood must be largely dependent upon the carbon dioxide production in the body and must fluctuate with the least change in activity. It would be impossible for the alveolar air to follow these changes immediately and accurately without the destruction of the rhythmicity of respiratory activity. In this case an instantaneous cross-section of the respiratory system such as is presented in these experiments might show considerable temporary disturbances of normal relations. It is inevitable also that these should be most striking in that element of the system which is susceptible to the most rapid changes, the blood; and

[2] The principle on which this formula depends has been employed for estimation of effective ventilation by Campbell, Douglas, Haldane, and Hobson (Campbell, J. M. H., Douglas, C. G., Haldane, J. S., and Hobson, F. G., *J. Physiol.*, 1913, xlvi, 301) and Haggard and Henderson (1).

least evident in the slowest moving portion of the system, the alveolar air.

This would be an entirely satisfactory explanation of temporary differences between alveolar and arterial tension found in single observations; *e.g.*, Case 2. But in Case 3 this difference was found on two different occasions and therefore appeared to be a constant and distinctive characteristic of the subject. The pressure difference necessary to effect the passage of carbon dioxide from the blood in the lungs into the alveolar air is greater than has been supposed, in this case at least. This may mean that the invasion coefficient for CO_2 is not what it has been supposed. It more probably means that the term "alveolar air" has not an exact anatomical significance and that the respiratory air, even from the deeper portions of the lungs, is not of uniform composition and is not all in close communication with the blood in the pulmonary circulation. It may indicate that even in normal persons part of the blood passes through unventilated or incompletely ventilated portions of the lung.

There must be material individual differences in reaction. If the charts of Case 1 and Case 3 (Charts 1 and 3) are compared closely, it is at once evident that every element of the latter lies considerably to the right of the corresponding element of the former. The mechanism seems to be set at a different point. One might say that the respiratory system of Case 3 was relatively insensible to acid. In substantiation of this idea it was found in some previous experiments of a different sort that this subject presented a far greater tolerance to carbon dioxide in rebreathing experiments than did Case 1. The latter exhibited marked distress when the CO_2 of the inspired air rose to about 8 per cent. At this point his respiratory rate became extremely rapid and his tidal air was maximal. At 8.5 per cent of CO_2, Case 3 had increased his tidal air to a maximum, but his respiratory rate was almost unchanged and he experienced only moderate discomfort.

Although it has been demonstrated that the difference in CO_2 tension between arterial and venous blood is very variable and may be larger than was indicated by indirect methods, the corresponding difference in pH is astonishingly small. In Case 3, May 14 (Chart 3, Experiment 2), the difference in carbon dioxide

tension even after correction for oxygen unsaturation was 9.5 mm. of Hg. The corresponding difference in pH was only 0.01. This is, of course, partly due to the slope of the absorption curve but far more to the effect of oxygen and the slope of the pH lines. When arterial blood is changed to venous blood the carbon dioxide increase must bear a definite relation to the oxygen reduction. It appears from the charts that, in persons with a normal hemoglobin, these two factors very nearly equalize one another. That the effect of oxygen on the carbon dioxide absorption curve should tend to diminish the difference in hydrogen ion concentration between arterial and venous blood has already been pointed out by Christiansen, Douglas, and Haldane (9), Hasselbalch (10), Parsons (12), and L. J. Henderson (11). The quantitative relations of the reaction have not been previously established.

By comparison of the charts of Cases 1 and 3 it appears that the ability to maintain the hydrogen ion concentration unchanged in the face of alterations in the carbon dioxide is a variable one. Thus Case 1, May 19 (Chart 1, Curve 2), for a change of 0.8 mm. in CO_2 tension shows no change in the pH; while Case 3, May 14 (Chart 3, Experiment 2), for 9.5 mm. of CO_2 tension shows a change of only 0.01 in pH. This is partly due to the fact that the pH lines, passing through the origin, become more nearly horizontal as the pH diminishes. In order to compensate for large carbon dioxide changes it is of advantage to work at a low pH, or, in terms of the charts, at the right of the chart. This advantage is further increased by the fact that the curves more nearly approach the horizontal as they pass to the right, so that the effect of oxygen is much more marked. This may be a further explanation of the greater tolerance of Case 3 for carbon dioxide in the inspired air.

Haggard and Henderson (14) have taken the position that oxygen is without effect on the carbon dioxide absorption curve of blood and have placed their arterial points accordingly. The close agreement of the values obtained by Michaelis (21) on venous blood, by Hasselbalch (10) on blood equilibrated with alveolar CO_2, and by us on arterial blood is perfectly incomprehensible if Henderson is right. The average difference in carbon dioxide content of arterial and venous blood of normal resting subjects as determined by Harrop (25) and the authors from thirteen persons

is 5.5 volumes per cent. This would give a difference of 0.2 in pH on any normal curve. If the arterial pH is 7.4 to 7.2, the venous pH would then have to be 7.2 to 7.0, far below any values actually found.

Miscellaneous Cases.

Although our interest lay primarily in the study of cardiac decompensation and anemia, two patients with other conditions were subjected to complete experiments. As they belong to neither of our special pathological groups they are reported here. One of these had a moderate polycythemia without splenomegaly, Case 4 (Chart 4). This was not sufficient to alter his absorption curve in a very striking manner, although it shows a slope slightly in excess of the normal. In other respects his chart is indistinguishable from that of a normal person. Again we may note a difference of 10 mm. between the alveolar and arterial carbon dioxide tensions, in spite of the absence of signs of pulmonary and cardiac disease.

Chart 5 is obtained from a patient with advanced chronic nephritis and hypertension. His curve lies at the lowest limit of the normal range and may indicate some reduction of the alkali of the blood. In spite of Cheyne-Stokes respirations there is no evidence of any retention of carbon dioxide, as the arterial pH is 7.37. The arterial-venous difference in carbon dioxide is surprisingly small.

In both cases arterial and venous pH are identical.

SUMMARY AND CONCLUSIONS.

The carbon dioxide absorption curve of human blood from three normal subjects has been determined.

The limits of variation of curves obtained from the blood of normal persons agree with those reported by previous observers. The limits of the carbon dioxide capacity of normal blood at 40 mm. of CO_2 tension and 37.5°C. are 43 and 56 volumes per cent with an average of 49 volumes per cent. Curves of different individuals vary in height and to a lesser extent in pitch. The curve of any given individual is quite characteristic and remains constant over a considerable period of time, as has been previously demonstrated by Christiansen, Douglas, and Haldane.

A direct method for the estimation of the arterial and venous carbon dioxide tension of human blood is described. It has been employed on three normal subjects at rest. The hydrogen ion concentration of arterial and venous blood has been calculated by means of the H_2CO_3:$BHCO_3$ ratio. At the same time the alveolar carbon dioxide tension has been determined by the method of Haldane and Priestley and compared with the arterial carbon dioxide tension found by the direct method.

1. The alveolar carbon dioxide varies, in general, with the height of the absorption curve in such a way that the hydrogen ion concentration of the blood of an individual equilibrated with an air-CO_2 mixture of the same CO_2 tension as that of his alveolar air, will fall between the limits of pH 7.42 and 7.29.

2. Although the relation of alveolar CO_2 tension to height of absorption curve shows this variation in a study of a number of persons, it is constant and characteristic for any one individual.

3. The arterial carbon dioxide tension shows about twice as great a range of variation in relation to the height of the absorption curve. In consequence the arterial pH varies from 7.23 to 7.45. The pH of any one individual is constant and characteristic.

4. The alveolar CO_2 tension is not always the same as the CO_2 tension of the arterial blood. It may be as much as 10 or 11 mm. lower.

5. The carbon dioxide tension of venous blood from an arm vein is higher than the values which have been obtained by the rebreathing methods for mixed venous blood. The difference between arterial and venous tension is variable. It averaged 6 mm. but may be as low as 0.8 mm. or as high as 10 mm. Calculations based on the figures for CO_2 content of arterial and venous blood given by Harrop substantiate our results.

6. Values have been corrected for the effect of oxygen unsaturation on the carbon dioxide-combining capacity of the blood. An empirical equation for such correction is presented.

7. In spite of the large difference between arterial and venous carbon dioxide tension, the pH of the two is almost identical largely because of the effect of oxygen on the carbon dioxide-combining power of the blood. It is pointed out that unless such a mechanism were present to reduce fluctuations in hydrogen ion concentration, the values found by other observers for the pH of venous blood would be impossible.

BIBLIOGRAPHY.

1. Haggard, H. W., and Henderson, Y., *J. Biol. Chem.*, 1919, xxxix, 163.
2. Stadie, W. C., *J. Exp. Med.*, 1919, xxx, 215.
3. Haldane, J. S., and Priestley, J. G., *J. Physiol.*, 1904–05, xxxii, 225.
4. Means, J. H., Bock, A. V., and Woodwell, M. N., *J. Exp. Med.*, 1921, xxxiii, 201.
5. Straub, H., and Meier, K., *Biochem. Z.*, 1918, lxxxix, 156; *Deutsch. Arch. klin. Med.*, 1919, cxxix, 54.
6. Van Slyke, D. D., *J. Biol. Chem.*, 1917, xxx, 347.
7. de Corral, J. M., *Biochem. Z.*, 1915, lxxii, 1.
8. Joffe, J., and Poulton, E. P., *J. Physiol.*, 1920–21, liv, 129.
9. Christiansen, J., Douglas, C. G., and Haldane, J. S., *J. Physiol.*, 1914, xlviii, 244.
10. Hasselbalch, K. A., *Biochem. Z.*, 1917, lxxviii, 112. Hasselbalch, K. A., and Lundsgaard, C., *Biochem. Z.*, 1912, xxxviii, 77. Hasselbalch, K. A., and Warburg, E. J., *Biochem. Z.*, 1918, lxxxvi, 410.
11. Henderson, L. J., *Am. J. Physiol.*, 1908, xxi, 427.
12. Parsons, T. R., *J. Physiol.*, 1917, li, 440; 1919–20, liii, 42, 340.
13. Henderson, L. J., *J. Biol. Chem.*, 1920, xli, 401.
14. Haggard, H. W., and Henderson, Y., *J. Biol. Chem.*, 1920–21, xlv, 215.
15. Van Slyke, D. D., and Cullen, G. E., *J. Biol. Chem.*, 1917, xxx, 289.
16. Morawitz, P., and Walker, J. C., *Biochem. Z.*, 1914, lx, 395.
17. Liljestrand, G., and Lindhard, J., *J. Physiol.*, 1919–20, liii, 420.
18. Krogh, A., and Liljestrand, G., *Biochem. Z.*, 1920, civ, 300.
19. Davies, H. W., Haldane, J. B. S., and Kennaway, E. L., *J. Physiol.*, 1920–21, liv, 32.
20. Bohr, C., in Nagel, W., Handbuch der Physiologie des Menschen, Braunschweig, 1909, i, 55.
21. Michaelis, L., Die Wasserstoffionenkonzentration, Berlin, 1914.
22. Krogh, A., and Lindhard, J., *J. Physiol.*, 1917, li, 59.
23. Pearce, R. G., *Am. J. Physiol.*, 1917, xliii, 73.
24. Krogh, A., and Krogh, M., *Skand. Arch. Physiol.*, 1909–10, xxiii, 179.
25. Harrop, G. A., Jr., *J. Exp. Med.*, 1919, xxx, 241.
26. Henderson, Y., *J. Biol. Chem.*, 1917, xxxii, 325.
27. Lundsgaard, C., *J. Biol. Chem.*, 1918, xxxiii, 133.
28. Peters, J. P., Jr., and Barr, D. P., *Am. J. Physiol.*, 1920–21, liv, 307, 335. Barr, D. P., and Peters, J. P., Jr., *Am. J. Physiol.*, 1920–21, liv, 345.

EXPLANATION OF CHARTS.

In all charts the ordinates represent the total carbon dioxide content of the blood, expressed in volumes per cent of gas, dry, at 0°, 760 mm. The abscissa represents carbon dioxide tension in mm. of mercury.

That portion of the absorption curve which was experimentally determined is indicated by a solid black line. Extrapolated portions of the curve are indicated by broken lines. In all the pathological cases the limits of variation of the normal absorption curve are shown enclosed by broken lines.

The pH values are calculated by means of the formula of Y. Henderson.

The arterial points are shown as solid circles. The values uncorrected for oxygen are marked A; the corrected values are marked A'. The venous points are indicated by solid squares. The uncorrected points are marked V, the corrected points V'.

The alveolar carbon dioxide tension is indicated in those instances in which it was obtained. The plasma carbon dioxide-combining capacity is also shown in some cases, expressed in terms of the alveolar carbon dioxide tension with which it should agree in normal persons, according to Van Slyke. The case numbers and chart numbers follow the numbering of the protocols.

CHART 1.

Case 1. J. P., normal subject.

Experiment 1. Feb. 27, 1920.

CO_2 absorption curve:

CO_2 tension, *mm. Hg*	73.7	47.5	15.9
CO_2, *vol. per cent*	64.9	57.7	37.1
	66.5	56.9	37.6

CO_2 content of venous blood, *vol. per cent* 57.1
57.5

Experiment 2. Mar. 5.

CO_2 absorption curve:

CO_2 tension, *mm. Hg*	20.3	40.5	65.6
CO_2, *vol. per cent*	39.1	53.7	64.0
	40.0	56.3	

CO_2 content of venous blood, *vol. per cent* 64.3
64.3

Experiment 3. Mar. 12.

Minute volume of respirations, *cc* 7,150
Tidal air 520 cc. Respirations per min 13.7
CO_2 in expired air 4.29 per cent. CO_2 output per min., *cc.* 306
Alveolar CO_2 before determining minute volume, *mm*.... 41.4
" CO_2 " arterial puncture, *mm* 39.6
" CO_2 after " " " 37.7

CO_2 absorption curve:

CO_2 tension, *mm. Hg*	74.1	47.6	12.2
CO_2, *vol. per cent*	64.3	58.7	34.7
	67.4		

CO_2 content of arterial blood, *vol. per cent* 54.0
54.2

CO_2 " " venous " " " " 62.2
61.7

Difference between arterial and venous CO_2 content, *vol. per cent* 7.9
CO_2 capacity of venous plasma (50.2 mm. Hg), *vol. per cent* 73.0
O_2 content of arterial blood, *vol. per cent* 19.8
O_2 capacity of blood, *vol. per cent* 22.5
O_2 saturation of arterial blood, *per cent* 88

Experiment 4. May 19.

Alveolar CO_2 before arterial puncture, *mm* 38.2
" CO_2 after " " " 37.2
37.0

CO_2 content of arterial blood, *vol. per cent* 54.4
53.7

CO_2 " " venous " " " " 55.7
55.5

Difference between arterial and venous CO_2, *vol. per cent*.	1.5
O_2 capacity of blood, *vol. per cent*	22.1
	22.5
O_2 content of arterial blood, *vol. per cent*	21.5
O_2 " " venous " " " "	18.3
	18.7
O_2 consumption, *vol. per cent*	3.0
O_2 saturation of arterial blood, *per cent*	96
O_2 " " venous " " "	83
Respiratory quotient of blood	0.50

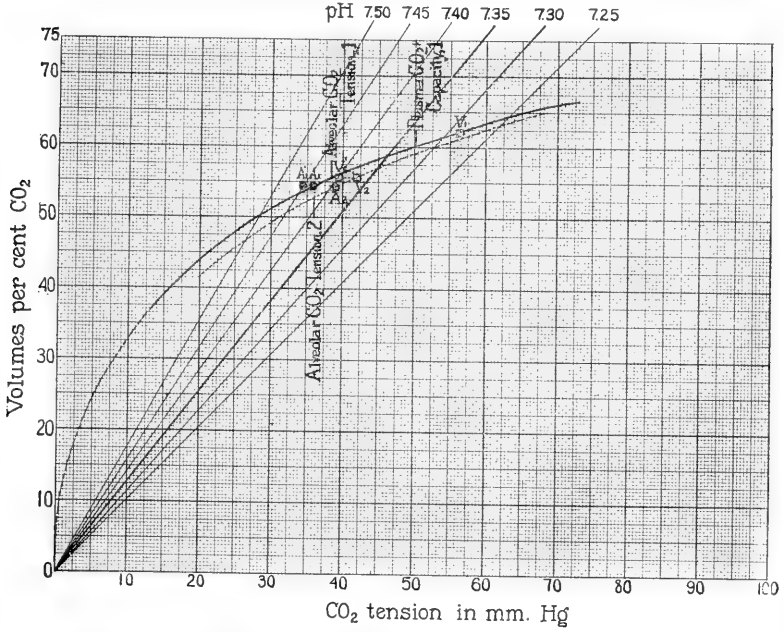

CHART 1. 1. Experiment of Mar. 12.

2. Experiment of May 19. The absorption curve was not determined with this experiment. The absorption curve employed is the average of three determinations made on J. P. on different occasions.

CHART 2.

Case 2. W. S. M. Normal subject.
Experiment 1. Feb. 13, 1920.

CO_2 absorption curve:

CO_2 tension, *mm. Hg*	13.7	80.6	52.1
CO_2, *vol. per cent*	32.5	66.6	54.5
	35.5	70.0	

CO_2 content of venous blood, *vol. per cent* 63.8
64.3

Experiment 2. May 21. The arterial puncture was rendered difficult by an arterial anomaly. Three punctures were necessary before blood was obtained, causing considerable pain and discomfort. The arm was held rigid during the venous puncture.

Alveolar CO_2 before arterial puncture, *mm* 33.5
" CO_2 after " " " 34.3
36.7

CO_2 absorption curve:

CO_2 tension, *mm. Hg*	94.0	60.2	36.3
CO_2, *vol. per cent*	68.1	56.6	47.2
	68.3		46.1

CO_2 content of arterial blood, *vol. per cent* 50.5
51.3
CO_2 " " venous " " " " 58.9
59.9
Difference between arterial and venous CO_2, *vol. per cent* ... 8.5
O_2 capacity of blood, *vol. per cent* 24.9
O_2 content of arterial blood, *vol. per cent* 21.9
O_2 " " venous " " " " 5.8
O_2 consumption of blood, *vol. per cent* 16.1
O_2 saturation of arterial blood, *per cent* 88
O_2 " " venous " " " 23
Respiratory quotient of blood 0.53

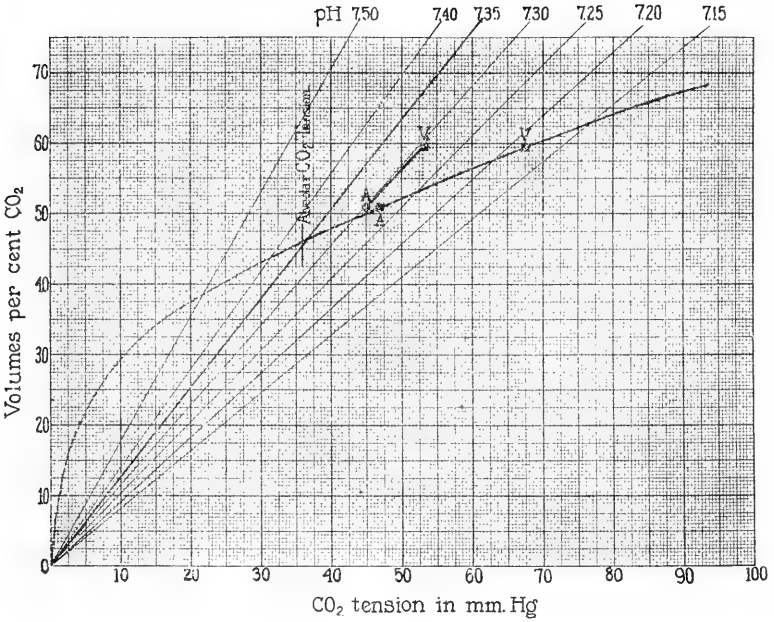
pH 7.50
7.40
7.35
7.30
7.25
7.20
7.15
Volumes per cent CO_2
CO_2 tension in mm. Hg
0
10
20
30
40
50
60
70
80
90
100

CHART 2.

Chart 3.

Case 3. D. P. B. Normal subject.

Experiment 1. Mar. 26, 1920.

Alveolar CO_2 before arterial puncture, *mm*			38.5
			38.8
" CO_2 after " " "			38.2
CO_2 absorption curve:			
CO_2 tension, *mm. Hg*	85.1	45.0	8.9
CO_2, *vol. per cent*	60.9	45.4	25.1
	61.3		26.0
CO_2 content of arterial blood, *vol. per cent*			45.1
			45.9
CO_2 " " venous " " " "			53.1
			53.6
Difference between arterial and venous CO_2, *vol. per cent*			7.8
CO_2 capacity of venous plasma (43.3 mm. Hg) " " "			62.9

Experiment 2. Mar. 29.

Alveolar CO_2 before venous puncture, *mm*			39.5
			38.7
" CO_2 after " " "			36.0
CO_2 absorption curve:			
CO_2 tension, *mm. Hg*	70.1	41.5	20.1
CO_2, *vol. per cent*	59.9	45.2	36.7
	59.9	47.0	37.9
CO_2 content of venous blood, *vol. per cent*			57.7
			57.3
CO_2 capacity of venous plasma (45.4 mm. Hg), *vol. per cent*			65.9

Experiment 3. May 14.

Alveolar CO_2 after arterial puncture, *mm*			37.4
CO_2 absorption curve:			
CO_2 tension, *mm. Hg*	45.2	23.5	96.0
CO_2, *vol. per cent*	45.3	35.0	61.7
	46.1	35.2	61.9
CO_2 content of arterial blood, *vol. per cent*			47.6
			48.1
CO_2 " " venous " " " "			56.2
			56.2
Difference between arterial and venous CO_2, *vol. per cent*			8.3
O_2 capacity of blood, *vol. per cent*			21.4
O_2 content of arterial blood, *vol. per cent*			19.3
O_2 " " venous " " " "			6.9
O_2 consumption, *vol. per cent*			12.4
O_2 saturation of arterial blood, *per cent*			90
O_2 " " venous " " "			32
Respiratory quotient of blood			0.67

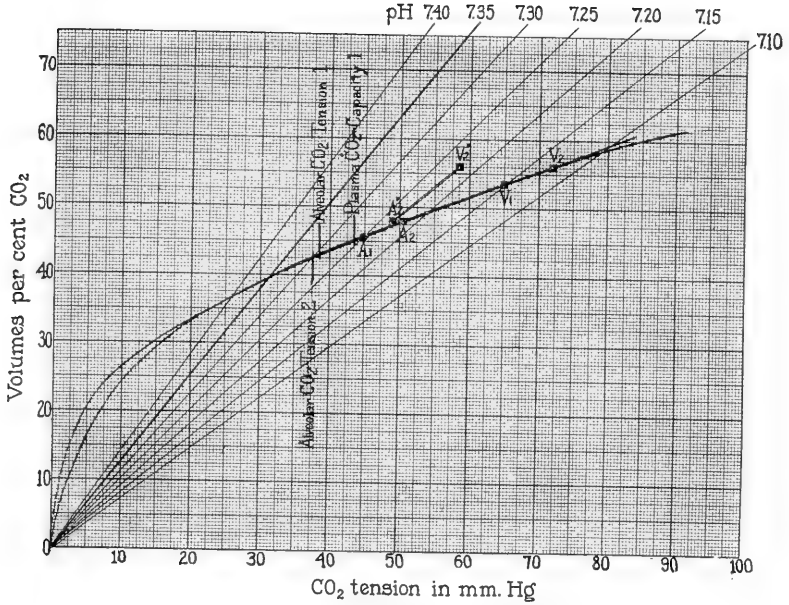

CHART 3. 1. Experiment of Mar. 26.
2. Experiment of May 14.

Chart 4.

Case 4. H. R. Polycythemia. Male, age 36 years. Russian, fireman. History of attacks of dizziness and fainting at intervals for 18 months. For 1 year slight dyspnea on exertion and sharp pains in muscles. Feels lethargic and sleepy in the day time, but restless and wakeful at night. Has to get up two or three times every night to urinate.

Very large man with florid complexion, whose respirations seem somewhat labored. Does not appear to be ill and lies quietly in bed. Tonsils very large and ragged. Lungs clear.

Blood Wassermann negative. Urine negative on two examinations. Fasting blood sugar 130 mg. per 100 cc. 2 hours after 100 gm. glucose by mouth, the blood sugar was still 235 mg. per 100 cc. and he excreted sugar continuously for 24 hours.

Several blood counts gave red blood cell figures varying from 7,800,000 to 8,100,000 in both venous and finger blood. Temperature and pulse normal.

Experiment 1. Apr. 12, 1920.

CO_2 absorption curve:

CO_2 tension, *mm. Hg*	76.5	67.9	53.6	5.4
CO_2, *vol. per cent*	60.6	56.8	50.3	20.7
		58.6		

O_2 capacity of blood, *vol. per cent* 23.4

Experiment 2. Apr. 28. Has been on a low calory diet with low carbohydrate for 1 week. Respirations quieter than at last observation. Slight cyanosis of finger-tips.

Alveolar CO_2 before arterial puncture, *mm* 34.7
35.9

" CO_2 after " " " 33.6

CO_2 absorption curve:

CO_2 tension, *mm. Hg*	50.2	18.5
CO_2, *vol. per cent*	54.6	31.8
	52.8	32.9

CO_2 content of arterial blood, *vol. per cent* 50.9
49.9

CO_2 " " venous " " " " 60.5
59.9

Difference between arterial and venous CO_2, *vol. per cent* ... 9.8
CO_2 capacity of venous plasma (47.7 mm. Hg), *vol. per cent*. 68.6
O_2 " " blood, *vol. per cent* 23.9
O_2 content of arterial blood, *vol. per cent* 22.6
O_2 " " venous " " " " 8.1
O_2 consumption, *vol. per cent* 14.5
O_2 saturation of arterial blood, *per cent* 95
O_2 " " venous " " " 38
Respiratory quotient of blood 0.68

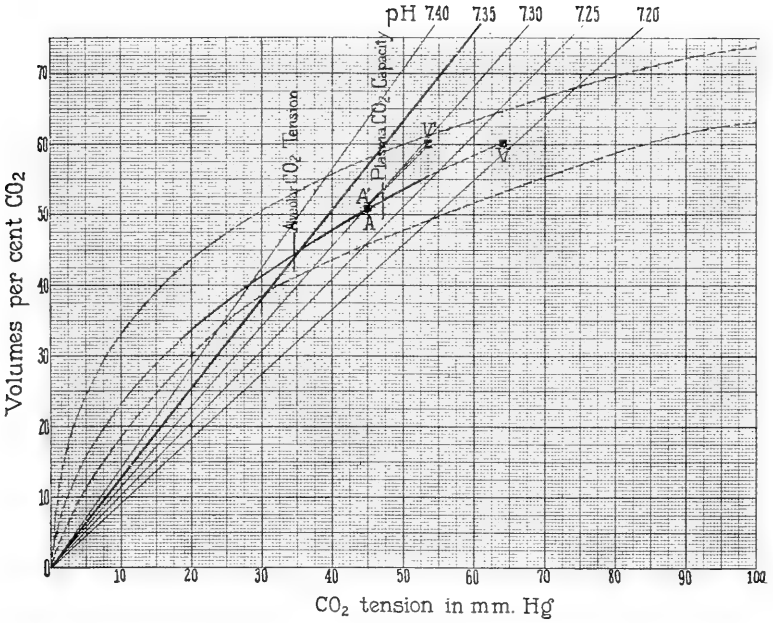

CHART 4. Experiment of Apr. 28.

CHART 5.

Case 5. H. G. Chronic cardionephritic. Male, age 60 years, real estate agent. Looks chronically ill. Quite pale. Cheyne-Stokes breathing with very short periods of apnea. Spastic paralysis of left leg and weakness of muscles of right side of face. Somewhat irrational with loss of emotional control.

Fine râles over left lung at end of inspiration. Heart enlarged to the left, with diastolic murmur over the aortic area and at the apex. Frequent extra systoles. Pulse 64 to 84. Blood pressure: systolic 160, diastolic 90 (has been above 200). Urine shows trace of albumin, no casts. Specific gravity 1.008. Non-protein nitrogen of blood 53 mg. per 100 cc.

Blood count: 4,700,000 red blood cells.

Experiment 1. May 24, 1920. Moderate cyanosis of face and hands. Some dyspnea with Cheyne-Stokes breathing. Arterial blood withdrawn during about equal portions of dyspneic and apneic periods.

CO_2 absorption curve:

CO_2 tension, *mm. Hg*	82.1	28.6	45.3
CO_2, *vol. per cent*	54.8	38.9	46.9
	53.5	38.3	48.8

CO_2 content of arterial blood, *vol. per cent*	40.4
	41.4
CO_2 " " venous " " " "	42.7
	44.0
Difference between arterial and venous CO_2, *vol. per cent*	2.4
CO_2 capacity of venous plasma (36.6 mm. Hg), *vol. per cent*	53.3
O_2 " " blood, *vol. per cent*	16.4
O_2 content of arterial blood, *vol. per cent*	13.8
O_2 " " venous " " " "	9.3
O_2 consumption of blood, *vol. per cent*	4.5
O_2 saturation of arterial blood, *per cent*	84
O_2 " " venous " " "	57
Respiratory quotient of blood	0.53

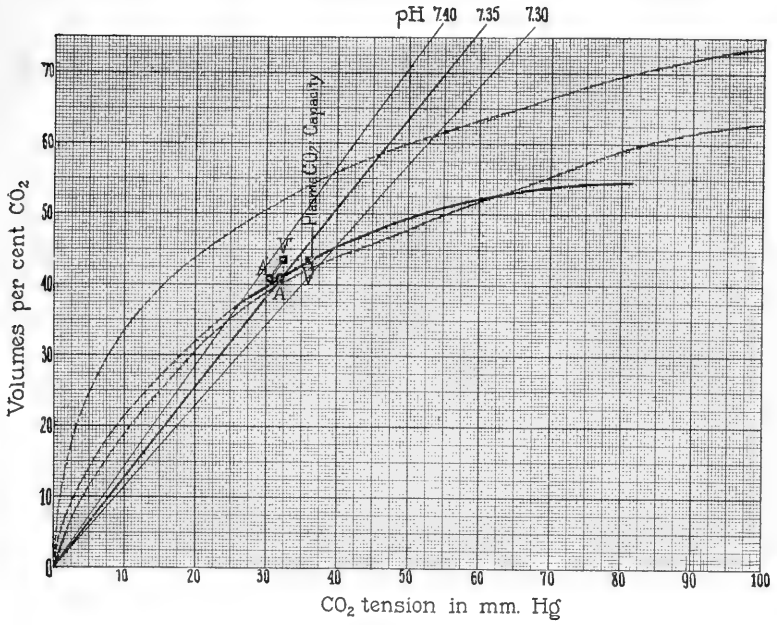

CHART 5.

Miscellaneous Protocols.

Case 21. Jno. K. Diabetes mellitus. Male, age 21 years. Diabetes mellitus of about 7 months duration, moderately severe. Admitted Apr. 5, 1920, with blood sugar of 256 mg. per 100 cc. and marked glycosuria. Emaciated. Skin dry. Cheeks flushed. No dyspnea nor hyperpnea. No cyanosis. Heart and lungs negative to physical examination.

Experiment 1. Apr. 6.

Alveolar CO_2, *mm*. 30.0
29.7
30.8

CO_2 absorption curve:

CO_2 tension, *mm. Hg*	82.9	68.1	32.7	14.1
CO_2, *vol. per cent*	58.2	53.1	37.6	27.5

CO_2 capacity of venous plasma (34.9 mm. Hg), *vol. per cent*. . 50.7

O_2 " " blood, *vol. per cent* 19.2

Case 22. I. M. Diabetes mellitus. Male, age 52 years, married, suit presser. Diabetes of about 7 months duration, rather mild. Well nourished and developed. No dyspnea or hyperpnea. No cyanosis. Blood sugar 202 mg. per 100 cc. Heart negative. Blood pressure: systolic 140,

diastolic 88. Lungs show few, scattered, moist râles. Discharged from hospital sugar-free on diet containing 1,650 calories.

Experiment 1. Apr. 14, 1920.

CO_2 absorption curve:

CO_2 tension, *mm. Hg*	71.1	18.1	58.4
CO_2, *vol. per cent*	56.1	29.4	51.1
	59.0	32.3	

CO_2 capacity of venous plasma (40.9 mm. Hg), *vol. per cent.* 59.5

O_2 " " blood, *vol. per cent* 24.4

Case 23. I. S. Diabetes mellitus. Male, age 42 years, married, cutter. Diabetes of moderate severity for 2½ years. Emaciated, drowsy. No visible signs of dyspnea or hyperpnea. No cyanosis. Lungs and heart negative. Slight pitting edema of the ankles. Blood sugar 274 mg. per 100 cc. Urine shows considerable sugar and acetone. Temperature and pulse normal.

Experiment 1. Mar. 31, 1920.

CO_2 absorption curve:

CO_2 tension, *mm. Hg*	76.0	49.3	28.3
CO_2, *vol. per cent*	62.0	49.3	39.5
		48.0	38.5

CO_2 capacity of venous plasma (43.7 mm. Hg), *vol. per cent.* 63.5

O_2 " " " blood, *vol. per cent* 17.9

18.2

Case 24. D. A. Diabetes mellitus with vulvar abscess. Old woman with senile dementia and diabetes of long standing, complicated by an extensive cellulitis of the vulva. Breathing quietly. Slightly cyanotic. Blood sugar 333 mg. per 100 cc.

Experiment 1.

CO_2 absorption curve:

CO_2 tension, *mm. Hg*	78.4	42.3	17.8
CO_2, *vol. per cent*	61.6	42.4	27.4
	61.5	44.0	28.9

CO_2 content of venous blood, *vol. per cent* 56.8

57.5

CO_2 capacity of venous plasma (46.8 mm. Hg), *vol. per cent.* 68.0

O_2 " " blood, *vol. per cent* 23.8

Case 25. E. C. Emphysema with slight cardiac decompensation.

Experiment 1.

CO_2 absorption curve:

CO_2 tension, *mm. Hg*	85.2	25.5
CO_2, *vol. per cent*	70.2	44.5
	68.8	44.6

O_2 capacity of blood, *vol. per cent* 22.3

22.4

II. THE CARBON DIOXIDE ABSORPTION CURVE AND CARBON DIOXIDE TENSION OF THE BLOOD IN CARDIAC DYSPNEA.

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(Received for publication, December 2, 1920.)

INTRODUCTION.

In cardiac dyspnea the carbon dioxide tension of the alveolar air as determined by the Haldane method is lower than normal in comparison with the plasma bicarbonate as measured by means of the Van Slyke pipette (1, 2). The ratio between these two in health and in most pathological conditions has been shown by Van Slyke, Stillman, and Cullen (3), Walker and Frothingham (4), and Peters (1, 5) to be very close, varying from the mean by only 10 per cent. The existence of such a close relation might well be expected in the light of current theories with regard to the physicochemical control of the respiratory mechanism. That this relation should be disturbed in cardiac dyspnea is hardly extraordinary.

In the average normal person at rest carbon dioxide content, carbon dioxide tension, and hydrogen ion concentration of arterial and of venous blood all vary within comparatively narrow limits both individually and in relation to one another. Furthermore, the physicochemical regulatory mechanism is so delicately adjusted that changes in any of these factors are reflected in an immediate response on the part of the respiratory mechanism, which produces a compensatory alteration of the alveolar CO_2. As long as the sensibility of the respiratory center and the mechanical facilities for the exchange of gases between the blood and the air in the lungs remain unaffected, changes in any of these factors are instantly compensated by changes in one or all of the others. If either the

sensibility of the respiratory center or the mechanical facilities for the elimination of CO_2 in the lungs are impaired, one or all of the normal interrelations must be disturbed.

In cardiac decompensation, with dyspnea, there is evidence that the efficiency of the lungs as a means for the oxygenation of the blood and the elimination of CO_2 is greatly reduced (2, 6). There is also a possibility that the circulation is retarded.

In an attempt to throw some light on the exact cause of the discrepancy between alveolar and plasma values we first devoted our attention to a study of those factors of the respiratory system that may be termed mechanical: the function of the lungs and the upper respiratory tract. The results of this study have been published. It was found that air obtained by the Haldane method from patients with cardiac dyspnea was comparable in a functional sense to that obtained from trained normal subjects (2). That is, the air so obtained was the air employed for the exchange of gases between the blood and the outside air and was the only air available for this purpose. Realizing the objections that might be raised to the use of the term "alveolar" air in this sense, we suggested the terms "effective" or "exchange" air. By this means we evaded the criticisms which had been advanced by Siebeck (6). The latter, in the course of some studies of the lung volume in cardiac decompensation, came to the conclusion that the lungs in this condition failed to function efficiently as a tonometer. He decided that the mixing of gases in the lungs was imperfect and that methods for obtaining alveolar air were inapplicable. However, he considered the alveolar air only as a means of ascertaining the carbon dioxide tension of the arterial blood. Although our findings were not entirely in accord with Siebeck's, no attempt to controvert his statement was made. The question of the relation of alveolar CO_2 to arterial tension was entirely ignored, although its importance was fully recognized. Further studies convinced us that the effective lung volume was diminished (7) and the effective ventilation increased (8) in cardiac decompensation. Pearce (9) had proposed as an explanation of the disturbance of the alveolar: plasma ratio a retarded circulation. In view of the changes in the lung volume and the findings of Siebeck, however, this could not be definitely proved by the ordinary respiratory methods. Pearce assumed what Siebeck denied,

the applicability of alveolar methods as a means of determining arterial CO_2 tension. Even in normal subjects such an assumption seemed to be open to some criticism. The only direct evidence of a definite relation between alveolar and arterial CO_2 tension was given by Krogh and Krogh (10) in some tonometric experiments on animals. There was no evidence of any kind that a similar relation existed in diseases involving disturbances of the respiratory mechanism. Obviously it was necessary to find a means to measure the arterial CO_2 tension and to compare this with the alveolar CO_2 tension. For this reason the method described in the first paper was employed in the study of patients with cardiac decompensation. The CO_2 absorption curves of seven patients were obtained one or more times. Complete experiments were made on four cases out of seven. The results of these experiments are collected in Table III, arranged as were those of the normal subjects in Table VI of Paper I. Detailed protocols of all seven cases are presented at the end of this paper.

Level of the Absorption Curve in Cardiac Dyspnea.

The absorption curves of normal individuals fall within certain definite limits and are constant and characteristic for any given individual. This is far from the case in pathological subjects. The variations in both group and individual are considerable. The limits of normal variations in height are about 43 to 56 volumes per cent. The limits of variation of twenty-two patients with various pathological conditions were 32.4 to 70.2 volumes per cent, about three times as great. It is probable that a study of more patients with a greater variety of pathological conditions would show a still greater range of variation. In relatively few cases has more than one observation been made; but in these few the changes in height at different times are also quite striking. The maximum change observed was in Case 11, whose curve rose from 36.2 to 51.1 in the course of 4 days. Such fluctuations are not restricted to conditions of recognized acidosis, such as is found in diabetes mellitus or in nephritis. They occur also in cardiac valvular disease and in severe anemia.

The height of the absorption curves of seven decompensated cardiac patients, on whom eleven observations were made, varied from 32.4 to 52.0 volumes per cent, with an average value of 44.7

TABLE I.

Absorption Curve at 40 Mm. CO_2 Tension in Seven Patients with Cardiac Decompensation and Dyspnea.

Case.	Diagnosis.	CO_2 content.
		vol. per cent
6. G. B.	Paroxysmal tachycardia. Some dyspnea; considerable cyanosis; fluid in right pleural cavity and in abdomen; edema of both ankles; temperature 103°.....	45.4
7. J. D. B.	Aortic regurgitation. Mar. 19. Able to be up and about ward, but showed moderate dyspnea and orthopnea, with faint cyanosis of lips and finger-tips even while at rest.....	45.0
	Apr. 30. Condition practically unchanged.....	47.5
8. E. H.	Mitral stenosis; auricular fibrillation. Marked dyspnea and orthopnea; considerable cyanosis; general edema; hydrothorax and ascites..	47.5
11. J. K.	Mitral stenosis; auricular fibrillation.	
	Apr. 16. Extreme cyanosis; moderate hyperpnea and edema.....	36.2
	Apr. 20. Cyanosis and hyperpnea improved....	51.1
10. J. M.	Mitral stenosis; auricular fibrillation.	
	Apr. 9. Extreme cyanosis; moderate dyspnea and edema.....	38.7
	Apr. 23. Cyanosis continues; dyspnea and edema gone; still some hyperpnea.....	48.1
9. P. O. S.	Aortic regurgitation.	
	Mar. 9. Marked dyspnea with irregular breathing. Moderate cyanosis; considerable edema with hydrothorax.....	52.0
	Mar. 23. Symptoms steadily increasing.....	48.1
12. C. C.	Mitral stenosis. Marked cyanosis; moderate hyperpnea.....	32.4
Maximum.....		52.0
Minimum.....		32.4
Average.....		44.7

volumes per cent (see Table I and Fig. A). Of the seven patients three showed a definite reduction of the alkali reserve of the blood during the stage of most severe decompensation. Later observations on two of these, at the time when compensation was becoming reestablished, gave normal values. The other four cases showed no departure from the normal as regards the height of their curves.

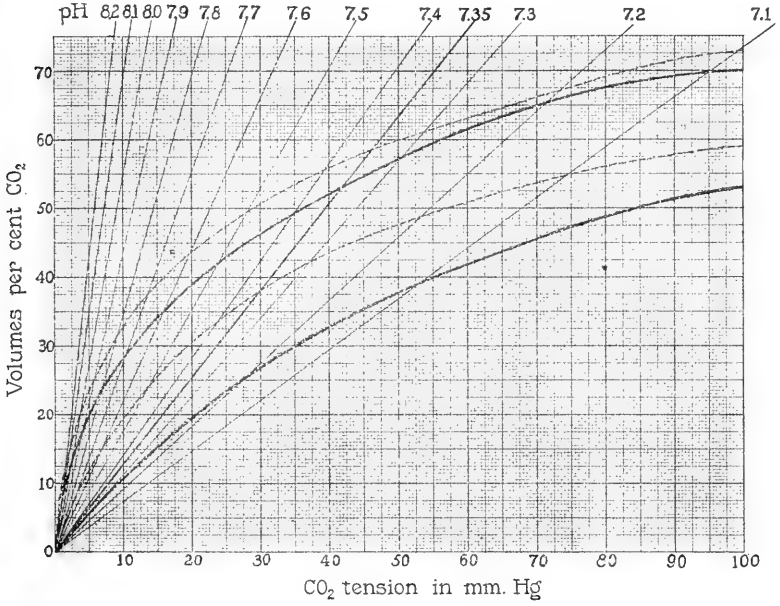

Fig. A. A comparison of limits of absorption curves of normal and cardiac subjects.

———— Limits of cardiac curves.

..... Limits of normal curves.

A low absorption curve is not a characteristic of the condition of cardiac decompensation. What factors determine the presence of such a curve in certain cases is not clear. As we shall show later, there is a relative retention of carbon dioxide in the arterial and venous blood of the cases with acidosis. This, in itself, should tend to produce an increase rather than a reduction of the alkali reserve, according to Henderson and Haggard (11).

It has been demonstrated by several observers, using various methods, that reduction of the partial pressure of oxygen in the blood produces a reduction of carbon dioxide tension and carbon dioxide content of the blood (12–15). However, in these studies the reduction of oxygen tension was produced in such a way that CO_2 was not allowed to accumulate in the blood. Whether a similar reduction in the level of the absorption curve will be produced by low oxygen in the presence of CO_2 accumulation has not been determined. That there is a certain amount of oxygen

TABLE II.

Comparison of CO_2 Capacity of Whole Blood and Oxygen Unsaturation of Blood.

Subject and diagnosis.	Date.	CO_2 capacity of blood.	Oxygen capacity of blood.	Oxygen content.		Oxygen saturation.	
				Arterial blood.	Venous blood.	Arterial blood.	Venous blood.
	1920	*vol. per cent*	*vol. per cent*	*vol. per cent*	*vol. per cent*	*per cent*	*per cent*
1. J. P. Normal.....	Mar. 12	55.9	22.5	19.8		88.0	
2. D. P. B. "	May 14	43.6	21.4	19.3	6.9	90.2	32.2
3. W. S. M. "	" 21	48.2	24.9	21.9	5.8	88.0	23.3
4. M. C. Asthma and emphysema.....	" 12	49.0	21.3	17.2	11.0	80.8	51.7
5. H. R. Polycythemia.	Apr. 28	47.9	23.9	22.6	9.0	94.6	37.7
6. J. K. Cardiac.....	" 20	51.1	26.5	25.0	22.5	94.3	84.9
7. J. M. "	" 23	48.1	20.8	22.0	11.0	100.0	52.9
8. J. D. B. "	" 30	47.5	13.9	14.3	3.7	100.0	26.6
9. G. B. "	Mar. 24	45.4	21.7		5.0		23.0
10. J. M. "	Apr. 9	38.7	20.2	16.7	8.5	82.7	42.1
11. J. K. "	Mar. 16	36.2	26.5	24.2	9.9	91.3	37.4
12. C. C. "	Apr. 9	32.4	18.3	18.0	3.2	98.4	17.5

unsaturation in the arterial blood and a very marked degree of oxygen unsaturation in the venous blood of some patients with cardiac decompensation has been shown by Harrop (16). As the most notable feature of the three patients with low absorption curves was a degree of cyanosis out of all proportion to the degree of dyspnea and hyperpnea, it seemed possible that the height of the absorption curve might bear some relation to the degree of oxygen unsaturation of the arterial and the venous blood.

That no such relation can be clearly established in our cases is evident from Table II, in which are shown the height of the carbon

dioxide absorption curve, and the oxygen content and saturation of both arterial and venous blood. At least, if the low curve is due to oxygen unsaturation, this is not the only active factor.

Although the restoration of compensation was associated with a return of the absorption curve to its normal level in Cases 10 and 11, the height of the absorption curve is no indication of the severity of the cardiac decompensation. The three cases with low curves were discharged from the hospital improved, while Cases 6, 8, and 9 with normal curves, failed to improve and died shortly after the observations reported.

There was nothing in the clinical picture to suggest a nephritic acidosis in any of these cases as the cause of the low absorption curves. Rather complete renal functional studies were made in Case 10. His blood non-protein nitrogen was only 43 mg. per 100 cc. and his phenolsulfonephthalein excretion 60 per cent in 2 hours.

We are then left to the conclusion that cardiac decompensation is sometimes associated with a real reduction of the alkali reserve of the blood, which disappears when compensation is reestablished. Why this should occur in some instances and not in others is not explained.

Arterial and Venous Carbon Dioxide Tension and Hydrogen Ion Concentration and Alveolar Carbon Dioxide Tension in Cardiac Dyspnea.

Complete experiments were made on four out of the seven cases with cardiac decompensation. The results of these experiments appear in Table III (arranged as were those of the normal subjects in Table VI, Paper I), and in Charts 7, 10, 11, and 12 inclusive. If the general averages in Table III are compared with those in Table VI, Paper I, the following points of distinction appear:

1. Although there is little difference in the range of variation of arterial CO_2 tension in the two tables, the CO_2 tension during cardiac dyspnea is high in relation to the level of the absorption curve because the absorption curves are low in three of the four subjects.

2. The consequence is that the arterial pH values in cardiac dyspnea are on the average lower than normal, indicating a carbon dioxide retention in the arterial blood.

TABLE III.

Results of the Study of Seven Cardiac Patients with Dyspnea.

Subject.	Date.	Alveolar CO_2 tension. (1)	Alveolar reaction. (2)	Arterial CO_2 content. (3)	Arterial CO_2 tension. Uncorrected. (4)	Arterial CO_2 tension. Corrected. (5)	Arterial reaction. Uncorrected. (6)	Arterial reaction. Corrected. (7)	Venous CO_2 content. (8)	Venous CO_2 tension. Uncorrected. (9)	Venous CO_2 tension. Corrected. (10)	Venous reaction. Uncorrected. (11)	Venous reaction. Corrected. (12)	Difference between arterial and alveolar CO_2 tension. (13)	Difference between venous and arterial CO_2 content. (14)	Difference between venous and arterial CO_2 tension. (15)	Difference between venous and arterial reaction. (16)
	1920	*mm. Hg*	*pH*	*vol. per cent*	*mm. Hg*	*mm. Hg*	*pH*	*pH*	*vol. per cent*	*mm. Hg*	*mm. Hg*	*pH*	*pH*	*mm. Hg*	*vol. per cent*	*mm. Hg*	*pH*
6. G. B.	Mar. 24								54.8	61.0	47.2	7.19	7.26				
7. J. D. B.	" 19	25.2	7.42						49.8	50.5		7.23					
	Apr. 30	27.6	7.42	49.1	42.5	42.5	7.30	7.30	52.7	53.0	46.5	7.25	7.30	14.9	3.6	4.0	0.00
8. E. H.	Mar. 10								51.1	46.5		7.29					
9. P. O. S.	" 9								62.1	61.0		7.25					
	" 23								56.9	65.5		7.18					
10. J. M.	Apr. 9	26.0	7.34	39.6	40.5	38.0	7.23	7.25	49.8	61.0	52.3	7.15	7.22	13.0	10.2	14.3	0.03
	" 23	33.0	7.37	54.0	52.2	52.2	7.26	7.26	63.4	75.0	65.2	7.16	7.23	19.2	9.4	13.0	0.03
11. J. K.	" 16	22.0	7.32	35.9	39.7	38.0	7.20	7.20	45.5	62.3	47.5	7.10	7.21	16.0	9.6	9.5	−0.01
	" 20*	(32.5)	(7.41)	(52.4)	(42.25)	(41.25)	(7.34)	(7.35)						(8.75)			
12. C. C.		27.4	7.22	33.5	42.5	42.5	7.13	7.13	42.9	62.5	50.0	7.06	7.17	15.1	9.4	7.5	−0.04
Maximum*.....		33.0	7.42	54.0	52.2	52.2	7.30	7.30	63.4	75.0	65.2	7.29	7.30	19.2	10.2	14.3	0.03
Minimum*.....		22.0	7.22	33.5	39.7	38.0	7.13	7.13	42.9	46.5	46.5	7.06	7.17	13.0	3.6	4.0	−0.04

3. The alveolar CO_2 tension is, as has been previously pointed out, lower than normal. It is variable in relation to the absorption curve and may be, as in Case 12 (Chart 12), comparatively high, giving an alveolar pH far below the normal limits.

4. The difference between alveolar and arterial CO_2 tension is consistently increased. In five experiments on dyspneic cardiac patients this difference never fell below 13 mm. and in one case it reached the astonishing figure of 19 mm.

5. The venous CO_2 tension falls within normal limits, but, like the arterial, is high in relation to the level of the absorption curve.

6. There is no consistent increase in the difference between arterial and venous CO_2 content or tension, although in one or two instances both were slightly above the normal limits.

When each of these factors is studied in relation to the individual subjects of these experiments, it is apparent that only one is definitely and consistently present in every instance of cardiac dyspnea: this is the increase in the difference between alveolar and arterial CO_2 tension.

Although the average arterial CO_2 tension is relatively high and the arterial pH, in consequence, lower than normal, in Cases 7 (Chart 7) and 10 (Chart 10) the pH of the arterial blood is quite within normal limits. Can one say, then, that a carbon dioxide retention is characteristic of cardiac dyspnea, or does it occur only in certain cases, possibly those who show a considerable reduction in the height of the absorption curve, as do Cases 11 and 12? Unfortunately the range of variation in the normal arterial pH makes this question hard to answer. Absolute values are of little assistance in judging relative factors. Our results suggest that different normal subjects maintain the pH of their arterial blood at different levels. Furthermore the resting arterial pH level seems to be characteristic and constant for a given individual, as is the level of the absorption curve itself. In this case an arterial pH which is normal for one individual might mean a considerable retention of carbon dioxide in the case of another.

The statement was made above that there was nothing characteristic about the height of the absorption curve of the blood of patients with cardiac dyspnea, but that in certain cases low curves were found, indicating a diminution of the available alkali of the blood. We were then considering the absolute level of the

absorption curve. Again attention must be called to the fact that we are dealing in relative values only and that absolute levels, although satisfactory for purposes of clinical study, give little information with regard to functional changes in physiology or pathology. In order to ascertain whether a given pathological condition has changed the level of either the absorption curve or the arterial pH of an individual, it is not enough to know the limits of variation of normal individuals as a group. It is necessary to know the normal resting absorption curve and arterial pH of the given subject under investigation. In clinical investigations this is not always possible. Patients with severe cardiac dyspnea do not invariably recover. Of our seven cases three died without any intervals of improvement (Cases 6, 8, and 9); one (Case 7) had a chronic cardiac decompensation which resisted treatment, increasing gradually but steadily. Cases 10 and 11 improved. The former never completely recovered compensation; the latter made a very rapid recovery and was discharged without dyspnea or hyperpnea. Two determinations of the absorption curve were made on the blood of four of the seven patients: Cases 7, 9, 10, and 11.

In Case 9 the second observation, made 2 weeks after the first, revealed a drop of 4 volumes per cent in the height of the absorption curve. During this time the condition of the patient had continuously grown worse. The two observations on Case 7, at an interval of almost 6 weeks, were only about 2 volumes per cent apart. The second was the higher. There was little change in Case 7's condition on these two occasions. Possibly his dyspnea was a little more severe in the earlier experiment; his general condition seemed a trifle less favorable at the time of the second observation. The changes in absorption curve and in condition are, in any event, of no significance.

The rise in level of the absorption curves that occurred during the clinical improvement of Cases 10 and 11 is very striking. Although it is impossible, then, to say that cardiac dyspnea is always attended by a lowering of the absorption curve, there is a suggestion that such an association is not uncommon.

For the arterial pH our data are much more meager. This was determined in only four cases and in only two of these were two determinations obtained. In one of these, Case 10, the pH

remained unchanged in spite of a rise in the level of the absorption curve; in the other, Case 11, both pH and absorption curve rose. The latter regained compensation more completely than did the former in the interval between observations. The second experiment on Case 11 gives normal results in all essentials. Even the difference between alveolar and arterial CO_2 tension has returned to normal limits. It is quite conceivable that the picture presented by Case 10 on April 23 represents a preliminary step on the way to compensation and that, with complete recovery, which never occurred, the arterial pH would also have risen. There is no doubt that Case 12 had an uncompensated acidosis because his arterial pH lay below the normal limits. A definite carbon dioxide retention or uncompensated acidosis[1] occurred in two subjects, then. In the others a similar condition cannot be excluded as the result of our experiments although the arterial pH lay within normal limits.

There is nothing questionable about the increased difference between arterial and alveolar CO_2 tension. It occurred consistently in the presence of dyspnea and returned to normal in the one instance in which compensation was definitely reestablished. It is inconceivable that such a difference is due merely to errors in analytical methods or in calculation. It has been shown that the assumption that the alveolar air is in carbon dioxide equilibrium with the arterial blood is not always in keeping with the facts. That such differences as occurred in normal resting subjects might be only temporary was pointed out. But in cardiac dyspnea the differences are far greater and are invariably present.

Such a difference in itself might be produced by a diminution of the rate of the circulation. The venous blood might return to the lungs with an accumulation of carbon dioxide too great to permit complete removal in the lungs. The CO_2 remaining would pass on into the arteries and produce an increase in ventilation by its action on the respiratory center. In this case the difference between arterial and venous CO_2 tension and arterial and venous CO_2 content should be increased. Such an increase

[1] By a carbon dioxide retention or uncompensated acidosis we mean an accumulation of carbon dioxide sufficient to produce a real change in the pH of the blood.

is sometimes found, but it is not consistent and bears no relation to the arterial-alveolar discrepancy. The differences between arterial and venous CO_2 tension and arterial and venous CO_2 content in Case 7, for instance, are only 4.0 mm. and 3.6 volumes per cent respectively, while the arterial-alveolar difference is 14.9 mm. In addition to our own experiments we have calculated the differences between arterial and venous CO_2 content and CO_2 tension in ten of Harrop's (16) cardiac cases who showed definite evidences of decompensation. The calculations were made by the same formula employed in the normal cases (see Paper I). The results appear in Table IV and can be compared with Harrop's normal cases in Table VIII, Paper I. In his cases, as in ours, there is little evidence of a diminished circulation rate. A retarded circulation would seem to be, therefore, at the most only an occasional minor cause of the difference between arterial and alveolar CO_2 tension.

The predominant cause must lie in the lungs themselves. There must be an interference with the escape of CO_2 from the blood in the pulmonary circulation. This seems the more probable because of the known changes in the effective lung volume (7, 17). There may be portions of the lungs in which the circulation is more or less intact, but which contain no air. Or there may be portions of the lungs which are air-containing, but immobile and not adequately ventilated by the respirations. The latter is the view of Siebeck (6). Present methods of measuring lung volume have failed to settle the question because they are capable of measuring only the portions of the lungs that contain air available for respiratory purposes. Whether or not there is a true carbon dioxide retention in all cases of cardiac dyspnea, there is always an interference with the elimination of CO_2 from the blood and, therefore, a compensated or potential acidosis.

If the alveolar air is considered from the standpoint of respiratory mechanics, as a measure of the effective ventilation, a greater effective ventilation is necessary in order to maintain a given arterial carbon dioxide tension in the case of the patient with cardiac dyspnea than is necessary in the case of the normal person. To what extent does this account for the increase in the hydrogen ion concentration of the arterial blood? Does the alveolar carbon dioxide tension always indicate a ventilation in

excess of the normal in relation to the height of the absorption curve? If this were true the alveolar pH should always be considerably above 7.30. This is not true in Case 10, April 9, Case 11, April 16, and Case 12. In these instances the alveolar carbon dioxide is at or below the normal limit in relation to the height of

TABLE IV.

Difference between Arterial and Venous CO_2 Tension.

(Calculated from observations on patients with cardiac decompensation, presented by Harrop.)

Case No.	Arterial CO_2 content.	Arterial oxygen content.	Venous CO_2 content.	Venous oxygen content.	Difference between arterial and venous CO_2 content.	Difference between arterial and venous CO_2 tension.	Oxygen capacity.
	vol. per cent	*vol. per cent*	*vol. per cent*	*vol. per cent*	*vol. per cent*	*mm. Hg*	*vol. per cent*
1	46.4	17.2	54.0	6.9	7.6	15.4	20.3
	46.3	19.6	48.7	16.3	2.4	4.9	21.8
	44.3	22.3	56.1	15.5	11.8	27.2	23.1
2	45.4	17.1	48.3	9.3	2.9	4.6	19.0
	48.2	17.9	52.7	9.5	4.5	8.4	18.7
	49.6	17.8	53.1	12.6	3.5	6.9	18.5
4	41.4	13.9	44.2	10.8	2.8	5.9	16.8
	43.5	15.2	46.5	9.6	3.0	5.6	16.6
5	38.9	17.7	41.8	6.3	2.9	3.3	19.9
	42.7	18.2	47.1	10.4	4.4	8.3	19.7
	43.8	17.9	47.6	13.1	3.8	7.8	19.6
7	38.7	16.5	43.2	7.3	4.5	7.5	18.9
	42.9	20.5	46.5	16.1	3.6	7.5	22.9
	43.6	21.1	49.0	15.4	5.4	5.5	22.5
8	31.4	19.3	37.5	11.1	6.1	12.4	20.7
	42.8	21.3	45.7	11.6	2.9	3.9	22.5
	41.5	22.5	46.5	17.6	5.0	10.8	23.3
Maximum..	49.6		56.1		11.8	27.2	
Minimum..	31.4		37.5		2.4	3.3	
Average....	43.0		47.6		4.5	8.6	

the corresponding absorption curves. Although these three patients showed some dyspnea the ventilation was insufficient to compensate for the low absorption curves they presented. Nor was this due to the fact that they had passed the limits of compensation and were unable to increase their ventilation

further. In none of these cases was dyspnea a prominent feature. The actual minute volume of respiration of Case 10 was only 8,650 cc. with a respiratory rate of 18 per minute. This suggests that the uncompensated acidosis found in these cases was not due entirely to an inability of the injured respiratory mechanism to effect the proper carbon dioxide elimination, but to the fact that the respiratory center was not so sensitive as normal to its natural stimulus, the hydrogen ion concentration of the blood. As the comparative lack of dyspnea shown by these patients is not at all typical of cardiac decompensation, this insensibility of the respiratory center to the natural acid stimulus is not necessarily a characteristic of cardiac dyspnea *per se*.

In the normal subjects the pH of the arterial and venous blood was found to be almost identical in spite of a considerable difference in CO_2 content. This was possible because of the effect of oxygen on the absorption curve. The same holds true of the cardiac cases, in some of whom even larger differences of CO_2 content occur. The difference in pH of arterial and venous blood never exceeds 0.04 and is, therefore, within the limits of error of the method.

One may pause for a moment to consider what bearing the presence of a carbon dioxide retention in association with a low absorption curve may have on the work of Henderson and Haggard (11). They claim that any damming back of carbon dioxide causes the blood to abstract alkali from the tissues and produces a rise in the level of the absorption curve. The facts here established for cardiac dyspnea are quite at variance with such a theory unless one argue from a teleological standpoint that the damming back of carbon dioxide is produced in an attempt to withdraw alkali from the tissues and thus restore the alkali of the blood to its normal level. Aside from the fact that this ascribes to the blood a purposeful effort at compensation, this is not the usual reaction to a reduction in the level of the absorption curve as is evidenced by the curves of nephritis patients (see Case 5, Paper I, and Case 16, Paper III).

It is interesting to compare the relations that obtain in cardiacs with those found by Means, Bock, and Woodwell (18) in pneumonia and with those found by us in one case of severe asthma and emphysema with extreme cyanosis (Case 13, Chart 13).

These investigators found a carbon dioxide acidosis in three pneumonia patients, without any reduction in the level of the absorption curve. They, however, accept Henderson's view that oxygen does not affect the absorption curve of blood. Fortunately they have published figures which allow us to make corrections by our formula. As we have calculated their results the arterial pH values in their three cases are: for E. D., 7.23 uncorrected, 7.28 corrected; for G. A., 7.30 uncorrected, 7.35 corrected; for N. D., 7.27 uncorrected, 7.30 corrected. Because of the degree of oxygen unsaturation in the arterial blood of pneumonia patients these corrections are much larger than the corresponding corrections in cardiac dyspnea, where the oxygen unsaturation of the arterial blood is usually comparatively slight. If oxygen does affect the CO_2-combining capacity of blood there is little evidence of a carbon dioxide acidosis in these figures alone.

Case 13 had an asthmatic condition of long standing with an extreme emphysema and the most extraordinary cyanosis. His face and extremities were at all times a deep purple and during his acute attacks were almost black. The experiment was performed during one of these attacks. His absorption curve lies at a normal level. His arterial tension is quite high in proportion and about 18 mm. higher than the alveolar tension, which lies at a pH of 7.33. The corrected arterial pH is 7.22. The most striking thing about his chart is the enormous venous carbon dioxide tension. Although the difference between the arterial and venous carbon dioxide content is only 5.7 volumes per cent, the portion of the absorption curve on which the arterial and venous points lie is so flat that this represents a change of CO_2 tension of about 40 mm. uncorrected, as far as we can tell by extrapolating the venous point. The compensating effect of oxygen is, however, also increased by the flatness of the curve sufficiently to reduce the venous tension about 35 mm. (In calculating the effect of oxygen on the venous point in this case we were unable to use our formula because the CO_2 tension was above 70 mm. A value for K of 0.414 was used instead of the usual 0.34. This is the average value of K between 70 and 90 mm. From Table I it appears that there is a considerable variation in the value of K above 70 mm. and there may be a large error in this correction.) In spite of this there is still a very

considerable difference in pH between the arterial and venous blood. This difference is, however, much less than would occur if the arterial tension were lower on a curve of the same level, because of the difference in the slope of the pH lines. In a study of these factors may lie an explanation of the relatively great tolerance for carbon dioxide found by Scott (19) in emphysema patients. No general conclusions can be drawn from this one patient as to the respiratory mechanism in emphysema or asthma. The picture is similar to that of cardiac dyspnea in the fact that there is a marked difference between the alveolar and the arterial CO_2 tension, suggesting again a functional impairment of the ventilating power of the lungs. Furthermore, there is a carbon dioxide retention, as evidenced by the low arterial pH.

Plasma Bicarbonate as a Measure of the Alkali Reserve.

As a result of a large series of observations of the carbon dioxide-combining capacity of the venous plasma of decompensated cardiac patients made in 1916 and subsequently, we were led to conclude that the alkali reserve of the blood of these patients, although quite variable, lay for the most part within normal limits. The same thing is true of the present cases, if we consider the carbon dioxide capacity of the plasma. Six determinations in this series vary from 55.0 to 75.7 volumes per cent. Among these is a determination made on Case 11 from the same specimen of blood that showed such a marked reduction in the height of the absorption curve.

The contradictory results obtained from whole blood and from venous plasma may be explained by variations in the carbon dioxide content of the venous blood. As the tension of carbon dioxide in blood is increased the bicarbonate of the blood also increases. This is evidenced by the fact that the slope of the absorption curve is greater than that of the curve which represents the solubility of carbon dioxide in the blood. The increase in the bicarbonate of blood in response to increases of CO_2 tension is associated with a transfer of base from cells to plasma. Joffe and Poulton (20) have demonstrated that the change in bicarbonate content of whole blood is reflected in the plasma. This had already been clearly demonstrated by Van Slyke and Cullen (21). Van Slyke and Cullen, Straub and Meier (22), and

others have shown that the CO_2 absorption curve of plasma is much flatter than that of whole blood, approaching more nearly the curve of a simple bicarbonate solution.

In this case the carbon dioxide-combining capacity of the venous plasma must be dependent, to some extent at least, upon the carbon dioxide tension or content of the venous blood at the time that it is withdrawn. Table V shows this relation clearly. In this table are represented the carbon dioxide content of venous blood, drawn directly, without stasis, beneath albolene; the

TABLE V.

Relation between CO_2 Content of Venous Blood and CO_2 Capacity of Venous Plasma.

Subject.	CO_2 capacity of whole blood.	CO_2 content of venous blood.	CO_2 capacity of venous plasma.
	vol. per cent	*vol. per cent*	*vol. per cent*
A. B.	60.8	63.9	69.1
J. M.	48.1	63.3	75.7
M. C.	49.0	63.2	74.5
P. O. S.	52.0	62.1	74.2
J. P.	55.9	62.0	73.0
H. R.	47.7	60.2	68.6
N. B.	52.7	60.1	66.0
D. P. B.	47.0	57.5	66.0
D. A.	42.1	57.2	68.0
D. P. B.	43.3	53.3	62.9
E. H.	47.5	51.1	64.0
J. D. B.	45.0	49.8	60.7
J. M.	38.7	49.8	59.4
E. S.	47.8	46.8	49.1
J. K.	36.2	45.5	55.0
H. G.	45.4	43.3	53.3

carbon dioxide capacity of whole blood at 40 mm. of CO_2 tension and 37.5°C.; and the carbon dioxide capacity of venous plasma determined according to the technique of Van Slyke (23). (For the last, venous blood was withdrawn under albolene, centrifuged in this condition, and the plasma removed to a separatory funnel and saturated with alveolar CO_2.) The observations are arranged according to the magnitude of the CO_2 content. The plasma capacity parallels the CO_2 content rather more closely than it does the CO_2 capacity of whole blood. But, if the CO_2 capacity

of the plasma is to be used as a measure of the bicarbonate content of the blood, it should vary directly as the CO_2 capacity of the whole blood.

The cardiac cases are not the only ones in which the plasma method fails to agree with the whole blood method. H. G. shows a slight acidosis on the basis of his plasma figures, although the whole blood CO_2 capacity is normal. In this case the venous carbon dioxide content is very low. The discrepancy is slight, but is quite striking in comparison with J. D. B., a cardiac patient with an absorption curve at the same level as that of H. G. Even in normal subjects estimations of the bicarbonates of whole blood from those of plasma may prove inaccurate. This is well illustrated in the case of D. P. B., who on several occasions showed a CO_2 capacity relatively higher in the plasma than in the whole blood.

In severe anemia the use of plasma may lead to misinterpretations. In this condition, as we shall show, the whole blood curve becomes relatively flat, approaching that of plasma. Changes in CO_2 tension, therefore, produce relatively small alterations in CO_2 content. The consequence is that the values obtained from whole blood are high in comparison with those obtained from plasma. This is especially noticeable in Cases A. B. and E. S. (The corresponding hemoglobin values were 32 and 35 per cent respectively.) This may explain the fact that Kahn and Barsky (24), studying the plasma in pernicious anemia, found a reduction of the alkali reserve in three cases, while the four cases in our series gave consistently high values for the CO_2-combining capacity of whole blood.

Finally, a further source of error lies in the fact that the absorption curves of plasma from different specimens of blood may differ. This has been demonstrated by Straub and Meier (22).

The use of the CO_2-combining power of the plasma as a measure of the alkali reserve will cause little or no error in most cases if it is employed only in those conditions in which the respiratory and circulatory systems are undisturbed and react in the normal manner to the natural stimuli. It was to subjects of this type that Van Slyke and coworkers (25) first applied it with such success that his method has become the standard method for the measurement of the alkali reserve. Henderson and Haggard

(26) have contended that whole blood is preferable to plasma. The latter, however, was chosen by Van Slyke with full recognition of the error involved, because of technical reasons, especially the fact that whole blood demanded immediate analysis, whereas plasma could be kept for a considerable length of time without deterioration. Furthermore, the coagulum produced by the addition of acid to whole blood rendered the cleansing of his pipette difficult. With the substitution of tartaric acid for sulfuric in the Van Slyke method one of the technical reasons for preferring plasma has been removed. The impossibility of keeping whole blood for any length of time remains an objectionable feature.

If, for this reason, the use of plasma is found necessary, variations in carbon dioxide saturation, the chief cause of error in the use of plasma, must be avoided. This can easily be effected by bringing the whole blood into equilibrium with a standard air-CO_2 mixture, *e.g.* alveolar air, before removing the plasma. If this were done at least the chief objection to the use of plasma would be removed. However, the air-CO_2 mixture used for saturation of whole blood must be more carefully standardized than is the case when plasma is employed. This is evident from the differences in the absorption curves of whole blood and plasma. Small variations in carbon dioxide tension have a much greater effect on the carbon dioxide content of whole blood. The use of alveolar air, which is the usual procedure with plasma might, with whole blood, introduce a perceptible error. Under resting conditions the alveolar CO_2 tensions of normal individuals should not vary by more than a few millimeters. As the corresponding change in the carbon dioxide content of the blood is only half as great, the use of alveolar air by an operator trained in respiratory methods should produce no serious errors.

Under these conditions plasma should give values that are dependable as far as the ability of the blood to neutralize acids other than carbonic is concerned. For the study of the respiratory function of the blood, on the other hand, whole blood alone can be used. In order to avoid unnecessary complications, the normal limits of variation at room temperatures will have to be more definitely established, as whole blood work up to the present has been confined almost entirely to physiological studies and saturation has been effected at body temperature.

Just as this paper was approaching completion the final report of the work of Joffe and Poulton (20) on the absorption curves of whole blood and plasma appeared. On the basis of their findings they also conclude that the carbon dioxide capacity of plasma separated from venous blood is not a proper measure of the available alkali of the blood. They advise a method identical with that here proposed.

Up to the time that Van Slyke published his method the presence of a carbon dioxide retention had not yet been demonstrated in any pathological condition, and, although the possibility of such an occurrence was clearly recognized by him, it did not seem of sufficient practical importance to outweigh the obvious technical advantages of plasma over whole blood. As applied to the study of diabetes the use of plasma probably introduced no serious error. When we presented our data to Dr. Van Slyke we found that he had come to practically the same conclusion as a result of some studies on anesthesia.

Carbon Dioxide Content of the Venous Blood in Cardiac Dyspnea.

It is clear that a discrepancy between the CO_2 capacity of whole blood and that of venous plasma may be produced by at least three factors: (1) An abnormal venous carbon dioxide tension; (2) a disturbance of the normal proportion of cells to plasma; (3) an abnormality in the carbon dioxide absorption curve of the plasma itself. The second of these we may assume to be unimportant in cardiac dyspnea, although we have not investigated the matter carefully. In the majority of cases hemoglobin determinations were made by means of the oxygen capacity and revealed no striking changes. The plasma absorption curve has not been determined. We have, however, studied the venous carbon dioxide content of a number of subjects.

The determination of the presence or absence of carbon dioxide retention in the venous blood is not so simple a matter as it may appear. The results obtained by previous observers are not at all consistent. Harrop (16) found that the difference between arterial and venous carbon dioxide content was sometimes increased in cardiac dyspnea, indicating a relative retention of carbon dioxide in the venous blood. Table IV gives a summary of Harrop's findings. The results obtained from the five cases

in our series appear in Table III. The two sets of observations are in essential agreement. The difference between arterial and venous blood was much more variable in decompensated cardiac cases than in normal subjects, and in some of the former was relatively increased. Scott (27) has recently reported the CO_2 content of the plasma of both arterial and venous blood lower than normal in cardiac decompensation. A few unpublished determinations made by one of us on venous plasma in 1916 were not in agreement with his findings. The values obtained were more variable than those obtained from normal subjects, but the variations were not predominantly low. In some cases they were quite high. The same is true of the values obtained from whole blood. Their only characteristic is a great range of variation (see Tables I and III). By comparison with Table V it will be seen that these variations bear a rough relation to the height of the absorption curves.

Neither Harrop's nor Scott's method can throw much light on the question of the presence or absence of a carbon dioxide retention. The absolute value for carbon dioxide content of either whole blood or plasma, whether arterial or venous, although of interest, cannot be accepted as a satisfactory criterion. The CO_2 content which is normal for a blood with a high absorption curve is excessive for a blood with a low absorption curve, if the reaction of the blood is to be maintained constant. The locus of points of equal pH is represented by straight lines passing through the origin. As the curves become lower, therefore, equal amounts of CO_2 have a greater effect in changing the pH. The presence of a carbon dioxide retention can only be determined, then, by a comparison of the carbon dioxide content of the blood with the height of the absorption curve. Such a comparison appears in the venous pH values in Table III. In the case of the three cardiac patients with low absorption curves (Cases 10, 11, and 12) there is a definite carbon dioxide retention which explains the falsely high values for bicarbonate obtained by the plasma CO_2 capacity method of Van Slyke (23).

SUMMARY AND CONCLUSIONS.

1. A study has been made of the carbon dioxide absorption curve of the blood of seven patients with cardiac decompensation

and dyspnea. In three out of the seven low curves were found. In two of these cases the curves returned to the normal level when compensation was reestablished. Clinically these patients were distinguished by the presence of an extreme degree of cyanosis with little dyspnea. No relation could be established between the reduction of the absorption curve and the oxygen unsaturation of the arterial or venous blood. A low absorption curve was not an indication of the severity of the condition. No signs of renal involvement were discovered in any of these cases.

2. The reduction of the alkali reserve indicated by these low absorption curves was not reflected in the plasma. The CO_2 capacity of the venous plasma is determined by the carbon dioxide tension at which it existed in the body rather than by the carbon dioxide-combining capacity of the blood. For this reason the determination of the plasma bicarbonates by the technique originally proposed is not applicable as a measure of the alkali reserve in conditions associated with CO_2 accumulation in the venous blood. A true measure of the bicarbonate content of blood is obtained from the carbon dioxide capacity of whole blood. As whole blood deteriorates when kept, while plasma does not, it may still be necessary to employ plasma for purposes of routine procedure. In this case errors due to variations in carbon dioxide saturation may be avoided by saturating the whole blood with a standard air-CO_2 mixture before separating the plasma. With this modification the method may be rendered applicable to the determination of the alkali reserve of the blood. For the study of the respiratory function of the blood, however, only whole blood should be used.

3. (*a*) In four cases arterial and venous carbon dioxide tension were determined. The alveolar carbon dioxide tension was also determined. The only consistent and characteristic finding was an increase in the difference between alveolar and arterial CO_2 tension. This difference varied from 13 to 19 mm.

(*b*) In two cases there was a definite CO_2 retention in the arterial and venous blood with a consequent lowering of the pH.

(*c*) No consistent increase in the difference between arterial and venous CO_2 content or tension could be demonstrated, although in one or two instances it was slightly greater than normal.

4. The causes of the discrepancy between the alveolar carbon dioxide tension and the carbon dioxide-combining capacity of the venous plasma in cardiac dyspnea are:

(*a*) The fact that the carbon dioxide capacity of the plasma gives values that are too high in the presence of carbon dioxide retention such as is sometimes found in cardiac dyspnea.

(*b*) The alveolar carbon dioxide is very low in proportion to the carbon dioxide content of the venous blood and does not bear a definite relation to the height of the absorption curve.

5. The causes of cardiac dyspnea seem to be: The fact that a greater ventilation is necessary to effect the normal carbon dioxide elimination. This is largely brought about by an impairment of the efficiency of the pulmonary mechanism for the exchange of gases between the blood and the outside air. This necessitates the maintenance of a greater difference in carbon dioxide pressure between the blood in the pulmonary circulation and the alveolar air in order to effect the normal carbon dioxide output. To maintain the carbon dioxide tension and the hydrogen ion concentration at the proper level the alveolar carbon dioxide tension must be abnormally low. In some cases a diminution of the circulation rate may be an additional factor in the production of a carbon dioxide acidosis. Finally in a certain proportion of the cases, at least, there is a real reduction of the available alkali of the blood. In at least two cases with a reduction of the available alkali of the blood were found indications of a comparative insensibility of the respiratory center to its natural physicochemical stimulus, the hydrogen ion concentration of the blood.

BIBLIOGRAPHY.

1. Peters, J. P., Jr., *Am. J. Physiol.*, 1917, xliii, 113.
2. Peters, J. P., Jr., and Barr, D. P., *Am. J. Physiol.*, 1920–21, liv, 307.
3. Van Slyke, D. D., Stillman, E., and Cullen, G. E., *J. Biol. Chem.*, 1917, xxx, 401.
4. Walker, I. C., and Frothingham, C., Jr., *Arch. Int. Med.*, 1916, xviii, 304.
5. Peters, J. P., Jr., *Am. J. Physiol.*, 1917, xliv, 84.
6. Siebeck, R., *Deutsch. Arch. klin. Med.*, 1912, cvii, 253.
7. Peters, J. P., Jr., and Barr, D. P., *Am. J. Physiol.*, 1920–21, liv, 335.
8. Barr, D. P., and Peters, J. P., Jr., *Am. J. Physiol.*, 1920–21, liv, 345.
9. Pearce, R. G., *J. Lab. and Clin. Med.*, 1916–17, ii, 867.
10. Krogh, A., and Krogh, M., *Skand. Arch. Physiol.*, 1909–10, xxiii, 179.
11. Henderson, Y., and Haggard, H. W., *J. Biol. Chem.*, 1919, xxxix, 163.

12. Douglas, C. G., Haldane, J. S., Henderson, Y., and Schneider, E. C., *Phil. Tr. Roy. Soc. London, Series B*, 1912, cciii, 271.
13. Barcroft, J., The respiratory function of the blood, Cambridge, 1914, 243–265.
14. Lutz, B. R., and Schneider, E. C., *Am. J. Physiol.*, 1919, l, 280.
15. Parsons, T. R., Parsons, W., and Barcroft, J., *J. Physiol.*, 1919–20, liii, p. cx.
16. Harrop, G. A., Jr., *J. Exp. Med.*, 1919, xxx, 241.
17. Peabody, F. W., *Arch. Int. Med.*, 1917, xx, 433, 443.
18. Means, J. H., Bock, A. V., and Woodwell, M. N., *J. Exp. Med.*, 1921, xxxiii, 201.
19. Scott, R. W., *Arch. Int. Med.*, 1920, xxvi, 544.
20. Joffe, J., and Poulton, E. P., *J. Physiol.*, 1920–21, liv, 129.
21. Van Slyke, D. D., and Cullen, G. E., *J. Biol. Chem.*, 1917, xxx, 289.
22. Straub, H., and Meier, K., *Biochem. Z.*, 1918, lxxxix, 156; *Deutsch. Arch. klin. Med.*, 1919, cxxix, 54.
23. Van Slyke, D. D., *J. Biol. Chem.*, 1917, xxx, 347.
24. Kahn, M., and Barsky, J., *Arch. Int. Med.*, 1919, xxiii, 334.
25. Stillman, E., Van Slyke, D. D., Cullen, G. E., and Fitz, R., *J. Biol. Chem.*, 1917, xxx, 405.
26. Henderson, Y., and Haggard, H. W., *J. Biol. Chem.*, 1918, xxxiii, 333.
27. Scott, R. W., *Proc. Soc. Exp. Biol. and Med.*, 1919–20, xvii, 19.

EXPLANATION OF CHARTS.

Chart 7.

Case 7. J. D. B. Syphilitic aortitis. Dilatation of ascending aorta. Aortic insufficiency. Male, age 50 years, single, pastry cook.

Looks chronically ill. Moderate dyspnea even while at rest, but able to walk around ward. Faint cyanosis of lips and finger-tips. Lungs clear.

Heart very much enlarged, with loud systolic and diastolic murmurs, maximum over the aortic area. X-ray revealed aneurysmal dilatation of the ascending aorta.

Wassermann ++++.

Patient improved but slightly and is still in the hospital at the time of publication.

Experiment 1. Mar. 19, 1920.

Alveolar CO_2 before venous puncture, *mm*			25.5
			25.0
" CO_2 after " " "			25.2
CO_2 absorption curve:			
CO_2 tension, *mm. Hg*	77.2	47.7	23.8
CO_2, *vol. per cent*	62.9	48.7	37.1
	59.7		36.5
CO_2 content of venous blood, *vol. per cent*			49.8
CO_2 capacity of venous plasma (41.7 mm. Hg) *vol. per cent*			60.7
O_2 capacity of blood, *vol. per cent*			16.4

Experiment 2. Apr. 30. Condition practically unchanged, although cyanosis may be slightly increased.

Alveolar CO_2 before arterial puncture, *mm*			27.3
			27.1
			27.7
" CO_2 after " " "			27.6
CO_2 absorption curve:			
CO_2 tension, *mm. Hg*	59.8	47.2	28.4
CO_2, *vol. per cent*	55.6	52.9	39.8
	56.1	50.7	40.3
CO_2 content of arterial blood, *vol. per cent*			49.3
			48.9
CO_2 " " venous " " " "			52.0
			53.4
Difference between arterial and venous CO_2, *vol. per cent*			3.6
O_2 capacity of blood, *vol. per cent*			13.9
O_2 content of arterial blood, *vol. per cent*			14.3
O_2 " " venous " " " "			3.7
O_2 consumption, *vol. per cent*			10.6
O_2 saturation of arterial blood, *per cent*			100
O_2 " " venous " " "			27
Respiratory quotient of blood			0.34

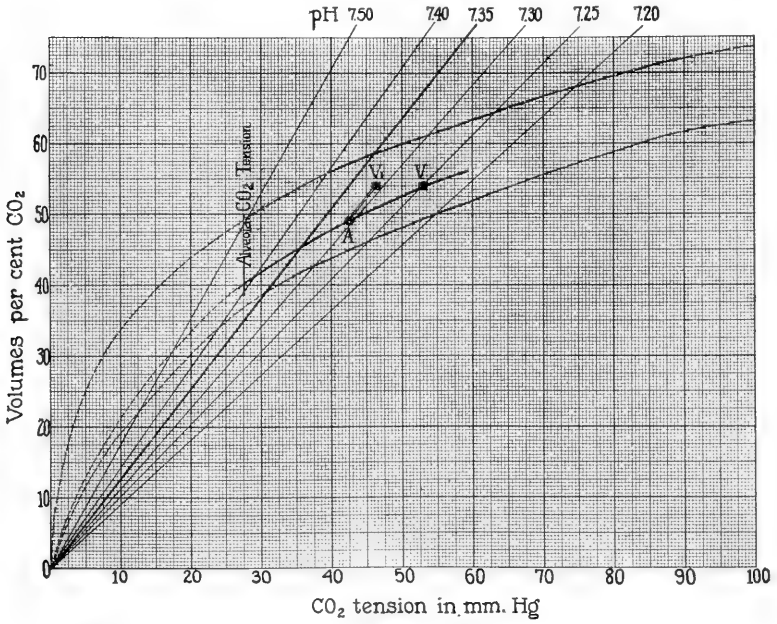

Chart 7. Experiment of Apr. 30. Directions for interpretation of charts are given in Paper I.

CHART 10.

Case 10. J. M. Mitral stenosis. Male, age 60 years, single, engineer. Looks chronically ill. Lies quietly in bed, without dyspnea or orthopnea. Very marked cyanosis of face and extremities. Veins of neck engorged, but not visibly pulsating. Brawny, pitting edema of feet and legs. Signs of moderate amount of fluid in the right pleural cavity. Liver very much enlarged and pulsating.

Temperature normal. Pulse absolutely irregular; rate 72 to 100. Blood pressure: systolic 138; diastolic 86. Heart moderately enlarged both to the right and left. Loud blowing, systolic murmur at apex, accompanied by rough thrill. No murmurs at base. Electrocardiogram shows auricular fibrillation.

Urine contains considerable albumin, with a few granular and hyaline casts. Specific gravity 1.023 to 1.027. Non-protein nitrogen of blood 43 mg. per 100 cc. Phenolsulfonephthalein excretion 60 per cent in 2 hrs. Discharged from hospital, improved, 1 week after last experiment.

Experiment 1. Apr. 8, 1920.

Minute volume of respirations, *cc.*	8,652
Tidal air 475 cc. Respirations per min.	18.2
CO_2 in expired air 2.40 per cent. CO_2 output per min., *cc.*	208
Vital capacity of the lungs, *cc.*	1,197

Experiment 2. Apr. 9. Condition unchanged.

Alveolar CO_2 before arterial puncture, *mm.*				25.0
CO_2 absorption curve:				
CO_2 tension, *mm. Hg*	75.8	54.4	44.4	
CO_2, *vol. per cent*	56.6	46.0	41.7	
CO_2 content of arterial blood, *vol. per cent*				39.6
				39.6
CO_2 " " venous " " " "				49.5
				50.0
Difference between arterial and venous CO_2, *vol. per cent.*				10.2
CO_2 capacity of venous plasma (40.9 mm. Hg), *vol. per cent.*				59.4
O_2 " " blood, *vol. per cent*				20.7
O_2 content of arterial blood, *vol. per cent*				16.7
O_2 " " venous " " " "				8.5
O_2 consumption, *vol. per cent*				8.2
O_2 saturation of arterial blood, *per cent*				83
O_2 " " venous " " "				42
Respiratory quotient of blood				1.24

Experiment 3. Apr. 23. Condition greatly improved. Quite comfortable. No edema. Signs of fluid in chest gone. Cyanosis still well marked. Heart action good, rate about 60, with practically no pulse deficit. Is able to be out of bed and to walk about ward without difficulty or discomfort.

Vital capacity of lungs, *cc.*	2,463
Minute volume of respirations, *cc.*	9,854
Tidal air 498 cc. Respirations per min.	19.8

CO_2 in expired air 2.72 per cent. CO_2 output per min., *cc.* 268
Alveolar CO_2 before arterial puncture, *mm.*..... 34.2
" " 35.3
" CO_2 after " " " 31.2
CO_2 absorption curve:

CO_2 tension, *mm. Hg*.....	80.4	26.6
CO_2, *vol. per cent*.....	64.6	38.0
	63.9	40.5

CO_2 content of arterial blood, *vol. per cent*..... 53.8
" " 54.2
CO_2 " " venous " " " " 63.0
" " 63.7
Difference between arterial and venous CO_2, *vol. per cent*.. 9.3
CO_2 capacity of venous plasma (52.0 mm. Hg), *vol. per cent.* 75.7
O_2 " " blood, *vol. per cent*..... 20.8
O_2 content of arterial blood, *vol. per cent*..... 22.0
O_2 " " venous " " " " 11.1
" " 11.0
O_2 consumption, *vol. per cent*..... 10.9 or 9.7
O_2 saturation of arterial blood, *per cent*..... 100
O_2 " " venous " " " 53
Respiratory quotient of blood..... 0.85 or 0.96

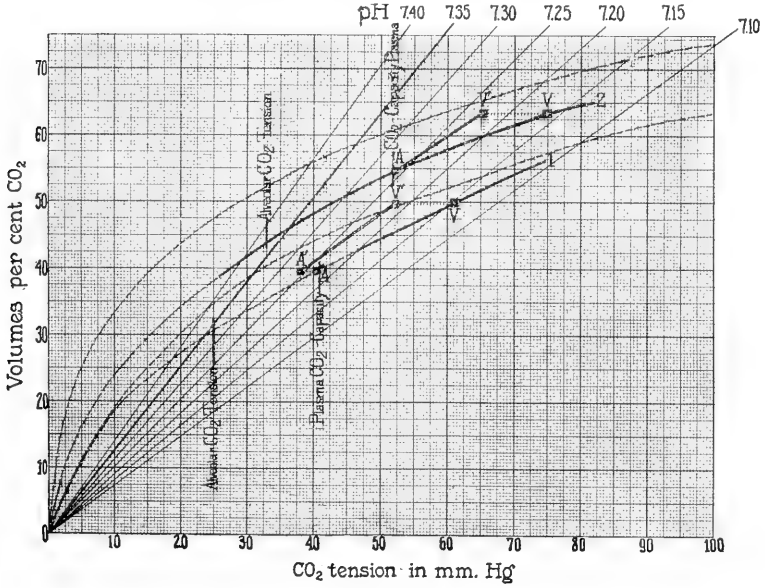

Chart 10. 1. Experiment of Apr. 9.
2. Experiment of Apr. 23.

Chart 11.

Case 11. J. K. Mitral stenosis. Male, age 35 years, single, elevator operator. Looks chronically ill. Lies quietly in bed with only slight dyspnea and orthopnea. Face and extremities are a livid purple. Slight, pitting edema of feet, ankles, and legs. Few sibilant and sonorous râles scattered throughout lungs, but no signs of pleural effusion. Liver enlarged and pulsating. Temperature normal. Pulse totally irregular, radial rate 55, heart rate 92. Blood pressure: systolic 140, diastolic 100. Heart enlarged both to left and right. Loud systolic and soft diastolic murmurs, maximum over apex. Pulmonic second sound accentuated. Electrocardiograms show auricular fibrillation.

Urine shows cloud of albumin, no casts. Specific gravity 1.018.

Discharged from hospital improved, 9 days after last experiment.

Experiment 1. Apr. 16, 1920.

Alveolar CO_2 before arterial puncture, *mm*			23.0
			22.1
" CO_2 after " " "			21.4
			21.4
CO_2 absorption curve:			
CO_2 tension, *mm. Hg*	68.0	47.1	36.7
CO_2, *vol. per cent*	47.7	38.3	35.8
	47.4	39.4	
CO_2 content of arterial blood, *vol. per cent*			36.8
			35.0
CO_2 " " venous " " " "			44.8
			46.1
Difference between arterial and venous CO_2, *vol. per cent*			9.6
CO_2 capacity of venous plasma (37.8 mm. Hg), *vol. per cent.*			55.0
O_2 " " blood, *vol. per cent*			26.3
			26.7
O_2 content of arterial blood, *vol. per cent*			24.2
O_2 " " venous " " " "			9.9
O_2 consumption of " " " "			14.3
O_2 saturation of arterial blood, *per cent*			91
O_2 " " venous " " "			37
Respiratory quotient of blood			0.67

Experiment 2. Apr. 20. Greatly improved. No dyspnea. Cyanosis less marked, but still considerable. Edema gone. Lungs clear. Pulse good quality. Heart rate about 40. No pulse deficit (digitalis heart block). Arthritis of left foot with temperature of 102°F.

Vital capacity of the lungs, *cc*	1,800
Alveolar CO_2 before arterial puncture, *mm*	30.2
	34.7
" CO_2 after " " "	32.5

CO_2 absorption curve:

CO_2 tension, *mm. Hg*	37.8
CO_2, *vol. per cent*	49.1
	50.7
CO_2 content of arterial blood, *vol. per cent*	53.7
	51.0
O_2 " " " " " " "	25.0
O_2 " " venous " " " "	22.5
O_2 consumption, *vol. per cent*	2.5
O_2 saturation of arterial blood, *per cent*	94
O_2 " " venous " " "	85

(O_2 capacity of Apr. 16 used for calculations.)

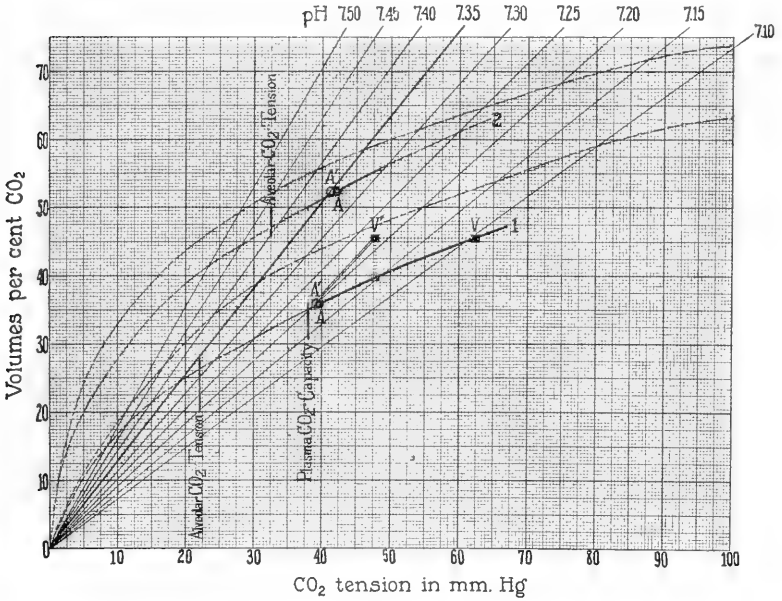

CHART 11. 1. Experiment of Apr. 16.
2. Experiment of Apr. 20.

CHART 12.

Case 12. C. C. Mitral stenosis. Looks chronically ill. Lies quietly in bed, with only slight dyspnea and no orthopnea. Very marked cyanosis of head and extremities. Somewhat irrational.

Experiment 1.

Alveolar CO_2 before arterial puncture, *mm*				28.0
				26.1
				27.8
" CO_2 after " " "				27.5
CO_2 absorption curve:				
CO_2 tension, *mm. Hg*	66.2	47.6	39.0	
CO_2, *vol. per cent*	43.7	34.8	33.0	
	45.3	35.9	33.0	
CO_2 content of arterial blood, *vol. per cent*				33.4
				33.6
CO_2 " " venous " " " " "				43.1
				42.6
Difference between arterial and venous CO_2, *vol. per cent*				9.4
O_2 capacity of blood, *vol. per cent*				18.3
O_2 content of arterial blood, *vol. per cent*				18.0
O_2 " " venous " " " "				3.2
O_2 consumption, *vol. per cent*				14.8
O_2 saturation of arterial blood, *per cent*				98
O_2 " " venous " " "				18
Respiratory quotient of blood				0.64

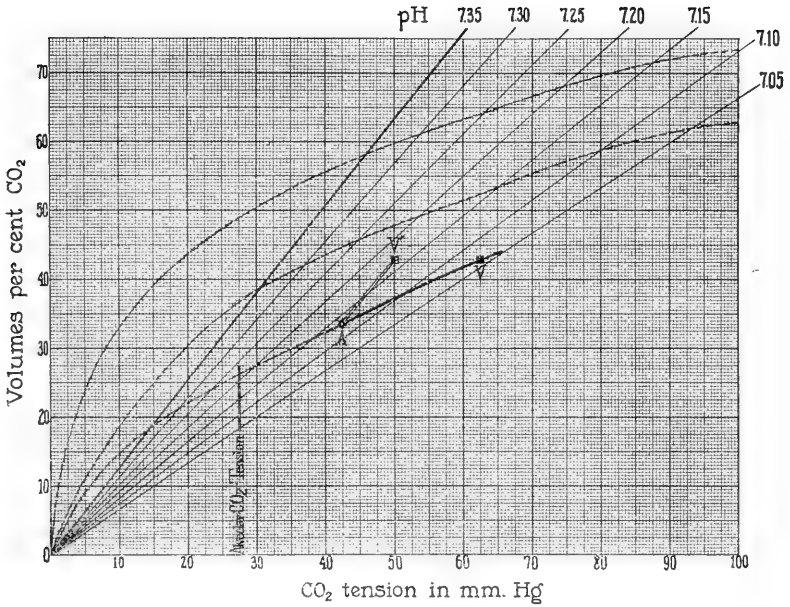
pH
7.35
7.30
7.25
7.20
7.15
7.10
7.05
Volumes per cent CO_2
0
10
20
30
40
50
60
70
Water CO_2 Tension
10
20
30
40
50
60
70
80
90
100
CO_2 tension in mm. Hg

Chart 12.

Chart 13.

Case 13. M. C. Bronchial asthma with severe emphysema. Male, age 40 years, single, hospital orderly.

Complains of severe dyspnea and cough. Has been admitted to the hospital several times. Looks acutely distressed. Is sitting up in bed. Respirations are rapid and labored, with prolonged, wheezing expiration. Most intense cyanosis of head and extremities, which are a deep purple. Veins of neck engorged. Lungs show boardy resonance everywhere. Expiratory breath sounds prolonged, high pitched, and wheezing. Rhonchi and râles of all kinds heard all over chest. Heart: apex and borders of dulness not made out. Sounds distant. No murmurs made out. Pulse about 100.

Experiment 1. May 12, 1920.

Alveolar CO_2 before arterial puncture, *mm*.....				39.8
				44.1
				41.2
" CO_2 after " " "				40.9
CO_2 absorption curve:				
CO_2 tension, *mm. Hg*.....	61.9	15.1	75.1	39.6
CO_2, *vol. per cent*.....	57.0	32.2	56.9	48.7
	56.3	31.0	58.2	50.1
CO_2 content of arterial blood, *vol. per cent*.....				58.4
				56.7
CO_2 " " venous " " " " "				63.9
				62.5
Difference between arterial and venous CO_2, *vol. per cent*...				5.7
CO_2 capacity of venous plasma (51.2 mm. Hg), *vol. per cent.*				74.5
O_2 " " blood, *vol. per cent*.....				21.3
O_2 content of arterial blood, *vol. per cent*.....				17.2
O_2 " " venous " " " "				11.0
O_2 consumption, *vol. per cent*.....				6.2
O_2 saturation of arterial blood, *per cent*.....				81
O_2 " " venous " " "				52
Respiratory quotient of blood.....				0.92

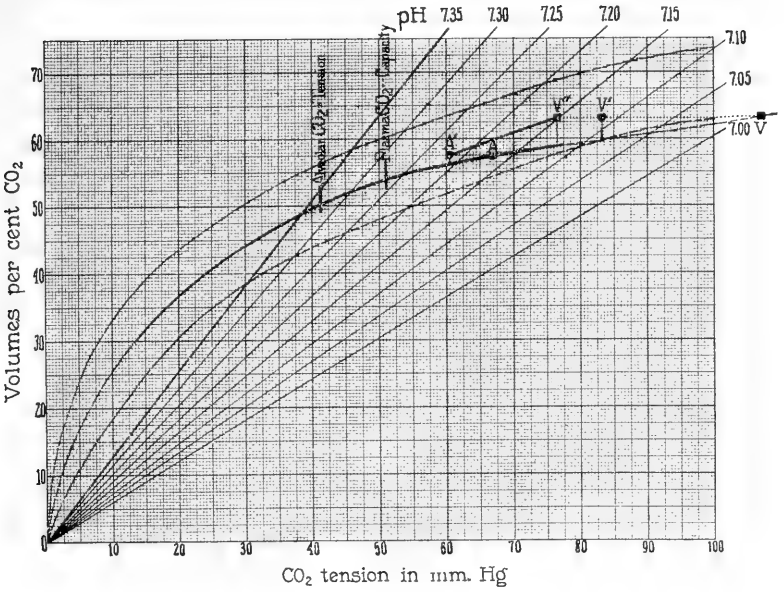

CHART 13. V″ represents the venous CO_2 tension corrected for oyxgen unsaturation, using the value of 0.414 for K (see text).

Additional Protocols.

Case 6. G. B. Paroxysmal tachycardia. Male, age 32 years, single, cook. Acutely ill. Some dyspnea, orthopnea, and cyanosis. Edema of both ankles. Signs of moderate amount of fluid in the right pleural cavity and in the abdomen. Liver enlarged. Temperature about 103° F. Pulse regular, rate about 180. Heart enlarged in both directions, but without signs of a valvular lesion.

Condition did not respond to treatment and patient died suddenly 6 days after the experiment here reported.

Experiment 1. Mar. 24, 1920.

CO_2 absorption curve:

CO_2 tension, *mm. Hg*	78.7	47.2	11.6
CO_2, *vol. per cent*	61.1	49.1	27.4
			26.4

CO_2 content of venous blood, *vol. per cent* 54.8
O_2 capacity of blood, *vol. per cent* 21.7
O_2 content of venous blood, *vol. per cent* 5.0
O_2 saturation of venous blood, *per cent* 23

Case 8. E. H. Mitral stenosis and insufficiency. Male, age 39 years, butcher. Looks acutely and chronically ill. Sitting, propped up in bed, with considerable dyspnea and orthopnea, pallid, cyanotic, and in acute distress. Edema of both lower extremities, abdominal wall, and back. Double hydrothorax. Moderate ascites. Very large, pulsating liver.

Temperature about 100. Pulse absolutely irregular, rate slow as the result of digitalis therapy.

Heart enlarged to the right and left, with systolic and diastolic thrill and shock, maximum over the midcardiac area. Auricular fibrillation. Patient died 2 days after the experiment here reported.

Experiment 1. Mar. 10, 1920.

CO_2 absorption curve:

CO_2 tension, *mm. Hg*	16.0	34.8	69.5
CO_2, *vol. per cent*	19.0	46.2	59.2
			61.1

CO_2 content of venous blood, *vol. per cent*..... 51.1

CO_2 capacity of " plasma (44.0 mm. Hg) *vol. per cent*... 64.0

Case 9. P. O. S. Syphilitic aortitis. Aortic insufficiency. Male, age 49 years, single, laborer. Looks acutely and chronically ill. Marked dyspnea and orthopnea with irregular respirations. Moderate cyanosis. Edema of lower extremities, back, and abdominal wall. Signs of fluid in both pleural cavities. Liver very much enlarged. Temperature 100. Pulse regular, rate 90, collapsing in type. Blood pressure: systolic 140, diastolic 50. Heart very much enlarged, especially downward and to the left. Soft, systolic and loud, buzzing, diastolic murmurs, maximum over the base of the heart. Diastolic murmur accompanied by a thrill.

Wassermann ++++.

Patient died 3 weeks after the last experiment without having left the hospital.

Experiment 1. Mar. 9, 1920.

CO_2 absorption curve:

CO_2 tension, *mm. Hg*	66.8	59.9	20.6
CO_2, *vol. per cent*	63.9	60.7	39.0
	65.4		39.7

CO_2 content of venous blood, *vol. per cent*..... 62.1

CO_2 capacity of venous plasma (51.1 mm. Hg), *vol. per cent*.. 74.2

Experiment 2. Mar. 23. Continues to grow worse. Hydrothorax increasing. Irrational. Dyspnea is less, possibly because of morphine, but respirations are more irregular. Considerable cyanosis.

CO_2 absorption curve:

CO_2 tension, *mm. Hg*	(26.4)	93.7	51.8
CO_2, *vol. per cent*	42.8	65.8	52.5
	42.8		

CO_2 content of venous blood, *vol. per cent*..... 56.9

O_2 capacity of blood, *vol. per cent*..... 22.3

III. THE CARBON DIOXIDE ABSORPTION CURVE AND CARBON DIOXIDE TENSION OF THE BLOOD IN SEVERE ANEMIA.

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(Received for publication, December 2, 1920.)

Carbon Dioxide Absorption Curve in Severe Anemia.

An analysis of the CO_2 absorption curve of normal blood has been made in Paper I of this series (1). In the course of the investigation we had occasion to study the blood of a case of pernicious anemia and found such marked variation from the normal in the shape and character of the absorption curve that we were stimulated to study the bloods of other cases of anemia as they presented themselves in the wards. We later found that the CO_2 absorption curves from the blood of one case of pernicious anemia and from one case of moderate secondary anemia had been constructed in 1917 by Hasselbalch (2) who used them as collateral evidence in his argument concerning the true nature of the supposed acidosis of newly born children. In this paper we wish to present the absorption curves of several patients with varying degrees of anemia, to discuss the factors which may cause the curve of anemic blood to differ from the normal, and finally to show the bearing of the findings upon the respiratory mechanism and particularly upon the dyspnea of severe anemia.

The methods employed have been described in detail in Paper I. The absorption curve of fully oxygenated blood and the CO_2 content of venous blood have been determined. Upon three of the patients arterial puncture was performed and the CO_2 content of arterial and of venous blood was ascertained. The percentage of hemoglobin was obtained in all cases by the oxygen capacity method of Van Slyke (3). In one case the alveolar CO_2 tension was also determined.

TABLE I.

Subject.	Date.	Tension of CO_2. (1)	CO_2 absorbed. (2)	Venous CO_2 content. (3)	Arterial CO_2 content. (4)	CO_2 capacity of plasma. (5)	Venous oxygen content. (6)	Arterial oxygen content. (7)	Oxygen capacity. (8)	Hemoglobin. Haldane scale. (9)	Diagnosis and remarks. (10)
	1920	*mm. Hg*	*vol. per cent*	*vol. per cent*	*vol. per cent*	*mm. Hg*	*vol. per cent*	*vol. per cent*	*vol. per cent*	*per cent*	
Jas. M.	June 10	0.1	45.60	61.28	57.93		0.54	2.92	3.41	18.9	Probable pernicious anemia, male, age 62. Red blood count 710,000. Pulse 88–120. Respirations 18–28. Temperature 97–99°.
			45.60	61.08	57.34				3.28	17.7	
		39.8	58.12								
			59.66								
		78.4	67.38								
			65.90								
C. P.	May 28	24.0	47.30	55.18	52.24		1.55	4.37	4.34	23.1	Pernicious anemia, male, age 55. Red blood count 1,000,000. Pulse 108–112. Respirations 24–28. Temperature 100–101°.
			48.34	54.50	51.55						
		46.8	55.59								
			55.87								
		93.1	63.00								
			64.60								
R. H.	June 10	30.2	46.00	52.08	50.87		0.00	4.76	4.36	23.5	Gastric carcinoma, female, age 44. Red blood count 2,300,000. Pulse 80–104. Respirations 20–32. Temperature 99–102°.
		47.9	51.90	53.43	50.47						
			55.12								
		82.2	60.13								
			61.27								

A. B.	Mar. 16	18.0	51.10	63.99		47.54			6.17	33.3	Pernicious anemia, female, age 47. Red blood count 1,500,000–1,000,000. Pulse 120–128. Respirations 20–24. Temperature 98–102°.
		46.3	64.60	63.91					5.76	31.1	
			60.89								
		80.4	70.00								
	Apr. 1	20.6	48.35						4.80	25.90	
		52.2	58.10								
		91.0	65.10								
E. S.	Mar. 3	6.4	33.52	46.77		33.78	3.66		6.90	37.4	Chronic nephritis, hypertension, and uremia, female, age 22. Red blood count 4,000,000. Pulse 84–108. Respirations 20–28. Temperature 98–99.2°. Blood pressure: systolic 190, diastolic 110.
			33.15						6.45	35.4	
		30.5	44.80								
		53.6	50.67								
			52.08								
C. A.	Mar. 22	25.8	61.40						9.88	53.3	Gastric carcinoma, female.
		38.8	69.80								
		61.0	74.60								
N. B.	Mar. 15	7.1	31.60	60.13		45.26			10.08	54.7	Pernicious anemia, female, age 53. Red blood count 2,700,000. Pulse 92. Temperature 98–103°.
			32.27			45.47			9.61	52.2	
		42.2	53.81								
			52.95								
		71.9	63.46								
			63.18								

The cases upon which this study is based include three patients with pernicious anemia, one with a severe anemia probably pernicious, two suffering from gastric carcinoma, and one from chronic nephritis. A protocol of the combined data of all observations is given in Table I. In Column 1 are included the tensions of CO_2 to which the blood was exposed and in Column 2 the volume of CO_2 absorbed at each tension is given. From these data the CO_2 absorption curves were constructed. The other columns of the table are self-explanatory.

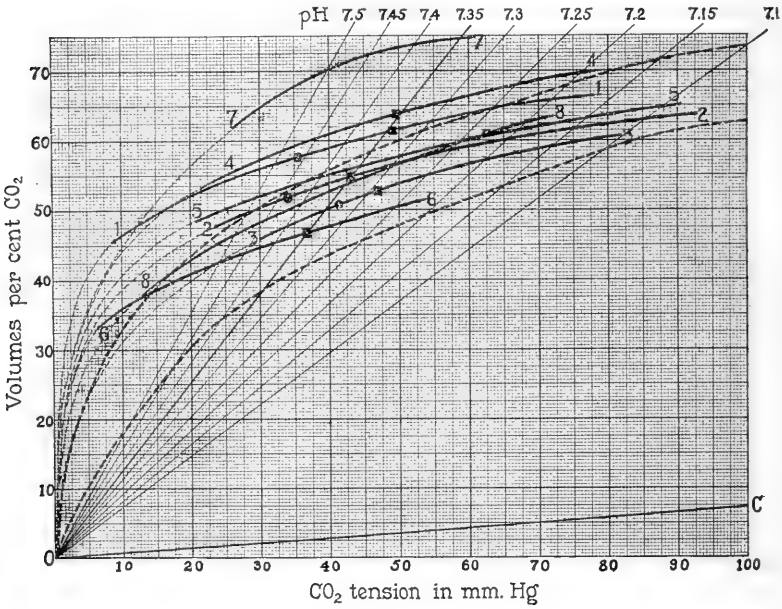

FIG. A. Comparison of CO_2 absorption curves of blood in severe anemia with curves of normal blood.

Solid curved lines: curves from blood of anemia. 1. Jas. M., 18 per cent hemoglobin. 2. C. P., 23 per cent hemoglobin. 3. R. H., 23 per cent hemoglobin. 4. A. B., Mar. 16, 1920, 32 per cent hemoglobin. 5. A. B., Apr. 1, 1920, 26 per cent hemoglobin. 6. E. S., 36 per cent hemoglobin. 7. C. A., 53 per cent hemoglobin. 8. N. B., 53 per cent hemoglobin.

Broken curved lines: limits of dissociation curves from blood of normal persons.

Straight diagonal line *OC* represents the CO_2 in solution and in form of H_2CO_3.

Other diagonal lines represent pH of blood.

● = Arterial CO_2 content.

■ = Venous CO_2 content.

The absorption curves are represented in Fig. A. The solid curved lines are curves of blood from patients with anemia. Each of these is numbered and the numbers, the initials of the patients to which they refer, and the percentage of hemoglobin contained in the blood are given in the legend. The curved broken lines represent the upper and lower limits of the absorption curve for the blood of normal individuals (1). The diagonal, *OC*, gives the amount of CO_2 in physical solution and in the form of carbonic acid for each tension. The other diagonal lines indicate the hydrogen ion concentration in terms of the negative logarithm, pH, of Sörensen (4).

From a superficial survey of the figure it will be seen that the curves of blood in anemia differ markedly from the normal. They are more nearly parallel to the base line. The volume of CO_2 absorbed at low tensions is relatively large while the volume at high tensions is less than normal. In these respects our results are entirely in keeping with those of Hasselbalch (2). The curve of blood from severe anemia resembles the curve of plasma quite as closely as it does that of normal whole blood.

In Fig. B the CO_2 absorption curves of 0.03 M sodium carbonate solution, of 0.1 M sodium phosphate solution, of plasma, and of normal blood have been drawn for the purpose of comparing their slopes with that of the blood of Jas. M., a patient with severe anemia. The curves of phosphate solution and of plasma have been constructed from the figures given by Van Slyke and Cullen (5). The curve of carbonate solution is derived from the formula of Parsons (6).

The difference in the slope of the curves can be explained by the presence and character of the acids in the various solutions. In a solution of sodium carbonate where H_2CO_3 is the only acid, essentially all the alkali combines with CO_2 at low tensions. Below 10 mm. of CO_2 tension the curve is steep, while at all tensions above 10 mm. the curve is flat and parallels the line of physical absorption, *OC*. In plasma, in whole blood, and in the phosphate solutions, all of which contain both H_2CO_3 and other potential acids, the alkali is divided between the acids in proportions depending upon their relative strength and molecular concentration. The amount of alkali which may combine with carbonic acid between any two tensions, and hence the slope of

the CO_2 absorption curve, will depend upon the character and concentration of the other acid substances. For this reason it is important to consider the potential acids which exist in normal blood.

The plasma contains serum proteins which may have acid properties under conditions existing within the body. Attempts

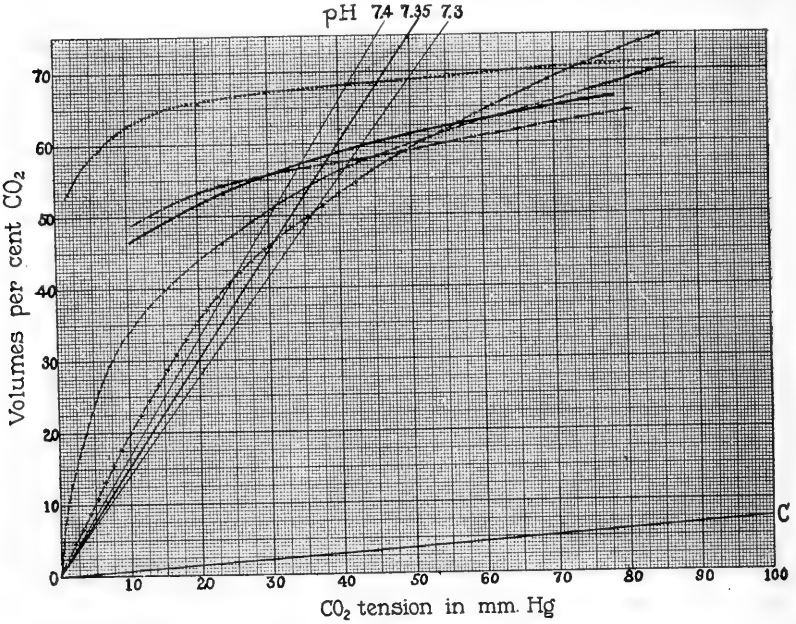

FIG. B. Comparison of curve of blood of severe anemia with other CO_2 absorption curves.

..... = 0.03 M Na_2CO_3 solution.
--x--x--x-- = 0.1 M Na_2HPO_4 solution.
—·—·—·— = Plasma.
---------- = Normal blood.
———— = Blood of Jas. M.; severe anemia.

to measure the magnitude of their acid action have given rise to conflicting results (7–10). The close resemblance between the curve of plasma and that of bicarbonate indicates that their influence is not great under normal conditions. Within the red blood cells there are two substances of potentially acid character, the phosphates and hemoglobin. The influence of the former

cannot be estimated quantitatively. It will be seen, however, that the curve of phosphate solution resembles closely that of normal whole blood. The presence of phosphates might from theory play a considerable rôle in the production of the typical blood curve (11). The action of hemoglobin as a potential acid has been emphasized recently by many investigators (12–15). It has been found to be an amphoteric substance which at the hydrogen ion concentrations existing within the body may have the properties of a weak acid.

In anemia the red blood cells are diminished in number and the reduction is accompanied by a decrease in the concentration of hemoglobin. If the action of hemoglobin as an acid is of importance, a marked reduction in its concentration should produce a flattening of the slope of the curve, which, in the most severe anemias, should resemble the curve of plasma. It has been seen that this actually occurs. The absorption of CO_2 by the blood of Jas. M. with 18 per cent hemoglobin occurs in a manner more like plasma than normal whole blood.

If hemoglobin were the only substance of importance, the slope of the curve should vary directly with the concentration of the pigment. Our results do not show this. The flattening of the curve is detectable only in severe anemia. Curves of blood containing 80 per cent of hemoglobin may have as steep a slope as those with 120 per cent (1). Careful measurements of the curves have failed to disclose any exact relation between the two phenomena. In this respect our curves are not in accord with those of Hasselbalch, who found the slope of the curve to vary directly with the percentage of hemoglobin. The most striking relation between the factors was apparent at low tensions, below those at which most of our curves were constructed. Hasselbalch's curves indicate that the blood of severe anemia does not lose all its CO_2 at 0 mm. of CO_2 tension. If this should be true, the extrapolations, the dotted portions of the curves in Fig. A, may be misleading.

In our data there is no exact relation between the percentage of hemoglobin and the slope of the absorption curve. Other factors must exert an influence. What these factors may be is, for the present, a matter for speculation only. In anemia plasma constitutes a large proportion of the total blood volume. Under

these circumstances the influence of the serum proteins upon the slope of the curve may vary in the different bloods studied. Straub and Meier (14) presented a plasma curve from a patient with polycythemia which differed from the normal and indeed resembled the curve of normal whole blood. It is possible that differences in color index, variations in the relation of the number of red blood cells to the percentage of hemoglobin, may exert some influence upon the shape of the curve. The curve of E. S. indicates that this factor is not of great importance. Although the blood of E. S. contains 4,000,000 red blood cells per cubic millimeter, the curve is as flat as that of the blood of A. B. with 1,500,000 red blood cells.

TABLE II.

Subject.	CO_2 absorbed at 40 mm. tension.
	vol. per cent
Jas. M.	58.4
R. H.	49.2
C. P.	53.0
A. B.	60.0
	54.2
E. S.*	47.0
C. A.	69.0
N. B.	51.5
Average	56.5
" of normal bloods	48.95

* E. S., a nephritic with edema and reduction of plasma bicarbonate. Omitted in calculation of average.

The slope of the curves of blood in anemia implies that at low CO_2 tensions the content of CO_2 will be relatively high. Furthermore, in anemia, plasma constitutes a large proportion of the total blood volume. Per unit volume, plasma contains more alkali than the red blood cells. This also will have the effect of making the CO_2 content of the blood higher than that of normal blood. That the curve in anemia, however, is not invariably high is shown by the curve of the blood of E. S., a chronic nephritic, whose alkali reserve, measured by the plasma method of Van Slyke, was diminished. The CO_2 content of the blood at the physiologically important tension of 40 mm. is shown in Table II.

The difference between the CO_2 content of arterial and venous blood in the three cases studied is much smaller than is usually observed in normal individuals.

Probably the chief physiological significance of the flat absorption curve of blood in anemia is the information which it gives concerning the combining power of blood between any two CO_2 tensions. A flat curve expresses diagrammatically the fact that the blood which it represents will combine with less CO_2 for a given rise in CO_2 tension and will lose less CO_2 for a given fall in tension.

The comparatively low combining power of blood in severe anemia between the tensions of 40 and 60 mm. is represented in a roughly quantitative way in Table III.

TABLE III.

Subject.	CO_2 content at 40 mm.	CO_2 content at 60 mm.	Difference.
	vol. per cent	*vol. per cent*	
Jas. M.	58.4	63.6	5.2
C. P.	53.0	58.8	5.8
R. H.	49.2	55.9	6.7
A. B.	60.0	66.0	6.0
	54.2	58.7	4.5
E. S.	47.0	51.7	4.7
C. A.	69.0	74.0	5.0
N. B.	51.5	58.4	6.9
Average difference			5.6
" normal difference			6.8

The decreased power of the blood in anemia to absorb or dissociate CO_2 with varying CO_2 tensions implies that changes in hydrogen ion concentration will be greater for each shift in tension. This is shown diagrammatically in Fig. A by the relation of the flat curves of anemia to the diagonal pH lines. The actual changes in hydrogen ion concentration between arterial and venous blood within the body are, however, influenced by other factors. The curves of blood in anemia are higher than the normal. This tends to lessen the effect of the flat curves upon hydrogen ion concentration, a fact indicated in the charts by the divergence of the pH lines as they pass from the origin. At 40 volumes per cent of CO_2 the pH 7.30 and 7.40 lines are

TABLE IV.

Results of the Study of Six Patients with Severe Anemia.

Subject.		Date.	Alveolar CO_2 tension.	Alveolar reaction.	Arterial CO_2 content.	Arterial CO_2 tension.		Arterial reaction.		Venous CO_2 content.	Venous CO_2 tension.		Venous reaction.		Difference between arterial and alveolar CO_2 tension.	Difference between arterial and venous CO_2 content.	Difference between arterial and venous CO_2 tension.	Difference between arterial and venous reaction.
						Uncorrected.	Corrected.	Uncorrected.	Corrected.		Uncorrected.	Corrected.	Uncorrected.	Corrected.				
			(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)	(11)	(12)	(13)	(14)	(15)	(16)
		1920	*mm. Hg*	*pH*	*vol. per cent*	*mm. Hg*	*mm. Hg*	*pH*	*pH*	*vol. per cent*	*mm. Hg*	*mm. Hg*	*pH*	*pH*	*mm. Hg*	*vol. per cent*	*mm. Hg*	*pH*
18.	N. B.	Mar. 15								60.1	60.0		7.24					
19.	A. B.	" 16 Apr. 1								63.9	50.0		7.35					
14.	R. H.	June 10			50.7	41.0	41.0	7.34	7.34	52.8	47.0	43.0	7.30	7.33		4.8	2.0	0.01
15.	Jas. M.	May 5			57.6	35.0	35.0	7.47	7.47	61.2	49.0	45.0	7.35	7.38		3.9	10.0	0.09
17.	C. P.	" 28	28.2	7.50	51.9	33.5	33.5	7.44	7.44	54.9	42.5	41.0	7.35	7.37	5.3	3.0	6.5	0.07
16.	E. S.*	Mar. 3								46.8	45.0	41.5	7.28	7.32				
Maximum*.....					57.6	41.0	41.0	7.47	7.47	63.9	60.0	45.0	7.35	7.38		4.8	10.0	0.09
Minimum*.....					50.7	33.5	33.5	7.34	7.34	52.8	42.5	41.0	7.24	7.33		3.0	2.0	0.01

* The values obtained from the experiment on E. S., Case 16, have been omitted from the summary of maximum and minimum values because the anemia in this case was complicated by a severe chronic nephritis with uremia.

separated only about 4 mm. of CO_2 tension. At 60 volumes per cent this difference has increased to 10 mm. of CO_2 tension. This attempt at compensation is, however, counterbalanced by the reduction of the effect of oxygen on the curve. It will be remembered that the curves of Fig. A are those of blood containing oxygenated hemoglobin. The curve of reduced blood is always higher than that of the same blood fully oxygenated. Since the hemoglobin of venous blood is partially reduced, the venous points are not correctly represented on the curves of Fig. A, but should lie on a higher curve. If, as was indicated in Paper I of this series, the effect of oxygenation or reduction of the blood upon the height of the CO_2 absorption curve is a direct function of the hemoglobin, it is evident that the effect of oxygen unsaturation on blood from anemia will be less than on normal blood. The complete oxygen unsaturation of a blood with 25 per cent hemoglobin will increase the height of the absorption curve no more than will a 25 per cent unsaturation of a blood with 100 per cent hemoglobin. The net result of these various factors upon the changes in hydrogen ion concentration between arterial and venous blood is shown in Charts 14 to 17 and in Column 16 of Table IV. In spite of the small difference in carbon dioxide content between arterial and venous blood there is a considerable difference in pH, amounting in Cases 15 and 17 to 0.07. This is partly due to the diminished slope of the absorption curve, but even more to the loss of the oxygen effect. Even complete oxygen unsaturation cannot compensate a carbon dioxide change of more than 1 volume per cent in a subject with only 25 per cent hemoglobin.

Carbon Dioxide Tension and Hydrogen Ion Concentration of the Arterial and Venous Blood.

The hydrogen ion concentration of the arterial blood in the three cases studied lies at or above pH 7.34, in Case 15 (Chart 15) as high as 7.47. Means and coworkers (16) have also found the arterial pH quite high. In Case 17 (Chart 17) the alveolar carbon dioxide tension is correspondingly low, giving an alveolar pH of 7.50. In another case of anemia Means found the alveolar pH to be 7.43. This would indicate a tendency toward an arterial

alkalosis and a ventilation somewhat in excess of that which should be expected from the height of the absorption curve.

Although the arterial pH is in general rather high, the venous pH falls surprisingly close to the 7.35 line. In the four instances in which we have sufficient data to calculate the corrected venous pH the limits of variation are 7.32 to 7.38. In Means' case and our Case 19 it is also evident that the venous pH must be much closer to the average normal than is that of the arterial blood. Of course this may be due only to a peculiar coincidence, but it appears too frequently to pass without speculation.

It is generally stated that the respiratory center is controlled by the hydrogen ion concentration of the arterial blood and, in turn, responds in such a way as to maintain the arterial hydrogen ion concentration constant. It seems more probable, however, that the true controlling factor is the reaction of the tissue fluids in the respiratory center. Presumably this reaction is dependent upon carbon dioxide tension, just as it is in the blood. The carbon dioxide tension of the tissues must, however, always exceed that of the arterial blood and must, during the greater part of the circulation at least, be higher than that of the venous blood. In general it must be much closer to that of the venous blood. If the general tendency of the respiratory mechanism, then, is to maintain the pH of the tissue fluids in the respiratory center constant, one should expect the pH of the venous blood from the center to remain constant rather than that of the arterial blood which passes to the center.

This is not apparent in normal persons because, with a normal concentration of hemoglobin, the effect of oxygen on the carbon dioxide absorption curve renders the pH of arterial and venous blood practically identical.

DISCUSSION.

The influence of these factors upon the mechanism of respiration is apparent. The amount of CO_2 carried from the tissues for each change in tissue tension is less than normal. Unless the blood flow is increased, this will tend to result in accumulation of CO_2 within the tissues. With each increase in CO_2 tension, the hydrogen ion concentration rises with relative rapidity. Further-

more, the same difficulty which the blood has in absorbing CO_2 from the tissues is experienced in dissociating it in the lungs. For a given fall of tension the blood of anemia will dissociate less CO_2 than normal. Unless the ventilation is increased the CO_2 will tend to accumulate in the arterial blood and a vicious circle will be established. There is present in severe anemia a tendency to accumulation of CO_2 in the tissues, a diminished ability of the blood to lose CO_2 in the lungs, and a relatively rapid change in hydrogen ion concentration with any change in the CO_2 tension, all factors which tend to excite the respiratory center and produce dyspnea.

It is not surprising, therefore, to find that in severe anemia hyperpnea is present. It is probably not necessary to draw a causal relation between dyspnea and oxygen want even though the presence of the latter should be established. In regard to the question of oxygen consumption, however, the findings here presented suggest an interesting possibility. Barcroft (17) found that *in vitro* the last portions of oxygen were separated from hemoglobin with great difficulty. It is here shown in Cases 14 and 15 that the last bit of oxygen can be removed during the passage of the blood through the tissues. This has been shown previously by Lundsgaard (18) and others. Barcroft also found that the last portions of oxygen were more readily given off if the hydrogen ion concentration of the solution was increased. It seems possible that the more rapidly changing hydrogen ion concentration of the blood of severe anemia may explain why the last portions of oxygen can be freed during the comparatively brief interval of the passage of the blood through the tissues.

CONCLUSIONS.

1. The CO_2 absorption curves of blood from six patients with severe anemia have been studied. They have been found to have a flatter slope than have the curves of normal blood.

2. This is explained in part by the low percentage of hemoglobin. The flatness of the curves, however, is not exactly proportional to the diminution in the percentage of hemoglobin.

3. The blood of severe anemia has a diminished power of absorbing or dissociating CO_2 with changes of CO_2 tension.

Absorption of CO_2 from the tissues or loss of CO_2 from the lungs is probably accomplished with difficulty.

4. In three cases the hydrogen ion concentration of the arterial blood was determined and found to be relatively low, the pH being as much as 7.47 in one instance.

5. The venous pH was surprisingly constant, varying in four cases between 7.32 and 7.38.

6. In spite of the relatively small difference in carbon dioxide content between arterial and venous blood, the corresponding difference in pH is considerable, reaching 0.07 in two cases. This is due to the flat absorption curve and the loss of the compensating effect of oxygen unsaturation.

7. In severe anemia changes in CO_2 tension produce relatively small changes in CO_2 content and relatively great variations in hydrogen ion concentration. The fault in the CO_2-carrying power and the greater changes in hydrogen ion concentration may explain the dyspnea of anemia.

BIBLIOGRAPHY.

1. Peters, J. P., Jr., and Barr, D. P., *J. Biol. Chem.*, 1920–21, xlv, 489.
2. Hasselbalch, K. A., *Biochem. Z.*, 1917, lxxx, 251.
3. Van Slyke, D. D., *J. Biol. Chem.*, 1918, xxxiii, 127.
4. Sörensen, S. P. L., *Ergebn. Physiol.*, 1912, xii, 393.
5. Van Slyke, D. D., and Cullen, G. E., *J. Biol. Chem.*, 1917, xxx, 289.
6. Parsons, T. R., *J. Physiol.*, 1919–20, liii, 42.
7. Moore, B., McQueen, J. M., and Webster, T. A., *J. Physiol.*, 1919–20, liii, p. xxvii.
8. Milroy, T. H., *J. Physiol.*, 1917, li, 259.
9. Bayliss, W. M., *J. Physiol.*, 1919–20, liii, 162.
10. Campbell, J. M. H., and Poulton, E. P., *J. Physiol.*, 1920–21, liv, 152.
11. Henderson, L. J., *J. Biol. Chem.*, 1920, xli, 401.
12. Parsons, T. R., *J. Physiol.*, 1917, li, 440.
13. Parsons, T. R., *J. Physiol.*, 1919–20, liii, 340.
14. Straub, H., and Meier, K., *Deutsch. Arch. klin. Med.*, 1919, cxxix, 54.
15. Joffe, J., and Poulton, E. P., *J. Physiol.*, 1920–21, liv, 129.
16. Means, J. H., Bock, A. V., and Woodwell, M. N., *J. Exp. Med.*, 1921, xxxiii, 201.
17. Barcroft, J., The respiratory function of the blood, Cambridge, 1914.
18. Lundsgaard, C., *J. Exp. Med.*, 1919, xxx, 147.

CHARTS

EXPLANATION OF CHARTS.

CHART 14.

Case 14. R. H. Gastric carcinoma with severe anemia. Female, age 44 years, married. Looks chronically ill. Somewhat irrational. Well nourished, but shows some evidence of loss of weight. Skin pale and yellowish. Lips pale, with slight cyanotic tinge; similar cyanotic tinge in finger-nails. No signs of dyspnea or hyperpnea while at rest in bed.

Lungs show few, crackling râles at bases, especially on left side. Heart not enlarged. Blowing, systolic murmur and soft, diastolic murmur, maximum over aortic area. Pulse soft, regular, rate about 90. Blood pressure: systolic 150, diastolic 40. X-ray examination showed a small effusion in left pleural cavity. Thoracentesis obtained a small amount of viscid, bloody fluid.

Urine showed occasional trace of albumin, no casts. Specific gravity 1.010 to 1.018.

Non-protein nitrogen of blood 43.5 mg. per 100 cc.

Phenolsulfonephthalein excretion 25 per cent in 2 hrs.

Temperature 100–102° F.

Blood counts:

June 5. Leucocytes 20,000; polymorphonuclear 80 per cent; lymphocytes 5 per cent; large mononuclear 10 per cent; eosinophils 5 per cent.

Erythrocytes 1,200,000. A few normoblasts, but no megaloblasts were found.

June 10. Erythrocytes 2,400,000.

Patient died in hospital and autopsy revealed the presence of gastric carcinoma.

Experiment 1. June 10, 1920.

CO_2 absorption curve:

CO_2 tension, *mm. Hg*	82.2	47.9	30.2
CO_2, *vol. per cent*	60.1	51.9	46.0
	61.3	55.1	

CO_2 content of arterial blood, *vol. per cent*	50.9
	50.5
CO_2 " " venous " " " "	52.1
	53.4
Difference between arterial and venous CO_2, *vol. per cent.*	2.1
O_2 capacity of blood (24 per cent hemoglobin) *vol. per cent.*	4.4
O_2 content of arterial blood, *vol. per cent*	4.8
O_2 " " venous " " " "	0.0
O_2 consumption of blood, *vol. per cent*	4.8
O_2 saturation of arterial blood, *per cent*	100
O_2 " " venous " " "	0
Respiratory quotient of blood	0.44

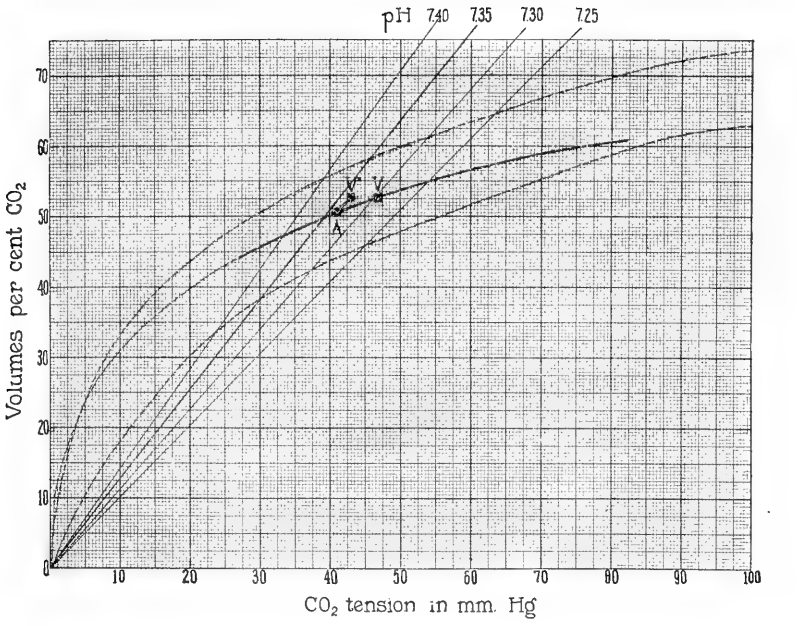
pH 7.40
7.35
7.30
7.25
Volumes per cent CO_2
CO_2 tension in mm. Hg
0
10
20
30
40
50
60
70
80
90
100
A
V
V'

Chart 14.

Chart 15 and 16.

Case 15. Jas. M. Severe anemia (probably pernicious). Male, age 62 years, married, fireman. Looks chronically ill. Complains only of pains in the stomach. Mentally sluggish and unable to give any information. Extremely weak. Vomiting greenish brown fluid, streaked with mucus, which contains much bile, 10 per cent acid, no free HCl, and many pus cells. Thin and emaciated, extremely pale. No signs of dyspnea or hyperpnea while at rest in bed. Teeth all gone. Lungs clear. Heart normal. Pulse 58 to 108.

Blood counts:

May 4. Leucocytes 6,200; polymorphonuclear 39 per cent; lymphocytes 59 per cent; large mononuclear 2 per cent; erythrocytes 700,000.

May 6. Leucocytes 2,400; polymorphonuclear 50 per cent; lymphocytes 42 per cent; large mononuclear 2 per cent; erythrocytes 700,000; a few normoblasts and megaloblasts found.

May 8. Leucocytes 3,000; polymorphonuclear 55 per cent; lymphocytes 43 per cent; large mononuclear 2 per cent; erythrocytes 1,900,000; many megaloblasts and a few normoblasts found.

Patient received a transfusion of 630 cc. of citrated blood on May 7. During the night his temperature rose to 103° F., his pulse became very weak, and his respirations shallow and labored. He died at noon the next day. Blood grouping had been carried out with care and no evidence of hemolysis was found.

Experiment 1. May 5, 1920.

CO_2 absorption curve:

CO_2 tension, *mm. Hg*.....	39.8	9.1	38.3	78.4
CO_2, *vol. per cent*.....	58.1	45.6	57.7	67.4
	59.7	45.6	57.6	65.9

CO_2 content of arterial blood, *vol. per cent*.....	57.9
	57.3
CO_2 " " venous " " " "	61.1
	61.3
	62.2
Difference between arterial and venous CO_2, *vol. per cent*...	3.9
O_2 capacity of blood, *vol. per cent*.....	3.4
	3.3
O_2 content of arterial blood, *vol. per cent*.....	2.9
O_2 " " venous " " " "	0.5
O_2 consumption, *vol. per cent*.....	2.4
O_2 saturation of arterial blood, *per cent*.....	87
O_2 " " venous " " "	15
Respiratory quotient of blood.....	1.62

Case 16. E. S. Chronic nephritis with severe anemia. Female, age 21 years, married, housewife. Acutely ill. Fairly well developed and nourished. Very pale. Sitting up in bed, with dyspnea, orthopnea, and slight cyanosis. Moderate edema of feet and legs. Lungs show signs of fluid in both pleural cavities. Heart somewhat enlarged, without murmurs. Rate 96. Blood pressure: systolic 190, diastolic 115. Temperature normal.

Urine shows very heavy cloud of albumin, with many casts, many red blood cells, and some pus. Specific gravity 1.006 to 1.011.

Non-protein nitrogen of blood 165 mg. per 100 cc.

Phenolsulfonephthalein excretion about 5 per cent in 2 hrs.

Blood count:

Mar. 2. Leucocytes 19,200; polymorphonuclear 82 per cent; erythrocytes 4,200,000.

Experiment 1. Mar. 3, 1920.

CO_2 absorption curve:

CO_2 tension, *mm. Hg*.....	30.5	53.6	6.4
CO_2, *vol. per cent*.....	44.8	50.7	33.5
		52.1	33.2

CO_2 content of venous blood, *vol. per cent*..... 46.8
46.8

CO_2 capacity of venous plasma (33.8 mm. Hg), *vol. per cent*.. 49.1

O_2 content of venous blood, *vol. per cent*..... 3.7
3.7

Experiment 2. Mar. 5.

O_2 capacity of venous blood (35 per cent hemoglobin), *vol. per cent*..... 6.5

O_2 content of venous blood, *vol. per cent*..... 3.1

O_2 saturation of venous blood, *per cent*..... 48

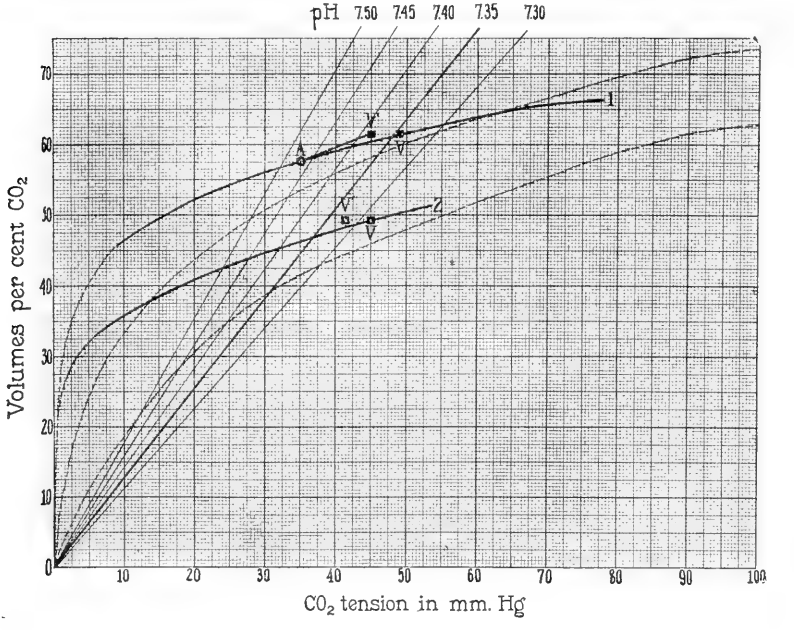

Chart 15 and 16. 1. Case 15.
2. Case 16.

CHART 17.

Case 17. C. P. Pernicious anemia. Male, age 55 years, single, stable worker. Looks chronically ill. Very weak and emaciated. Extremely pale, with lemon-yellow tinge. Some retinal hemorrhages. No signs of dyspnea or hyperpnea while at rest in bed. No cyanosis. Lungs clear. Heart not enlarged. Blowing, systolic murmur at apex. Pulse regular, 90 to 100. Blood pressure: systolic 145, diastolic 60. Temperature 100–102° F.

Blood counts:

May 24. Leucocytes 3,000; polymorphonuclear 50 per cent; lymphocytes 48 per cent; large mononuclear 2 per cent; erythrocytes 700,000. Many normoblasts and megaloblasts found.

June 2. Polymorphonuclear 60 per cent; lymphocytes 25 per cent; large mononuclear 14 per cent; eosinophils 2 per cent; erythrocytes 1,100,000; many normoblasts and megaloblasts found.

Experiment 1. May 28, 1920.

Alveolar CO_2 before arterial puncture, *mm.*				28.3
				27.8
" CO_2 after " " "				28.6
CO_2 absorption curve:				
CO_2 tension, *mm. Hg*	93.1	46.8	24.0	
CO_2, *vol. per cent*	63.0	55.6	47.3	
	64.6	55.9	48.3	
CO_2 content of arterial blood, *vol. per cent*				52.2
				51.6
CO_2 " " venous " " " "				55.2
				54.5
Difference between arterial and venous CO_2, *vol. per cent.*				2.2
O_2 capacity of blood, *vol. per cent*				4.3
O_2 content of arterial blood, *vol. per cent*				4.4
O_2 " " venous " " " "				1.6
O_2 consumption, *vol. per cent*				2.8
O_2 saturation of arterial blood, *per cent*				100
O_2 " " venous " " "				37
Respiratory quotient of blood				0.79

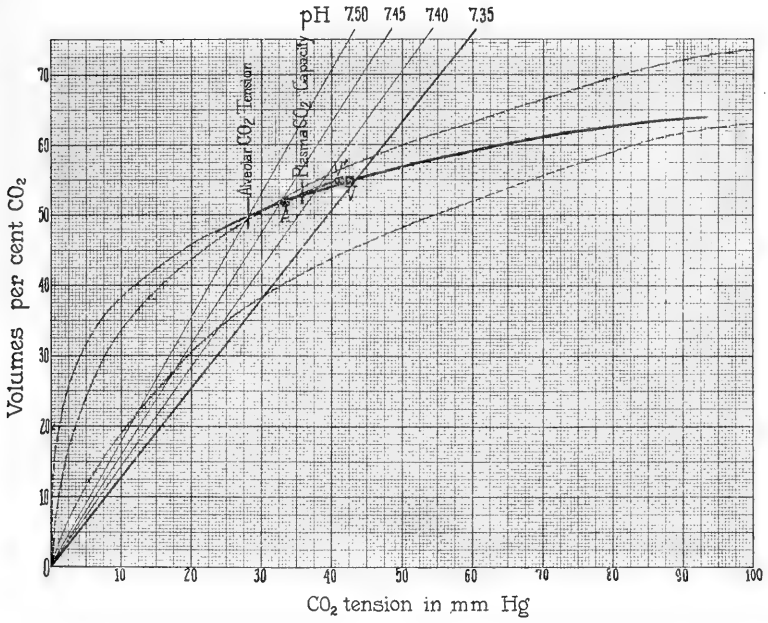

CHART 17.

Additional Protocols.

Case 18. N. B. Pernicious anemia. Female, age 53 years, married, servant. Looks chronically ill. Fairly well nourished and developed, but flabby. Pale, lemon-yellow color. Small hemorrhagic spots in roof of mouth, retinal hemorrhages, and purpuric spots on extremities. No dyspnea or visible hyperpnea while patient is at rest in bed. Lungs clear. Heart slightly enlarged to the left with a soft systolic murmur and accentuation of the pulmonic second sound.

Gastric contents show no free HCl, total acid 25 per cent, no blood. Stools show no gross or occult blood. Gastrointestinal examination by means of x-ray reveals a prepyloric filling defect persisting throughout examination, but no definite evidence of carcinoma.

Temperature varied between 99 and 103. Pulse about 90. Urine showed occasional trace of albumin and a few granular casts; specific gravity 1.006 to 1.015.

Blood counts:

Mar. 11. Leucocytes 5,600; polymorphonuclear 40 per cent; erythrocytes 2,200,000; no nucleated red blood cells.

Mar. 17. Erythrocytes 2,700,000.

Experiment 1. Mar. 15, 1920.

CO_2 absorption curve:

CO_2 tension, *mm. Hg*	71.9	7.1	42.2
CO_2, *vol. per cent*	63.5	31.6	53.8
	63.2	32.3	53.0

CO_2 content of venous blood, *vol. per cent* 60.1

CO_2 capacity of venous plasma (45.4 mm. Hg), *vol. per cent*.. 66.0

O_2 " " " blood (54 per cent hemoglobin), *vol. per cent* 10.1
9.6

Case 19. A. B. Pernicious anemia. Female, age 47 years, single, housewife. Looks chronically ill. Slightly emaciated, very pallid, with lemon-yellow color. Petechial hemorrhages on hard palate. No signs of dyspnea or hyperpnea while lying quietly in bed. Thyroid distinctly enlarged. Lungs clear. Heart slightly enlarged to left, with soft, systolic murmur at base, not transmitted. Pulse rapid. Spleen not felt.

Gastrointestinal x-ray examination reveals no evidence of new growth. Urine shows moderate amount of albumin, no casts. Specific gravity 1.015 to 1.021. Temperature 99–102° F.

Blood counts:

Mar. 16. Erythrocytes 1,500,000.

Apr. 1. Erythrocytes 1,000,000.

Experiment 1. Mar. 16, 1920.

CO_2 absorption curve:

CO_2 tension, *mm. Hg*	80.4	46.3	18.0
CO_2, *vol. per cent*	70.0	64.6	51.1
		60.9	

CO_2 content of venous blood, *vol. per cent* 63.8
64.0

CO_2 capacity of venous plasma (47.5 mm. Hg), *vol. per cent*.. 69.1

O_2 " " blood (26 per cent hemoglobin), *vol. per cent*. 6.2
5.8

Case 20. G. A. Gastric carcinoma with anemia.

Experiment 1. Mar. 22, 1920.

CO_2 absorption curve:

CO_2 tension, *mm. Hg*	61.0	25.8	38.8
CO_2, *vol. per cent*	74.6	61.4	69.8

O_2 capacity of blood (53 per cent hemoglobin), *vol. per cent*. 9.9

ON A POSSIBLE ASYMMETRY OF ALIPHATIC DIAZO COMPOUNDS.

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(Received for publication, December 31, 1920.)

In 1915 Levene and La Forge made an observation which pointed to the formation of a diazo compound as an intermediary phase in the process of transformation of glucosaminic acid ethyl ester into anhydro-manonic ethyl ester.[1, 2] This observation brought forth the possibility of asymmetry in the α-carbon atom of the diazo compounds of the sugar acids. Since it was known that also esters of aliphatic α-amino-acids on treatment with nitrous acid were convertible into optically active oxy-acids, and since it was known that under certain conditions the treatment of the amino-acids resulted in the formation of aliphatic diazo esters, it seemed to the authors possible that also in the latter there existed an asymmetry. In 1916, Levene and Senior undertook to test experimentally the existence of optical isomerism in the aliphatic diazo compounds. The work was interrupted because of the conditions of war, Dr. Senior having accepted a commission in the Chemical Warfare Service. The work was resumed in September, 1920, by the present writers. In November there appeared a publication by Marvel and Noyes[3] on the subject of the present article. The experimental results reported by these writers were negative.

Our own work is not yet completed, but in view of the just mentioned publication we wish to record results which point favorably towards the possibility of asymmetry in these diazo compounds.

[1] Levene, P. A., and La Forge, F. B., *J. Biol. Chem.*, 1915, xxi, 345.
[2] Levene, P. A., *J. Biol. Chem.*, 1918, xxxvi, 89.
[3] Marvel, C. S., and Noyes, W. A., *J. Am. Chem. Soc.*, 1920, xlii, 2259.

Dextro aspartic acid was converted into diazosuccinic acid ethyl ester. It was found possible to purify the ester by fractional distillation under a pressure varying from 0.08 to 0.12 mm.

The analytical data of the samples are given in the following table.

	N	Impurity.	$[\alpha]_D^{20}$
	per cent	*per cent*	
Experiment 1.			
First fraction	10.06	30.0	+1.27
Second "	13.68	5	+1.25
Third "	14.10	0	+1.34
Experiment 2.			
First fraction			
Second "	13.64	2.5	
Third "	13.33	5	
Second and third fractions redistilled.			
First fraction	13.76	1.7	+1.27
Second "	13.64	2.5	
Experiment 3.			
First fraction			
Second "	13.64	2.5	+1.00
Third "	14.20	0	+0.85

The theory for $C_8H_{11}O_4N_2$ requires N = 14.02 per cent.

The accuracy of the analytical method was tested on the crystalline diazosuccinic acid monoamide monoethyl ester and on diazoacetic acid ethyl ester.

The impurity of the highest optical activity that could be present in our material is *d*-malic diethyl ester. The optical rotation of this ester is $[\alpha]_D^{20} = +10.18$. It would require the presence of from 8 to 13 per cent of the latter compound if the rotation of our samples were brought about by impurities and not by the diazo compound. Impurities of such proportions could not have escaped detection by the analytical method employed in this work.

On hydrolysis of the diazo esters, optically active material was formed with a magnitude of rotation which did not differ essentially from that of the original material.

The details of the experiments will be reported in a subsequent communication. Further work is in progress.

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