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THE ASSIMILATION OF CHOLESTEROL AND ITS ESTERS.

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(Received for publication, June 9, 1915.)

Although it is now a generally accepted fact that the cholesterol of the body has its origin in the food, and not, as has been held by some investigators, in an actual synthesis within the body, there is yet no very definite information as to the mode of absorption, or the form in which it is assimilated. It was with a view to clearing up these two points that the work represented in this paper was undertaken. The presence in the body of the free cholesterol, an alcohol, together with its esters of the higher fatty acids, oleic, palmitic, and stearic, makes it desirable to get a better understanding of the relations of these two forms in order to approach more nearly a solution of the problem of their physiology.

That cholesterol, when fed, is actually absorbed, and that the absorption is followed by a rise in blood cholesterol, has long been established. Jankau[1] found that when fed to rabbits and dogs, only a part could be recovered from the feces, although he was unable to recognize an increase in the blood. This was presumably due to the very imperfect methods of analysis available at that time. Pribram[2] demonstrated an increase in blood cholesterol after feeding, and believed that the increase was in part at least due to free cholesterol, since the serum from such animals showed an increased inhibitory power toward the hemolytic action of saponin, the free cholesterol only being active as an inhibiting agent. Kusumoto[3] also found that only a part of the cholesterol of the diet could be recovered from the feces. Gardner and coworkers[4] have shown definitely that feed-

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[1] Jankau, L., *Arch. f. exper. Path. u. Pharmakol.*, 1892, xxix, 237.

[2] Pribram, H., *Biochem. Ztschr.*, 1906, i, 413.

[3] Kusumoto, C., *ibid.*, 1908, xiv, 411.

[4] Dorée, C., and Gardner, J. A., *Proc. Roy. Soc., Series B*, 1908, lxxx, 212; 1909, lxxxi, 109. Fraser, M. T., and Gardner, J. A., *ibid.*, 1909, lxxxi, 230; 1910, lxxxii, 559. Gardner, J. A., and Lander, P. E., *Biochem. Jour.*, 1913, vii, 576; *Proc. Roy. Soc., Series B*, 1913–14, lxxxvii, 229.

ing cholesterol, either free or as esters, results in an increase in the blood content both in the free and combined forms. Their work has been done on dogs, cats, rabbits, and chicks, using in part at least, the digitonin method of Windaus[5] for analysis, and there seems to be no reason to question their findings or conclusions.

Since most fatty material which is absorbed goes into the body through the lymphatics by way of the thoracic duct, it seemed probable that cholesterol would also be absorbed in this way because of its solubility in such material, and insolubility in aqueous media. Consequently a series of experiments was made on dogs for the purpose of determining whether or not a marked increase of cholesterol could be obtained in the chyle following a meal rich in cholesterol.

A thoracic duct fistula was produced in each case by ligating the external jugular vein at a point 2 or 3 cm. distal to the entrance of the duct, the innominate vein proximal to the duct, the subclavian vein, and several other small veins which enter the segment thus cut off. The jugular vein was then cut near the distal ligature, and its proximal end sewed into the corner of the incision. Chyle could be collected from a fistula so produced, by means of a cannula, or by allowing it to drop directly into a test-tube when the dog is standing up. Unfortunately the chyle coagulates rather readily and could not be made to flow for more than about twenty-four hours. Consequently it was usually practicable to make but a single experiment, or at most two, on each dog. I wish here to express my thanks to Prof. W. G. MacCallum for his kindness in doing these operations.

The plan followed was to allow the dog to recover from the etherization in the course of three or four hours, then to give the test meal, allowing the dog to eat voluntarily when possible, and at other times feeding first 10 to 15 grams of cooked lean meat in order to stimulate the digestive organs to normal activity, and then giving the desired material by stomach tube. A 10 cc. sample of chyle was taken either before or at the time of feeding, and afterwards at varying intervals, and a comparison of the cholesterol content of the specimens made by means of a modification of the Windaus method. The details of this method I

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[5] Windaus, A., *Ztschr. f. physiol. Chem.*, 1910, lxv, 110.

have given in another place.[6] The ether extract of the chyle was obtained by mixing the sample with anhydrous sodium sulphate, allowing to dry, and grinding fine in a mortar, then extracting thoroughly with ether in a continuous extraction apparatus. A description of the experiments follows.

Experiment I.—Immediately after the completion of the fistula a sample of chyle was collected. Four and one-half hours later the dog was fed the yolks of four eggs mixed with a small amount of raw meat (20 to 30 gm.). This meal, although not analyzed, contained probably about 1 gm. of cholesterol, of which 90 per cent or more was free.[7] The material was eaten voluntarily by the dog. Table I shows the details of the experiment and analysis. The last column, headed "per cent," gives here, and in the succeeding tables, the per cent of the total cholesterol which is in the uncombined form.

TABLE I.

Dog 1.

Fistula Completed at 1 p.m.

Chyle.		Free cholesterol.		Cholesterol esters.		Per cent.
Time collected.	Amount.	Amount.		Amount.		
p.m.	*gm.*	*gm.*	*per cent*	*gm.*	*per cent*	
1.00	13.4	0.0049	0.036	0.0088	0.066	35.29
*5.30	15.2	0.0060	0.039	0.0100	0.066	37.62
7.30	9.6	0.0059	0.062	0.0115	0.119	33.91
8.30	10.0	0.0088	0.088	0.0207	0.207	29.83
9.30	9.8	0.0111	0.113	0.0270	0.276	28.32

* Time of feeding.

Experiment II.—This experiment was the second carried out on Dog 2, the first being reported as Experiment IV. The test meal consisted of 30 gm. of lean meat and three raw eggs, with which was thoroughly mixed 0.5 cc. of oleic acid. A small aliquot of this was analyzed, and the whole found to contain 0.88 gm. of cholesterol, of which 88.15 per cent was free. Owing to the fact that the digitonin method gives low values for free cholesterol in the presence of much oily material, the amount of free cholesterol was probably something over 90 per cent of the total cholesterol. The dog ate the food voluntarily. The results follow.

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[6] Mueller, J. H., *Jour. Biol. Chem.*, 1915, xxi, 23.

[7] Mueller, *loc. cit.*

TABLE II.

Dog 2.

Chyle.		Free cholesterol.		Cholesterol esters.		Per cent.
Time collected.	Amount.	Amount.		Amount.		
a. m.	*gm.*	*gm.*	*per cent*	*gm.*	*per cent*	
7.30	10.5	0.0043	0.041	0.0097	0.092	30.83
*8.30						
11.30	9.3	0.0058	0.062	0.0164	0.177	23.94
p. m.						
12.30	10.4	0.0067	0.065	0.0162	0.155	29.54
1.30	11.2	0.0062	0.055	0.0159	0.139	28.35

* Time of feeding.

Experiment III.—In order to see whether pure free cholesterol dissolved in some neutral solvent would also be assimilated in the same way, this dog was fed first a very small piece of lean meat, which he ate, and was then given by stomach tube 0.75 gm. of cholesterol (Merck) dissolved in about 10 cc. of cottonseed oil and emulsified in 250 cc. of water by means of white of egg. No trouble was experienced, either in this or in the following experiments where a similar procedure was followed, in the dog's vomiting the liquid so administered.

TABLE III.

Dog 3.

Fistula Completed at 11.30 a.m.

Chyle.		Free cholesterol.		Cholesterol esters.		Per cent.
Time collected.	Amount.	Amount.		Amount.		
p. m.	*gm.*	*gm.*	*per cent*	*gm.*	*per cent*	
*3.15	11.4	0.0049	0.043	0.0092	0.081	34.67
5.15	10.7	0.0076	0.071	0.0164	0.153	31.70
7.15	11.0	0.0083	0.076	0.0193	0.175	30.28
11.15	10.8	0.0145	0.134	0.0415	0.385	25.82

* Time of feeding.

Experiment IV.—In order to investigate the absorption of cholesterol esters it was necessary to resort entirely to the use of synthetic compounds, fed by the method used successfully in the case of free cholesterol in Experiment III. The esters used, the palmitate and oleate, were prepared according to the method recommended by Hürthle,[8] by heating cholesterol with five times its weight of the acid to be used to about 200° for two hours, and purifying the esters so produced by alcohol, in which they are very difficultly soluble. In this experiment the dog was given first 17 gm. of lean meat, which had been found to contain about 0.015 gm. of cholesterol, mostly free, and then by stomach tube 1.5 gm. of cholesterol palmitate, dissolved in cottonseed oil, and emulsified in water with egg white. The cottonseed oil was found on analysis to contain in 1 cc., 0.0010 gm. of material precipitated by digitonin before saponification, and 0.0016 gm. after saponification of the filtrate from this. This substance is probably the so called phytosterol, which evidently exists as an ester, as well as free, although I am not aware that this point has been brought out before. It is closely related chemically to cholesterol, and is converted into it when fed to animals.[9] The results follow.

TABLE IV.

Dog 2.

Fistula Completed at 11.30 a.m.

Chyle.		Free cholesterol.		Cholesterol esters.		Per cent.
Time collected.	Amount.	Amount.		Amount.		
a. m.	*gm.*	*gm.*	*per cent*	*gm.*	*per cent*	
11.30	7.8	0.0013	0.016	0.0029	0.037	30.19
p. m.						
*3.40						
5.30	9.8	0.0025	0.025	0.0039	0.040	38.46
6.40	9.9	0.0026	0.026	0.0038	0.038	40.62
7.40	9.9	0.0027	0.027	Lost		

* Time of feeding.

Experiment V.—The chyle obtained in this experiment was very bloody a small vein having evidently been left opening into the jugular. This accounts for the relatively high proportion of free cholesterol found, the cholesterol of dog blood being in the neighborhood of 80 per cent uncombined. The dog was given the same test meal as the preceding one; *viz.*, 1.5 gm. of cholesterol palmitate dissolved in cottonseed oil and emulsified in water.

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[8] Hürthle, K., *Ztschr. f. physiol. Chem.*, 1895–96, xxi, 331.

[9] Fraser and Gardner, *Proc. Roy. Soc., Series B*, 1909, lxxxi, 230.

TABLE V.

Dog 4.

Fistula Completed at 11.15 a.m.

Chyle.		Free cholesterol.		Cholesterol esters.		Per cent.
Time collected.	Amount.	Amount.		Amount.		
p. m.	*gm.*	*gm.*	*per cent*	*gm.*	*per cent*	
4.30	10.1	0.0082	0.081	0.0036	0.035	69.83
*5.15						
7.15	10.0	0.0116	0.116	0.0048	0.048	70.73
9.15	11.7	0.0129	0.110	0.0067	0.057	65.87

* Time of feeding.

Experiment VI.—Cholesterol oleate was substituted in this experiment for the palmitate, the same amount being used.

TABLE VI.

Dog 5.

Fistula Completed at 1.30 p.m.

Chyle.		Free cholesterol.		Cholesterol esters.		Per cent.
Time collected.	Amount.	Amount.		Amount.		
p. m.	*gm.*	*gm.*	*per cent*	*gm.*	*per cent*	
*4.30	10.7	0.0026	0.024	0.0045	0.042	36.36
6.45	9.8	0.0093	0.095	0.0133	0.136	41.13
8.45	9.7	0.0065	0.067	0.0100	0.103	39.41
a. m.						
12.30	10.0	0.0067	0.067	0.0106	0.106	38.73

* Time of feeding.

Experiment VII.—Chyle was still running from the fistula used in the preceding experiment the next morning, and another experiment was undertaken on the same dog, using this time 1.5 gm. of cholesterol palmitate. Unfortunately only one sample of chyle could be obtained after feeding, the fistula becoming permanently thrombosed.

TABLE VII.

Dog 5.

Chyle.		Free cholesterol.		Cholesterol esters.		Per cent.
Time collected.	Amount.	Amount.		Amount.		
a.m.	*gm.*	*gm.*	*per cent*	*gm.*	*per cent*	
9.00	9.9	0.0032	0.033	0.0042	0.043	43.42
*9.30						
p.m.						
12.30	9.4	0.0073	0.078	0.0106	0.113	40.84

* Time of feeding.

The figures quoted leave little doubt but that cholesterol is absorbed in the chyle together with other lipoidal material. It is of course not certain that no cholesterol is taken up by the blood stream directly. Unfortunately no determinations were made on the blood itself during the course of these experiments. It might be argued that an increase in the cholesterol of the lymph would not necessarily indicate an absorption through the lacteals, but that if the absorption were primarily by way of the blood stream, an increase would naturally follow in the lymph of the thoracic duct, since the general lymph has its origin ultimately in the blood plasma. Such a supposition, however, is not borne out by the figures, the increase in some, at least, of the experiments being too great to be accounted for on such a basis. The dogs used were all fair sized; one (No. 2), which was afterward bled to death for a different purpose, yielded about a liter of blood. Of this, perhaps 750 cc. were composed of plasma. In order to account for the increase which was shown in Experiment II on this dog, over 0.7 gram of cholesterol would have been required for this amount of plasma alone, without taking into consideration the blood remaining in the dog, the lymph and intercellular fluid, or the tissues themselves, which have been shown to share in the increase.[10] Since by actual analysis, less than 0.9 gram of cholesterol was fed in this experiment, and since it has been repeatedly shown that there is not a complete absorp-

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[10] Gardner and Lander, *Biochem. Jour.*, 1913, **vii**, **576**.

tion of cholesterol, much that is fed appearing in the feces either as such or as coprosterol, its reduction product, it is obvious that the increase shown by the chyle could not be explained on such a basis. In other experiments the rate of increase would have required a still larger quantity of cholesterol.

It is evident, further, from the figures, that the intestine itself is a most important site for the conversion of free cholesterol into its esters or *vice versa.* This is probably the first direct evidence of this process by any particular tissue, although it has, of course, been known that such changes take place in the organism. In those experiments in which a meal was given containing almost entirely free cholesterol, the increase is evident, not only in the free cholesterol of the chyle, but also, and to about twice the amount, in the combined cholesterol. On the other hand, when cholesterol almost entirely in the combined form is fed, there is likewise an increase in both fractions, about one-third being attributable to free cholesterol. Evidently during the absorption, the trend of the process is to preserve the ratio between free and esterified cholesterol which is normal to the chyle, and either an esterification or a hydrolysis may take place, according to the character of the material supplied. It is odd that in all the experiments in which a preponderance of free cholesterol was fed, there appears to have been a slight decrease in the relative amount of free cholesterol in the total, while in two of the experiments in which the esters were fed (IV and VI), the reverse is true. The significance of this fact, if any, is not apparent.

In Experiment II it was attempted to show that the formation of esters from the free cholesterol might be due to the liberation, during the digestion, of free fatty acids. By adding oleic acid to the food it was thought that a considerably greater increase in esters might be shown than in Experiment I, the two being in other respects similar. The results did not in any way bear out the hypothesis. It is possible, of course, that the amount of oleic acid used (0.5 cc.) was too little to give a decided effect.

In Experiment III, the finding of such a large amount of cholesterol in the last sample of the chyle eight hours after feeding, which contained also considerably more oily ether extract than the preceding ones, may be explained in part by slow absorption owing to the weak condition of the dog, but probably more by the blocking of the chyle in the intestinal lymphatics, following the temporary closing of its exit. The chyle never ran continuously, but coagulated in the orifice of the duct soon after each sample was taken. As stated above, it was always possible to start the flow again without any difficulty by inserting a cannula until about twenty-four hours after the operation, when permanent clotting usually took place.

The experiments on cholesterol palmitate were not, on the whole, as satisfactory as those on free cholesterol and cholesterol oleate, in all of which very marked absorption was indicated. Experiment IV is almost entirely negative, even the apparent increase in free cholesterol in the second sample being probably due to a false value for the first determination, since figures of this kind were not obtained in any other case. The extremely small size of the first sample renders this likely. It may be noted, however, in this connection that this is the only ambiguous figure in the whole series, the work taken altogether indicating that the modification of the digitonin method of analysis used here is remarkably accurate, even for much smaller quantities of material than has been generally believed.

Experiment V, in spite of the large amount of blood present, shows a distinct increase in cholesterol content, while the increase shown in Experiment VII is equal to that obtained in the feeding with free cholesterol.

CONCLUSIONS.

1. Cholesterol is readily absorbed from the intestinal tract through the chyle and may be recognized in the contents of the thoracic duct.

2. When cholesterol is fed entirely in the free condition, a portion is esterified during the absorption and appears so in the chyle, and *vice versa*. Hence either in the intestinal contents or in the wall of the intestine, processes of esterification or of hydrolysis may take place which result in the absorption of the cholesterol in approximately the same proportion of free to ester found normally in the chyle.

3. This ratio seems not to be affected by the presence of small quantities of fatty acids in the food.

THE RATE OF THE PASSAGE OF FATTY ACID OF FOOD INTO THE MAMMARY GLANDS OF THE GOAT.

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(*From the Department of Physiology, Columbia University, New York.*)

(Received for publication, June 10, 1915.)

In connection with other studies on milk secretion, which are being pursued by the writer in the Department of Biological Chemistry, it seemed desirable to study the rate at which the fat of the food could be metabolized and excreted by the mammary glands. The following observations were made under the direction of Dr. E. L. Scott, and for his assistance and helpful advice I wish to express my indebtedness.

Mendel[1] has shown that by staining food fat with Sudan III, stained fats will appear in the lacteals of the dog in from four to five hours after feeding. There is no assurance, however, that recombinations may not take place in the intermediary metabolism and cause the dye to be excreted before or after the fat with which it was originally combined. Winternitz,[2] in feeding iodized fats to a goat, found traces of iodine in the milk in from fifteen to twenty hours after feeding. Owing to the occurrence in the liver of acids which are more unsaturated than those contained in the food, there is a possibility of the iodine recombining with these acids and consequently being found in the milk in a new combination. If a substance could be fed which could be identified in the milk unchanged it would seem to afford a more satisfactory method for the study of the activity of the mammary glands.

My observations were made on a single goat. The substance selected for feeding was arachis oil, which normally contains about 5 per cent of arachidic acid. I am aware that arachidic acid is sometimes found in butter, due probably to the practice of feed-

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[1] Mendel, L. B., *Am. Jour. Physiol.*, 1909, xxiv, 493.

[2] Winternitz, H., *Deutsch. med. Wchnschr.*, 1897, xxiii, 477; *Ztschr. f. physiol. Chem.*, 1898, xxiv, 425.

ing arachis meal to the cows. Although in my experiment the normal milk was examined for arachidic acid for the five successive days just previous to the experiment, none was found to be present. Then, after emptying the glands on the evening of July 27, 1914, 25 cc. of arachis oil were given in the feed and 25 cc. were given twice daily thereafter until the morning of August 1. The morning milk of July 28, twelve and one-half hours after the first feeding, was found to contain arachidic acid in abundance. The evening milk of July 28 contained appreciably more, and from then on to the end of the experiment, August 1, a.m., the amount remained apparently constant.

The analytical procedure in this case was to extract the fat from the milk by Meigs'[3] method. A weighed quantity of milk is diluted with an equal volume of distilled water and then shaken for five minutes with a volume of ether equal to the original volume of milk; then alcohol equal in volume to the amount of ether is shaken with the milk-ether mixture and, after settling, the ether layer is pipetted off and the fat is dried to constant weight in a vacuum desiccator over sulphuric acid. The arachidic acid was determined by Renard's[4] method as modified by Lewkowitsch.[5] By this method the unsaturated fatty acids are separated from the saturated acids by extracting the lead salts with ether.. The remaining salts are then decomposed with hydrochloric acid and the fatty acids are collected. The fatty acids are washed several times with hot water and finally dissolved in boiling 90 per cent alcohol. Arachidic acid crystallizes out upon cooling the solution to 15°C.

While no attempt was made at exact quantitative estimation, as it was the purpose only to see whether any arachidic acid were present in the milk, it may be of interest to note that the total weight of the crystals from samples Nos. 2, 3, and 4 was 1.2878 grams. The fact that these crystals became almost transparent at 77°C. and wholly transparent at 81°C. indicates that they were a mixture. Since arachidic acid has a melting point of 77°C., and lignoceric acid a melting point of 80.5°C., and since both these acids are characteristic of arachis oil, it is fair to presume that the crystals were composed of these acids. The microscopic appearance of the crystals was such as to confirm this assumption.

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[3] Meigs, A. V., *Philadelphia Med. Times*, 1881–82, xii, 660.

[4] Renard, A., *Compt. rend. Acad. d. sc.*, 1871, lxxiii, 1330.

[5] Lewkowitsch, J., Chemical Technology and Analysis of Oils, Fats, and Waxes, London, 1909, 4th edition, i, 132–33; ii, 252.

From these facts it is evident that the time required for the ingested fatty acids to appear in the milk is at least not over twelve and one-half hours and probably less. No attempt was made to determine how long after discontinuing the feeding of arachis oil the arachidic acid continues to be excreted.

ANIMAL CALORIMETRY.

TWELFTH PAPER.

THE INFLUENCE OF THE INGESTION OF FAT.[1]

By J. R. MURLIN and GRAHAM LUSK,

With the technical assistance of J. A. Riche.

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(Received for publication, June 4, 1915.)

I. INTRODUCTION.

Work of fundamental character by Magnus-Levy[2] showed the influence of the ingestion of very fat bacon upon the metabolism of the dog. Respiration experiments lasting about thirty minutes each, using the Zuntz method, were made upon a dog breathing through a tracheal cannula. These showed that after giving 140 grams of fat bacon the metabolism increased during the fifth to ninth hours to a height which was about 10 per cent above the original basal level as measured twenty-four hours after the last ingestion of food. After 320 grams of fat bacon had been taken the metabolism showed a maximal increase of 19 per cent during

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[1] The urinary analyses were performed by Mr. R. H. Harries.

[2] Magnus-Levy, A., *Arch. f. d. ges. Physiol.*, 1894, lv, 1.

the fourth to seventh hours. The increased metabolism extended from the fourth to the thirteenth hours after food ingestion and then subsided to the original basal level. The total increase in heat production could be estimated as 2.5 per cent of the energy content of the fat ingested. The environmental temperature of the dog varied between 16° and 19°, and all extraneous movements were avoided.

In 1910 O. Müller[3] sought to compare the relative specific dynamic action of starch and glucose. However, when he gave starch he also administered 40 grams of fat with it, thereby introducing a technical error, as will appear later.

In the Third and Eleventh Papers of this series the manner of the carbohydrate metabolism has been discussed. In the present paper the course of metabolism after fat ingestion will be described, as well as that observed after combining fat with glucose and with glycocoll in various ways in the ingesta.

The work of Bloor[4] has shown that after giving fat to a dog there is a gradual rise in the fat content of the blood, the maximum being attained in the sixth hour, after which there is a fall. Furthermore, when fat was injected intravenously so that the fat content of the blood was doubled, the excess disappeared within five minutes after the cessation of the injection. Should the course of metabolism after fat ingestion follow the curve of fat content in the blood, this would tend to establish the standpoint that the heat production may be increased by increasing the metabolites available for cell nutrition.

II. EXPERIMENTAL PROCEDURE.

The two dogs which were used in these experiments were subjected to the same experimental procedure described in former papers. The "standard diet" was given at 5.00 p.m. and eighteen hours thereafter the basal metabolism was determined. On other experimental days the material the dynamic action of which was to be tested was given about 9.00 a.m., or eighteen hours after food, and the course of the heat production noted. The food when ingested had a temperature of 38°. The experiments were

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[3] Müller, O., *Biochem. Ztschr.*, 1910, xxviii, 427.

[4] Bloor, W. R., *Jour. Biol. Chem.*, 1914, xix, 1.

conducted at an environmental temperature of 26°. The methods of calculation followed were the same as those used in the Eleventh Paper.[5]

The fat emulsion was made by Fairchild Brothers and Foster and contained:

	Per cent
Lecithin	1
Peanut oil	37
Lard oil	37
Water	25

100 grams contained 694 calories as determined in the bomb calorimeter. The glucose administered was anhydrous and chemically pure, manufactured by Merck, and showed a heat value as determined in the bomb of 3.70 calories per gram.

III. EXPERIMENTAL PART.

These experiments have analyzed the course of the heat production (1) after the ingestion of 75 grams of emulsified fat; (2) after the ingestion of 75 grams of fat followed by 70 grams of glucose; (3) after the ingestion of 50 grams of glucose plus 20 grams of glycocoll four hours after the administration of 75 grams of emulsified fat. The results of these experiments are epitomized in Tables I, II, and III and in Charts I, II, and III, whereas the full details are appended in Tables IV and V.

The experiments on Dogs III and XIV showed the following comparisons between indirect and direct calorimetry.

Dog.	No. of experiments.	Indirect.	Direct.	Difference.		No. of experiments in which error was	
						under 2 per cent.	over 2 per cent.
		ccl.	cal.	cal.	per cent		
III	10	996.71	975.05	21.66	2.2	5	5
XIV	22	2,244.20	2,230.18	14.02	0.6	14	8

In two of the thirty-two experiments the difference between indirect and direct calorimetry was less than 1 per cent. There

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[5] Lusk, G., *Jour. Biol. Chem.*, 1915, xx, 555.

is nothing to indicate that any particular foodstuff causes a discrepancy between the forms of measuring heat, but probably that a

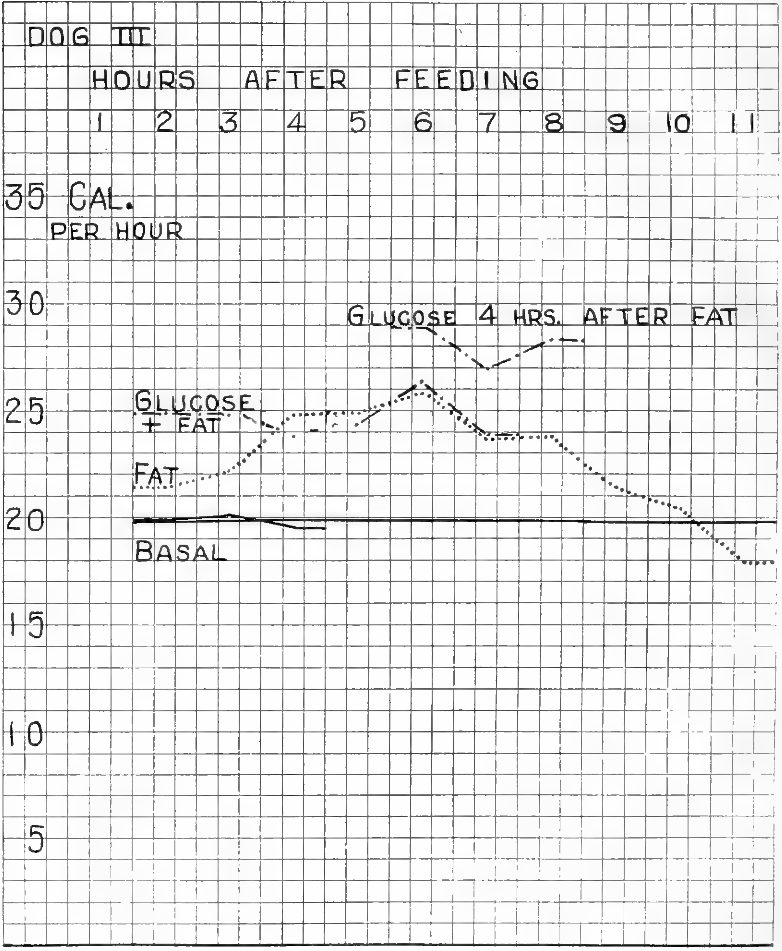

CHART I. The effect of fat and of glucose plus fat upon the heat production.

change in the position of the dog itself, or of the position of the rectal thermometer within the dog, may so affect the reading of the body temperature as to modify materially the calculations of direct calorimetry. Experiments of Mr. Riche indicate this to be true. Such errors would tend to equalize each other in the long run. Alcohol checks in general show more accurate agreement than work on dogs, thus proving the excellence of the calorimeter itself.

TABLE I.

The Influence of Fat, 75 Grams (694 Calories), and Glucose, 70 Grams (259 Calories), upon the Metabolism of Dog III in Calories per Hour.

Experiment No.		R. Q. average.	Hrs. after food.*									
			2	3	4	5	6	7	8	9	10	11
Basal	94	0.82	19.0	19.7	18.9							
	98	0.86	19.7	20.2	19.7							
	100	0.84	20.9	20.3	20.2							
Average		0.84	19.9	20.1	19.6	......	..Ave	rage	19.9			
Fat 75 gm	93	0.81	23.0	22.7	24.0	23.7						
	95	0.79	20.4	22.4	24.1	26.2	26.4	23.7				
	99	0.78					25.4	23.8	23.8	21.3	20.4	17.9
	101	0.78	20.9	21.3	26.3							
Average		0.79	21.4	22.1	24.8	24.5	25.9	23.7	23.8	21.3	20.4	17.9
Increase in per cent			7	11	25	23	30	19	19	7	2	
Fat 75 gm. + glucose 70 gm	96	0.98	24.6	26.0	24.1	25.1	29.6	26.1				
	97	1.00	25.3	23.4	23.7	23.6	23.0	21.6				
Average			24.9	24.7	23.9	24.3	26.3	23.8				
Increase in per cent			25	20	20	22	32	20				
Glucose 70 gm. 4 hrs. after fat 75 gm	101	0.94					29.0	27.0	28.3			
Increase in per cent							45	36	42			

* Except when the basal metabolism was determined.

A. The Administration of Fat, 75 Grams.

The experiments upon Dog III show that after the ingestion of fat the heat production gradually rises till the sixth hour to a maximum 30 per cent above the basal metabolism and then falls slowly to the basal level which is reached ten hours after the fat has been taken (see Chart I). In Dog XIV the increase appeared earlier, did not reach the same height and was not followed to its conclusion (see Charts II and III). *In Dog III the total increase in heat production amounted to 28.8 calories, or 4.1 per cent of the 694 calories of energy contained in the 75 grams of ingested fat.*

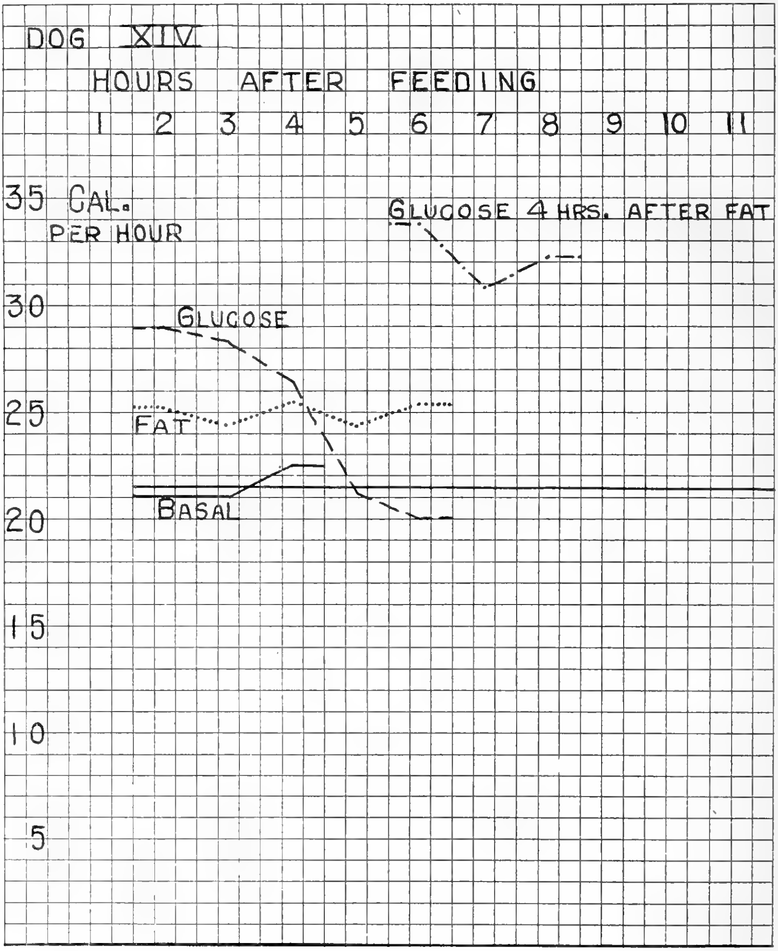

CHART II. The effect of fat, of glucose, and of glucose plus fat upon the heat production.

In Dog III the respiratory quotients during three experiments in which the basal metabolism was determined averaged 0.84, and in four experiments after fat ingestion the average was 0.79. In Dog XIV (First Series) the quotients of five basal metabolism experiments averaged 0.85, and of four after fat ingestion, 0.80; in the Second Series three basal metabolism experiments showed an average quotient of 0.86, and one after fat ingestion of 0.81. The average quotient of each basal metabolism experiment was therefore always higher than any of the quotients found after giving fat. *It appears, therefore, that the administration of fat*

TABLE II.

Dog XIV. First Series.

The Influence of Fat, 75 Grams (694 Calories), and Glucose, 70 Grams (259 Calories), Severally and Together in Calories per Hour.

Experiment No.	R. Q. average.	Hrs. after food.*						
		2	3	4	5	6	7	8
Basal.......... 2	0.83	22.2	20.7	22.0				
6	0.85	19.8	19.0	22.5				
8	0.91	21.2	22.4	20.7				
12	0.85	21.8	21.2	25.0				
14	0.82	20.2	21.6					
Average.........	0.85	21.0	21.0	22.5	......	Aver	age 21	.5
Fat............ 3	0.79	25.0	25.5	25.9	24.4			
7	0.80	25.9	24.6	28.7				
9	0.80	24.9	25.1	23.5				
13	0.80	25.2	23.0	24.3	24.3	25.3		
Average.........	0.80	25.2	24.0	25.6	24.4	25.3		
Increase in per cent.		17	14	19	13	18		
Glucose 70 gm.. 4	0.99	29.0	28.4	28.1				
5	1.02	29.0	28.0	24.7	21.2	20.0		
Average.........	1.00	29.0	28.2	26.4	21.2	20.0		
Increase in per cent.		35	31	23				
Glucose 70 gm.. 7 4 hrs. after fat....	0.93					33.8	30.9	32.2
Increase in per cent.						57	39	50

* Except when the basal metabolism was determined.

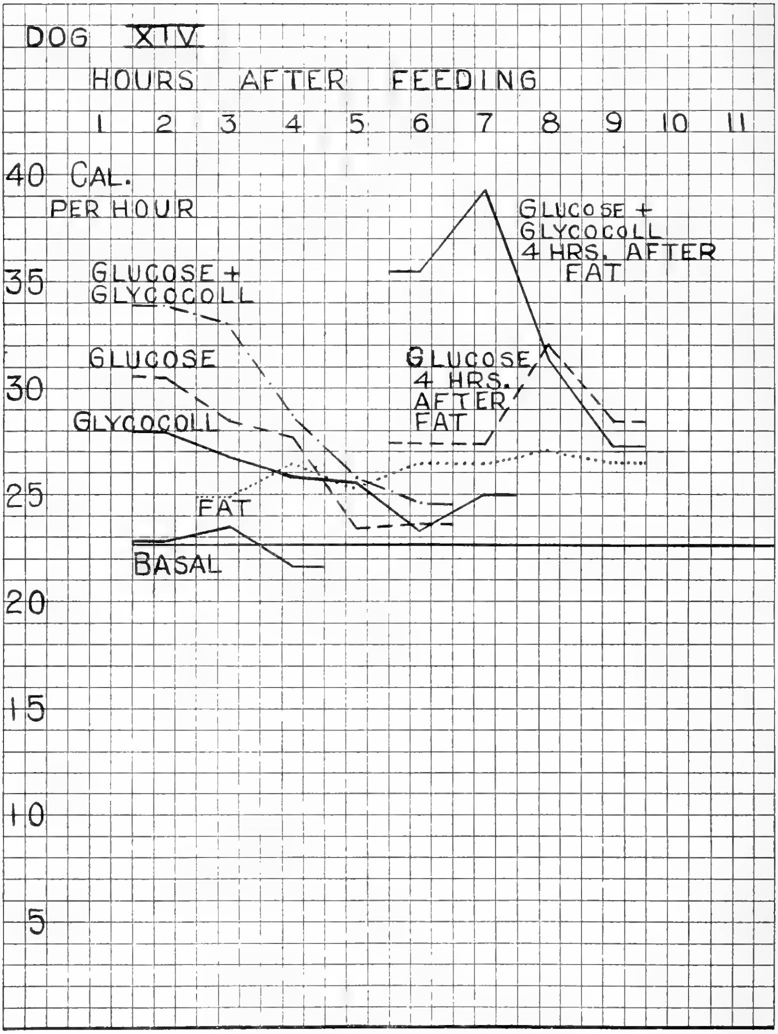

Chart III. The effect of fat, of glycocoll, of glucose, of glucose plus glycocoll, and of glucose plus glycocoll plus fat upon the heat production.

causes a relative increase in the metabolism of fat itself. Thus, an analysis of Experiments 6 and 12 on Dog XIV in which the basal metabolism was determined and in both of which the respiratory quotients averaged 0.85, shows that 43 and 40 per cent of the

TABLE III.

Dog XIV. Second Series.

Influence of Fat, 75 Grams (694 Calories), Glucose, 70 Grams (259 Calories), Glycocoll, 20 Grams (42 Calories), Glucose, 50 Grams + Glycocoll, 20 Grams (227 Calories), Severally and Together, upon the Metabolism of Dog XIV in Calories per Hour.

Experiment No.		R. Q. average.	Hrs. after food.*							
			2	3	4	5	6	7	8	9
Basal	15	0.85	24.1	23.9	20.7					
	17	0.90	21.1	24.5	22.7					
	21	0.84	22.1	22.1	21.8					
Average		0.86	22.8	23.5	21.7	......	Average 22.7			
Fat 75 gm	20	0.81		24.9	26.4	25.2	26.4	26.4	27.0	26.4
Increase in per cent				10	16	11	16	16	19	16
Glucose 70 gm	16	1.00	31.4	30.3	28.5	24.0	23.7			
	19	1.00	30.2	27.0	27.0	22.8				
Average			30.6	28.6	27.7	23.4	23.7			
Increase in per cent			35	26	22	3	4			
Glycocoll 20 gm	18	0.88	28.0	26.8	25.8	25.5	23.2	25.0		
Increase in per cent			23	18	14	12	2	10		
Glycocoll 20 gm. + glucose 50 gm	23	1.01	33.9	33.0	28.7	25.7	24.6			
Increase in per cent			49	45	26	13	9			
Glucose 70 gm. 4 hrs. after fat	22	0.93					27.4	27.4	32.0	28.4
Increase in per cent							20	20	41	25
Glucose 70 gm. + glycocoll 20 gm. 4 hrs. after fat	24	1.00					35.4	39.2	31.4	27.2
Increase in per cent							56	73	38	20

* Except when the basal metabolism was determined.

energy was derived from fat, whereas in Experiment 13, after fat ingestion, the energy from fat was 56 per cent of the total. Furthermore, in the two experiments in which the basal metabolism was determined the protein plus carbohydrate calories were 11.5 and 10.0 per hour, whereas in the fat experiment the protein plus carbohydrate calories were 10.6 per hour. In the Second Series of experiments on Dog XIV analogous relations exist. *The increase in heat production is therefore derived from the increased oxidation of incoming fat.*

B. The Administration of Fat and Glucose.

In order to determine whether there was a summation of effect when fat and glucose were given together, two such experiments (Nos. 96 and 97) were accomplished on Dog III. In these experiments the heat production rose in the first two hours of experimentation to a higher level than when fat alone was given. The respiratory quotients during this period indicated the combustion of the readily absorbable glucose. In Experiment 96 there appeared to be a decided secondary rise in metabolism during the fifth and sixth hours, indicating the maximal rise due to fat absorption (see Chart I).

C. The Administration of Glucose Four Hours after Fat Had Been Given.

Since the maximal effect of fat upon metabolism takes place six hours after its ingestion and that of carbohydrate during the second and third hours after it has been given, the experiment was varied so that 70 grams of glucose were given to Dog III four hours after 100 grams of fat emulsion had been given. It was noted that during the fifth to seventh hours the metabolism was increased 41 per cent above the normal in contrast with a 23 per cent increase when fat alone was given. The relations are shown below:

Dog III.

Experiment Nos.		R. Q.	Cal. per hr.	No. of hrs.	Increase above normal.	
					cal.	*per cent*
94, 98, 100	Basal	0.84	19.9	3		
93, 95, 99, 101	Fat 75 gm.	0.79	24.5	3*	4.6	23
101	Glucose 70 gm. 4 hrs. after fat 75 gm.	0.94	28.1	3*	8.2	41

*6, 7, and 8 hrs. after fat.

Still more striking were experiments made upon Dog XIV (First Series). Here the effects of carbohydrate and fat alone and together were determined and with the following results:

Dog XIV. First Series.

Experiment Nos.		R. Q.	Cal. per hr.	No. of hrs.	Increase above normal.	
					cal.	*per cent*
2, 6, 8, 12, 14	Basal............	0.85	21.5	3		
3, 7, 9, 13	Fat 75 gm.......	0.80	25.0	5	3.5	16
4, 5	Glucose 70 gm...	1.00	28.6	2	7.1	33
7	Glucose 70 gm. 4 hrs. after fat 75 gm..........	0.93	32.3	2	10.8	50

This table shows that after giving fat the metabolism rose 16 per cent above the normal and the respiratory quotient fell from 0.85 to 0.80. After giving glucose the metabolism rose 33 per cent during the two hours of maximal effect, and the respiratory quotient was 1.00. However, when 70 grams of glucose were given four hours after the ingestion of 75 grams of fat the heat production rose to a level 50 per cent above the basal, or the arithmetical sum of the increases which fat and carbohydrate alone would have produced. In this the results are comparable to those observed when glucose and alcohol were given alone or together.

When glucose was given after fat ingestion the respiratory quotient was 0.93 in Dog XIV and 0.94 in Dog III, instead of unity when carbohydrate was given alone. The decided lowering of the respiratory quotient has its counterpart in a similar fall when alcohol and glucose are given together.[6] It is perhaps impossible to calculate exactly the relative quantities of fat and carbohydrate oxidized under these circumstances, for a probable conversion of carbohydrate into fat would raise the respiratory quotient, a complication for which correction is impossible. Be that as it may, the quotients of 0.93 and 0.94 which were found show that there can be no question that *with an ample influx of both fat and glucose at the same time, both food substances are simultaneously oxidized and with a large increase in the heat production.*

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[6] Lusk, *loc. cit.*, 1915, xx, 555.

Two sets of experimental conditions occurred in which the summation is not evident: (1) when fat and carbohydrate are given at the same time and the carbohydrate is more rapidly absorbed, a high respiratory quotient betokens its almost exclusive utilization and there is no summation of effect (see Experiments 96 and 97 on Dog III); (2) when glucose is given after fat, and low quotients like 0.86 and 0.91 indicate its slow absorption, due possibly to prolonged retention in the stomach (Experiment 22, Dog XIV).

In a former article Lusk[7] has held that there was no evidence to show that the increased metabolism after giving carbohydrate and fat was due to acid stimulation of the cells. If it were true that the high heat production after giving fat and glucose together was due to an increased production of acids in metabolism, then one would naturally expect to find an increased quantity of ammonia in the urine. The following table shows the quantities of ammonia nitrogen eliminated after the ingestion of 75 grams of fat and after giving 70 grams of glucose four hours after fat ingestion.

Date.	Experiment No.	Length of urine collection in hrs.	N in mg. per hr.	
			Total N.	NH_3N.
1915				
Apr. 19...	20 Fat 75 gm....................	8 hrs. 20 min.	112	7
" 28...	4 hrs. after 75 gm. fat.......	5 hrs.		15
" 21...	22 Glucose 70 gm. 4 hrs. after fat 75 gm..................	5 hrs. 9 min.	95	4
" 29...	Glucose 70 gm. 4 hrs. after at 75 gm.................	5 hrs.		3

It is apparent from these results that when glucose and fat are oxidized together in the organism the quantity of ammonia eliminated in the urine is much less than when fat alone is given. As ammonia elimination is held to be an index of acid formation the reduction in ammonia output may be considered as an added indication that the increased heat production which follows carbohydrate ingestion is not due to acid stimulation.

It may be further stated that in Experiment 18 following the ingestion of glycocoll the ammonia excretion was 20 mg. per hour, and in Experiment 23 after glycocoll and glucose were given

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[7] Lusk, *loc. cit.*, 1915, xx, 555.

together it amounted to 23 mg. per hour. In this instance in which a stimulation is effected through the metabolism of an amino-acid the ammonia output appears to be uninfluenced by the administration of carbohydrate.

Although it may be possible that the increased heat production after administration of carbohydrate is due to acid stimulation, no convincing evidence in favor of this view exists at present. Of theoretical significance might be mentioned the possible synthetic production of alanine from methyl glyoxal, which substance would then act as a stimulus to metabolism.

D. The Effect of Glycocoll, Glucose, and Fat, Severally and Together.

A second series of experiments performed upon Dog XIV was designed to show the results of the simultaneous effect of glycocoll, glucose, and fat upon the metabolism. To this end the influence of each of these three substances was determined separately, then that of glucose and glycocoll given together, and finally the effect of a mixture of all three materials.

After giving fat the metabolism was found at its maximum in the sixth to ninth hours, showing at this time an average increase of 17 per cent above the normal. When glucose and glycocoll were given together with fat the first two were administered at the beginning of the fifth hour after the fat had been ingested, in order to obtain a maximal effect. Since the effect of glucose or of glycocoll reaches a maximum during the second and third hours after their ingestion, these hours are taken for comparison. The essential results may be thus briefly summarized.

Dog XIV. Second Series.

Experiment Nos.		R. Q.	Cal. per hr.	No. of hrs.	Increase over normal.	
					cal.	*per cent*
15, 17, 21	Basal	0.86	22.7	3		
20	Fat 75 gm.	0.80	26.6	4*	3.9	17
18	Glycocoll 20 gm.	0.90	27.6	2	4.9	25
16, 19	Glucose 70 gm.	1.02	29.6	2	6.9	30
23	Glucose 50 gm. + glycocoll 20 gm.	1.03	33.5	2	10.8	48
24	Glucose 50 gm. + glycocoll 20 gm. given 4 hrs. after fat 75 gm.	1.02	37.3	2	14.6	64

* Hours 6, 7, 8, 9 after fat ingestion.

Analysis of these figures reveals a very close approximation to a summation of effect as the different substances are brought into play among the metabolizing cells. This is illustrated as follows:

	Increase.	
	cal.	*per cent*
Glycocoll 20 gm.	4.9	25
Glucose 70 gm.	6.9	30
Sum of both	11.8	55
Glucose 50 gm. + glycocoll 20 gm.	10.8	48

This accords with former results given in the Eleventh Paper.[8] Further analysis shows the following relations:

	Increase.	
	cal.	*per cent*
Glycocoll 20 gm.	4.9	25
Glucose 70 gm.	6.9	30
Fat 75 gm.	3.9	17
Sum of all	15.7	72
Glycocoll 20 gm. + glucose 50 gm. 4 hrs. after fat 75 gm.	14.6	64

It has previously been shown that there is little difference in the heat production after giving 50 and 70 grams of glucose during the first two experimental hours. Bearing this in mind, it is evident from the above table that *after giving 20 grams of glycocoll + 50 grams of glucose during the height of the absorption of 75 grams of fat, the increase in metabolism is nearly the sum of the increases brought about by giving each substance separately.* Reasoning from analogy based on work published in the Eleventh Paper, alcohol, if it had been added to the mixture, would have raised the metabolism to even a higher level.

IV. SUMMARY.

This paper extends to fat metabolism the consideration given to that of amino-acids, carbohydrates, and alcohol presented in the Eleventh Paper.

1. One must picture the large influx of fat globules into the blood stream through the thoracic duct after partaking of fat, an influx which reaches its maximum during the sixth hour. The

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[8] Lusk, *loc. cit.*, 1915, xx, 555.

heat production of the organism may gradually rise, reach its maximum during the sixth hour, and may fall to the basal level ten hours after fat has been ingested (as in Dog III). It appears that the ingestion of fat does not change the quantity of heat produced from protein and glycogen, but that the increased metabolism is at the expense of the fat ingested.

2. If glucose be ingested at the time of the highest fat metabolism, the heat production undergoes a second increase by the same quota which glucose alone would have increased metabolism. At this level of higher metabolism the respiratory quotient is 0.93 or 0.94, instead of unity, which it would have been if glucose had been given alone. Hence, glucose and fat were being oxidized together. This is the metabolism of plethora. As concerns fat and carbohydrate, the opinion of Carl Voit that the height of the heat production is determined by the quantity and quality of the materials brought to the cells by the blood appears to be vindicated.

3. When glucose and glycocoll are given together so that their molecules enter the circulation at the time of the height of fat absorption, the increase in metabolism is very nearly equal to the sum of the increases which each of the three materials would have induced alone.

Date.	Exper. No.	Time.	CO_2.	O_2.	R.Q.	H_2O.	Urine N.	Non-protein. R.Q.	Calories.	
									Protein.	Carbohydrate into fat.
1914		*a.m.*	*gm.*	*gm.*		*gm.*	*gm.*			
Oct. 27..	93	10.50–11.50	7.86	6.86	0.83	10.01	0.148	0.84	3.92	
		11.50–12.50	7.46	6.85	0.79	10.87	0.148	0.79	3.92	
		p.m.								
		12.50– 1.50	7.91	7.23	0.80	10.52*	0.148	0.79	3.92	
		1.50– 2.50	8.00	7.10	0.82	10.21*	0.148	0.82	3.92	
		a.m.								
Oct. 28..	94	10.07–11.07	6.44	5.68	0.82	8.65	0.133	0.83	3.53	
		11.07–12.07	6.65	5.92	0.82	8.11	0.133	0.82	3.53	
		p.m.								
		12.07– 1.07	6.43	5.64	0.83	7.77	0.133	0.84	3.53	
		a.m.								
Oct. 29..	95	10.20–11.20	6.77	6.14	0.80	8.87	0.128	0.80	3.39	
		11.20–12.20	7.45	6.74	0.80	8.21	0.128	0.80	3.39	
		p.m.								
		12.20– 1.20	8.00	7.22	0.81	8.34	0.128	0.81	3.39	
		1.20– 2.20	8.34	7.94	0.76	8.66	0.128	0.76	3.39	
		2.20– 3.20	8.54	7.99	0.78	8.48	0.128	0.77	3.39	
		3.20– 4.20	8.13	7.06	0.79	7.97	0.128	0.84	3.39	
		a.m.								
Oct. 30..	96	10.25–11.25	10.03	7.00	1.04	8.22	0.113	1.08	3.00	0.26
		11.25–12.25	11.16	7.28	1.12	9.18	0.113	1.16	3.00	0.57
		p.m.								
		12.25– 1.25	8.91	7.02	0.92	9.03	0.113	0.94	3.00	
		1.25– 2.25	8.68	7.42	0.85	8.39	0.113	0.86	3.00	
		2.25– 3.25	11.37	8.47	0.98	8.89	0.113	1.00	3.00	
		3.25– 4.25	9.78	7.54	0.94	9.28	0.113	0.96	3.00	
		a.m.								
Nov. 5..	97	10.21–11.21	10.16	7.26	1.02	8.83	0.132	1.06	3.50	0.20
		11.21–12.21	10.42	6.54	1.16	9.08	0.132	1.23	3.50	0.71
		p.m.								
		12.21– 1.21	9.04	6.85	0.96	8.50	0.132	0.99	3.50	
		1.21– 2.21	9.37	6.75	1.01	8.50	0.132	1.05	3.50	0.16
		2.21– 3.21	8.58	6.68	0.93	7.89	0.132	0.96	3.50	
		3.21– 4.21	8.00	6.30	0.92	7.76	0.132	0.95	3.50	

* Estimated.

alories.		Body temperature.			Morning weight.	Behavior of dog.	Food.
ect.	Direct.	Start.	End.	Dif.			
					kg.		
.97	21.25	37.47	37.58	+0.11	12.85	Quiet	100 gm. fat emulsion at 10 a.m.
69	22.84		37.78	+0.20		“	
.97	22.76		37.90	+0.12		“	
.68	23.24		37.83	−0.07		“	
.31	90.09						
.97	18.76	37.65	37.52	−0.13	12.70	Quiet	Basal.
.75	19.43		37.60	+0.08		“	
.86	19.21		37.63	+0.03		“	
.58	57.40						
.40	20.47	37.56	37.60	+0.04	12.75	Quiet	100 gm. fat emulsion at 9.25 a.m.
.41	22.22		37.81	+0.21		“	
.08	22.65		38.00	+0.19		Slight movement	
.21	23.72		38.11	+0.11		Quiet	
.43	26.29		38.39	+0.28		“	
.69	21.94		38.26	−0.13		“	
.22	137.29						
.64	25.36	37.82	38.08	+0.26	12.90	Quiet	100 gm. fat emulsion + 70 gm. glucose at 9.25 a.m.
.94	24.66		38.09	+0.01		“	
.14	24.61		38.06	−0.03		“	
.06	25.27		38.30	+0.24		“	
.55	24.64		38.24	−0.06		“	
.07	24.58		38.15	−0.09		“	
.40	149.12						
.39	22.93	37.86	37.80	−0.06	13.15	Quiet	100 gm. fat emulsion + 70 gm. glucose at 9.25 a.m.
.36	25.48		37.92	+0.12		“	
.70	23.82		37.94	+0.02		“	
.55	23.35		37.94	+0.00		“	
.96	22.18		37.94	+0.00		“	
.58	22.00		38.03	+0.09		“	
.54	139.76						

TABL

Date.	Exper. No.	Time.	CO₂.	O₂.	R. Q.	H₂O.	Urine N.	Non-protein. R.Q.	Calories. Protein.	Calories. Carbohydrate into fat.
1914		a.m.	gm.	gm.		gm.	gm.			
Nov. 6..	98	10.10–11.10	7.07	5.82	0.88	9.44	0.135	0.90	3.58	
		11.10–12.10	7.06	6.02	0.85	8.55	0.135	0.86	3.58	
		p.m.								
		12.10– 1.10	6.81	5.88	0.84	7.93	0.135	0.85	3.58	
		p.m.								
Nov. 9..	99	3.34– 4.34	8.23	7.64	0.78	9.56	0.086	0.78	2.28	
		4.34– 5.34	7.83	7.15	0.80	9.21	0.086	0.80	2.28	
		5.34– 6.34	7.65	7.17	0.78	8.98	0.086	0.77	2.28	
		6.34– 7.34	6.98	6.41	0.79	8.11	0.086	0.79	2.28	
		7.34– 8.34	6.11	6.26	0.71	7.79	0.086	0.70	2.28	
		8.34– 9.34	6.17	5.33	0.84	7.46	0.086	0.85	2.28	
		a.m.								
Nov. 11..	100	10.31–11.31	7.15	6.21	0.84	8.23	0.113	0.84	3.00	
		11.31–12.31	7.05	6.02	0.85	7.42	0.113	0.86	3.00	
		p.m.								
		12.31– 1.31	6.79	6.06	0.82	7.02	0.113	0.82	3.00	
		a.m.								
Nov. 12..	101	10.10–11.10	6.67	6.35	0.76	9.55	0.113	0.76	3.00	
		11.10–12.10	7.02	6.41	0.80	8.42	0.113	0.79	3.00	
		p.m.								
		12.10– 1.10	8.39	7.96	0.77	8.66	0.113	0.76	3.00	
		2.10– 3.10	10.78	8.39	0.94	10.43	0.097	0.95	2.57	
		3.10– 4.10	10.16	7.79	0.95	9.48	0.097	0.97	2.57	
		4.10– 5.10	10.57	8.19	0.94	9.00	0.097	0.95	2.57	

uded.

Calories.		Body temperature.			Morning weight.	Behavior of dog.	Food.
irect.	Direct.	Start.	End.	Dif.			
					kg.		
.72	21.11	37.63	37.61	−0.02	13.25	Quiet	Basal.
.25	17.41		37.42	−0.19		"	
.72	22.10		37.68	+0.26		Slight movement	
.69	60.62						
.40	25.88	37.96	38.07	+0.11	13.35	Quiet	100 gm. fat emulsion at 9.25 a.m. 6th to 11th hrs.
3.83	24.81		38.11	+0.04		Slight movement	
3.78	22.44		37.96	−0.15		Quiet	
1.33	22.20		37.84	−0.12		Movements	
.42	20.80		37.86	+0.02		Quiet	
7.94	17.45		37.68	−0.18		"	
2.70	133.58						
0.87	21.14	37.57	37.40	−0.17	13.35	Quiet	Basal.
0.30	21.40		37.39	−0.01		"	
0.24	21.69		37.52	+0.13		"	
1.41	64.23						
0.94	21.72	37.36	37.32	−0.04	13.35	Movement	100 gm. fat emulsion at 9.25 a.m.
1.32	22.18		37.54	+0.22		"	
6.32	23.92		37.69	+0.15		"	
8.58	67.82						
8.98	24.14	38.21	38.12	−0.09		Quiet	70 gm. glucose at 1.25 p.m.
6.99	25.43		38.18	+0.06		Movement	
8.31	25.57		38.11	−0.07		Quiet	
4.28	75.14						

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Date.	Exper. No.	Time.	CO_2.	O_2.	R.Q.	H_2O.	Urine N.		Non-protein R. Q.	Calories.	
										Protein.	Car hyd in fa
1914		a.m.	gm.	gm.		gm.	gm.				
Nov. 13..	2	11.00–12.00	7.60	6.59	0.84	13.99	0.130		0.85	3.45	
		p.m.									
		12.00– 1.00	7.21	6.13	0.86	12.01	0.130		0.87	3.45	
		1.00– 2.00	7.18	6.61	0.79	10.16	0.130		0.79	3.45	
		a.m.									
Nov. 17..	3	10.54–11.54	8.46	7.45	0.83	8.73	0.111		0.83	2.94	
		11.54–12.54	8.50	7.62	0.81	8.49	0.111		0.81	2.94	
		p.m.									
		12.54– 1.54	8.27	7.83	0.77	7.68	0.111		0.76	2.94	
		1.54– 2.54	8.55	7.20	0.86	7.54	0.111		0.87	2.94	
		2.54– 3.54	9.10	8.52	0.78	7.77	0.111		0.77	2.94	
		3.54– 4.54	10.06	9.40	0.78	8.09	0.111		0.78	2.94	
		a.m.									
Nov. 18..	4	10.10–11.10	11.31	8.32	0.99	7.63	0.118		1.01	3.12	0.
		11.10–12.10	11.08	8.13	0.99	8.30	0.118		1.02	3.12	0.
		p.m.									
		12.10– 1.10	10.98	8.05	0.99	8.05	0.118		1.02	3.12	0.
		a.m.									
Nov. 19..	5	10.20–11.20	11.45	8.27	1.01	8.40	0.114		1.03	3.02	0.
		11.20–12.20	11.13	7.99	1.01	8.78	0.114		1.04	3.02	0.
		p.m.									
		12.20– 1.20	11.10	6.88	1.17	8.58	0.114		1.23	3.02	0.
		1.20– 2.20	7.71	6.22	0.90	7.49	0.114		0.92	3.02	
		2.20– 3.20	8.02	5.71	1.02	7.03	0.114		1.06	3.02	0.
		a.m.									
Nov. 24..	6	10.30–11.30	7.12	5.80	0.89	7.34	0.082		0.91	2.17	
		11.30–12.30	6.75	5.62	0.87	7.21	0.082		0.89	2.17	
		p.m.									
		12.30– 1.30	6.84	6.22	0.80	6.75	0.082		0.80	2.17	

Calories.			Body temperature.			Morning weight.	Behavior of dog.	Food.
in.	Indirect.	Direct.	Start.	End.	Dif.			
						kg.		
72	22.17	21.87	37.80	37.68	−0.12	13.27	Quiet	Basal.
25	20.70	21.31		37.69	+0.01		"	
51	21.96	20.76		37.80	+0.11		"	
	64.83	63.94						
05	24.99	25.20	38.19	38.13	−0.06	13.35	Quiet	100 gm. fat emulsion at 9.56 a.m.
51	25.45	25.76		38.14	+0.01		"	
92	25.86	25.42		38.46	+0.32		"	
42	24.36	24.18		38.56	+0.10		"	
28	28.22	28.61		38.68	+0.12		Movement	
30	31.24	29.13		38.75	+0.07		"	
	160.12	158.30						
36	29.04	28.06	38.45	38.43	−0.02	13.35	Quiet	70 gm. glucose at 9.23 a.m.
19	28.38	29.92		38.50	+0.07		"	
91	28.11	26.49		38.60	+0.10		Movement	
	85.53	84.47						
83	28.98	24.72	38.56	38.60	+0.04	13.45	Quiet	70 gm. glucose at 9.35 a.m.
84	28.02	29.10		38.56	−0.04		"	
92	24.72	29.71		38.56	±0.00		"	
21	21.23	23.65					"	
78	19.97	23.01					"	
	122.92	130.19						
66	19.83	19.65	38.48	38.17	−0.31	13.35	Quiet	Basal.
82	18.99	23.21		38.19	+0.02		"	
38	22.55	20.94		38.29	+0.10		"	
	61.37	63.80						

Date.	Exper. No.	Time.	CO_2.	O_2.	R.Q.	H_2O.	Urine. N.		Non-protein. R. Q.	Calories	
										Protein.	Ca hy i f
1914		*a.m.*	*gm.*	*gm.*		*gm.*	*gm.*				
Nov. 27..	7	10.30–11.30	8.55	7.79	0.80	9.97	0.126		0.80	3.34	
		11.30–12.30	8.35	7.33	0.83	9.01	0.126		0.83	3.34	
		p.m.									
		12.30– 1.30	9.04	8.70	0.76	9.19	0.126		0.75	3.34	
		2.30– 3.30	12.21	9.86	0.90	10.90	0.126		0.91	3.34	
		3.30– 4.30	11.67	8.92	0.95	10.35	0.126		0.97	3.34	
		4.30– 5.30	12.02	9.33	0.94	9.75	0.126		0.95	3.34	
		a.m.									
Nov. 28..	8	9.45–10.45	7.78	6.18	0.92	6.89	0.117		0.94	3.10	
		10.45–11.45	7.94	6.62	0.87	7.26	0.117		0.89	3.10	
		11.45–12.45	7.79	6.02	0.94	6.38	0.117		0.97	3.10	
		p.m.									
Dec. 3..	9	1.00– 2.00	8.20	7.46	0.80	12.63	0.102		0.80	2.70	
		2.00– 3.00	8.20	7.51	0.79	11.20	0.102		0.79	2.70	
		3.00– 4.00	7.77	7.06	0.80	9.96	0.102		0.80	2.70	
1915											
Feb. 15..	12	12.24– 1.24	7.82	6.45	0.88	13.40	0.163		0.90	4.32	
		1.24– 2.24	7.48	6.29	0.87	11.81	0.163		0.88	4.32	
		2.24– 3.24	8.36	7.52	0.81	9.89	0.163		0.81	4.32	
		a.m.									
Feb. 16..	13	10.07–11.07	8.24	7.59	0.79	10.94	0.120		0.79	3.18	
		11.07–12.07	7.74	6.86	0.82	9.96	0.120		0.82	3.18	
		p.m.									
		12.07– 1.07	8.20	7.24	0.82	8.57	0.120		0.83	3.18	
		1.07– 2.07	8.13	7.28	0.81	8.29	0.120		0.81	3.18	
		2.07– 3.07	8.26	7.60	0.79	7.97	0.120		0.79	3.18	
Feb. 17..	14	1.00– 2.00	7.18	5.95	0.88	6.64	0.095		0.89	2.52	
		2.00– 3.00	7.13	6.47	0.80	6.98	0.095		0.80	2.52	
		3.00– 4.00	7.82	7.41	0.77	6.85	0.095		0.76	2.52	

nued.

Calories.			Body temperature.			Morning weight.	Behavior of dog.	Food.
on-ein.	In-direct.	Direct.	Start.	End.	Dif.			
						kg.		
.60	25.94	28.06	38.76	38.81	+0.05	13.50	Quiet	100 gm. fat emulsion at 9.20 a.m.
.25	24.59	22.61		38.67	−0.14		"	
.33	28.67	27.47		38.74	+0.07		"	
	79.20	78.14						
.43	33.77	31.51	39.01	39.03	+0.02		Quiet	70 gm. glucose at 1.40 p.m.
.57	30.91	32.40		39.03	±0.00		"	
.88	32.22	32.11		39.09	+0.06		"	
	96.90	96.02						
.06	21.16	21.27	38.62	38.41	−0.21	13.75	Slight movement	Basal.
.34	22.44	22.53		38.40	−0.01		Quiet	
.64	20.74	21.46		38.58	+0.18		"	
	64.34	65.26						
.18	24.88	24.99	38.82	38.79	−0.03	13.80	Quiet	100 gm. fat emulsion at 9.15 a.m.
.31	25.01	25.70		38.89	+0.10		Movement	
.84	23.54	22.30		38.89	±0.00		Quiet	
	73.43	72.99						
.49	21.81	22.29	39.59	38.57	−0.02	12.60	Quiet	Basal.
.85	21.17	21.81		38.57	±0.00		"	
.69	25.01	24.02		38.58	+0.01		"	
	67.99	68.12						
.04	25.22	25.01	38.75	38.77	+0.02	12.60	Quiet	100 gm. fat emulsion at 9.25 a.m.
.78	22.96	24.04		38.70	−0.07		"	
.08	24.26	22.43	38.72	38.83	+0.11		"	
.14	24.32	23.07		38.94	+0.11		"	
.08	25.26	25.31		39.03	+0.09		"	
	122.02	119.86						
.70	20.22	18.52	38.59	38.39	−0.20	12.65	Quiet	Basal.
.06	21.58	21.64		38.52	+0.13		"	
.01	24.53	23.10		38.67	+0.15		Movement	
	66.33	63.26						

TABLE

Date.	Exper. No.	Time.	CO_2	O_2.	R.Q.	H_2O.	Urine. Protein N.	Urine. Amino-acid N.	Non-protein. R.Q.	Calories Pro-tein.	Ca hy i f.
1915		*a.m.*	*gm.*	*gm.*		*gm.*	*gm.*	*gm.*			
Apr. 13..	15	10.41–11.41	8.24	7.16	0.84	10.31	0.127		0.84	3.37	
		11.41–12.41	8.11	7.14	0.83	9.13	0.127		0.83	3.37	
		p.m.									
		12.41– 1.41	7.34	6.11	0.87	8.34	0.127		0.89	3.37	
		a.m.									
Apr. 14..	16	10.08–11.08	12.48	8.96	1.01	9.95	0.105		1.04	2.78	0.
		11.08–12.08	12.16	8.63	1.03	9.88	0.105		1.05	2.78	0.
		p.m.									
		12.08– 1.08	10.94	8.17	0.97	9.16	0.105		1.00	2.78	
		1.08– 2.08	9.47	6.87	1.00	7.94	0.105		1.03	2.78	0.
		2.08– 3.08	9.09	6.81	0.97	7.40	0.105		1.00	2.78	
		a.m.									
Apr. 15..	17	10.43–11.43	8.05	6.10	0.96	8.85	0.106		0.99	2.81	
		11.43–12.43	8.41	7.27	0.84	8.53	0.106		0.85	2.81	
		p.m.									
		12.43– 1.43	8.30	6.65	0.91	7.60	0.106		0.93	2.81	
											Am ac
		a.m.									
Apr. 16..	18	10.46–11.46	10.20	8.44	0.88	10.43	0.106	(0.82)	0.82	2.81	9.
		11.46–12.46	9.98	8.05	0.90	9.74	0.106	(0.82)	0.86	2.81	9.
		p.m.									
		12.46– 1.46	9.32	7.91	0.86	8.86	0.106	(0.56)	0.81	2.81	6.
		1.46– 2.46	9.37	7.55	0.90	8.16	0.106	(0.41)	0.89	2.81	4.
		2.46– 3.46	8.61	6.84	0.92	7.80	0.106	(0.30)	0.92	2.81	3.
		3.46– 4.46	8.65	7.53	0.84	8.00	0.106	(0.11)	0.83	2.81	1.
											Car hyd in f
		a.m.									
Apr. 17..	19	10.00–11.00	12.00	8.64	1.01	10.79	0.150		1.05	3.98	0.
		11.00–12.00	11.20	7.67	1.06	9.97	0.150		1.11	3.98	0
		p.m.									
		12.00– 1.00	10.91	7.71	1.03	8.76	0.150		1.05	3.98	0.
		1.00– 2.00	8.38	6.66	0.92	7.96	0.150		0.94	3.98	

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	Calories.		Body temperature.			Morning weight.	Behavior of dog.	Food.
ein.	In-direct.	Direct.	Start.	End.	Dif.			
						kg.		
.69	24.06	23.81	37.81	37.67	−0.14	14.45	Quiet	Basal.
.55	23.92	24.14		37.71	+0.04		“	
.32	20.69	21.37		37.70	−0.01		“	
	68.67	69.32						
51	31.45	28.80	38.11	38.11	±0.00	16.40	Quiet	70 gm. glucose at 9.21 a.m.
35	30.35	28.35		38.00	−0.11		“	
69	28.47	29.31	37.83	37.98	+0.15		“	
13	24.01	23.69		38.07	+0.09		“	
90	23.68	22.66		38.19	+0.12		“	
	137.96	132.81						
31	21.12	21.90	37.83	37.69	−0.14	14.65	Quiet	Basal.
67	24.48	24.27		37.68	−0.01		“	
94	22.75	22.19		37.79	+0.11		“	
	68.35	68.36						
99	27.97	28.02	37.96	37.93	−0.03	14.65	Quiet	20 gm. glycocoll at 9.48 a.m.
81	26.79	27.36		37.94	+0.01		“	
75	25.82	24.67		37.96	+0.02		“	
07	25.46	23.85		38.21	+0.25		“	
01	23.17	22.38		38.11	−0.10		“	
17	25.01	22.92		37.96	−0.15		“	
	154.22	149.20						
04	30.21	26.93	37.95	37.95	±0.00	14.50	Quiet	70 gm. glucose at 9.02 a.m.
61	27.00	27.36		37.95	±0.00		“	
75	27.00	25.74	37.76	37.76	±0.00		“	
77	22.75	22.87		37.76	±0.00		“	
	106.96	102.90						

TABLE

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Date.	Exper. No.	Time.	CO_2.	O_2.	R.Q.	H_2O.	Urine. Protein N.	Urine. Amino-acid N.	Non-protein R.Q.	Calories. Protein.	Calories. Ami aci
1915		*a.m.*	*gm.*	*gm.*		*gm.*	*gm.*	*gm.*			
Apr. 19..	20	10.30–11.30	8.60	7.40	0.85	9.59	0.112		0.85	2.97	
		11.30–12.30	8.83	7.91	0.81	8.71	0.112		0.81	2.97	
		p.m.									
		12.30– 1.30	8.43	7.54	0.81	7.89	0.112		0.81	2.97	
		1.30– 2.30	8.89	7.89	0.82	7.69	0.112		0.82	2.97	
		2.30– 3.30	8.98	7.93	0.82	7.40	0.112		0.83	2.97	
		3.30– 4.30	8.70	8.16	0.78	7.10	0.112		0.77	2.97	
		4.30– 5.30	8.56	7.95	0.78	7.26	0.112		0.78	2.97	
		a.m.									
Apr. 20..	21	11.36–12.36	7.62	6.57	0.84	10.30	0.140		0.85	3.71	
		p.m.									
		12.36– 1.36	7.58	6.59	0.84	9.35	0.140		0.84	3.71	
		1.36– 2.36	7.41	6.51	0.83	8.19	0.140		0.83	3.71	
Apr. 21..	22	1.15– 2.15	9.54	8.09	0.86	9.18	0.095		0.86	2.52	
		2.15– 3.15	9.99	7.96	0.91	8.30	0.095		0.92	2.52	
		3.15– 4.15	11.97	9.23	0.94	8.86	0.095		0.96	2.52	
		4.15– 5.15	11.28	8.09	1.01	8.86	0.095		1.04	2.52	
		a.m.									
Apr. 22..	23	9.42–10.42	14.18	9.83	1.05	11.35	0.140	(0.82)	1.12	3.71	9.
		10.42–11.42	13.36	9.64	1.01	11.80	0.140	(0.82)	1.05	3.71	9.
		11.42–12.42	11.28	8.38	0.98	10.85	0.140	(0.56)	1.01	3.71	6.
		p.m.									
		12.42– 1.42	10.53	7.44	1.03	10.06	0.140	(0.41)	1.09	3.71	4.
		1.42– 2.42	9.77	7.16	0.99	8.52	0.140	(0.30)	1.04	3.71	3.
Apr. 23..	24	1.10– 2.10	14.87	10.24	1.06	14.53	0.140	(0.82)	1.13	3.71	9.
		2.10– 3.10	15.38	11.44	0.98	17.24	0.140	(0.82)	1.00	3.71	9.
		3.10– 4.10	11.45	9.37	0.89	15.33	0.140	(0.56)	0.87	3.71	6.
		4.10– 5.10	11.77	7.76	1.10	12.75	0.140	(0.41)	1.20	3.71	4.

Calories.			Body temperature.			Morning weight.	Behavior of dog.	Food.
Non-protein.	Indirect.	Direct.	Start.	End.	Dif.			
						kg.		
21.96	24.93	25.38	37.94	37.89	−0.05	14.85	Quiet	100 gm. fat emulsion at 8.30 a.m.
23.47	26.44	26.43		37.92	+0.03		Quiet	
22.23	25.20	26.13		38.09	+0.17		Quiet	
23.46	26.43	25.86		38.16	+0.07		Slight movement	
23.62	26.39	25.37		38.20	+0.04		Quiet	
24.06	27.03	24.30		38.23	+0.03		Quiet	
23.41	26.38	25.42		38.31	+0.08		Quiet	
	182.80	176.89						
18.35	22.06	24.25	37.82	37.82	±0.00	14.80	Quiet	Basal.
18.38	22.09	23.05		37.70	−0.12		"	
18.06	21.77	23.13		37.73	+0.03		"	
	65.92	70.43						
24.90	27.42	28.12	38.09	38.14	+0.05	14.85	Quiet	100 gm. fat emulsion at 8.15 a.m.
24.83	27.35	29.21		38.40	+0.26		"	
29.46	31.98	28.29		38.36	−0.04		"	70 gm. glucose at 12.16 p.m.
25.76	28.43	28.54		38.36	±0.00		"	
	115.18	114.16						
20.63	33.91	32.57	38.16	38.31	+0.15	15.15	Quiet	20 gm. glycocoll + 50 gm. glucose at 8.50 a.m.
19.96	33.01	32.55		38.36	+0.05		"	
18.69	28.68	28.64		38.21	−0.15		"	
17.17	25.71	27.38		38.10	−0.11		"	
17.49	24.65	25.64		38.12	+0.02		"	
	145.96	146.78						
22.08	35.41	32.26	38.18	38.29	+0.11	15.10	Quiet	100 gm. fat emulsion at 8.10 a.m.
26.30	39.18	41.18		38.64	+0.35		Panting 15 min.	20 gm. glycocoll + 50 gm. glucose at 12.10 a.m.
21.48	31.45	32.10		38.50	−0.14		Quiet	
18.30	27.16	29.44		38.39	−0.11		Quiet	
	133.20	134.98						

THE MINERAL EXCRETION OF THE MONKEY.[1]

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Preliminary to the study of the mineral excretion of the diseased monkey, it was desirable to obtain normal figures for the same animal. These figures may possibly supplement those tabulated by Hunter and Givens[2] for the nitrogen excretion of this animal. As they may be of service to future workers we have decided to place them on record.

A young, apparently healthy male of the genus *Macacus*, weighing 2.7 kilos, was placed in an Abderhalden metabolism cage. His daily ration which was quantitatively consumed consisted of 260 grams of a mixture of 1,000 grams of skinned bananas, 1,275 cc. of evaporated milk, and 1,275 cc. of distilled water. The milk in 260 cc. of this mixture contained 1.38 grams of nitrogen and furnished 313 calories.

The urine was collected in forty-eight hour periods under toluene and acetic acid. The cage was washed with distilled water and the washings were added to the urine after the determination of the ammonia.

The feces passed each day were gathered quantitatively and preserved in alcohol. At the end of the experiment the feces for the entire period together with the alcohol were placed in a tared desiccator and dried to constant weight *in vacuo* at a water bath temperature not exceeding 55°. The desiccator was connected with a distilling flask and this in turn with a suction flask containing sulphuric acid. The nitrogen content of the distillate and sulphuric acid was separately determined and added to that of the dried feces.

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[1] The experimental data are taken from the dissertation submitted by E. Oviatt as a partial requirement for the degree of Master of Science, State University of Iowa, Iowa City.

[2] Hunter, A., and Givens, M. H., *Jour. Biol. Chem.*, 1914, xvii, 55.

Analytical Methods.

Urine.—Nitrogen was determined according to Kjeldahl; ammonia according to Benedict and Osterberg's[3] modification of the Folin method; inorganic and total sulphates were determined according to Folin; total sulphur was determined according to Benedict; chlorine according to Clark's modification of Dehn's[4] method; total phosphorus according to the method of Neumann; potassium and sodium were determined according to a method described in Abderhalden's Handbuch der biochemischen Arbeitsmethoden;[5] calcium and magnesium according to McCrudden.[6]

Food and Feces.—Nitrogen, phosphorus, potassium, and sodium were determined as above. Total sulphur was determined according to the Denis[7] modification of the Benedict process after oxidation with fuming nitric acid. Chlorine was determined gravimetrically on the aqueous extract of the ash. The ashing was carried out in a platinum dish in the presence of pure sodium carbonate. Calcium and magnesium were determined according to McCrudden on the dry ash.

All the analyses were carried out in duplicate and rejected unless the variations were well within the experimental error. The average of the duplicates was selected as the basis for calculations. The analyses are charted in Tables I and II and these are self-explanatory.

DISCUSSION.

The animal maintained his weight throughout the experiment and retained 3.5 per cent of the ingested nitrogen. The average ammonia nitrogen forms 1.4 per cent of the total nitrogen excreted. This coincides with the average obtained by Hunter and Givens. The inorganic sulphate sulphur forms 60.9, the ethereal sulphate sulphur 16.9, and the unoxidized sulphur 22.2

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[3] Benedict, S. R., and Osterberg, E., *Biochem. Bull.*, 1913, iii, 41.

[4] Clark, S., in Hawk, P. B., Practical Physiological Chemistry, 3rd edition, Philadelphia, 1910, p. 388.

[5] Baumann, L., *Handb. d. biochem. Arbeitsmethoden*, 1912, vi, 291. An omission has occurred on page 292, line 16 from the bottom. A few drops of dilute hydrochloric acid should be added before volatilization of the ammonium salts.

[6] McCrudden, F. H., *Jour. Biol. Chem.*, 1909–10, vii, 83.

[7] Denis, W., *ibid.*, 1910–11, viii, 401.

per cent of the total urinary sulphur as compared with 87.8, 6.8, and 5.1 per cent respectively, for man.[8]

The chlorine was almost entirely excreted by the kidneys. 98 per cent of the phosphorus, approximately 80 per cent of the calcium and magnesium, and 25 per cent of the sodium and potassium was found in the feces.

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[8] Folin, O., *Am. Jour. Physiol.*, 1905, xiii, 62.

TABLE I.

Date.	Volume of urine.	Total Nitrogen.				Ammonia nitrogen of the urine.	Sulphur.						
		Intake.	Output.		Balance.		Intake.	Output.				Balance.
			Urine.	Feces.				Inorganic.	Ethereal.	"Neutral."	Feces.	
Jan., 1915.												
21 and 22	394	2.8144	2.1518	0.5575	0.1051+	0.0334	0.1708	0.0746	0.0212	0.0290	0.0570	0.0110−
23 and 24	477	2.8144	2.1575	0.5575	0.0994+	0.0321	0.1708	0.0764	0.0195	0.0297	0.0570	0.0118−
25 and 26	431	2.8144	2.1488	0.5575	0.1081+	0.0301	0.1708	0.0755	0.0208	0.0229	0.0570	0.0054−
27 and 28	426	2.8144	2.1750	0.5575	0.0819+	0.0262	0.1708	0.0744	0.0221	0.0279	0.0570	0.0106−
Daily average	**216**	**1.4072**	**1.0791**	**0.2787**	**0.0493+**	**0.0152**	**0.0854**	**0.0376**	**0.0104**	**0.0137**	**0.0285**	**0.0046−**

TABLE II.

Date.	Chlorine.				Phosphorus.				Sodium.			
	Intake.	Output.		Balance.	Intake.	Output.		Balance.	Intake.	Output.		Balance.
		Urine.	Feces.			Urine.	Feces.			Urine.	Feces.	
Jan., 1915.												
21 and 22	0.5919	0.5942	0.0031	0.0054−	0.4203	0.0087	0.4110	0.0006+	0.3214	0.2311	0.0903	0.0000
23 and 24	0.5919	0.5617	0.0031	0.0271+	0.4203	0.0084	0.4110	0.0009+	0.3214	0.2210	0.0903	0.0101+
25 and 26	0.5919	0.5098	0.0031	0.0791+	0.4203	0.0079	0.4110	0.0014+	0.3214	0.2259	0.0903	0.0052+
27 and 28	0.5919	0.5398	0.0031	0.0490+	0.4203	0.0078	0.4110	0.0015+	0.3214	0.2440	0.0903	0.0129−
Daily average	**0.2959**	**0.2757**	**0.0015**	**0.0187+**	**0.2101**	**0.0041**	**0.2055**	**0.0005+**	**0.1607**	**0.1152**	**0.0451**	**0.0004+**

Date.	Potassium.				Calcium.				Magnesium.			
	Intake.	Output.		Balance.	Intake.	Output.		Balance.	Intake.	Output.		Balance.
		Urine.	Feces.			Urine.	Feces.			Urine.	Feces.	
Jan., 1915												
21 and 22	1.4500	1.1070	0.4200	0.0770−	0.4983	0.0693	0.4295	0.0005−	0.1332	0.0281	0.1072	0.0021−
23 and 24	1.4500	1.0252	0.4200	0.0048+	0.4983	0.0854	0.4295	0.0166−	0.1332	0.0279	0.1072	0.0019−
25 and 26	1.4500	1.0317	0.4200	0.0017−	0.4983	0.0830	0.4295	0.0142−	0.1332	0.0278	0.1072	0.0018−
27 and 28	1.4500	1.0300	0.4200	0.0000	0.4983	0.0879	0.4295	0.0191−	0.1332	0.0274	0.1072	0.0014−
Daily average	**0.7250**	**0.5242**	**0.2100**	**0.0092−**	**0.2491**	**0.0407**	**0.2147**	**0.0063−**	**0.0666**	**0.0139**	**0.0536**	**0.0009−**

ON THE ORIGIN OF CREATINE.[1]

By L. BAUMANN and J. MARKER.

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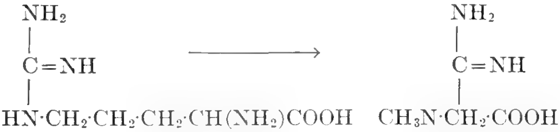
(Received for publication, June 12, 1915.)

The search for possible precursors of creatine has always led to a consideration of substances containing the guanidine radicle, as arginine and glycocyamine, or methylamino groups, as sarcosine, betaine, and choline.

Van Hoogenhuyze and Verploegh[2] found no increase in the elimination of creatinine, after the ingestion of proteins relatively rich in arginine. Jaffé[3] did not observe an increase in the urinary creatinine after the injection of arginine into rabbits. Thompson[4] found that the bulk (73 to 96 per cent) of the administered arginine nitrogen appears as urea in the urine. Inouye,[5] however, believes that an increase in creatine occurs when arginine is added to cat liver or is perfused through the organ.

The present investigation was concerned with possible creatine formation resulting from the action of voluntary muscle on arginine and methylureidoacetic acid. The conversion of arginine into creatine would probably involve a deamination, oxidation, and methylation of the base.



$$\begin{array}{lcl} NH_2 & & NH_2 \\ | & & | \\ C{=}NH & \xrightarrow{\qquad\qquad} & C{=}NH \\ | & & | \\ HN{\cdot}CH_2{\cdot}CH_2{\cdot}CH_2{\cdot}CH(NH_2)COOH & & CH_3N{\cdot}CH_2{\cdot}COOH \end{array}$$

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[1] The experimental data are taken from a dissertation submitted by J. Marker for the degree of Master of Science, State University of Iowa, Iowa City.

[2] Van Hoogenhuyze, C. J. C., and Verploegh, H., *Ztschr. f. physiol. Chem.*, 1905, xlvi, 415.

[3] Jaffé, M., *ibid.*, 1906, xlviii, 430.

[4] Thompson, W. H., *Jour. Physiol.*, 1905, xxxii, 137; 1905-06, xxxiii, 106.

[5] Inouye, K., *Ztschr. f. physiol. Chem.*, 1912, lxxxi, 71.

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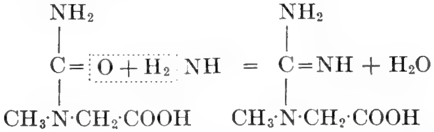
Methylureidoacetic acid has not been found in animal tissues, but recently Ackermann[6] demonstrated the presence of its anhydride *N*-methylhydantoin among the putrefaction products of creatinine. The transformation of methylureidoacetic into methylguanidinoacetic acid would be effected by substitution of the keto oxygen by the imino group. This might possibly occur *in vivo* by reaction of the oxygen atom with ammonia.



$$\begin{array}{c}\mathrm{NH_2}\\ |\\ \mathrm{C}=\boxed{\mathrm{O}+\mathrm{H_2}}\,\mathrm{NH}\\ |\\ \mathrm{CH_3{\cdot}N{\cdot}CH_2{\cdot}COOH}\end{array} = \begin{array}{c}\mathrm{NH_2}\\ |\\ \mathrm{C}=\mathrm{NH}+\mathrm{H_2O}\\ |\\ \mathrm{CH_3{\cdot}N{\cdot}CH_2{\cdot}COOH}\end{array}$$

The substances under investigation were placed in contact with hashed muscle and perfused through this tissue.

EXPERIMENTAL DATA AND RESULTS.

The arginine was obtained from edestin by the method of Kossel and Kutscher.[7] The pure copper nitrate salt was converted into the carbonate and this was used in our experiments.

The methylureidoacetic acid was prepared from sarcosine and urea[8] and employed as the sodium or ammonium salt.

Muscle creatine was determined according to Baumann,[9] and the creatine in the blood and perfusates by an adaptation of this method. 25 cc. of blood or perfusate are boiled for three hours under reflux with 62 cc. of 5 N sulphuric acid. The filtrate is diluted to 150 cc. 10 cc. are pipetted into a 50 cc. volumetric flask and exactly neutralized with 2.5 N sodium hydrate solution (litmus paper). About 7.9 cc. of alkali are required for this purpose. The solution is brought to the mark by the addition of 30 cc. of saturated picric acid and water. 25 cc. of the clear filtrate are employed for the creatinine determination. The standard contains 0.1 mg. of creatinine in 5 cc. of solution; this was diluted with 15 cc. of saturated picric acid and 5 cc. of water. 1.25 cc. of 2.5 normal alkali are added to both the standard and the unknown, and ten minutes allowed for the color to develop.

The water content of the muscle was determined by noting the loss after drying to constant weight *in vacuo* over concentrated sulphuric acid and phosphorus pentoxide at room temperature. The nitrogen was determined in 5 cc. of the muscle extract according to Kjeldahl.

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[6] Ackermann, D., *Ztschr. f. Biol.*, 1913, lxii, 208; 1913, lxiii, 78.

[7] Kossel, A., and Kutscher, F., *Ztschr. f. physiol. Chem.*, 1900–01, xxxi, 165.

[8] Baumann, L., *Jour. Biol. Chem.*, 1915, xxi, 563.

[9] Baumann, *ibid.*, 1914, xvii, 15.

Experiment A.—This experiment is representative of a series of similar ones. 25 gm. of hashed muscle, 25 cc. of defibrinated blood, and 10 cc. of toluene were placed in each of four flasks. Flask 3 contained in addition 1 gm. of arginine carbonate and Flask 4 1 gm. of methylureidoacetic acid neutralized with sodium hydroxide solution. Creatine determinations were made on the contents of Flask 1 immediately, and on the contents of Flasks 2, 3, and 4 at the end of approximately twenty-four hours at 37°. The muscle and blood were obtained from a dog just prior to the experiment. The absence of bacterial growth was demonstrated by smears and cultures.

Flask	1(Control).	2(Control).	3(Arginine).	4(Methylureidoacetic acid).
Creatine content	64 mg.	66 mg.	66 mg.	112-50=62 mg.

A previous trial showed that 1 gram of methylureidoacetic acid when subjected to the process employed for the determination of creatine in muscle produced a color equivalent to 50 mg. of creatine. Perfusion of the limb seemed to offer more nearly physiological conditions for studying the behavior of muscle tissue towards the substances in question, and an apparatus was constructed for this purpose. This apparatus is patterned after one which Brodie[10] has described.

The technique of perfusion was as follows: A dog was anesthetized and prepared for operation. The femoral vessels of one leg and the carotid artery were exposed, a cannula was inserted into the latter, and the dog was exsanguinated. The blood is defibrinated and filtered through cheesecloth. A measured quantity of the blood and a known volume of 0.9 per cent sodium chloride containing the substance to be perfused are well mixed. A tourniquet is placed about the limb above the point of exposure of the femoral vessels to prevent loss of fluid through the collateral vessels. The femoral vessels are then tied off and cannulas inserted. Ringer's solution is now perfused for a short time until the fluid returns fairly colorless; this removes traces of blood which might give rise to clots and so interfere with the subsequent circulation of the perfusate. The perfusate is then placed in the receiving bottle at once and the apparatus started. The volume of the perfusate in our experiments was usually 150 cc., but a smaller quantity may be employed. An incandescent lamp placed under the limb and a reflector above it help to supply the necessary warmth to the parts. The perfusate was supplied to the limb at a temperature of 37°. This was accomplished by immersing both the receiving and pressure

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[10] Brodie, T., *Jour. Physiol.*, 1903, xxix, 266.

bottles in a constant temperature bath. A liberal supply of oxygen was bubbled through the fluid, so that the blood to the limb was a bright red, whereas the "venous" blood was colored purplish. The pressure of the perfusate fluctuated between 80 and 140 mm. of mercury.

Experiment Q.—The right hind leg of a dog weighing 5 kilos was perfused for one and a half hours with a mixture of 100 cc. of his own defibrinated blood and 50 cc. of 0.9 per cent sodium chloride solution containing 2 gm. of methylureidoacetic acid neutralized with ammonia. The pressure of the perfusate fluctuated between 80 and 100 mm. 95 cc. of fluid were regained. The semi-membranosus, tibialis anticus, and gastrocnemius muscles were removed from both sides and hashed immediately. 50 gm. samples were analyzed for creatine, and from 1 to 2 gm. served for the water determination. Creatine was also determined in the blood and perfusate. Nitrogen was determined in the muscle extract.

	Creatine. *mg.*	Water. *per cent*	Extractive nitrogen. *gm.*
50 gm. of unperfused muscle	124	70.98	1.61
50 " " perfused "	108	74.88	1.498
1 gm. of dry unperfused muscle	8.54		0.1109
1 " " " perfused "	8.60		0.1192
100 cc. of defibrinated blood	9.7		
95 " " perfusate	32.6		

Experiment R.—A dog weighing 7 kilos was prepared as above for perfusion of the right hind limb. A mixture of 125 cc. of defibrinated blood and 25 cc. of 0.9 per cent sodium chloride solution containing 2 gm. of arginine carbonate was perfused for 1 hour and 50 minutes. 81 cc. of fluid were recovered. For analysis the hamstring, tibialis anticus, and gastrocnemius muscles were taken.

	Creatine. *mg.*	Water. *per cent*	Extractive nitrogen. *gm.*
50 gm. of unperfused muscle	116	64.26	1.274
50 " " perfused "	90	72.57	1.063
1 gm. of dry unperfused muscle	6.49		0.0713
1 " " " perfused "	6.56		0.0775
125 cc. of defibrinated blood	16.8		
81 " " perfusate	9.1		

Experiment S.—The right hind leg of a dog weighing 19 kilos was perfused for 1 hour and 15 minutes with a mixture of 150 cc. of his own defibrinated blood, 1.56 gm. of acid potassium phosphate, 2 gm. of arginine carbonate, and 30 cc. of 0.9 per cent sodium chloride solution. The pressure during the perfusion ranged from 120 to 140 mm. 200 cc. of fluid were recovered. The muscles of the entire leg from both sides were removed for analysis.

	Creatine. *mg.*	Water. *per cent*	Extractive nitrogen. *gm.*
50 gm. of unperfused muscle..............	129	75.36	1.500
50 " " perfused "	127	75.82	1.500
1 gm. of dry unperfused muscle.........	10.5		0.1217
1 " " " perfused "	10.5		0.1240
150 cc. of defibrinated blood.....	20.2		
200 " " perfusate......	24.4		

DISCUSSION AND CONCLUSIONS.

The increase in the creatine content of the perfusate in Experiment Q is probably due to the presence of *N*-methylhydantoin formed from the ureido acid by heating with sulphuric acid. None of the experiments show a decided increase in the creatine content of the perfused muscle or of the perfusate.

We conclude that neither arginine nor methylureidoacetic acid, under the experimental conditions outlined above, gives rise to an *appreciable increase* in muscle creatine.

In closing we wish to express our indebtedness to Prof. J. McClintock, for his advice and assistance.

THE USE OF ALUMINIUM HYDROXIDE CREAM FOR THE REMOVAL OF ALBUMIN IN NITROGEN PARTITION IN URINARY ANALYSIS.

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(Received for publication, April 22, 1915.)

For nitrogen partition in specimens of urine containing albumin it is necessary to remove this protein before proceeding with the analysis. The albumin interferes directly or indirectly with the application of several of the methods employed. It is usually removed by coagulation and filtration. This method is tedious because of the time element involved in restoring the original concentration of the urine. With relatively high percentages of albumin, the coagulation and quantitative removal of the same are readily carried out. With small percentages of albumin, this becomes difficult and it is sometimes impossible to get rid of a more or less marked opalescence due to some of the albumin which did not flocculate out of the solution.

Marshall and Welker[1] recently pointed out that aluminium hydroxide cream prepared under certain conditions has the power of quantitatively removing colloids from their solutions. If this insoluble material when added to urine would remove all the colloids, and only the colloids, it might prove a valuable reagent for the removal of the albumin.

This investigation was undertaken to study the effect of aluminium cream on normal and albuminous urine as far as the nitrogenous constituents were concerned.

The aluminium cream was prepared by adding a slight excess of a 1 per cent solution of ammonium hydroxide to a 1 per cent solution of ammonium alum, at room temperature. The precipitated cream was washed by decantation until the wash water

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[1] Marshall, J., and Welker, W. H., *Jour. Am. Chem. Soc.*, 1913, xxxv, 820.

showed only the faintest trace of residue on evaporation. It is important that relatively dilute solutions be used in making the aluminium cream. Stronger solutions give a precipitate which does not have the power to precipitate colloids to any marked degree.

A mixed specimen of normal urine was obtained. One liter of the same was diluted to two liters with distilled water and filtered. This is designated as the untreated specimen. Another liter of the normal urine was treated with a liter of aluminium cream, well mixed, and filtered. The following quantitative methods were used: the Kjeldahl method for total nitrogen, the Benedict method for urea, the Folin methods for ammonia and creatinine, the Folin-Shaffer method for uric acid, and the Welker modification of Salkowski-Arnstein methods for purine bases. Three pairs of filtrates were analyzed. The results of these analyses, expressed in grams per liter of diluted urine, are shown in Table I.

TABLE I.

	Specimen.	Total nitrogen.	Nitrogen of				
			Ammonia.	Urea.	Uric acid.	Purine base.	Creatinine.
		gm.	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*
I	Untreated	4.984	0.0202	4.024	0.063	0.052	0.256
I	Al-treated	4.974	0.0202	4.018	0.067	0.045	0.259
II	Untreated	4.704	0.168	3.772	0.068	0.035	0.256
II	Al-treated	4.704	0.168	3.722	0.066	0.037	0.255
III	Untreated	3.874	0.171	3.189	0.047	0.031	0.173
III	Al-treated	3.857	0.151	3.176	0.044	0.029	0.174

The results for total nitrogen show that none of the nitrogen of normal urine was removed by aluminium cream. The various methods were carried out for the different nitrogen fractions in spite of these findings, because it was desirable to know that no change had been effected in the urine that would interfere with the application of any of the different methods. We observed only one interference. The ammonium urate in the uric acid determination does not crystallize out as rapidly from the aluminium-treated urine as it does from the untreated urine. This is probably due to the lack of crystallization points in the former. The figures in the tables are in all cases the averages of two or more closely agreeing determinations. The absolute differences between the amounts of nitrogen in the treated and untreated specimens are within the limits of error of the methods employed.

In the case of albuminous urines, our procedure was a little different. 900 cc. of urine were mixed with 100 cc. of an albumin solution, and these with one liter of aluminium cream. The other sample was made up of 900 cc. of urine and 1,100 cc. of water. The filtrates from the albuminous urines were tested for albumin and the tests were negative. The results of these analyses, expressed in grams per liter of diluted urine, are shown in Table II.

TABLE II.

	Specimen.	Total nitrogen.	Nitrogen of				
			Ammonia.	Urea.	Uric acid.	Purine base.	Creatinine.
		gm.	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*
IV	Untreated	4.872	0.288	3.897	0.070	0.027	0.263
IV	0.5 per cent albumin. Al-treated	4.886	0.283	3.876	0.073	0.026	0.266
V	Untreated	3.078	0.188	2.500	*0.066		0.177
V	1 per cent albumin. Al-treated	3.078	0.165	2.535	*0.065		0.177

* These figures include uric acid and purine base nitrogen.

A study of the precipitating power of a number of different aluminium cream preparations on hand gave results which would indicate that an equal volume of cream will remove all the albumin from urines containing 1 per cent or less of the protein.

Samples from a number of different aluminium cream preparations, when evaporated to dryness on the steam bath and ignited to constant weight before the blast lamp, showed an aluminium oxide content varying between 0.45 and 0.6 per cent.

The results of our experiments show:

I. That aluminium cream does not remove any appreciable quantity of nitrogen from normal urine.

II. That aluminium cream can be used for the removal of small quantities of albumin from urine without any appreciable effect on the normal nitrogenous constituents.

ON THE FORMATION OF SPECIFIC PROTEOLYTIC FERMENTS IN RESPONSE TO THE PARENTERAL INTRODUCTION OF FOREIGN PROTEIN.

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(*From the Department of Physiological Chemistry, University of Pennsylvania, Philadelphia.*)

(Received for publication, June 9, 1915.)

The theory of Abderhalden, now particularly under discussion as the foundation for specific diagnosis of pregnancy and malignant neoplasm, predicates in general terms the formation of proteolytic enzymes in response to the parenteral introduction of foreign proteins. Under the terms of this hypothesis, it is immaterial if the protein be foreign in the total sense—derived from another species—or foreign in the narrower sense of being a protein of the same body but displaced in the body, as in the case of placental cells distributed through the general circulation. Most of the methods of testing the occurrence of the Abderhalden reaction are more or less minutely microchemical. By the use of prepared and purified foreign proteins it is possible to apply the test in a macrochemical manner. In this way it is believed that many of the difficulties of the microchemical investigations may be eliminated and the results placed upon a more interpretable and indubitable basis. The present paper contains the results obtained in the study of one protein; other investigations are to follow.

The protein employed in this study was the protamine of the Quinnat salmon of the Pacific Coast. This protein may be prepared in a state of high purity, without denaturation. We have used the sulphate of salmin. This protein when mixed with the blood serum of the animal used, the rabbit, forms a colloidal combination only in part soluble in the same serum. A portion of this bicolloidal combination flocculates as a coarse agglutination; a portion remains in solution. Apparently there is a station of equilibrium; the amount of the combination that separates out may be increased or decreased by dilution, addition of more protamine or of more of the serum proteins. On boiling, this combination is split, the coagulable proteins of the serum are coagulated at the appropriate reaction, while the protamine, apart from a small amount held by adsorption in the coagulated protein, remains in solution in the fluid. This precipitation of higher proteins by protamines, observed by the earliest students of protamines, is probably related to the toxicity of protamines. For the rabbit, this toxicity becomes marked if much more than 0.1 gram be injected per kilo of weight of animal.

The cleavage of protamine yields arginine, proline, serine, and valine, the arginine in the proportion of over 85 per cent. Under these circumstances, one would be able to confirm in a qualitative manner any noteworthy digestion of the protamine revealed by quantitative analysis. The normal trypsin of blood serum digests protamine, slowly but yet demonstrably, in a twenty-four hour test. If, therefore, an animal, following injections of protamine, should display in its circulation blood an added power of digestion of the protamine, this might be due to increase of the normal trypsin of the blood, of non-specific type, or to the formation of a specific protaminolytic ferment, or possibly to the removal of some antitryptic substance from the blood.

Two rabbits were given three injections of protamine, at intervals of ten days. Each rabbit received 100 mg. per kilo, dissolved in Ringer's solution. Such a solution is slightly acid, but it was injected in that state. The rabbits displayed no signs of intoxication or injury. One week after the last injection the rabbits were etherized, bled to death, and for control a normal rabbit was bled to death in a similar manner. The blood from each rabbit, amounting in each instance to between 50 and 55 cc., was kept from clotting by stirring, placed in flasks, protamine solution containing 0.65 gram of protamine added, then alkali enough (as previously determined) to make the final reaction about one ten-thousandth normal, covered with toluene and incubated for twenty-four hours at 38°. The content of each flask was then heated to the boiling point, the blood protein coagulated by appropriate addition of acetic acid, the coagulated protein removed by filtration, and the clear filtrate concentrated on the hot plate at 70° to about 40 cc. This small concentrate was then mixed with nine volumes of absolute alcohol, which precipitated the protamine and any traces of protein that may have remained, the amino-acids remaining in solution. Portions of this alcoholic filtrate were then subjected to the Kjeldahl test for nitrogen. Had the injections of the protamine provoked the formation of a specific protaminolytic trypsin, or augmented the amount of circulating trypsin of normal type, a larger digestion should have occurred in the bloods of the test animals than in the blood of the control, and the nitrogen contents would have yielded the direct evidence of this. This was, however, not the case; the nitrogen contents of the three filtrates were about the same. Calculated for the total mass of filtrate, the results were as follows:

Control.	Test Animal 1.	Test Animal 2.
0.048	0.039	0.042

That the blood of the control animal contained the highest value, was of course only a coincidence. The correct interpretation is that the nitrogen content in all was merely the expression of the usual content of non-protein nitrogen of blood, plus the amino-nitrogen set free by the usual digestion of protamine by the blood of the normal rabbit.

This negative result demonstrates merely that the rabbit does not form a protective ferment in response to the injection of the particular protein, protamine of the salmon. The hypothesis of Abderhalden has suffered from dogmatic generalization; the negative result should not be thus abused. This result does not prove that a protective ferment is not formed in response to the incorporation of placental protein. But it does indicate definitely that the dogmatic statement that the body responds with protective ferments to the injection of foreign proteins as a class reaction cannot be maintained.

ON THE ESTIMATION OF NON-PROTEIN NITROGEN AND GLUCOSE IN FINGER BLOOD.

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(From the Department of Physiological Chemistry, University of Pennsylvania, Philadelphia.)

(Received for publication, June 9, 1915.)

A modification of the Folin method for the estimation of non-protein nitrogen that would make it applicable to a small amount of blood drawn from the finger would serve a useful purpose in many experimental and clinical studies. It is sometimes not possible to secure permission to enter the vein for blood; and frequently it is not possible to draw blood from the vein as often as might be desired. The following modification of the Folin method yields approximate results with the use of a few drops of blood taken from the finger. It is based upon the suggestion several times advanced,[1] that the oxidized blood be Nesslerized directly, without driving over the ammonia.

The finger is cleaned and a strip of bandage or handkerchief tied above the elbow to increase the venous pressure in the hand. The finger is then pricked with a sharp lance, just as in the method of securing blood for counting the corpuscles. The blood should drop freely when the hand is held down. Four to eight drops are needed, depending upon the amount of non-protein nitrogen; in terms of weight, we use from 0.2 to 0.5 gram of blood. In a small Erlenmeyer flask are placed 10 cc. of absolute alcohol-ether in the proportions of 3:1. This is weighed just before being used. The dropping blood is caught in this flask, the stopper inserted, and the flask again weighed. We have tried many methods and substances for deproteinization of the blood, and have found this simple mixture of alcohol and ether to give the best results. Within a half hour, the mixture may be filtered into the digestion flask. The filtrate is clear; it is practically free of protein; although

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[1] Compare Gulick, A., *Jour. Biol. Chem.*, 1914, xviii, 541.

it probably contains traces of nitrogen-containing lipoids. The Kjeldahl digestion is carried out in a 25 cc. long necked, round bottomed Kjeldahl flask, the neck having a crook near the top. The mixture is filtered directly into the digestion flask, and the filter paper washed with 5 cc. of the alcohol-ether. Then a small piece of acid potassium sulphate is added, and several glass beads, and the alcohol-ether is driven off by heating on a hot plate. When the flask is practically dry, 1 cc. of strong sulphuric acid is added, and not over 300 or 400 mg. of potassium sulphate. Then the flask is placed over the direct flame of a small burner, the flask resting upon an asbestos board that has a perforation just a little smaller than the diameter of the circle of 1 cc. of sulphuric acid in the digestion flask. It is then heated until the contents are colorless. The digestion fluid is now transferred to a 100 cc. flask, the sulphuric acid neutralized by the addition of about 3 cc. of a 30 per cent sodium hydroxide solution, the flask filled to over three-quarters full and then 5 cc. of Winkler's modification of Nessler's fluid are poured in, and the flask is filled to the mark with water. The color of this unknown is compared in a Duboscq colorimeter with the color of a standard, containing in the 100 cc. flask 2 cc. of an ammonium sulphate solution, of which 2 cc. contain 0.0002 gram N.

A number of points require amplification. The flask is shaken after each drop of blood falls into the alcohol-ether. The filtrate must be entirely clear. Duplicates must always be done. Blanks must be done with each set of reagents, to demonstrate freedom from ammonia. The digestion is rather slow, on account of the absence of any catalyser. We have not been able to find any catalyser that does not later cloud the Nessler test or change the color. No more than the denominated amount of potassium sulphate can be used, on account of danger of clouding the Nessler test. The glass beads must be free of lead. The amount of alkali added should about equal the sulphuric acid remaining in the digestion flask, as alteration in alkalinity of the diluted fluid is apt to cloud the Nessler test. The final solution to be compared for color must be absolutely clear; if in the least cloudy, too high results will be secured. If the blood be high in nitrogen, over 0.075 per 100 cc. of blood, the digestion residue should be diluted in a 200 cc. flask. The water used in dilution must be free of ammonia. The flasks used for dilution should have their marks calibrated or certified flasks should be obtained. Occasionally, without apparent cause, the Nessler test clouds and cannot be used; usually one obtains perfectly clear solutions if an excess of ammonia is not present.

The solution of ammonium sulphate is measured with a 2 cc. Ostwald pipette. Recently Bock and Benedict[2] have criticized these pipettes, stating that with their use an error of as high as 1 per cent or more may be committed. This does not agree at all with the experience of one of us (T.) who has often employed these pipettes in physicochemical work that made their calibration and exact control necessary. The statements of Bock and Benedict do not agree with the current statements in the technical literature. For example, in the 1910 edition of the Ostwald-Luther Hand- und Hilfsbuch, on page 169 are given, as an illustration of the evenness of sampling with these pipettes, four successive weighings, whose mean variations are less than one-tenth of the degree of error claimed by Bock and Benedict.

Four samples of blood of normal men gave the following figures in grams of non-protein nitrogen per 100 cc. of blood: 0.023; 0.029; 0.026; 0.030. The blood of five normal women gave for 100 cc.: 0.027; 0.019; 0.024; 0.027; 0.023. These figures correspond with those obtained with the original Folin method. The following figures were obtained in analyses of blood of patients in the University Hospital, controlled by estimations of venous blood done according to the Folin method by Dr. L. Jonas.

Non-protein nitrogen.		Non-protein nitrogen.	
Method of Folin. *gm. per 100 cc.*	Method of authors. *gm. per 100 cc.*	Method of Folin. *gm. per 100 cc.*	Method of authors. *gm. per 100 cc.*
0.028	0.027	0.077	0.071
0.046	0.045	0.087	0.088
0.056	0.052	0.074	0.075
0.028	0.026	0.057	0.046
0.020	0.019	0.049	0.039
0.023	0.022	0.031	0.034

Apart from two estimations, the agreements are satisfactory. The agreements are most satisfactory when several duplicates are used, and as the above figures are the mean of two or three du-

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[2] Bock, J. C., and Benedict, S. R., *Jour. Biol. Chem.*, 1915, xx, 47.

plicates, the figures as stated are better than are the results of single estimations. The duplicates do not agree as closely as in the case of the original Folin method, being sometimes 3 to 5 mg. apart. Under these circumstances, the method yields only a good approximation, and when the figures are tabulated it would be best to round the figures into even decimals and fives of mg. in the 100 cc. of blood. The method is not adapted to measure small variations in the non-protein nitrogen under experimental conditions. But it is adapted to follow the ups and downs of a case of nephritis or an experiment from day to day, since the figures expressed in round numbers will still fulfill all clinical and many experimental desiderata. The highest figure we have noted with the use of this method was 0.151, in a case of poisoning with corrosive sublimate.

The Bang method for the microchemical estimation of glucose in the blood drawn from the finger is being widely used, and will yield satisfactory results if carefully employed. It is also capable of yielding aberrant results if not carried out in an invariable and rigid manner. During the past two years many estimations of glucose have been made in this laboratory with this method, and a great many control tests have been done, in order that the limitations and requirements of the method might be fixed. The results are presented in a summarized form, familiarity with the technique of the original method of Bang being presupposed.

(a) Whenever a fresh set of papers or reagents is taken in hand, the method is to be checked up on known solutions of pure *d*-glucose, controlled by gravimetric and polariscopic estimations. The sugar solution is to be dropped on the filter papers, and the latter carried through all the steps of the method. Such tests indicate that when applied to a sugar solution corresponding to 0.100 gram of glucose in 100 cc. of blood, the original Bang method yields a deficit—from 10 to 30 per cent low figures being obtained. With increasing amounts of sugar the loss becomes less until there is little loss with a solution of a strength corresponding to 0.200 per 100 cc. of blood. Such check tests should be done from time to time on stock solutions.

(b) A blank check must be done on the reagents each day that an estimation is done. Since the figure for the blank for the reagents is to be subtracted, it is important to know how the blanks are running, since they are subject to considerable variation. In a controlled series of twenty-three estimations, the following blanks were obtained, in terms of cc. of hundredth normal iodine solution:

Five times	0.05
Four times	0.07
Twice	0.08
Once	0.09
Twice	0.10
Twice	0.12

With such variations in the blank (and these we have frequently found), it will not do to make an average of a series of blanks and use the same average subtraction in each estimation. A blank should be done each day that the method is used.

(c) Glucose is not the only reducing substance in the blood. Under normal conditions, the non-glucose reducing substances in the blood probably do not amount to more than the equal of 0.05 cc. of the iodine solution. It is optional whether one adds to the blank a fixed sum to allow for the uric acid and creatinine (say 0.08 cc.) or disregards it; in the one case the figures will be slightly but uniformly higher, in the other lower. In pathological instances of retention of excessive amounts of uric acid and creatinine, such retention might occasion a large error and lead to the suggestion of a hyperglycemia that did not actually exist.

(d) The blood must flow freely from the finger and must spread smoothly on the papers. The amount of blood must be fairly constant, not under 0.10 and not over 0.15 gram for each paper. Slight pressure about the elbow gives free dropping of blood from the finger without influence on the sugar content.

(e) The proteins must be completely removed, since they influence the processes of reduction and also obscure the end-point in titration. We have found the heating of the blood-impregnated papers in the hot-air oven at 100°, as recommended by Gardner and McLean,[3] to be the only certain and satisfactory method of coagulation of the proteins. If the corks are inverted, three to five minutes' heating is sufficient; if the corks are allowed to remain in the flasks, fifteen minutes are necessary.

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[3] Gardner, A. D., and McLean, H., *Biochem. Jour.*, 1914, viii, 391.

After proper heating, the protein is completely coagulated and it is never necessary to filter the KCl extract.

(f) The time of boiling. We have not been able, in the tests where the blood is heated in the hot-air oven, to titrate the full amount of a known solution of sugar with less than four minutes of boiling to secure complete reduction. The stream of CO_2 should be vigorous throughout.

(g) The error in titration is considerable, even with the best reagents and most carefully prepared solutions. One has but to make a series of estimations of one known sugar solution to realize this. We use certified burettes, 1 cc. divided into hundredths. The smallest drop of the titration solution is about 0.02 cc. This is the smallest plus or minus error possible. One might use two-hundredth normal iodine solution, but one loses in distinctness of end-point in titration as much as one gains in volume measurement in the burette.

(h) The iodine solution must be fresh each day and must be checked each day.

(i) The larger the number of duplicates, the better the results. Two are the minimum, and these cannot be trusted unless they agree quite closely. Often one paper will give an aberrant result and this must be rejected. Therefore it is safest to have at least three samples, and if one is wide and the other two check, the mean of the two that agree should be the figure given, not the mean of the three. The insistence, in the literature, upon numerous duplicates indicates how frequent are variant results.

When all these possible and actual variations are checked up, as is the fashion in physicochemical investigations, and the results expressed in a plus or minus error, it is clear that this cannot, under the best of conditions, be less than 0.005 glucose in the 100 cc. of blood. In other words, if one obtains in a given analysis the figure of 0.085 glucose in the 100 cc. of blood, one could not be certain that it was not either 0.080 or 0.090. Under these circumstances, it would be best to use only the round figure and not split the tens at all. Instead of expressing glucose concentration in terms of percentage or in the ten thousand, it would be much better, in analogy with the figures for the non-protein nitrogen, to express the figures in grams per 100 cc. of blood; and the figures are indeed of the same general order of magnitude

The variations in the glucose content of the blood are in two directions in normal individuals; variations between persons and variations at different times in the same individual. The extensive data in the literature and our own experience lead us to conclude that in different normal individuals the blood sugar may vary from 0.050 to 0.150 in the 100 cc. of blood. Not less marked are the variations in the same individual, and this independent of variations in diet. The following figures were determined on different days, for a normal man, in blood drawn at 3.30, after luncheon taken at 12.30, the luncheon consisting each day of the same food, a constant weight of cinnamon roll with a quart of buttermilk. The figures are given in grams of glucose to 100 cc. of blood. 0.090; 0.110; 0.070; 0.110; 0.060; 0.050; 0.040; 0.070; 0.070; 0.100; 0.060; 0.110; 0.060; 0.050; 0.040; 0.060; 0.060; 0.040. Obviously, it would not be proper to take the mean of these many estimations, which happens to be 0.070, and state that the normal sugar content of this blood was 0.070 in the 100 cc. All the figures are normal for this individual. Such variations are very common. Under these circumstances, it is entirely improper to fix the figures for the blood sugar as rigidly as is being done in the literature. We may later learn that certain types of individuals and certain phases of metabolism incline to the figure of 0.050 or to the upper figure. We have learned how fluctuating, within a short time, are the figures for non-protein nitrogen, depending upon conditions in the intake and elimination of nitrogenous substances; that similar variations may exist in the carbohydrate metabolism cannot be reasonably doubted. It is an error to fix narrow limits for the sugar content of the blood, particularly when a method so readily affected as that of Bang is used to secure the data. This is particularly true for the data of normal states; the figures for the hyperglycemias of diabetes are much more certain, as the method yields more accurate results the higher the concentration of glucose.

A STUDY OF THE PROTEIN METABOLISM UNDER CONDITIONS OF REPEATED HEMORRHAGE.

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Two older theories of the protein metabolism have within recent years been clearly defined and placed in juxtaposition largely through the labors of Folin and Abderhalden. According to the hypothesis formulated by Abderhalden, the amino-acids, products of digestion of protein in the alimentary tract, are in the epithelium of the intestine converted into the proteins of the blood serum, stamped with the biological stamp of the species, and sent into the circulation to serve as the substrate for cellular anabolism. The utilization of the circulating proteins (corresponding to the "totes Eiweiss" of Pflüger) by the individual cells of the various tissues must of necessity be preceded by a digestion within the cells, and out of the resultant amino-acids the particular cell then constructs its individual proteins, leaving the superfluous amino-acids to be burned or cast out. The protein-forming function of the intestinal epithelium is thus regarded as a synthetic function of the highest grade. The synthesis of the proteins of the blood serum cannot, however, be regarded as vested exclusively in the intestinal epithelium; since these proteins are maintained in striking constancy during prolonged starvation, they must also be formed in the tissues of the body. The second hypothesis, to which the recent investigations of Folin and Denis[1] and of Van Slyke[2] have contributed much, postulates the passage of the amino-acids, products of digestion of protein, through the resorption wall into the venous circulation, to be carried to the

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[1] Folin, O., and Denis, W., *Jour. Biol. Chem.*, 1912, xi, 87, 161; 1912, xii, 141, 253. Folin, O., and Lyman, H., *ibid.*, 1912, xii, 259.

[2] Van Slyke, D. D., and Meyer, G. M., *ibid.*, 1913–14, xvi, 197, 213, 231.

tissues and there stored[3] (in a state not yet clearly defined) as substrates for the processes of anabolism. In this hypothesis although the meaning of the proteins of the blood serum is not defined, their place of origin would be located in the tissues. Into this hypothesis, the recent work of Lusk[4] on the stimulation of cellular reactions by amino-acids fits very well. In the nature of its formulation, the hypothesis of Abderhalden is scarcely capable of experimental demonstration; the hypothesis of Folin, however, lends itself to such demonstration.

We have attempted to secure data that might bear upon this general problem by a study of the blood following extreme depletion by repeated hemorrhage. Our plan of investigation has been as follows: Dogs were kept without food for thirty-six to forty hours. At the beginning of the experiment the animal was anesthetized with ether, and complete anesthesia was maintained until the end of the experiment or, in the case of Dog 2, until the animal died. A large volume of blood was then withdrawn from the femoral vein, and the volume of circulating fluid at once restored by the introduction of an identical volume of Ringer's solution, whose freezing point corresponded to that of an 0.85 per cent solution of sodium chloride, thus being isotonic to dog blood. After an hour another hemorrhage was done and the same volume of Ringer's solution introduced. Four or five such withdrawals of blood were made, the total volume of the circulating fluid being kept constant by injections of Ringer's solution. In the blood withdrawn analyses of the protein content, the sugar content, and the partition of nitrogen were made, as detailed in Table I. The effect upon the blood from the standpoint of the original composition may be seen from the fact that within four hours so much blood may be drawn as to reduce the volume of the corpuscles from over 50 per cent to less than 20 per cent. The constituents of the blood might be restored in one or both of two ways; *viz.*, a washing out of material already present in the tissues, and actual chemical formation within the tissues. It was foreseen that difficulties would inevitably arise in any attempt to define the source of any observed restoration or altera-

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[3] Compare also Wishart, M. B., *Jour. Biol. Chem.*, 1915, xx, 535.

[4] Lusk, G., *ibid.*, 1915, xx, 555.

tion, unless the data were very decisive. The data are contained in Table I. All results are expressed as grams per 100 cc. of serum. Total nitrogen was estimated by the Kjeldahl method; sugar, by the method of Vosburgh and Richards;[5] serum albumin and globulin were estimated gravimetrically after coagulation of the proteins following separation with magnesium sulphate; amino-nitrogen and urea by the methods of Van Slyke; non-protein nitrogen was estimated by the method of Folin applied macro-chemically.

TABLE I.

Animal No.	Venesection and injection.	Total N.	Total protein.	Serum globulin.	Serum albumin.	Non-protein N.	Amino N.	Urea N.	Sugar.
	cc.								
1 Weight 26 kg.	400	1.03	6.2	2.2	4.1	0.017			0.185
	400	0.93	5.5	1.1	4.3	0.018			0.165
	400	0.85	4.8	0.95	3.9	0.019			0.170
	350	0.69	3.9	1.2	2.7	0.025			0.220
	*300	0.48	2.7	1.1	1.6	0.027			0.310
2 Weight 41 kg.	560	1.16	7.05	2.9	4.2	0.019	0.0023		0.170
	550	1.01	6.7	2.6	4.1	0.018	0.0023		0.190
	550	0.94	5.7	2.4	3.3	0.021	0.0020		0.155
	550	0.91	5.4	2.2	3.2	0.028	0.0044		0.091
3 Weight 12.6 kg.	250	1.03	6.1	2.7	3.4	0.020		0.007	0.225
	300	0.99	5.3	2.1	3.2	0.024		0.009	0.250
	250	0.73	4.4	1.6	2.9	0.023		0.011	0.228
	250	0.61	3.7	1.4	2.3	0.028		0.011	0.245
	*200	0.48	3.3	0.9	2.4	0.041		0.014	0.265

*Dog bled to death. Corpuscles less than 20 per cent of blood.

Inspection of the data indicates the following findings. (a) There is a progressive decrease in the total nitrogen and total protein, most marked in Dogs 1 and 3, in which the venesections were relatively more severe. It is assumed that no notable number of red corpuscles would be added to the circulating blood in the time concerned. The reduction in the proteins of the serum is less than that required by the dilution of the blood as a

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[5] Vosburgh, C. H., and Richards, A. N., *Am. Jour. Physiol.*, 1903, ix, 35.

result of the substitution of Ringer's solution for whole blood, calculated on the assumption that the blood constitutes one-twelfth the body weight. This indicates that there has been a washing out of these proteins into the circulating blood, and this was, of course, to have been expected.

(b) The reduction in serum albumin and globulin is not the same, the concentration of serum albumin being disproportionately reduced in Dog 1, and of the serum globulin in Dogs 2 and 3. Absolute mass, however, is to be inquired into as well as concentration, and for this purpose one must compare the amounts in the serum of the total blood as calculated from the proportion of red cells and the assumed total amount of blood. This is of course a very rough calculation, but suggestive nevertheless. Table II presents the amounts of the two blood proteins thus calculated for the first and last venesections, for the assumed total mass of blood, in grams.

TABLE II.

	Globulin.	Albumin.
Dog 1		
First venesection	23	41
Last venesection	18	28
Dog 2		
First venesection	47	68
Last venesection	58	84
Dog 3		
First venesection	13	17
Last venesection	8	19

The figures are obviously not uniform, either in direction or amount of variation. In the second dog, the total serum albumin in the circulation at the close of the experiment was greater than at the beginning; in the other two dogs, there was no change or but little loss. In the second dog, there was actual gain in circulating globulin; in the first dog, little change; and in the third dog, decided loss. When one considers these figures and includes with them the known amounts of albumin and globulin removed in the venesections, one secures the impression that the reductions and changes can be explained simply as due to washing out of the tissues and lymph spaces. Certainly there is in these figures no warrant for the assumption of any synthesis of these proteins on the part of the tissues, nor indeed was any noteworthy synthesis to have been expected within the time of the experiments.

(c) The non-protein nitrogen rises progressively with each venesection, expressed in concentration, and, of course, more markedly when expressed in absolute terms. Table III expresses the figures for non-protein nitrogen, calculated for the first and last hemorrhages, on the stated assumption of the total mass of blood in the body in grams.

TABLE III.

	Non-protein nitrogen.
Dog 1	
First venesection	0.18
Last venesection	0.44
Dog 2	
First venesection	0.32
Last venesection	0.76
Dog 3	
First venesection	0.10
Last venesection	0.32

These are very large increases and when one adds to these figures the sums of non-protein nitrogen known to have been removed in the venesections, it does not seem possible to regard the results as due to any mere washing out of the tissues. Part of this nitrogen is, of course, urea nitrogen, and this also was increased as shown in the analyses of Dog 3. That the amino-nitrogen is also increased is indicated by the figures for Dog 2. It does not seem possible to resist the conclusion that the increase of non-protein nitrogen has been the result of an active process on the part of the tissues, due either to a setting free of stored amino-acids or to amino-acids derived from hydrolysis of tissue (cellular) protein or serum protein. To decide between these is mere conjecture, but the derivation from the liberation of stored amino-acids certainly seems the more likely, since we must assume that the greater part of the non-protein nitrogen has been derived from and represents amino-nitrogen.

The figures for blood sugar indicate that the commonly stated occurrence of hyperglycemia in the state of ether anesthesia is not invariable.

ON THE PREDOMINANCE OF THE LIVER IN THE FORMATION OF UREA.

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A review of the older literature relating to the question of the origin of urea in the animal body indicates that the commonly advanced view that urea was formed largely, or indeed entirely, in the liver was founded upon little direct experimentation, but upon indirect experimentation and more or less circuitous reasoning. Even the fact of the continuance of urea elimination by the frog following extirpation of the liver was interpreted as an adaptation rather than as an illustration of normal function. In the modern conception of the catabolism of protein in the body, the hydrolysis of the protein is held to occur within the cells. The resulting amino-acids are then subjected to deamination, the ammonia is combined to form carbonate or carbamate, and these are finally converted into urea. These reactions, under the older idea, were regarded as completed in the liver. Whether the entire series of reactions occurred in the liver, following the transportation there of the amino-acids, or whether only the final stage of conversion of ammonium salt into urea occurred in the liver, was a secondary question. In the Abderhalden hypothesis of synthesis of blood proteins from the amino-acids, products of the digestion of protein, the superfluous amino-acids were held to be deaminized in the liver and the ammonia was converted into urea. That amino-acids, however, may easily pass through the liver unscathed, is known.[1]

A direct test of the urea-forming function of the liver is possible. It is necessary to remove the alimentary tract, in order to exclude nitrogenous substances being resorbed from the tract.

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[1] Fiske, C. H., and Karsner, H. T., *Jour. Biol. Chem.*, 1913–14, xvi, 399.

Prolonged fasting cannot accomplish the same result, since bacterial formation of ammonia continues in the colon during fasting, the bacteria subsisting upon the nitrogenous secretions of the fasting alimentary tract. Following the complete removal of the alimentary tract, blood reaches the liver only through the hepatic artery. The liver is thus placed under the exact conditions of nutrition and metabolism that obtain in the other tissues. Analyses of the blood coming from the hepatic vein and from the inferior cava below the entrance of the renal veins ought to yield information bearing upon the urea-forming functions of the liver and the peripheral tissues. We have made this experiment four times. The removal of the whole alimentary tract is an entirely feasible operation,[2] with or without the retention of the spleen and pancreas. The operations were performed for us by Dr. Max M. Peet.

Under complete ether anesthesia, the tract was rapidly removed, every precaution being taken to guard against loss of blood, which was of course to a considerable degree inevitable. In no case did the time required for the operation exceed fifteen minutes. In the first two dogs, the spleen and pancreas were allowed to remain in the body. In the last two dogs these organs were removed with the alimentary tract. In Dog 4 a clamp was placed upon the hepatic veins at their junction with the cava. This was done as a control of the results of the first three tests. The hepatic veins of the dog are short. The blood was drawn by inserting a needle, directed towards the liver, and then drawing the blood into the syringe by slow and gentle suction. Nevertheless, it seemed possible that in thus drawing the blood from the hepatic vein, blood might also be drawn back from the cava, and thus vitiate the results of the experiment. In Dog 4, therefore, the veins were clamped, so that the blood drawn could have come only from the liver. In each instance, one-half hour elapsed after the removal of the alimentary tract before the samples of blood were drawn for analysis. In Experiment III second samples were taken after another half hour. The blood was drawn into potassium oxalate solution, and analyzed for urea according to the urease method

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[2] Taylor, A. E., *Jour. Biol. Chem.*, xv, 217.

of Van Slyke and Cullen, and for non-protein nitrogen according to the method of Folin, applied macrochemically. The figures for urea include the ammonia, which does not, however, alter the meaning of the findings. The results of the analyses are given in the following table.

	Non-protein N. Hepatic blood.	Urea N. Hepatic blood.	Non-protein N. Peripheral blood.	Urea N. Peripheral blood.
Dog 1....	0.022	0.0084	0.020	0.0061
Dog 2....	0.031	0.0226	0.029	0.0189
Dog 3....	0.030	0.0146	0.030	0.0129
	0.030	0.0114	0.027	0.0097
Dog 4....	0.018	0.0069	0.017	0.0065

All figures represent gm. in 100 cc. of blood.

The figures reveal a slightly higher concentration of non-protein nitrogen and urea (plus ammonia) in the blood coming from the liver than in the blood coming from the hind legs. Expressed as figures for the totals of the four dogs, the average for non-protein nitrogen was 0.026 for hepatic blood, 0.024 for peripheral blood; the average for urea N was 0.0128 for hepatic blood, 0.0108 for peripheral blood. These figures, while always positive in the one direction, are, however, subject to another consideration in interpretation. Urea is not the chief nitrogenous product of the muscular system, which is creatinine. It might therefore well be assumed that the intensity of metabolism in the unit of time might not be so high for urea formation in the muscle as in the liver, where urea represents apparently the chief nitrogenous catabolic end-product. Even disregarding this consideration, however, the slight excess of urea N present in hepatic blood over the urea N of peripheral blood certainly does not speak for any special predominance of the liver in the total function of formation of urea. The figures, instead, speak for the view that the formation of urea is the function of all tissues.

A possible objection to this experiment might lie in the question whether the removal of the portal circulation could disturb the nutrition and function of the liver. We do not regard this objection as well founded. The supply of oxygen comes to the liver through the hepatic artery. Certainly, the arterial circulation must be ample to care for the nutritional needs of the liver, considered as an organ alone, and especially during the short time concerned in these experiments. Experience with the Eck fistula does not yield any foundation for the idea that the nutrition of the liver is dependent upon the portal circulation.

EXPERIMENTAL STUDIES OF URINARY AND BLOOD NITROGEN CURVES AFTER FEEDING.

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The following studies were designed to show the hourly variations in the normal dog of the total non-protein nitrogen of the blood compared with the output of urine and of the nitrogen in the urine, and the effect upon these of various diets and of variations in the water intake.

The purpose was to establish normal curves under fixed conditions, which it is hoped will prove useful in a further study of the curves in the various forms of experimental nephritis and after the administration of diuretics.

Nine bitches have been placed on a diet consisting of meat, lard, sugar, sodium chloride, bone ash, and water, adequate in calories and containing about 0.4 gram of nitrogen per kilo of body weight. On the sixth day the ration was varied to suit the study, being richer in nitrogen in Dogs 2 to 6 while in Dogs 7, 8, and 9 no food was given on the days of the study. The water intake was varied as shown in the protocols. On the day of the study, the animals were catheterized at intervals and at the time of each catheterization 5 or 10 cc. of blood were taken from the jugular vein for estimation of the non-protein nitrogen by Folin's method. The urinary nitrogen was estimated by Kjeldahl. The protocols follow:

Moderate Nitrogenous Intake.—Dog 1. Female, weight 10,870. Placed for six days on a diet of beef heart 150 gm. (0.39 gm. N per kilo), lard 50 gm., sucrose 50 gm., bone ash 15 gm. sodium chloride 2 gm., water 200 cc. given daily at 8 a.m. On the sixth day there were obtained the urine by catheter and 5 cc. of blood from the jugular vein at 8 and 10 a.m., noon, 2, 4, 6, and 10 p.m., and 8 a.m. the next day. The results are shown in Chart I.

High Nitrogenous Intake.—Dog 2. Female, weight 6,200. Placed for five days on a diet of beef heart 90 gm. (0.4 gm. N per kilo), lard 30 gm., sucrose 30 gm., bone ash 10 gm., sodium chloride 2 gm., water 200 cc. given daily at 8 a.m. On the sixth day, given at 8 a.m. beef heart 230 gm. (1 gm. N per kilo), lard 12 gm., sucrose 12 gm., bone ash 4 gm., sodium chloride 1 gm., water 80 cc.; on this day there were obtained the urine by catheter and 5 cc. of blood from the jugular vein at 8 and 10 a.m., noon, 2, 4, 6, and 10 p.m., and 8 a.m. the next day. Results are shown in Chart I.

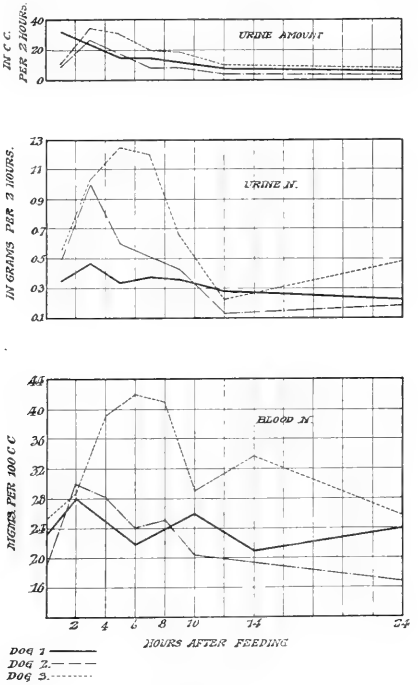

CHART I.

Very High Nitrogenous Intake.—Dog 3. Female, weight 7,600. Placed for five days on a diet of beef heart 110 gm. (0.4 gm. N per kilo), lard 35 gm., sucrose 35 gm., bone ash 20 gm., sodium chloride 2 gm., water 200 cc. given at 8 a.m. daily. On the sixth day, given at 8 a.m. the same diet except that of the beef heart 610 gm. (2.5 gm. N per kilo) were fed. Urine and blood were obtained on this day as in Dogs 1 and 2. Results are shown in Chart I.

Very High Nitrogenous Intake. Water throughout the Day.—Dog 4. Female, weight 12,150. Placed for five days on a diet of beef heart 165 gm. (0.4 gm. N per kilo), lard 55 gm., sucrose 55 gm., bone ash 20 gm., sodium chloride 2 gm., water 300 cc. given daily at 8 a.m. On the sixth day, given at 8 a.m. the same diet except that of the beef heart 1000 gm. (2.5 gm. N per kilo) were fed and of water only 40 cc. On this day there were obtained the urine by catheter and 5 cc. of blood from the jugular vein at 8 and 10 a.m., noon, 2, 4, 6, and 10 p.m., and 8 a.m. the next day. At these same periods 40 cc. of water were administered, mixed with the food at 8 a.m., given by stomach tube at the other intervals. Results of the urine and blood analyses are shown in Chart II.

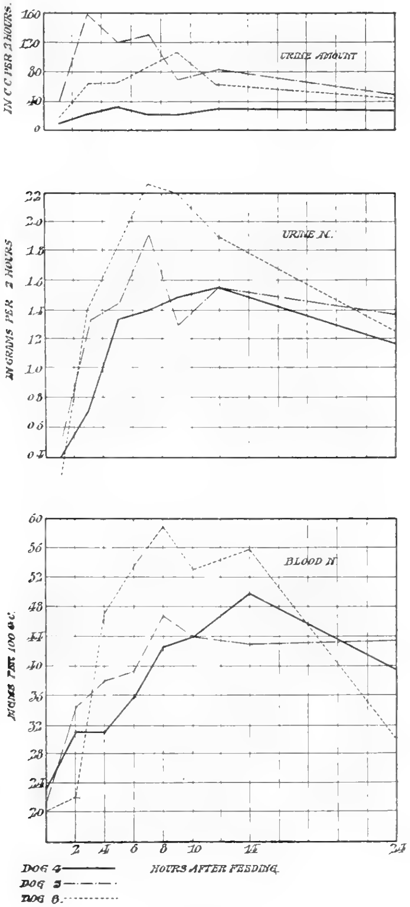

Chart II.

Very High Nitrogenous Intake. Water with Feeding and throughout the Day.—Dog 5. Female, weight 12,200. Placed for five days on same diet as Dog 4. On the sixth day, given at 8 a.m. the same diet except that of the beef heart 1000 gm. (2.5 gm. N per kilo) were fed and of water 300 cc. On this day urine and blood were obtained as in Dog 4 and 40 cc. of water were administered by stomach tube at each period as in Dog 4. Results are shown in Chart II.

Very High Nitrogenous Diet. Water Once Eight Hours after Feeding.—Dog 6. Female, weight 10,450. Placed for five days on a diet of beef heart 140 gm. (0.4 gm. N per kilo), lard 45 gm., sucrose 45 gm., bone ash 20 gm., sodium chloride 2 gm., water 300 cc. given daily at 8 a.m. On the sixth day, given at 8 a.m. the same diet except that of beef heart 900 gm. (2.5 gm. N per kilo) were fed and no water was added to the food. At 4 p.m., 300 cc. of water were given by stomach tube. Urine and blood were obtained as in Dogs 4 and 5. Results are shown in Chart II.

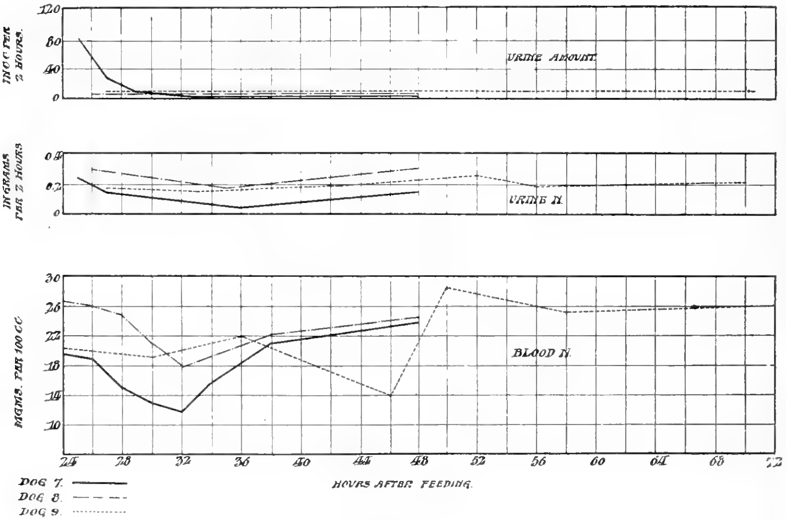

CHART III.

Fasting. Water at Beginning of Day.—Dog 7. Female, weight 7,360. Placed for five days on same diet as Dog 3. On the sixth day, given no food, but at 8 a.m., 200 cc. of water by stomach tube. Urine and blood obtained as in Dog 1. Results are shown in Chart III.

Fasting. No Water.—Dog 8. Female, weight 12,260. Placed for five days on same diet as Dog 4. On the sixth day, given no food or water; on this day there were obtained the urine by catheter and 5 or 10 cc. of blood from the jugular vein at 8 a.m., noon, 4, and 10 p.m., and 8 a.m. the next day. The results are shown in Chart III.

Fasting. Small Amounts of Water as Desired.—Dog 9. Same animal as in previous experiment. Allowed to feed freely at 10 a.m. for three days after previous experiment; then again fasted for two days, water being permitted freely (50 cc. taken each day). On these two fasting days there were obtained urine by catheter and 5 cc. of blood from the jugular vein at 10 a.m., 4 and 10 p.m., 8 a.m., noon, 4, and 8 p.m., and 8 a.m. the next day. Results are shown in Chart III.

CONCLUSIONS.

The daily variation in the non-protein blood nitrogen of the normal dog receiving a diet containing 0.4 gram of nitrogen per kilo is about 9 mg., the maximum being reached about two hours after feeding with a return to the original level in about ten to fourteen hours. By feeding excessive quantities of meat, the non-protein blood nitrogen may be increased 25 to 40 mg. in six to eight hours and the original level is usually not reached even at the end of twenty-four hours.

In one animal (Dog 4) the blood nitrogen did not reach its maximum until fourteen hours after feeding, the urinary nitrogen exhibiting a parallel gradual rise; this is probably only to be explained on the basis of slow absorption from the gastro-intestinal tract. It is to be observed that this animal received its allotment of water in small doses throughout the day by stomach tube instead of in one large dose with its feeding; this factor may have influenced the rate of absorption.

The curve of the non-protein blood nitrogen in the normal dog after feeding follows closely that of the urinary nitrogen. There is sometimes exhibited, however, a further or secondary rise in the blood nitrogen at the time that the diuresis and output of nitrogen in the urine is rapidly decreasing.

In the fasting dog there occurs a gradual fall in blood nitrogen to a minimum of 12 to 18 mg., reached thirty to forty-eight hours after the last feeding, and followed by a rise in the next few hours to about 25 to 28 mg. at which level it tends to persist. The urinary nitrogen shows a similar but less pronounced curve.

The amount of urine influences the urinary nitrogen to a much greater extent when the blood and urinary nitrogen values are high than when they are low. It may be seen by comparing the curves of Dogs 7 and 8 that free diuresis induced by the administration of water to the fasting animal has little effect upon the curve of nitrogen either of the urine or blood.

The application of Ambard's formula using total nitrogen instead of urea to these data fails to give a constant figure. Better results are obtained by the use of a somewhat similar but simplified formula, but here also considerable discrepancies occur and we have been unable to find any formula that will constantly express the relation between blood nitrogen, urinary nitrogen, and urinary amount.

THE FINAL HYDROGEN ION CONCENTRATIONS OF CULTURES OF BACILLUS COLI.[1]

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(Received for publication, June 23, 1915.)

INTRODUCTION.

In 1912 Michaelis and Marcora[2] showed that a certain culture of *Bacillus coli* ceased activity in a lactose bouillon at a hydrogen ion concentration of 1×10^{-5}N. This point Michaelis and Marcora considered to be a physiological constant of *Bacillus coli.*

Since these authors apparently worked with but one culture, and varied the medium only in its initial reaction, it remained to be determined if this point is attained by other cultures of *Bacillus coli* and in media of such widely varying composition that it may truly be called a physiological constant.

The solution of this question is an integral part of an extensive research upon the influence of hydrogen ion concentrations on bacterial metabolism. Sufficient data have now accumulated to justify a preliminary account of the final hydrogen ion concentrations attained by several cultures which have been extensively studied in this laboratory.

Methods.

For the determination of hydrogen ion concentrations use was made of the chain:

$$\text{Hg HgCl} \mid \tfrac{\text{N}}{10}\ \text{KCl} \mid \text{Saturated KCl} \mid \text{Solution} \mid \text{Pt } H_2$$

The calomel cells were prepared from mercury which had been

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[1] Published by permission of the Secretary of Agriculture.

[2] Michaelis, L., and Marcora, F., *Ztschr. f. Immunitätsforsch. u. exper. Therap.*, Orig., 1912, xiv, 170.

distilled *in vacuo* after several distillations according to Hulett's[3] method. The HgCl was prepared according to the procedure followed by Loomis and Acree.[4] The $\frac{N}{10}$ KCl solution was made from thrice recrystallized and ignited Kahlbaum KCl. Some of the electrodes were of platinized platinium, others of gold coated with palladium black. Both types agreed well. The hydrogen was generated electrolytically from 10 per cent NaOH, and was passed over concentrated sulphuric acid, then over heated palladium asbestos. The generator was kept running continuously, on a low current when not in use, in order to prevent a backward diffusion of the atmosphere. The electrode vessel will be described in another paper. In principle it is similar to that of Hasselbalch,[5] but particular attention was given to the elimination of dead spaces, to the provision of maximum solution surface, and to the recommendations of Cumming[6] in regard to the contact between solution and saturated KCl. By attention to details it was found that the liquid contact potential was reduced to a small value, and it was considered unnecessary to make the Bjerrum[7] extrapolation correction. Corrections were made for the barometric pressure including, of course, the vapor tension of the solution, which was assumed to be that of water at the same temperature, as well as an occasional small correction for the calomel electrode found by comparison with other assumed standards. The apparatus was held in an air thermostat with active air circulation. The thermostat was kept constant to within about ± 0.05 of a degree of 30.0°C. The fluctuations in temperature within periods of five minutes were not over 0.003 of a degree, according to readings taken with an eight pair copper constantine thermo element.

The equation for the calculation of hydrogen ion concentration from the potential of the chain used at 30°C. is:

$$\frac{E - 0.337}{0.0601} = \log. I/C_H = P_H$$

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[3] Hulett, G. A., *Physical Review*, 1911, xxxiii, 307.

[4] Loomis, N. E., and Acree, S. F., *Am. Chem. Jour.*, 1911, xlvi, 585.

[5] Hasselbalch, K. A., *Biochem. Ztschr.*, 1913, xlix, 451.

[6] Cumming, A. C., *Tr. Faraday Soc.*, 1907, ii, pt. iii, 213; 1912, viii, 86; 1913, ix, 174.

[7] Bjerrum, N., *Ztschr. f. Electrochem.*, 1911, xvii, 389.

The potentials were measured with a Leeds and Northrup potentiometer and with a galvanometer as zero point instrument. Two Weston cells kindly loaned by the Bureau of Standards furnished the known potentials.

Organisms Studied.

The organisms studied were those described by Rogers, Clark, and Davis[8] and by Rogers, Clark, and Evans.[9] They were all of that class of the colon-aerogenes family which, when grown *in vacuo* in a medium consisting of 1 per cent Witte peptone, 1 per cent dextrose, and 0.5 per cent K_2HPO_4, produce carbon dioxide and hydrogen in the ratio $CO_2/H_2 = 1.06$. Since the cultural characteristics of these organisms approach closely to those generally attributed to *Bacillus coli* we may, for the sake of brevity, call them *Bacilli coli,* although they are distinguished in this laboratory from other members of the colon-aerogenes family by calling them the "low ratio organisms."

The Progress of Acid Formation.

The progressive development of the acidity of the cultures is shown in Tables I and II. Were we to plot the P_H values against time we should find a form of curve which is very much the same whatever the medium used. The rate of acid development, however, when extricated from its apparent rate due to the character of the medium's buffer effect, is a subject worthy of separate treatment. For present purposes it is only necessary to state that the progress of the acid formation has been followed in sufficient detail to make sure that the final hydrogen ion concentrations when measured were practically final values.

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[8] Rogers, L. A., Clark, W. M., and Davis, B. J., *Jour. Infect. Dis.,* 1914, xiv, 411.

[9] Rogers, L. A., Clark, W. M., and Evans, A. C., *ibid.,* 1914, xv, 99; 1915, xvii, 137.

TABLE I.

Progressive Change in Hydrogen Ion Concentration of Colon Cultures. Medium: 1 per cent Witte Peptone, 1 per cent Dextrose, and 0.5 per cent K_2HPO_4. Cultures Held in Jena Test-Tubes at 30° C.

Time.	P_H. Organism hx.	P_H. Organism fg.
hrs.		
0	7.29	7.29
5	7.29	7.29
10	7.16	7.00
15	6.11	6.08
20	5.47	5.26
25	5.22	5.11
30	5.17	5.02
35	4.92	4.85
50	4.86	4.87
100	4.68	4.84
150	4.64	4.83

TABLE II.

Progressive Change in Hydrogen Ion Concentration of Cultures of Colon 22hx. Medium: Standard Meat Infusion Containing 1 per cent Peptone and 2 per cent Dextrose. Cultures Held in Jena Test-Tubes.

Time.	P_H. 30° C.	P_H. 37.5° C.
hrs.		
0	6.95	6.95
24	5.08	4.93
48	4.84	4.83
96	4.79	4.82
192	4.74	4.83
240	4.75	

Final Hydrogen Ion Concentrations.

In Tables III and IV are shown typical results obtained with several organisms grown under the same conditions. In studying sixteen cultures isolated from human feces, cow feces, and grain, similar results were obtained when the organisms were grown under the same conditions. Whether the difference shown by the grain cultures in Table III is significant remains to be determined.

TABLE III.

Final Hydrogen Ion Concentrations of Colon Cultures. Medium: Standard Beef Infusion Containing 1 per cent Peptone and 1 per cent Dextrose. After Adjustment of the Reaction and Sterilization $P_H = 7.35$. Incubation Temperature 30° C. Incubation Period 18 Days.

Organism.	Source.	Corrected potential.	P_H.	$C_H \times 10^{-5}$.
22fg............	Human feces	0.6260	4.82	1.5
22hw............	Bovine feces	0.6230	4.77	1.7
22hx............	" "	0.6242	4.79	1.6
22hy............	" "	0.6228	4.76	1.8
22hz............	" "	0.6220	4.75	1.8
22ic............	" "	0.6220	4.75	1.8
22ja............	" "	0.6240	4.78	1.6
22jl............	" "	0.6230	4.77	1.7
22jm............	" "	0.6235	4.77	1.7
22jn............	" "	0.6248	4.80	1.6
22jw............	" "	0.6222	4.75	1.8
22ma............	" "	0.6189	4.70	2.0
22nb............	" "	0.6248	4.80	1.6
22or............	Grain	0.6466	5.16	0.70
22st............	"	0.6378	5.01	0.99
22te............	"	0.6426	5.09	0.82

TABLE IV.

Final Hydrogen Ion Concentrations of Colon Cultures. Medium: 1 per cent Witte Peptone, 0.4 per cent Liebig's Extract, 1 per cent Lactose, and 1 per cent KH_2PO_4. Cultures held in Jena Test-Tubes at 30° C. for 12 Days. Original Sterile Medium $P_H = 5.48$.

Organism.	P_H.	Organism.	P_H.	Organism.	P_H.
22fg	4.80	22hx	4.68	22hz	4.89
22fg	4.86	22hx	4.67	22hz	4.89
22hw	4.92	22hy	4.94		
22hw	4.92	22hy	4.91	Average	4.85

In Table V an interesting instance is given of the effect of adding sterile lactic acid to a medium before inoculation.

TABLE V.

Final Hydrogen Ion Concentrations Attained by Colon 22fg in Acidified 1 per cent Witte Peptone and 1 per cent Dextrose. Cultures Held in Vacuum Bulbs at 30° C. 14 Days.

Medium.	Original $P_{H^{\cdot}}$	Final $P_{H^{\cdot}}$
No acid added	6.74	4.37
Made 0.0025 N lactic	5.95	4.29
Made 0.0050 N lactic	5.00	4.53
Made 0.0075 N lactic	4.55	4.55

The results obtained in about thirty different media confirm so well the main contention of Michaelis and Marcora that it is hardly necessary to tabulate the data in detail.

It is of course true that in different portions of the same medium the accumulation of the same molecular concentrations of the acids produced by the organisms would lead to the same hydrogen ion concentration. It is also true that certain media of different composition might arrive at the same hydrogen ion concentration through the addition of the same amount of acid. For instance a 1 per cent Witte peptone, 1 per cent dextrose solution with an initial hydrogen ion concentration of $P_H = 6.89$, and the same solution brought to a hydrogen ion concentration of $P_H = 5.39$ by the addition of 1 per cent KH_2PO_4, both arrive at very nearly the same hydrogen ion concentration when each is made 0.01 normal with lactic acid. It also happens that near this point, which is about $P_H = 4.2$, the fermentation of dextrose by *Bacillus coli* ceases. In such a case the constancy of the final hydrogen ion concentration might be considered as merely an index of a constancy in the total acid produced.

On the other hand this is only a special instance, and a consideration of the data as a whole leaves little doubt that the hydrogen ion concentration rather than the total acid produced is a limiting factor. For instance, *Bacillus coli* grown in 1 per cent peptone, 2 per cent lactose, raises the hydrogen ion concentration to $P_H = 4.56$. To do so the organism need only make the medium 0.007 normal with lactic acid. But if this medium contains 0.05 M Na_2HPO_4 the organism must make the medium 0.06 normal with lactic acid before the hydrogen ion concentration is raised to $P_H = 5.00$. Instead of ceasing with the production of 0.007 normal acid the organism only ceased its activity when the hydrogen ion concentration of $P_H = 5.00$ was reached. In these instances, then, the total acid produced amounted in one case to 0.007 N and in the other to 0.06 N, while the final hydrogen ion concentrations were $P_H = 4.56$ and $P_H = 5.00$[10]. That the latter values approach closer to what may be called a physiological constant than do the former is hardly open to dispute.

There are, however, appreciable and noteworthy differences in the values obtained in different media. It seems to be true, for instance, that higher points are attained in dextrose media than in corresponding media containing lactose. For instance hx brought a 1 per cent peptone solution to $P_H = 4.26$ with a dextrose fermentation while with lactose only 4.56 was reached. Corresponding differences were observed in a variety of media in which dextrose was fermented in one case and lactose in another. To a certain extent this is comparable with the differences in titratable acid found by Brown[11] and also with the differences found by the Winslows[12] in the fermentation of dextrose and lactose by the coccaceae. As soon as we can obtain a stock of other exceptionally pure sugars comparable with the recrystallized lactose and dextrose used in the present investigation this work will be extended to include a variety of carbohydrates and their derivatives.

One of the most striking relationships to be found in the data at hand is that which seems to hold very generally between the buffer effect of the media and the final hydrogen ion concentration. This can be illustrated in the following way. If we add successive portions of lactic acid to a medium and then measure the resulting hydrogen ion concentration we obtain a close approximation of the effect of the acids produced by the organism. With a series of such measurements we obtain a relationship between the added acid and the resulting hydrogen ion concentration which we may plot in the form of a "titration curve." Such curves are shown in Fig. 1 for the following media:

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[10] Note that as P_H increases C_H decreases. See p. 88.

[11] Brown, W. W., *Jour. Infect. Dis.*, 1914, xv, 580.

[12] Winslow, C.-E. A., and Winslow, A. R., The Systematic Relationship of the Coccaceae, New York, 1908.

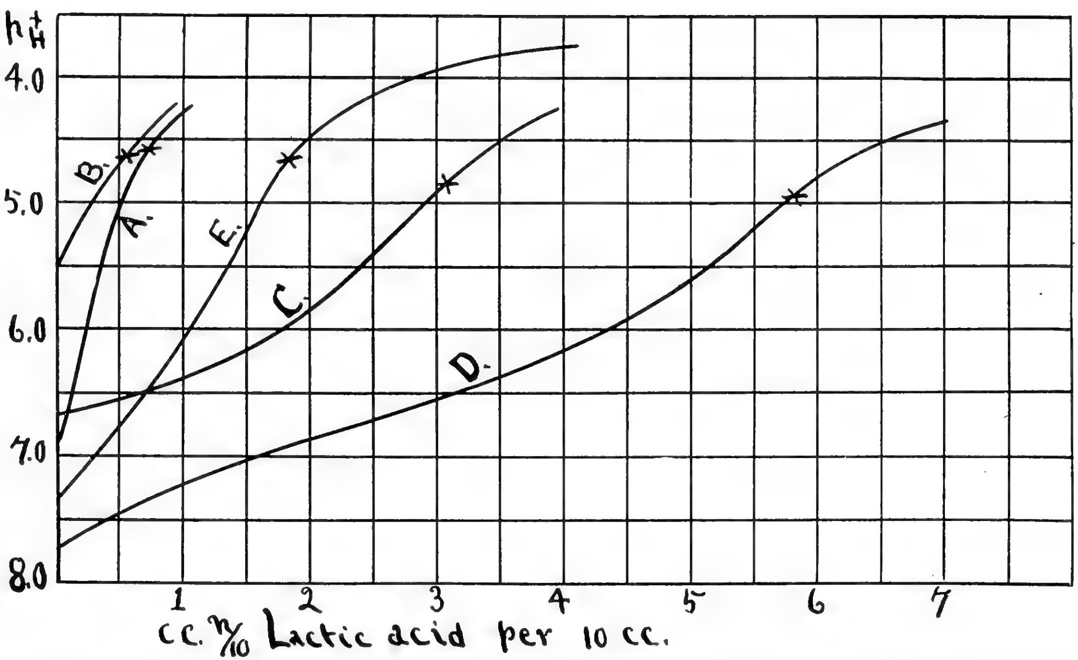

FIG. 1. Titration curves of media. The crosses show the final hydrogen ion concentrations, as P_H^{10}, attained by cultures of *B. coli*.

A. 1 per cent peptone
B. 1 per cent peptone, 0.05 M NaH_2PO_4
C. 1 per cent peptone, 0.05 M H_3PO_4, 0.075 N NaOH
D. 1 per cent peptone, 0.05 M Na_2HPO_4
E. 1 per cent peptone, 0.01 M Na_2HPO_4

When lactose was added to these media, and the final hydrogen ion concentrations attained through its fermentation were pointed off on the titration curves, they were found to fall at the positions designated by the heavy crosses. It appears at once that in the more highly buffered media, that is in the media with the greater neutralizing power, the final hydrogen ion concentrations are lower. That there is an orderly relationship is suggested by the fact that the points fall almost on a straight line.

The first conclusion to which this relationship points is, that although the hydrogen ion concentration is the chief limiting factor, other toxic bodies, perhaps the undissociated acids themselves, impose their own influence. Since the toxicity of these bodies would be proportional to their concentration we should expect to find their effect greater in media which permit the more extended fermentation, namely in the highly buffered media. In the cases just cited there seems to be a rather strict ratio. It is a curious fact that when we increase by $\frac{1}{7}$ the values of the normality of acid corresponding on the curves to the final hydrogen ion concentrations, we find that the hydrogen ion concentrations corresponding to these increased normalities are $P_H = 4.50$ for each of the three media which contain the same concentration of P_2O_5 (B, C, and D, Fig. 1). Whatever the meaning of this relationship may be it would be unprofitable to dwell too long upon it at this time for it cannot be extended with the same mathematical rigidity to other cases. Though a fair degree of proportionality is found to hold in many cases between the final hydrogen ion concentration and the buffer effect of the medium, we find in the case of milk, for instance, much lower final hydrogen ion concentrations attained than the titration curve would lead us to expect (see Table VI).

This and several other considerations have made it evident that we must proceed with caution if we are to deal rigidly with the specific effect of the hydrogen ion. No attempt will be made in this paper to treat of either the specific effect of the hydrogen ion

TABLE VI.

Final Hydrogen Ion Concentrations Attained by Colon Cultures in Sterilized Skimmed Milk. Cultures Held in Jena Test-Tubes at 30° C. for 5 Days. Original Sterile Milk $P_H = 6.18$.

Organism.	P_H.	Organism.	P_H.	Organism.	P_H.	Organism.	P_H.
22hg.........	5.16	22hp	5.04	22hu	5.03	22hy	5.14
22hm........	5.14	22hr	5.07	22hv	5.09	22hz	5.31
22hn........	5.14	22hs	5.12	22hw	5.09	22ib	5.10
22ho.........	5.16	22ht	5.13	22hx	5.06	22ij	5.30

or the specific effects of different acids. Data already accumulated on these questions must be reserved for separate treatment. The object of this paper is to show the bearing of the data presented upon a very important point in bacteriological technique. This may be stated very briefly as follows.

The Diagnostic Value of Final Hydrogen Ion Concentrations.

While the final hydrogen ion concentrations attained in cultures of *Bacillus coli* have been shown to vary somewhat with the medium, the variation is confined to a narrow range. This range is so narrow that for certain purposes the final hydrogen ion concentration may be considered a constant.

Now this limiting hydrogen ion concentration may be attained by very great differences in the total acid produced, and consequently, if we estimate the acid productivity of our cultures by titration, we may obtain widely different values. Unless the media employed by different laboratories are identical, at least in their buffer effect, the titratable acidity produced by the same organism may be found to be very different. The confusion becomes worse confounded when, as Miss Broadhurst[13] justly complains, different media are mentioned by the same name. In another paper[14] it is shown in more detail that present titrimetric methods are totally inadequate to meet the situation.

It may be well to add at this point that sufficient work has been done in this laboratory to indicate that the same phenomena described in this paper apply to many of the streptococci.

[13] Broadhurst, J., *Jour. Infect. Dis.*, 1913, xiii, 404.

[14] Clark, W. M., *ibid.*, 1915, xvii, 109.

Often the final hydrogen ion concentrations attained by different species are sufficiently wide to serve as differential characteristics; always the value attained by the same organism is far more constant than the titratable acid produced.

It appears then that by determining the final hydrogen ion concentration rather than the final titratable acidity we may expect to find values far more constant, of wider reproducibility, and of greater diagnostic worth.

If we can succeed in the endeavor now being made to simplify and systematize the colorimetric method of determining the hydrogen ion concentrations of bacterial cultures we shall have a method not only more valuable than present titrimetric procedures but one which may be made more rapid. A special case in which the colorimetric method has been successfully applied is in the differentiation of bacteria of the colon-aerogenes family. An account of this has recently been published.[15]

SUMMARY.

The final hydrogen ion concentrations attained in the fermentation of dextrose and lactose by several cultures of *Bacillus coli* in a variety of media have been measured electrometrically.

In any given medium the values obtained agree remarkably.

Distinct differences in the values obtained with the same organism in different media have been observed. These differences seem to follow in general this order: the greater the buffer effect of the medium the lower the final hydrogen ion concentration attained. This relationship suggests that as fermentation is prolonged other toxic bodies accumulate and superimpose their own relatively small effect.

The final hydrogen ion concentrations differ by such small amounts that the work here reported may be considered as a confirmation of the claim of Michaelis and Marcora that the final hydrogen ion concentrations are a physiological constant for *Bacillus coli*. It is shown, however, that if we are to deal with the specific effects of the hydrogen ion in any rigid way the differences in the final hydrogen ion concentration must be taken into serious consideration.

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[15] Clark, W. M., and Lubs, H. A., *Jour. Infect. Dis.*, 1915, xvii, 160.

It is shown that for certain practical purposes the final hydrogen ion concentrations furnish data of far greater significance than can possibly be obtained by measuring with titrimetric methods the acid productivity of these organisms.

THE RÔLE OF OXIDASES IN RESPIRATION.

PLATE I.

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(Received for publication, June 26, 1915.)

The suggestion has recently been made by R. S. Lillie[1] that "the oxidases play but an accessory part" in biological oxidation, while the chief rôle is assigned "to intracellular surfaces or phase boundaries," the electrical polarization of which is supposed to play an important part in the process. In support of this view he cites a series of experiments in which the oxidative activity of the cell was measured by the formation of granules of indophenol.

This theory is a new one and, if accepted, would make it necessary for us to modify greatly our ideas of respiratory processes. It seems desirable, therefore, to determine experimentally the relation of oxidases to the particular oxidation on which this theory is based, *i.e.*, the formation of indophenol. For this purpose the writer has repeated Lillie's experiments, using both plant and animal material, and has found that when the action of oxidases is prevented no oxidation occurs. It would seem, therefore, that the oxidases play a much more important part than Lillie has ascribed to them. The writer here presents an account of these experiments together with a discussion of some other work directly related to this subject.

The Indophenol Reaction.

The oxidation of a mixture of α-naphthol and paraphenylenediamine in dilute sodium carbonate solution was employed by Röhmann and Spitzer[2] some years ago, in a study of the oxidative activity of various organs of the

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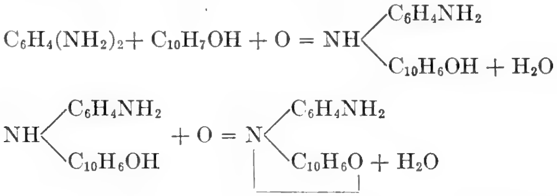
[1] Lillie, R. S., *Jour. Biol. Chem.*, 1913, xv, 237.

[2] Röhmann, F., and Spitzer, W., *Ber. d. deutsch. chem. Gesellsch.*, 1895, xxviii, 567.

animal body. According to these authors when the above compounds are brought together and exposed to the air oxidation proceeds in the manner represented by the following equation:



$$C_6H_4(NH_2)_2 + C_{10}H_7OH + O = NH\left\langle\begin{matrix}C_6H_4NH_2\\ C_{10}H_6OH\end{matrix}\right. + H_2O$$

$$NH\left\langle\begin{matrix}C_6H_4NH_2\\ C_{10}H_6OH\end{matrix}\right. + O = N\left\langle\begin{matrix}C_6H_4NH_2\\ C_{10}H_6O\end{matrix}\right. + H_2O$$

One atom of oxygen goes to effect the synthesis of a leuco-compound and a second atom of oxygen combines with this product to form a deep purple substance, known as indophenol. When solutions of these compounds are allowed to stand in contact with atmospheric oxygen the oxidation proceeds very slowly, but Röhmann and Spitzer found that in the presence of animal tissues the reaction was greatly accelerated.

More detailed observations on the course of this reaction have recently been made by Vernon.[3] When finely chopped tissues of the rabbit or rat were allowed to act on a mixture of α-naphthol, paraphenylenediamine, and sodium carbonate solutions he found that the amount of indophenol formed is in direct linear proportion to the time, though the reaction slows down toward the end of the process. This reaction Vernon believes to be due to the presence of oxidase ferments in the animal tissue; a belief held by former investigators who have employed the reaction as a test for oxidases in animal and plant material.

Following the method of Würster,[4] Lillie,[5] has employed in place of the paraphenylenediamine, dimethylparaphenylenediamine along with α-naphthol, which forms on oxidation dimethyl-indophenol. He observed that when animal cells were placed in either of these indophenol-forming solutions the oxidation product appeared in the form of minute rounded granules of a dark purple color. No suggestion is offered, however, as to whether they are amorphous particles of indophenol or depositions about previously existing granules in the cell.

Inasmuch as Lillie's contentions as to the nature of oxidation processes are based entirely on the location at which these bodies form in the cell, and particularly on the presence of membranes or surfaces such as are found around granules, it seemed desirable, before continuing the investigation, to determine the physical mechanism of their formation.

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[3] Vernon, H. M., *Jour. Physiol.*, 1911, xliii, 402.

[4] Würster, C., *Ber. d. deutsch. chem. Gesellsch.*, 1888, xxi, 1525.

[5] Lillie, *loc. cit.*; *Am. Jour. Physiol.*, 1902, vii, 412.

The Formation of Indophenol Granules.

A mixture consisting of watery solutions of paraphenylenediamine (1 per cent) and α-naphthol (1 per cent) was exposed to the air in a shallow watch glass for a few hours, until it assumed a deep purple color. A microscopic examination showed it to contain the characteristic long needle-shaped crystals of indophenol (Figure 1 A). In this reaction (which is well understood) the indophenol resulting from the oxidation is insoluble in water and appears in crystalline form.

To a similar indophenol-forming solution a small amount of gelatin was added. The reaction proceeded at approximately the same rate and formed a similar dark purple solution, but the indophenol was in the form of small oval granules rather than in crystals. Other colloids, as gum arabic or egg white, showed similar effects. The granules, however, varied considerably in size in different colloids and different concentrations of the same colloid. To portions of the diamine + naphthol solution, containing each 10 cc., sufficient gelatin was added to make the concentrations 0.1, 1.0, and 10.0 per cent. The granules which formed in the 0.1 per cent gelatin showed some resemblance to the normal crystals (Figure 1 B); yet while the normal are 100 to 200 μ in length these granules are only 5 to 10 μ long, with rounded ends, and show a marked tendency to aggregate into irregular spherites. But in the stronger gelatin the granules give no suggestion of the typical crystal form (Figure 1 C) but are rounded or slightly oval and, in the 10 per cent gelatin, only 3 to 5 μ in diameter.

The formation of dimethyl-indophenol was affected in exactly the same manner by the colloids.

Adding a small amount of a clear filtered extract of wheat bran, containing an active oxidase, to the indophenol-forming mixture the crystalline indophenol formed as before though much more rapidly. Also, in the presence of gelatin, the formation of granules was accelerated, yet the granules themselves had the same appearance as those formed more slowly.

It is apparent, therefore, that these granules observed to form in certain animal cells, and about to be described in plant cells, result from the oxidation of the naphthol-diamine mixture; that the indophenol or dimethyl-indophenol formed is prevented from crystallizing normally in the cell by the colloidal properties of the protoplasm, so that it is deposited in a granular form, and that it is not necessarily formed at the surfaces of cell granules or at other cell surfaces.

The Relation of Oxidation Processes to the Nucleus and Cell Membranes.

In an early paper by Loeb[6] it was pointed out that the inability of enucleated cells to regenerate was associated with what he believed to be a diminution of oxidative activity, and he concluded accordingly that the nucleus was the main (although not the only) oxidizing organ in the cell. Loeb has also recently suggested that the marked increase in the rate of oxidation in developing eggs of *Arbacia* on passing from the fertilized egg to the 32 cell stage may be due to the increase in the mass of the nuclei. Immediately following Loeb's original suggestion, considerable evidence was brought forward by Lillie[7] to show that intracellular oxidations take place particularly about the nuclei. In certain animal tissues, such as the testis, intestine, or kidney, where the nuclei show a definite arrangement, he found that indophenol granules and the oxidation products of various dyes were formed in largest amounts in those regions where the nuclei are chiefly situated. He also pointed out at this time that blood cells in an indophenol-forming solution develop granules most rapidly about their nuclei. Warburg[8] has shown that nuclei isolated from red blood cells of birds by alternate freezing and thawing (which destroys the cytoplasm but leaves the nuclei intact) absorb oxygen at a rate comparable with the absorption by uninjured cells.

Some more recent investigations, on the other hand, indicate that the nucleus plays only a secondary part. Wherry[9] finds that methyl green is oxidized in the cytoplasm of species of *Amoeba*, but never in the nucleus. Schultze[10] investigated various plant and animal cells and found several stains to be oxidized in the cytoplasm but not in the nucleus, as was shown by change in color. In Lillie's[11] more recent contribution to this subject he brings forward evidences to show that the oxidative activity of the nucleus, described by himself and other investigators, is due not to the activity of the nucleoplasm but to that of the nuclear membrane. Contrary to his previous conclusions, based on similar experiments, he now

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[6] Loeb, J., Artificial Parthenogenesis and Fertilization, Chicago, 1913.

[7] Lillie, *Am. Jour. Physiol.*, 1902, vii, 412.

[8] Warburg, O., *Ztschr. f. physiol. Chem.*, 1910–11, lxx, 413.

[9] Wherry, E. T., *Science*, 1913, xxxvii, 908.

[10] Schultze, W. H., *Verhandl. d. deutsch. path. Gesellsch.*, 1913, xvi, 161.

[11] Lillie, *Jour. Biol. Chem.*, 1913, xv, 237.

believes that other regions of the cell possess oxidative activity similar to that of the nucleus. He reports that the blood cells of the frog placed in dimethylparaphenylenediamine and α-naphthol solutions in alkaline physiological salt solution, form the first indophenol granules about the nuclei in both erythrocytes and leucocytes; somewhat later the granules are formed in the outer layer of the cytoplasm and finally throughout the cytoplasm. The fact that granules were first observed to form at the nuclear and plasma membranes he considers sufficient proof that the membranes are concerned in the oxidation processes leading to the formation of the indophenol. Lillie suggests that this action of the membrane may be due to variations in its electrical polarization; in support of this conclusion he states that by subjecting the blood preparations to an induction shock the rate of oxidation was increased to a marked degree in leucocytes and slightly in erythrocytes. He assumes that the granules which eventually appear throughout the cytoplasm form at colloidal surfaces or phase boundaries inasmuch as no definite membranes occur in the cytoplasm, the potentials of which would vary, as of the more conspicuous membranes. He accordingly concludes that the chief rôle in oxidation is to be assigned to these "intracellular surfaces or phase boundaries." And, though no direct evidence is brought forward, the assumption is made that "the oxidases play but an accessory part" in intracellular oxidations.

With the view of determining the correctness of this latter assumption various plant and animal cells were investigated. Plant cells, on account of their various conspicuous semi-permeable surfaces, are particularly suitable for such investigations. Epidermal and hair cells from various roots and leaves, cells and coenocytes of algae and fungi supplied suitable and convenient material.

The lower epidermis of onion bulb leaves was particularly good for this purpose on account of the large size of the cells. Small pieces of the tissue were stripped from the leaves and placed on a slide in a few drops of a mixture containing equal parts of a watery solution of 1 per cent paraphenylenediamine and saturated α-naphthol (referred to hereafter as 1 per cent Spitzer's reagent) to which was added sufficient neutral hydrogen peroxide to make the concentration 0.1 per cent. The reaction was then observed under a magnification of 250 or 500 diameters. In a few seconds the formation of dark purple granules of indophenol could be detected, appearing in a perfectly definite manner, first within the thin layer of protoplasm which surrounds the vacuole, particularly, it seemed, at the outside; and at the same time, or usually somewhat later, on the surface of the nucleus. Although exhibiting some Brownian motion the granules did not appear to migrate from their place of formation, yet the granulated areas gradually extended until all the cytoplasm became uniformly dotted with them in the course of five to ten minutes.

By tearing pieces of the epidermis straight across (at right angles to the long axis of the cells) various sized pieces of similar cells were obtained. These, except for the torn end, appeared normal; the cytoplasm remained pressed against the cell wall, and the nucleus, where it had not been removed, appeared in its normal position. On flooding these cells with the reagent and keeping them under observation, it was seen that granules began to appear in the torn cells instantly and long before they appeared in neighboring intact cells. Just at the tear there was a disorganized area where the granules formed somewhat irregularly; but a short distance within the cell (two or three times the thickness of the cytoplasmic layer) the granules made their first appearance just inside the exposed surfaces of the protoplasm and simultaneously on the surface of the nucleus. Apparently the penetration of the reagent to the nucleus was not delayed here, as in the case of intact cells. The photomicrograph (Figure 1 D) of such a piece of tissue shows granules equally abundant in intact cells and in cell fragments. This figure also shows granules in pieces of cells from which nuclei had been removed. These granules formed quite as rapidly and in the same definite positions as in torn cells containing a nucleus or in normal uninjured cells.

Epidermal cells from other leaves behaved like those of onion; particularly good results were obtained with those from *Iris* leaves, where it was also possible to separate cells into a nucleated and non-nucleated part. As in the former case, indophenol granules formed in the absence of the nucleus quite as rapidly as where it was present.

Similar results were obtained with various root-hair cells; after standing a few minutes in Spitzer's reagent, indophenol granules appeared in the thin peripheral layer of cytoplasm and distinctly in the cytoplasmic bands crossing the vacuole. When the nucleus occurred in the projecting portion of the cell, *i.e.*, in the hair (where it could be easily observed) it was apparent from the larger number of indophenol granules that the region of the nuclear wall possessed active oxidative properties. Since the nucleus is frequently, or usually, in the basal part of these hair cells, by cutting off the projecting part near the root proper large pieces of cells without nuclei were obtained. As would be expected from the previous experiments oxidations took place as in intact cells.

Filaments of different species of *Spirogyra*, when placed in the same concentrations of Spitzer's reagent and hydrogen peroxide as used in the former experiments, rapidly formed indophenol granules in the cytoplasmic layer and in some cases also about the surface of the nuclei, but in the majority of the cells examined practically no granules were formed in the cytoplasm adjoining the nucleus. Similarly in the coenocytes of *Vaucheria* the regions about the nuclei occasionally appeared most active in forming indophenol; but usually after a few seconds of exposure to the reagent the filaments became uniformly dotted with the purple granules. The mycelia of a species of *Saprolegnia* and one of *Mucor* rapidly effected the oxidation of the reagent, but the indophenol appeared so uniformly and densely throughout the cytoplasm that the nuclei could not be detected.

It would seem, therefore, that the function of the nucleus in respiratory processes has been over estimated. Since in those cells where the region about the nucleus was most active in bringing about oxidation the removal of the nucleus appeared in no way to diminish the general oxidative activity of the cytoplasm; and since in other cells the nuclear region possessed no oxidizing ability, it is evident that the nucleus may not be essential to or even play a primary part in cellular oxidations.

The fact that no oxidation takes place about certain nuclei is particularly important. If, as Lillie supposes, intracellular oxidations are effected by electrical phenomena at membranes, the question may be asked: Why do indophenol granules not form at all nuclear membranes, especially when this oxidation is proceeding in other parts of the cell?

In this connection it is an interesting fact that granules of indophenol have never been observed to form at plastid membranes; this is particularly interesting since the plastid surfaces, in many species of plants if not in all, are of a semi-permeable character similar to other membranes of the cell. In cells of *Spirogyra*, *Zygnema*, and *Vaucheria*, notwithstanding the large area of membrane exposed by the chromatophores, there appeared to be no tendency for the granules to form more rapidly or in larger numbers at the surfaces of the chromatophores than in other regions of the cytoplasm. Leaves of *Elodea* and *Bryum* and teased out mesophyll cells of leaves of *Tradescantia* gave a better opportunity for observation than the algae mentioned above, as the chloroplastids are more regular in outline; but here, as in the algae, the chloroplastid membrane is quite inactive in oxidation. Similarly in fresh sections of potato, indophenol appeared at the surface of leucoplastids and of starch grains only after the surrounding cytoplasm had become filled with the granules.

These facts indicate that if the cell membranes possess any oxidative activity their sphere must be limited to special cases. More illuminating evidence, however, was obtained by treating various cells with killing agents and then testing their ability to produce oxidation. A description of these experiments follows.

The Direct Relation of the Oxidases to Indophenol Formation.

The writer has observed that after standing for some minutes in 1 per cent solution of Spitzer's reagent many of the cells on which he was experimenting appeared more or less disorganized and apparently dead. In these cases the application of $Ca(NO_3)_2$ produced no plasmolysis, thus indicating that the plasma membrane had become perfectly permeable. On treating different preparations of the epidermal cells of onion with $Ca(NO_3)_2$, at frequent intervals after adding the indophenol-forming solution of the concentration usually employed (1 per cent of the combined salts and 0.1 per cent of hydrogen peroxide) it appeared that two minutes' exposure to the latter was sufficient to destroy the ability of the cells to undergo plasmolysis. But notwithstanding the great alteration of the plasma membrane and phase boundaries which is indicated by this failure to plasmolyze the oxidation continued quite as rapidly as before.

To test further the relation of the semi-permeability of the membranes to oxidation, cells were killed in a variety of ways before placing them in the oxidizable reagent. Pieces of onion epidermis were placed in chloroform, toluene, and in 1 per cent solutions of chloral hydrate and of urea for two minutes, then removed to distilled water for five minutes, and then placed in the indophenol solution previously used. In all these cases granules of indophenol formed in these cells exactly as they did in cells that were alive when the reagent was added. In some cases the reaction was even more rapid in the dead cells; this was probably due to increased permeability to the reagent. Figure 1E shows cells (which had previously been chloroformed) after standing five minutes in Spitzer's reagent. Root-hairs of wheat, barley, *Tradescantia*, filaments of *Spirogyra*, *Zygnema*, and *Vaucheria*, mycelia of *Mucor* and *Saprolegnia*, after treatment with chloroform or chloral hydrate formed indophenol granules just as rapidly as before treatment.

The above killing agents are known to have very little if any harmful action on the oxidases. Extracts of bran and potatoes in the presence of any one of these activate the oxidation of Spitzer's reagent to the same extent as the untreated extracts.

Certain other killing agents have quite the opposite effect on the formation of intracellular granules of indophenol. In all the experiments just described, in addition to using chloroform, ether, toluene, chloral hydrate, and urea, portions of all the material were also placed in 1 per cent potassium cyanide solution, 0.1 M HCl, 0.1 M NaOH, or in live steam, for two minutes, then removed to distilled water for five minutes, and finally placed in Spitzer's reagent and hydrogen peroxide. After this treatment none of the material, whether belonging to spermatophytes, algae, or fungi, formed indophenol granules in the cells. These killing agents, however, probably have no more destructive effects on the membranes and phase boundaries than those previously used, although both render them perfectly permeable; but potassium cyanide, mineral acids, strong alkalies, and high temperatures always completely inhibit the action of oxidases.

Summarizing these observations, it appears that the oxidation of Spitzer's reagent in plant cells frequently proceeds most rapidly in the region of semi-permeable membranes; yet many nuclear, and all the plastid surfaces observed showed no such activity; the oxidation is not prevented by the destruction of the membranes, but is completely stopped by the addition of agents which inhibit oxidases.

Evidence from Animal Tissue.

A. Nerve Tissue.—The ventral nerve cord of the crayfish supplied animal material in which comparatively large masses of tissue, almost free from nuclei, could be isolated by simply cutting the connecting nerve cord away from the ganglia. The entire nerve cord of an animal killed by bleeding was removed and placed in 1 per cent solution of paraphenylenediamine and α-naphthol (equal parts), in 0.125 M NaCl. The solution was placed in a shallow watch glass. In about half a minute the ganglia showed a faint purple color, soon followed by the appearance of a similar color in the connectives. The color in both regions rapidly deepened until at the end of thirty to forty-five minutes the entire cord presented a uniform deep purple appearance.

Frozen sections cut longitudinally through the ganglia and nerve cord show the granules to have formed throughout the tissue both in the nerve cells and fibers, and to some extent also in the connective tissue cells. The granules showed a marked tendency to aggregate in certain regions, but without reference, so far as could be detected, to any cellular or intracellular structures.

Subjecting the nerve cord to a rather vigorous induction shock (10 milliamperes) for two minutes after placing it in the indophenol-forming solution markedly stimulated the oxidation. While in the unstimulated condition thirty to forty-five minutes were required to reach the maximum color, fifteen to twenty-five minutes were sufficient after stimulation.

Segments of the cord from between the ganglia, under similar conditions of stimulation, formed indophenol just as rapidly as when the nerve fibers were connected with the nucleated nerve cells. The reaction was in fact somewhat more rapid; this was probably due to the greater ease with which the reagent penetrated, since the granules formed most rapidly at the cut ends of the cord.

As would be expected from the behavior of plant cells, it was found that if the nerve cord had been treated with chloroform or ether vapor for five minutes and had then been placed in Spitzer's reagent the formation of indophenol proceeded at about the same rate as in the normal nerve, although usually somewhat more rapidly. After treating with steam, potassium cyanide, hydrochloric acid, or sodium hydroxide, thereby destroying the oxidases, there was no formation of indophenol in any part of the nerve cord even after standing some hours in Spitzer's reagent.

These results have some bearing on the observations of Tashiro[12] regarding respiration in resting and stimulated nerves. By means of his delicate apparatus Tashiro was able to show that carbon dioxide is given off from both medullated and non-medullated nerve fibers and that the amount produced is greatest during stimulation and least under anesthesia. It was therefore concluded that conduction of nervous stimuli is of the nature of a propagated chemical change. The present experiments have shown that oxidation processes go on in both nerve cells and their fibers, under the activation of oxidases; and that the rate of this reaction is increased by stimuli which cause nervous excitation. But it was also found that the reaction proceeds without interruption after the application of an anesthetic which paralyzes the nerve as a conducting organ. Hence it can not be concluded that oxidation reactions (such as produce the CO_2 observed by Tashiro) are necessarily concerned in the transmission of nerve stimuli.

B. Blood.—Blood cells of the frog, upon the behavior of which Lillie bases his theory of oxidation, were examined to determine whether oxidases were concerned in their respiration.

The blood, immediately after shedding, was mixed with a solution consisting of nine volumes of $\frac{M}{8}$ sodium chloride and one volume of $\frac{M}{8}$ potassium oxalate, in order to prevent clotting and the breaking down of leucocytes. A few drops of the suspension of corpuscles were then mixed on a slide with an equal amount of Spitzer's reagent which was freshly prepared by mixing a saturated solution of α-naphthol in alkaline isotonic salt solution ($\frac{}{8}$ NaCl containing $\frac{M}{100}$ Na_2CO_3) with an equal volume of 1 per cent paraphenylenediamine in $\frac{M}{8}$ NaCl. In such a mixture reactions similar to those reported by Lillie[13] were observed to take place. On exposure to the air, without a cover glass, granules could be detected in the leucocytes after two or three minutes, forming first about the nucleus or the plasma membrane; in the erythrocytes after five or ten minutes forming in similar positions.

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[12] Tashiro, S., *Am. Jour. Physiol.*, 1913, xxxii, 107. Tashiro, S., and Adams, H. S., *Jour. Biol. Chem.*, 1914, xviii, 329.

[13] Lillie. *Jour. Biol. Chem.*, 1913, xv, 245.

After thirty minutes' exposure the leucocytes usually appeared quite black, or purple-black, being filled with the granules; while the erythrocytes showed a very distinct granulated area about the nucleus, or the cell surface, or frequently throughout the cytoplasm.

. On passing an induction shock of 10 milliamperes for three minutes through the preparation immediately after adding the reagent, the reaction in the leucocytes was accelerated so that five to ten minutes in the reagent sufficed to produce the same degree of color as thirty minutes in the unstimulated condition. The rate of reaction in the erythrocytes, however, was not accelerated to an appreciable extent.

Killing agents, as used on plant cells, were then applied. After exposing a drop of blood suspension to chloroform vapor for two minutes the greater part of the leucocytes broke down, but on mixing with Spitzer's reagent the erythrocytes formed indophenol rather more rapidly than when in their normal condition. Adding a trace of urea to a mixture of blood and Spitzer's reagent the formation of indophenol apparently proceeded as in the absence of the cell poison. On treating with steam, potassium cyanide, hydrochloric acid, or sodium hydroxide (for the destruction of the oxidases) before mixing the blood with the indophenol-forming solution, no reaction took place even after some hours.

It seems apparent, therefore, that in this material, as in that previously used, the reaction proceeds after the membranes have been destroyed but not after the oxidases have been inhibited. It is therefore evident that we are dealing with an oxidase reaction. The acceleration due to induction shocks may be explained in various ways without having recourse to the polarization of the membrane. If it is due to an increase of electrical depolarization of phase boundaries, as has been suggested by Lillie, one would expect the erythrocytes as well as the leucocytes to be appreciably affected, but this is not the case. Furthermore the writer has found that in plant cells, such as the epidermis of onion, the root-hairs of wheat, cells of *Spirogyra*, etc., induction shocks of 5 to 20 milliamperes and of various durations have no noticeable effects on the formation of indophenol.

These experiments apparently indicate in a convincing manner that oxidase enzymes are the active agents in this intracellular

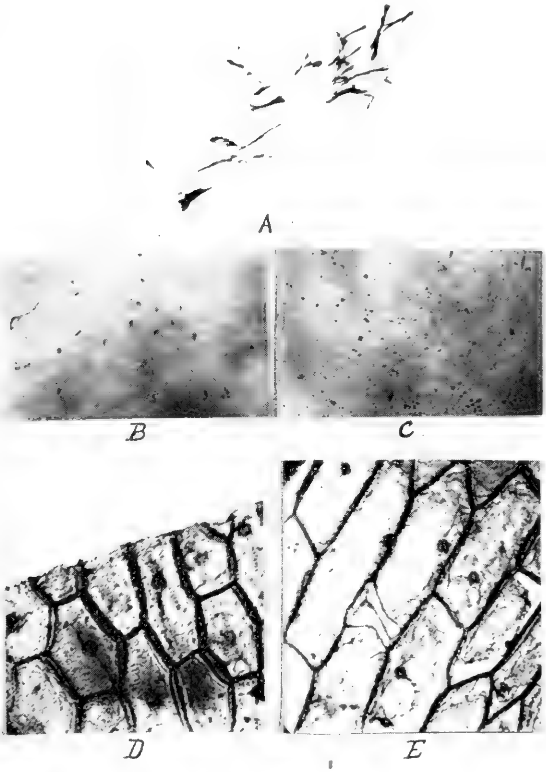

FIG. 1, A, B, and C. Photomicrographs showing the effects of a colloid on the formation of indophenol granules. A shows normal crystals formed from a watery solution. B and C show granules formed from solutions containing different amounts of gelatin.

D and E. Photomicrographs showing indophenol granules of cells of onion epidermis. D shows granules in intact cells and in cell fragments with and without nuclei. E shows granules in cells which had been chloroformed before adding the indophenol-forming reagent.

oxidation. Although Lillie supposed that electrical phenomena at phase boundaries may produce some oxidative activity, it is evident that in the oxidation of Spitzer's reagent these forces have little or no effect. If the contentions of Spitzer, Vernon, and Lillie be admitted; namely, that the formation of indophenol may be taken as a measure of the total oxidative activity of the cell, it may be concluded that the oxidases are the essential agents in bringing about this reaction.

STUDIES OF AUTOLYSIS.

II. THE ACCELERATION OF LIVER AUTOLYSIS.

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(Received for publication, June 9, 1915.)

In the previous paper[1] were presented the results of a study of the effect of manganous chloride upon liver autolysis. Several mechanisms by which this salt might bring about the increased speed and extent of digestion were suggested. The salt may have an activating effect upon a zymogen or already partially active enzyme, it may supply a favorable medium for proteolysis acting thus as a coferment analogous to the hydrochloric acid of gastric juice, or it may combine with the proteins of the liver and render some of them, normally undigested even in prolonged autolysis, available for the somewhat specific erepsin-like enzyme of the liver cell. While not conclusive, the evidence presented in the previous paper seemed to point to the last as the most probable mechanism. The evidence which is here presented is believed to confirm this hypothesis largely by a process of elimination of other possible mechanisms.

If an enzyme is activated by the salt, or provided with a coferment which renders it more active and less specific, various foreign proteins which are normally digestible should in the presence of manganous chloride digest more rapidly and perhaps more completely. Other foreign proteins, which are normally not digested by the liver enzymes, might be expected to digest in the presence of the salt. On the other hand, if the salt exerts its effect by combining with the liver proteins and thus rendering them more available for digestion, it should have no effect upon the rate or extent of digestion of such foreign proteins as are already digestible. Unavailable foreign proteins may or may not be rendered available by the salt.

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[1] Bradley, H. C., and Morse, Max, *Jour. Biol. Chem.*, 1915, xxi, 209.

The first experiments therefore concern themselves with a study of the action of the autolytic enzymes of the liver upon foreign proteins, with and without the presence of manganous chloride.

Experiment I. The Effect of Casein, Peptone, and Boiled Liver Proteins on the Autolytic Reaction.

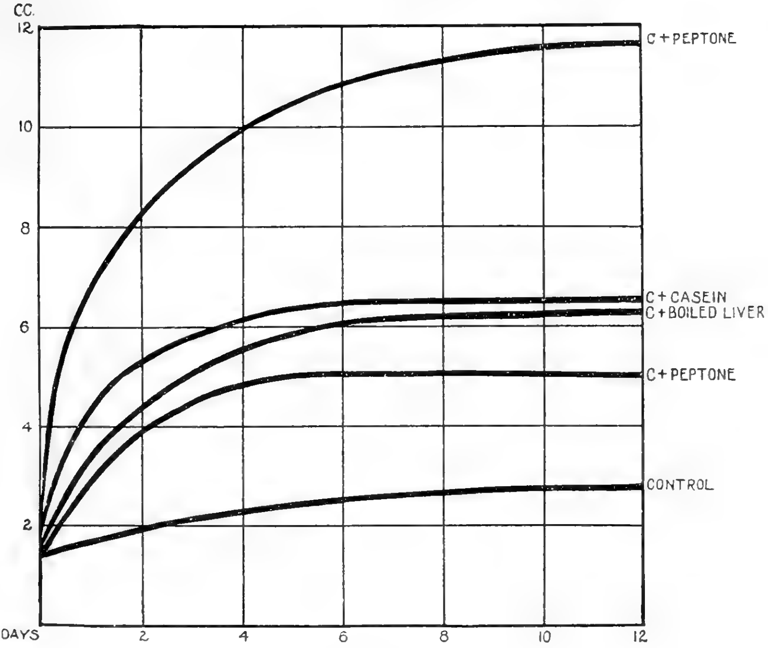
As before, 50 grams of the liver pulp were weighed out, the extra protein was added where indicated in the tables, and the whole made up to a volume of 250 cc. A mixture of chloroform and toluene preserved the digests, which were kept at a temperature of 37°C. The analytical procedure was exactly as described in the previous paper. Instead of reporting total nitrogen in the 10 cc. sample of the pulp analyzed, the value has been calculated for 6.25 cc. of the digest, the amount represented by the tannic acid filtrate analyzed. The titration figures of the tables show clearly what proportion of the protein present is digested to a form not precipitated by tannic acid. The figures in the tables representing digestions are in every case cc. of $\frac{N}{5}$ acid.

TABLE I.

Cc. $\frac{N}{5}$ *HCl.*

No.	Contents.	Days.			Total N in 6.25 cc.
		0	7	12	
I.......	Control, liver	1.4	4.2	4.4	11.8
II.......	" + casein	2.5	9.0	9.1	15.4
III.......	" + peptone	2.5	7.4	7.3	16.6
IV.......	" + peptone	3.8	15.7	15.4	29.4
V.......	" + boiled liver	2.6	8.9	8.8	23.3

It will be seen that the addition of these foreign proteins affects the reaction exactly like the addition of manganous chloride. The curves rise more rapidly than the control and reach equilibrium at a higher amino-acid level. And yet this change in the reaction is clearly not due to activations, or coenzymes, but to the increased mass of substratum present in the mixtures. The added 50 grams of boiled liver double the digested nitrogen, although no enzyme has been added.



Curve I.

Experiment II. The Effect of Foreign Proteins and Manganous Chloride on an Autolysis Which Has Reached Equilibrium.

Liver was allowed to autolyze three weeks, or long after approximate equilibrium had been attained. Various foreign proteins were added, with and without the manganous chloride. The results are shown in Table II.

The data admit of analysis by which it can be determined what effect the salt has on the extent of digestion of the foreign proteins, thus:

	cc.
1. Net gain of liver alone (control)	0.2
2. " " " " and $MnCl_2$	1.7
3. Increase due to $MnCl_2$	1.5
4. Net increase of liver and peptone	2.9
5. Increase due to digestion of peptone	2.7
6. Net increase of liver, $MnCl_2$, and peptone	4.2
7. Increase due to digestion of peptone, subtracting liver and $MnCl_2$, or 1.7	2.5

TABLE II.

Cc. $\frac{N}{5}$ *HCl.*

No.	Contents.				Net increase.		Total N in 6.25 cc.
		Days.					
		20	24	30	4	10	
I....	Control, liver	4.6	4.7	4.8	0.1	0.2	9.4
II....	C + $MnCl_2$	4.8	5.9	6.5	1.1	1.7	9.3
III....	C + peptone	6.0	8.5	8.9	2.5	2.9	13.7
IV....	C + peptone + $MnCl_2$	5.4	8.9	9.6	3.5	4.2	13.6
V....	C + albumin	3.9	4.4	4.7	0.5	0.8	13.8
VI....	C + albumin + $MnCl_2$	4.4	5.4	6.4	1.0	2.0	12.5
VII....	C + casein	4.5	6.8	7.8	2.3	3.3	11.3
VIII....	C + casein + $MnCl_2$	4.3	7.2	8.0	2.9	3.7	10.6
IX....	C + boiled liver	4.7	5.0	5.7	0.3	1.0	11.9
X....	C + boiled liver + $MnCl_2$	5.0	6.4	7.5	1.4	2.5	13.0
XI....	C + edestin	5.1	4.7	5.0	0.0	0.0	12.8
XII....	C + edestin + $MnCl_2$	5.1	6.2	7.8	1.1	2.7	12.8

In this case it is clear that the presence of manganous chloride has not increased the digestion of peptone in the least, the figures 2.5 and 2.7 being practically identical. Analysis of all the figures of the table shows that casein and boiled liver are both digested to the same extent whether manganous chloride is present or not. Edestin, on the other hand, while not attacked by the untreated liver, does appear to be digested by the combined action of liver and the salt. Ovalbumin is not digested in either case. Albumin has been tried a number of times, always with negative results, or with indications of inhibition of autolysis of the liver itself, as is shown in Table III.

This experiment also indicates that while albumin is not digested by the liver enzymes even in the presence of a favorable amount of the salt, it is digested quite considerably in the presence of hydrochloric acid.

	cc.
1. Net gain of liver alone	3.9
2. " " " " and $MnCl_2$	6.0
3. " " " " " HCl	5.5
4. Gain of liver, albumin, and $MnCl_2$	5.9
5. Digestion of albumin	0.0
6. Gain of liver, albumin, and HCl	10.0
7. Digestion of albumin	4.5

The fact that the acid renders the digestion of albumin possible, while the salt does not, although the salt does render the digestion of edestin possible, is difficult to understand on the basis of an activation phenomenon. It would seem to imply the activation of one set of enzymes by the salt, and another set by the acid. The first set can digest edestin and more of the liver than normally, while unable to catalyze the hydrolysis of albumin. The second set can in addition digest albumin. Neither of these sets of enzymes is active in autolysis under normal conditions.

TABLE III.

Cc. $\frac{N}{5}$ *HCl.*

No.	Contents.	Days.					Total N in 6.25 cc.
		0	1	3	6	12	
I....	Control, liver	1.6		3.2	3.5	5.5	9.75
II....	C + albumin	1.7		3.1	3.6	4.0	11.4
III....	C + albumin + $MnCl_2$	1.4	3.5	5.3	6.4	7.3	10.9
IV....	C + $MnCl_2$	1.7		4.6	6.9	7.7	9.7
V....	C + albumin + HCl	1.5	6.9	10.1	10.4	11.5	11.4
VI....	C + HCl	1.3	5.2	5.9	6.2	6.8	9.7

In the preceding figures and analyses comparisons of digestions have been made after autolysis has gone on for about ten days and equilibrium has been at least approximated in most cases. The results show that manganous chloride does not influence the amount of casein or peptone hydrolyzed at the end of that time. So far as the equilibrium between peptone or casein and their products is concerned, the salt is without effect. Does it have an effect upon the speed of the reaction? To determine this point an analysis of the earlier stages of the reaction must be made. Any change in the amount of the enzyme present, or in its activity, should appear most clearly during the first few days of the autolysis. While equilibrium is determined by the active masses of protein substratum, the speed will be expressive also of the amount of the enzyme or its activity. If the manganous salt activates a zymogen, that fact will be evident in the increased speed of the reaction at first in the case of peptone, casein, and boiled liver. In Table IV are tabulated the results of an experiment made to determine this point.

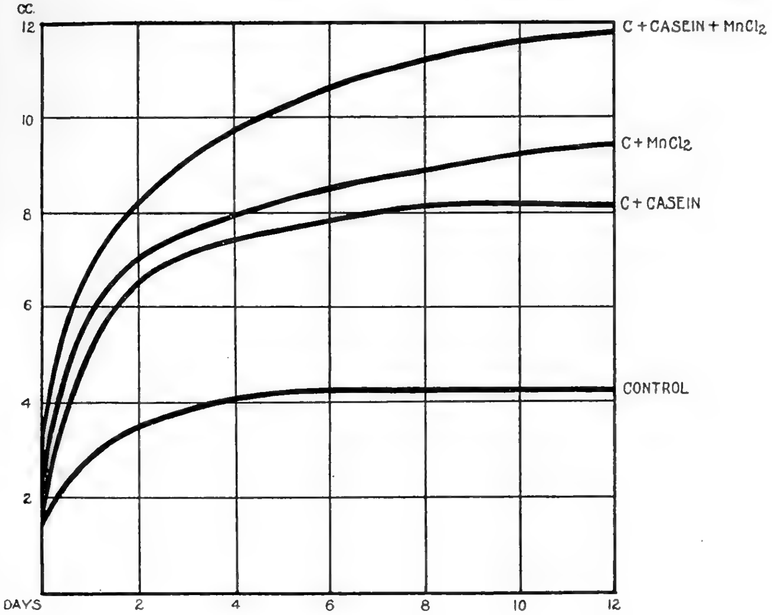
TABLE IV.

Cc. $\frac{N}{5}$ *HCl.*

No.	Contents.	Days.				Initial averages.	Net gains.			Total N in 6.25 cc.
							Days.			
		0	2	7	12		2	7	12	
I	Control, liver	1.4	3.5	4.2	4.4	1.5	2.0	2.7	2.9	11.8
II	C + $MnCl_2$	1.6	7.0	8.5	9.4	1.5	5.5	7.0	7.9	12.0
III	C + casein	2.5	7.8	9.0	9.1	2.5	5.3	6.5	6.6	15.4
IV	C + casein + $MnCl_2$	2.5	9.3	12.0	12.8	2.5	6.8	9.5	10.3	15.4
V	C + peptone	2.5	6.3	7.4	7.3	2.3	4.0	5.1	5.0	16.6
VI	C + peptone + $MnCl_2$	2.0	10.0	11.9	13.0	2.3	7.7	9.6	11.7	17.0
VII	C + boiled liver	2.6	7.0	8.9	8.8	2.5	4.5	7.4	7.3	23.3
VIII	C + boiled liver + $MnCl_2$	2.4	10.8	14.4	15.5	2.5	8.3	11.9	13.0	23.5

The curve illustrates what an analysis of the figures proves: There is no significant acceleration of the digestion of normally available foreign proteins in the presence of manganous chloride during the early stages of the reaction.

These results are important, since they strongly negate two of the hypotheses presented in the former paper to account for the accelerating action of manganous chloride. If an enzyme were activated from an otherwise inactive zymogen, we should find more rapid digestion of all fractions of the substratum—casein, peptone, and coagulated liver proteins. We should find the same if a coferment gave increased activity to the already active enzymes present. But we find that casein and peptone digest at the same rate and to the same degree whether manganous chloride



Curve II.

is present or not in the liver pulp. The effect, therefore, of the salt cannot be one of activation. On the other hand, these results tend to confirm our original hypothesis, that the action of the salt is wholly upon the liver proteins, converting them into proteins which, like peptone and casein, are readily hydrolyzed by the enzymes already present. It remains to determine the nature of this alteration of the proteins.

In the literature cited in the previous paper, the fact seems well established that the following electrolytes accelerate and increase the degree of autolysis of liver pulp: ferric chloride; ferrous sulphate, oxalate, and chloride; manganous sulphate, acetate, and lactate; lead acetate; the chlorides of platinum, gold; aluminum, and cobalt; and the nitrate of cobalt. We have shown in the previous paper that zinc sulphate accelerates within narrow limits of concentration, and we have confirmed the action of ferric chloride and of other salts of manganese. The latter have been investigated in detail by Mr. Robert Parsons in this laboratory and appear in his Bachelor's thesis. Some of these results are presented in Table V.

TABLE V.

No.	Contents, 1/25 mol.	Days.				Net gain.
		0	1	2	10	
I....	Control	1.4	4.2	4.3	4.5	3.3
II....	Manganous citrate	1.2	5.5	6.6	9.1	7.9
III....	" lactate	1.5	6.0	6.9	8.9	7.4
IV....	" tartrate	1.3	6.2	6.3	8.7	7.4
V....	" ammonium sulphate	9.5	15.0	15.3	16.5	7.0*
VI....	" nitrate	4.3	8.6	9.6	9.7	5.4*
VII....	" phosphate	1.2	3.8	4.5	5.3	3.1
VIII....	" acetate	1.3	4.8	5.4	6.1	4.8

* The high nitrogen level of these digests is due to the nitrogen added in the salt molecule.

While the maximum effects of the soluble salts of manganese may be produced at different concentrations, nevertheless the results as a whole are consistently alike. All of the soluble salts tried exert a profound effect upon the reaction, and under chosen conditions for each salt produce about the same optimum digestion. The nearly insoluble phosphate has little or no effect, while the carbonate inhibits autolysis. The action of these salts, together with those reported in the literature, suggests some common factor which acts upon the liver proteins and renders them available. The metallic ion appears to be one common factor, acidity the other. All of the salts mentioned as accelerating this reaction are electrolytes which hydrolyze in water with the production of free hydrogen ions, and either basic salts of the metals or hydroxides of low dissociating power. These salt solutions are thus all more or less distinctly acid. It is well known that acids accelerate the autolysis of certain tissues—notably the liver—and the single test reported in Table III illustrates this fact. Furthermore, the salts of calcium, sodium, potassium, and magnesium, which are reported in the literature as having no accelerating action on liver autolysis, are all salts which do not develop acidity by solution in water. It seemed probable therefore that the whole phenomenon of accelerating liver autolysis by metallic salts might be referred to their potential acidity. While the concentration of H ions in a 1/10 mol. solution of manganous chloride is exceedingly low, compared with an equimolecular solution of hydrochloric acid, any reaction which fixed the free ions—as by combination with the proteins, for example—would upset the equilibrium of the system and lead to further hydrolysis of the salt with the production of more H ions. In the presence of sufficient excess of the electrolyte this reaction should continue till the proteins become saturated with acid. If hydrochloric acid itself were used the same proteins should become saturated. If now the digestibility of these acid-combined proteins depends upon the combination, we should expect the optimum concentration of HCl in a given digest to give results identical with those produced by the optimum concentration of manganous chloride. That this is the case is shown by the next experiment.

TABLE VI.

Cc. $\frac{N}{5}$ *HCl.*

No.	Contents.	Days.						Total N in 6.25 cc.	Per cent digested in 12 days.
		0	1	3	7	12	48		
I	Control	1.6	4.1	5.6	6.4	6.7	6.7	11.4	58.5
II	1/10 M.MnCl$_2$	1.6	6.0	8.7	9.3	9.9	10.7		86.5
III	1/10 M.HCl	1.4	1.6	1.6	1.6	1.6	1.6	11.5	0.0
IV	1/20 "	1.5	6.8	8.7	9.7	9.9	10.0	11.5	86.5
V	1/50 "	1.6	6.7	8.0	10.0	10.0	10.0	11.3	87.4
VI	1/100 "	1.6	5.0	7.7	9.0	9.1			79.5
VII	1/200 "	1.6	4.7	6.7	7.6	8.0			69.9
VIII	1/500 "	1.6	4.2	5.9	6.5	6.9			60.3
IX	1/1000 "	1.6	4.2	6.2	6.8	6.7			58.5
X	1/2000 "	1.7	4.1	6.2	6.9	7.1			62.0

Average 1.6 Average 11.4

The identity of the results at optimum concentrations of salt and acid indicates an identity of the mechanism. The chloride ion and the acidity are common factors here. With the various salts of manganese, the metallic ion and the acidity were common factors. Chlorides of sodium and potassium have no action. Acidity appears therefore to be the only factor common to all the reagents thus far considered, and it is well known that acids combine with and alter proteins. It should be noted that a small excess of hydrochloric acid is sufficient to destroy the enzyme. Destruction occurs at about the level of H ions indicated by methyl orange and Congo red. At no concentration of the manganous salt is this H ion level approached. We get no marked inhibition therefore by increasing the concentration of the manganous salt far beyond the optimum for the reaction. Indeed, autolysis goes on very well in 1 mol. solutions, or about 20 per cent $MnCl_2 \cdot 4H_2O$.

It is evident that liver proteins are susceptible to alteration at a much lower concentration of H ions than such a protein as ovalbumin, for example. Liver proteins are altered by the acidity of manganous chloride; albumin requires hydrochloric acid itself before it is sufficiently modified to digest. The concentration of the hydrogen ion necessary for the alteration of tissue proteins is obviously of considerable physiological significance, as many different lines of investigation have recently shown. Fluctuations of acidity occur within the cells themselves; they are sensitive to reduced oxidation and react to injury with the production of acid. The blood itself fluctuates normally within limits, and under pathological conditions acidity may increase markedly. Such increased acidity is marked by a shift in the salt balance of the blood and tissue fluids toward an abnormal excess of the acid phosphates and bicarbonates, as has been shown by Henderson.[2] To what acidity the liver and other tissues are sensitive, as determined by increased autolysis, is one of the problems on which we expect to report shortly.

SUMMARY.

1. Casein, peptone, and coagulated liver proteins are digested by the autolytic enzymes of the liver. Their addition gives exactly the same type of curve of acceleration and altered equilibrium as the addition of manganous chloride.
2. The presence of manganous chloride does not lead to an increase in the rate or the extent of digestion of casein or peptone. The action of the salt cannot therefore be due to an activation phenomenon or to action as a coferment leading to greater enzyme activity.

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[2] Henderson, L. J., *Am. Jour. Physiol.*, 1908, xxi, 427.

3. Edestin is not digested by the liver enzymes under normal conditions, but is digested when manganous chloride is present.

4. Ovalbumin is not digested by the liver enzymes, with or without the presence of manganous chloride. It is digested, however, when hydrochloric acid is present.

5. All of the soluble salts of manganese investigated accelerate and increase liver autolysis. The phosphate which is practically insoluble does not. The carbonate, which prevents the formation of the normal acidity of the liver pulp, inhibits autolysis.

6. A low concentration of hydrochloric acid accelerates the autolysis of liver and leads to a shift of equilibrium identical with that induced by the optimum concentration of manganous chloride. The mechanism of this acceleration, and that produced by other electrolytes, is probably the same, the alteration of liver proteins by acids rendering them available substrata in the reaction.

7. A very low concentration of the H ion is sufficient to induce alteration of the indigestible liver proteins and render them digestible. A 1/200 mol. hydrochloric acid solution gives marked acceleration; a 1/100 mol. solution of manganous chloride does the same. While the H ion concentration of the latter is much less than that of the hydrochloric acid, fixation of the ions by proteins leads to further hydrolysis of the salt with the production of more acid.

8. Edestin, like liver proteins, appears to be sensitive to a low acidity. Ovalbumin requires a higher concentration of acid before it is altered sufficiently to be digested by the liver enzymes.

9. It is believed that many of the phenomena of autolysis in the body, such as atrophy, necrosis, involution, etc., will be found to depend upon the mechanism here described. In all such tissues as are altered by a low acid concentration in the same sense as is the liver, the development of such acidity will have two effects: (1) it will remove the inhibitory alkalinity, and (2) alter the proteins with a resulting increase in the mass of substratum. These two factors will lead to autolysis *in vivo* with diminution in size, or complete disappearance of cells, as the case may be.

THE RÔLE OF HALOGENS AS ACCELERATORS OF TISSUE ENZYME ACTION.

By MAX MORSE.

(*From the Department of Physiology, University of Wisconsin, Madison.*)

(Received for publication, June 8, 1915.)

It has been the experience of several workers that iodine and iodine compounds exert a more or less definite effect upon the rate of autolysis in various mammalian organs.

Thus Stookey[1] reported that when potassium iodide was fed to dogs and autolysis studied in the excised livers, an acceleration of rate was demonstrable. Kepinow[2] likewise found acceleration of rate in the case of intravenously injected potassium iodide in rabbits; but when this compound was added to a digest, no acceleration resulted. Iodine, however, when thus added gave evidence of inducing acceleration. Kaschiwabara[3] reviewed the work of Kepinow and after checking up the results in the light of more acceptable methods, subscribed to an acceleration such as the German worker had described. When Lugol's solution was injected intravenously, slight modification of rate was observed. Gideon Wells[4] came to the conclusion that when thyroid tissue was added to a digest of dog liver, no modification of rate was observed.

This observation, along with the others reported in the preceding paragraph was of interest in the studies which the writer[5] has been making upon the subject of atrophy, and has inspired the following experiments.

An examination of the effects of the following reagents has been made:

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[1] Stookey, L. B., *Proc. Soc. Exper. Biol. and Med.*, 1907–09, v–vi, 119.

[2] Kepinow, L., *Biochem. Ztschr.*, 1911, xxxvii, 238.

[3] Kaschiwabara, M., *Ztschr. f. physiol. Chem.*, 1912, lxxx, 45.

[4] Wells, H. G., *Am. Jour. Physiol.*, 1904, xi, 351.

[5] Morse, M., *Proc. Soc. Exper. Biol. and Med.*, 1912, x, 31; 1914, xii, 232; *Jour. Biol. Chem.*, 1914, xix, 421; *Am. Jour. Physiol.*, 1915, xxxvi, 145.

Iodine.
Iodine in potassium iodide.
Potassium iodide.
Thyroid tissue.
Iodized albumin from blood.
Bromine water.

In addition, thyroid was studied in its effects in dogs when fed *per os*.

Iodine.—Merck's resublimed iodine scales were used, these being added to pig liver hash[6] in percentages of from 0.5 to 5 per cent. When the scales were added at room temperature, and again if they were added to different hash at 0°C., no different results were obtained.[7] In percentages of 1 per cent or below, a small acceleration was observed; in larger amounts, distinct inhibition obtained. The effect of adding iodine to a protein digest has not been investigated, as far as I have been able to ascertain.

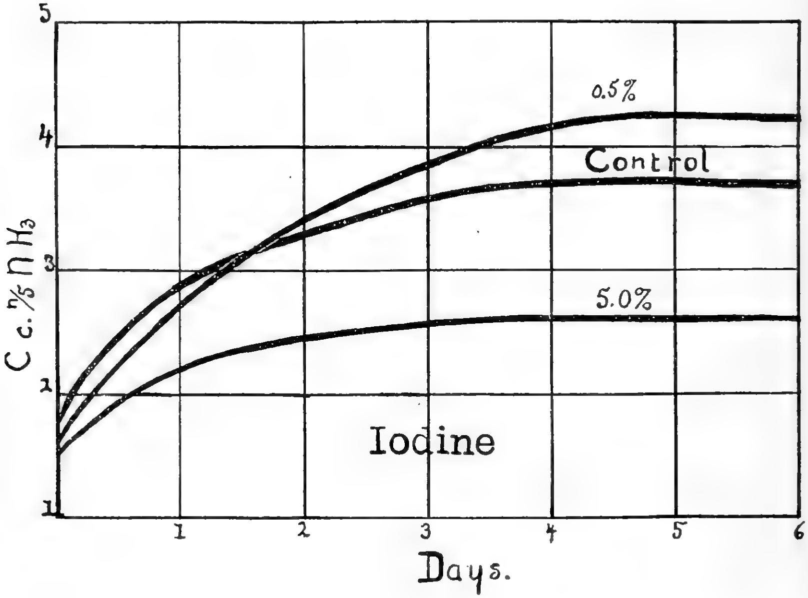

CHART I.

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[6] For the method consult Bradley, H. C., and Morse, M., *Jour. Biol. Chem.*, 1915, xxi, 209.

[7] Oswald, A., *Ztschr. f. physiol. Chem.*, 1909, lix, 321.

Iodine in Potassium Iodide.—When iodine is passed into a solution of potassium iodide in water, the following reversible reaction occurs:

$$I_2 + KI \leftrightarrows KI_3$$

There are, then, three possibilities for effects in Lugol's solution, besides the K·ion. In small amounts, where the calculated iodine content is at or below 1 per cent, a slight acceleration occurs, but in larger amounts inhibition takes place. There is good reason to believe that the same factor is operative here as in the case of the scales, and it is doubtful that any effect can be attributed to the molecular potassium tri-iodide, the molecular potassium iodide, or to the potassium ion.

Potassium Iodide.—In concentrations of KI from 0.2 to 1 per cent, a slight acceleration occurs, while in larger amounts (5 per cent = 3.75 per cent I), inhibition takes place as in the two preceding instances. Here, again, it appears that the modification in rate has to do with the iodine content.

Thyroid Tissue.—Armour's and Parke Davis and Co.'s desiccated thyroids were used as reagents. The rise in tannic acid non-precipitable nitrogen in all strengths of thyroid added to the pig liver hash was marked. A significant feature of the results was that with added percentages of thyroid, a corresponding increase in amount of the soluble nitrogen appeared.

This fact leads to the question whether the apparent acceleration is not due to the amount of added substrate. The same phenomenon obtains in the case of the iodized protein, to be mentioned in the following section. An experiment which to my mind is practically conclusive on the problem is the following:

A dog liver digest had been running in the thermostat until a plateau had been reached. On the fourth day after the equilibrium had been established, thyroid in known quantity was added (0.4 per cent of desiccated thyroid). Immediately a rise in the curve of soluble nitrogen was observed, which continued at uniform acceleration for four days.

In view of this result, and considering the slight effects which have been observed when iodides and iodine alone are used, it seems that the marked rise in thyroid digestion is due to the added substrate, rather than to any true acceleration of autolyzing enzyme. The results, then, would be in harmony with those obtained by Wells[4] upon different material and with different methods.

Iodized Blood Protein.—The commercial "Iodalbim" was used. The capsules were emptied, the contents weighed into the hash and the digest left at the temperature used throughout, 37°C. Here, as in the case of the

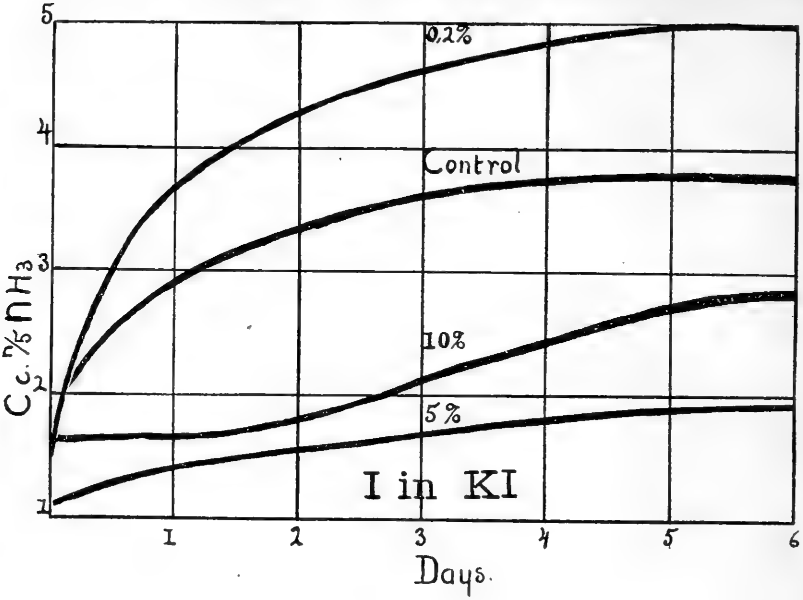

CHART II.

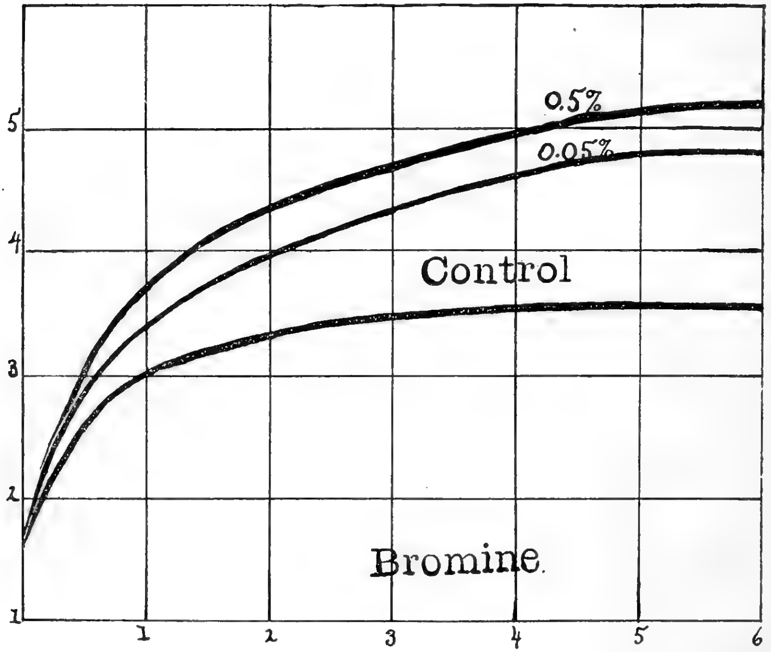

CHART III.

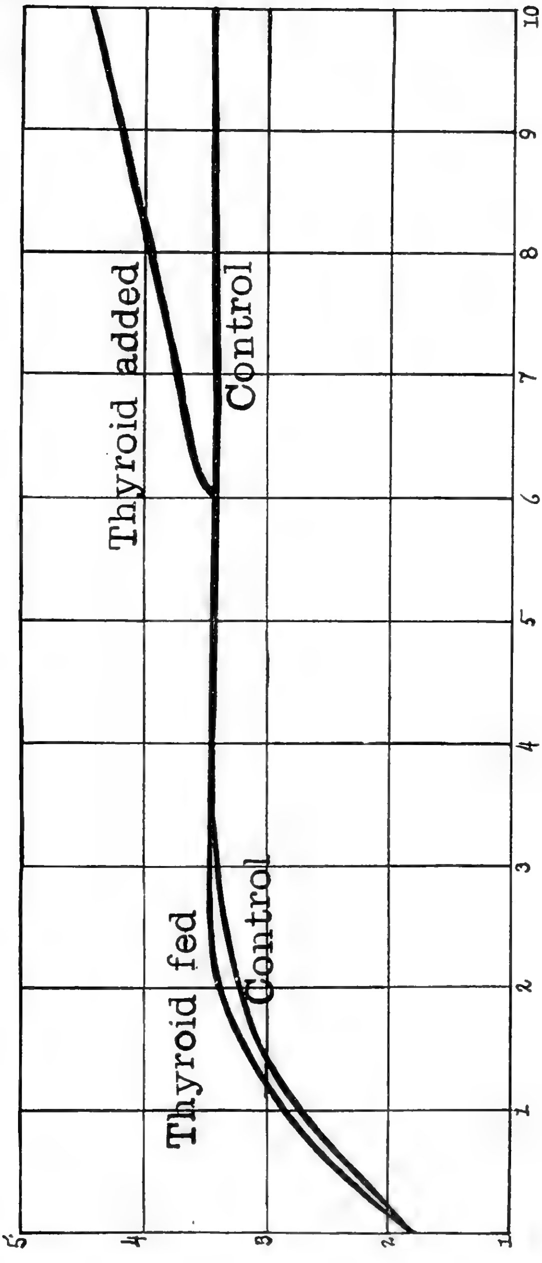
Thyroid added
Control
Thyroid fed
Control
1
2
3
4
5
6
7
8
9
10
1
2
3
4
5

CHART IV.

thyroid, a pseudo-acceleration was observed. Here, too, a concomitant rise in soluble nitrogen took place *pari passu* with the added substrate.

Bromine Water.—Liquid bromine was added in concentrations of from 0.05 to 0.5 per cent of the hash. The acceleration was consistent and varied directly with the concentrations.

Inasmuch as hydrobromic acid is formed when liquid bromine is added to aqueous solutions of proteins, and since increase in acidity seems everywhere to accelerate autolysis, it is to be expected that a true acceleration due to the increase of hydrogen ion concentration takes place here.[8] Hydrogen iodide likewise is formed in small quantities under similar circumstances, and this factor may be operative in the slight accelerations observed with iodine and its compounds; but there seems to be either adsorption or chemical combination of the acid; for the acidity of a protein aqueous solution to which iodine has been added is less than that of an aqueous solution of iodine alone. The well known masking of acid by gastric digests is a similar phenomenon. Iodine added to starch solutions, amino-acids, such as tyrosin, etc., produces hydrogen iodide and the latter also probably forms in the presence of proteins.

Thyroid Fed Per Os.—Two dogs of similar weight were selected and fed 250 gm. of lean meat daily for a period of two weeks. On alternate days 20 gm. of desiccated thyroid or the equivalent of fresh gland calculated for the same iodine content were added to the food of one of the dogs, designated "Experiment," the other being the "Control." At the end of the period the two dogs were simultaneously killed by chloroform, the livers excised as rapidly as possible, ground, meshed, weighed, and otherwise prepared for the digest. The rate of autolysis was followed in the same way in all the experiments of the series. The results show the correspondence to the results of Wells with thyroid added to the hash. There is, in other words, no apparent modification of rate.

CONCLUSIONS.

The following conclusions are warranted:

1. Iodine and its compounds of inorganic nature may slightly, but only slightly, modify the rate of autolysis; this slight modification may be due to the formation of HI in small amounts.

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[8] This suggestion was made to the writer by Dr. H. C. Bradley and is undoubtedly the valid explanation.

2. Organically bound iodine, such as thyroid and iodized blood protein, causes an apparent acceleration of autolysis, but this is doubtless due to increase of available substrate added.

3. Bromine accelerates quite markedly in minute amounts; this is probably due to the increase in hydrogen ion concentration when liquid bromine is added to an aqueous protein solution.

4. The feeding of thyroid for a period of two weeks does not lead to an acceleration of autolysis *in vitro* in dog livers.

5. The results of Wells, Kepinow, and Kaschiwabara are substantiated by the present experiments.

6. It is improbable that the acceleration of atrophy of the frog larva when fed thyroid and blood protein in iodized form can be accounted for on the assumption that there is an increase in rate of autolysis due to the thyroid added.[9]

Protocol of Experiments.

Designation.	Days.				
	0	1	3	5	10
Effect of adding 0.5 per cent iodine scales, 20°C.					
Control	1.7*	2.9	3.1	3.7	3.7
Experiment	1.5	2.7	3.5	4.2	4.2
Effect of adding 1.0 per cent iodine scales, 0°C.					
Control	1.2	3.2	4.2	4.5	5.1
Experiment	0.8	3.0	3.0	3.5	4.1
Effect of adding 5.0 per cent iodine scales, 20°C.					
Control	1.7	2.9	3.1	3.7	3.7
Experiment	1.5	2.0	2.2	2.5	2.5

*Figures denote cc. $\frac{N}{5}$ acid neutralized in the Kjeldahl determination.

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[9] Morse, *Jour. Biol. Chem.*, 1914, xix, 421.

Protocol of Experiments—Concluded.

Designation.	Days.				
	0	1	3	5	10
Effect of adding I in KI in various concentrations.					
Control	2.3	3.7			5.5
Lugol 1 per cent	2.3	3.6			5.7
Lugol 2 per cent	2.3	3.1			5.7
Lugol 5 per cent	2.3	3.0			3.6
Lugol 10 per cent	2.3	2.9			4.5
Control	1.7	2.9	3.1	3.7	3.7
Lugol 0.2 per cent	1.5	3.7	4.3	4.9	5.0
Effect of adding KI in various concentrations.					
Control	1.7	2.9	3.1	3.7	3.7
Potassium iodide 0.1 per cent	1.5	2.7	3.7	4.0	4.0
Potassium iodide 0.7 per cent	1.5	2.4	2.9	3.6	3.6
Potassium iodide 3.7 per cent	1.5	2.2	3.2	3.2	4.2
Effect of adding thyroid in various amounts.					
Control	1.2	3.2	4.2	4.5	5.1
Thyroid, 0.2 per cent	1.8	6.0	6.7	9.8	10.3
Control	1.7	2.9	3.1	3.7	3.7
Thyroid, 1 per cent	1.6	2.7	2.9	3.6	4.6
Thyroid, 5 per cent	2.2	2.5	2.7	5.0	5.9
Effect of adding iodized blood protein.					
Control	1.2	3.2	4.2	4.5	5.1
Iodalbim 0.2 per cent	1.5	4.3	5.4	6.0	6.2
Control	1.7	2.9	3.1	3.7	3.7
Iodalbim 1 per cent	1.7	3.6	4.9		6.9
Effect of feeding thyroid to dogs and also of adding thyroid to a digest after the plateau had been reached.					
Control	1.8	2.2	3.2	3.4	
Dog liver	1.6	2.2	3.4	3.4	
Thyroid added, Control	3.4	3.4			3.6
Thyroid added, Experiment	3.7	3.7			4.4

A METHOD FOR THE DETERMINATION OF "LECITHIN" IN SMALL AMOUNTS OF BLOOD.[1]

By W. R. BLOOR.

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(Received for publication, June 21, 1915.)

In the study of the physiological significance of the fatty substances of the blood, it was found necessary to have a scheme of analysis which would show with a fair degree of accuracy (within 5 per cent) the quantities of the main fatty constituents (fats, cholesterol, and phosphatides) in quantities of blood (2–3 cc.) which could be drawn at frequent intervals from even small animals without noticeably affecting the normal state of the animal. A method for total fat (fatty acids and cholesterol) has already been described.[2] The present work was undertaken to provide a method for the determination of the phosphatide fraction of the blood fat, and is intended to be used with the same alcohol-ether blood extract used for total fat.

During the time that work on this method was in progress no less than three methods have been published which may be used for the determination of the phosphatides of blood. The first of these, by Taylor and Miller,[3] depends on the colorimetric determination of the molybdenum precipitated as phosphomolybdate from a phosphate solution. The standard used contains 0.26 mg. of P (0.82 mg. H_3PO_4) and the results published show a variation from 9 to 20 per cent in the duplicates, the figures being always too high.

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[1] This work was begun in the Laboratories of Biological Chemistry of Washington University, St. Louis. A preliminary report of the method was made before the Northeastern section of the American Chemical Society in Boston, December, 1914.

[2] Bloor, W. R., *Jour. Biol. Chem.*, 1914, xvii, 377.

[3] Taylor, A. E., and Miller, C. W., *ibid.*, 1914, xviii, 215.

The second method is that of Raper[4] which appears to be more accurate for smaller quantities of phosphorus. With known phosphate solutions, variations in duplicates with the smallest amount of phosphorus (0.089 mg.) are about 3 per cent and the variation from the theoretical up to 5 per cent. With lecithin, containing about 0.14 mg. of P, the variation is about 6 per cent. In the Raper method, for the determination of the smaller quantities, it is necessary to add a known amount of phosphate solution in order to bring about complete precipitation.

Both methods involve a double procedure with double chance of loss or mistake and both are dependent on the molybdate precipitation with its admitted possibilities of error, especially where only traces of material are involved. Both appear to be more cumbersome and time-consuming as well as less sensitive than the method to be described.

The last method to appear, that of Greenwald,[5] is, however, apparently very well suited to the determination of phosphatides in small amounts of blood and would, therefore, seem to remove much of the necessity for another method for this purpose. For these reasons it calls for a rather full discussion. The method consists in the application of the ingenious method of Pouget and Chouchak[6] involving the precipitation of the phosphoric acid as the strychnine-molybdic acid double compound. The Pouget-Chouchak reagent is probably the most sensitive reagent for phosphoric acid known and therefore offers an excellent means of making accurate determinations of very minute quantities of phosphoric acid. The determination as carried out by Greenwald in his method seems, however, to be open to criticism on the following points.

First, it is desirable to point out that the method is a turbidity method and not a colorimetric one as it was originally called by Pouget and Chouchak. (Greenwald does not correct this mistake although he does not use the name colorimetric.) The yellowish brown color of thick layers of the solution is the same as that observed in any dense suspension which is of a sufficiently high degree of dispersion and is probably due to the scattering of the

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[4] Raper, H. S., *Biochem. Jour.*, 1914, viii, 649.

[5] Greenwald, I., *Jour. Biol. Chem.*, 1915, xxi, 29.

[6] Pouget, I., and Chouchak, D., *Bull. Soc. chim.*, series 4, 1909, v, 104.

rays of the blue end of the spectrum. The same tint may be observed in various suspensions, for example, silver chloride, fatty acids, soaps, mastic, etc.

The property made use of in turbidity determinations is the effect of the precipitate on light passing through the suspension. The effect is obviously proportional to the amount of substance precipitated only when the nature of the precipitate is the same in both standard and test solutions.

The nature of the precipitate, number and size of particles, their density, etc., is subject to the usual laws of precipitation and is therefore influenced by such factors as the amount of precipitable substance, amount of electrolytes or other foreign material in solution, volume of solution, speed of precipitation, amount of reagent, etc. The first two factors do not appear to have been controlled in Greenwald's method and that they are of importance is indicated by the experiments below.

a. Influence of Amount of Precipitable Substance.—Two suspensions prepared according to the directions, one containing 0.05 mg. H_3PO_4 and the other 0.075 mg., were compared in the colorimeter, the weaker solution being used as standard and set at 81 mm. The readings averaged 49 mm. in place of 54 mm. as would have been the case it the readings had been exactly proportional to the concentration. (All readings reported in this connection were checked by a member of this laboratory who has had experience in turbidity determinations.)

This variation calls for a correction similar to that found necessary in the nephelometric methods.[7] The correction is all the more necessary if the difference between standard and test is extended to double the standard as is claimed to be possible by Greenwald.

b. The Amount of Electrolytes.—Experiments were carried out as follows. As standard 0.05 mg. of H_3PO_4 treated according to Greenwald's directions was used. The test solution contained the same amount of phosphoric acid but contained in addition 0.5 cc. of concentrated sulphuric acid (half the amount used in the actual determination on blood serum) almost neutralized with sodium hydrate made from sodium. The solution was made up otherwise as in the standard. Both acid and alkali were free from anything which gave a precipitate with the reagent. With the standard

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[7] Kober, P. A., *Jour. Biol. Chem.*, 1912–13, xiii, 485. Marriott, W. M., *ibid.*, 1913–14, xvi, 289. Bloor, *ibid.*, 1914, xvii, 383.

set at 80 mm., the test solution readings averaged 65 mm. Since the amount of phosphate was the same in both, the difference could only have been due to the effect of the electrolyte on the nature of the precipitate. This fault of the method, while serious, may be easily remedied by adjusting the salt content of the standard to match that of the test as is done in the method to be described.

The precipitate produced by the reagent with phosphate has a slight inherent yellowish color which is enhanced by the length of column used and which makes the readings of even slightly different solutions difficult for the beginner. Even for the experienced person the limits within which accurate readings may be made seem to be considerably narrower than those given by Greenwald ("from a concentration of two-thirds to over twice the standard"). For the same reason more emphasis should be placed on the difficulty of driving off the picric acid. The great bulk goes off easily enough but the removal of the last traces requires time and care, otherwise enough will be left to give the solution a distinct yellow color.

Other minor objections to the method as described, which tend to limit its extended application, are the use of an uncommon size of colorimeter; also the great length of column, which is inconvenient with the ordinary narrow colorimeter cups, since it does not allow sufficient adaptability. Most of these objections, however, can be overcome and when these limitations are taken into account the method appears to offer an excellent means for determining traces of phosphoric acid in various substances.[8]

The work on the present method was completed before Greenwald's paper appeared. It presents a new and sensitive method for the precipitation of phosphoric acid, which while not as deli-

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[8] It is only fair to state that in view of the fact that silica is often a serious disturbing element in micro determinations of phosphoric acid (Schreiner, O., and Failyer, G. H., *U. S. Dept. Agriculture, Bureau of Soils, Bull. 31,* 1906) and since sodium hydrate, even that prepared from sodium, very soon becomes contaminated with silica when kept in glass, also since the digestion with sulphuric acid quickly etches the glass beads, an examination was made to learn the effects of silica on this determination. It was found that while silica does produce a precipitate with the strychnine molybdate reagent, the amount required was far greater than could possibly be present in the determination and that therefore the presence of silica need not be considered.

cate as the strychnine molybdate precipitation, is still adequate for the purpose and has the advantage that the turbidities produced are very easily compared. A different instrument (the nephelometer) is used. The treatment of the blood in this method is believed to be especially well suited to the purpose, since it gives an adequate extraction without being severe enough to harm the phosphatides. Moreover, the bulk of the organic material of the blood is left behind in the precipitate, which renders the ashing of the phosphorus-containing material easy and rapid. Also, the extract may be used for determining the other fatty constituents.

In view of these facts it is believed that there is still a place for the method and that it will be found useful for the determination of phosphoric acid in various organic materials.

The Method.

The method consists in the extraction of the phosphatides from the blood or serum by treatment with warm alcohol-ether and the determination of their amount by precipitation of the phosphoric acid after ashing, as the silver salt (or the silver ammonium salt). The phosphate is precipitated by silver nitrate in faintly alkaline solution in the presence of ammonium salts and the amount of precipitate is measured by the nephelometer. The procedure is as follows:

a. Preparation of the Phosphatide Extract.—Three cc. of blood are drawn directly from the vein by means of a needle connected with a pipette (containing a little powdered oxalate) by a short length of rubber tubing. It is run slowly (a slow stream of drops) into a 100 cc. graduated flask containing 70–80 cc. of a mixture of three parts of alcohol and one part of ether (both redistilled), which is constantly shaken. Fresh plasma or serum may be used in the same way. The mixture is slowly raised to boiling in the water bath, being kept constantly in motion to prevent superheating, then quickly cooled to room temperature, made up to volume with alcohol-ether, mixed, and filtered. The extract should be clear and practically colorless and when well stoppered will keep for several months.

b. The Digestion.—From 10–25 cc. of the extract (containing about 1.2 mg. of "lecithin" or 0.15 mg. of H_3PO_4) are measured with a pipette into a 200 x 25 mm. Jena test-tube, two or three glass beads of about 3 mm. diameter added, and the solution is evaporated to dryness by immersion of the tube in the water bath. It should be watched and shaken frequently until boiling has begun, after which the evaporation will generally proceed to dryness without further attention. The material in the tube should be dried for about fifteen minutes in the water bath to remove traces of alcohol, which would interfere with the completeness of the subsequent oxidation. The oxidation is essentially Neumann's procedure on a small scale. 1.5 cc. of a mixture of equal parts of concentrated sulphuric and nitric acids are added to the tubes and the whole is digested by heating with a micro burner in the hood or with a Folin draught apparatus.[9] The heating is done in two stages. During the first the mixture is gently boiled with a low flame until the red fumes have ceased to come off. This should take about five minutes. The tubes should be inclined at an angle of about 30° to prevent spattering. The heat is then gradually increased until the nitric acid is completely driven off, after which the sulphuric acid is boiled for eight to ten minutes. The mixture is then cooled somewhat and two drops of 0.25 per cent cane-sugar solution are added. The heating is resumed for one minute, after which the tube is cooled and 3 cc. of water are added. The object of the treatment with the sugar is to remove traces of HNO_3 which are in part combined with the phosphoric acid and which prevent its complete precipitation (see discussion below).

c. The Determination.—A drop of phenolphthalein (0.3 per cent) is added to the solution and it is exactly neutralized, the process being conveniently accomplished as follows. 20 per cent NaOH (free from chlorides and containing but little carbonate) is added to neutralization, noting the amount added, $\frac{N}{2}$ sulphuric added until just acid, the solution cooled, and $\frac{N}{10}$ NaOH added by drops until it is just alkaline again. The solutions are conveniently kept in burettes, and need only be approximate. 1 cc. of 10 per cent $(NH_4)_2SO_4$ and 1.5 cc. of $\frac{N}{10}$ NaOH are added and the

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[9] Folin, O., and Denis, W., *Jour. Biol. Chem.*, 1911–12, xi, 503.

solution is made to 10 cc. (indicated with sufficient accuracy by a scratch on the tube).

A standard phosphate solution is similarly prepared as follows. Three cc. of a phosphate solution,[10] containing 0.15 mg. of H_3PO_4, are measured into a similar Jena test-tube, one drop of phenolphthalein is added and then the amount of 20 per cent NaOH that was added to the test solution is run in. Concentrated sulphuric acid is added to neutralization, the excess of sulphuric removed by a drop or two of the strong alkali, the solution cooled, and then exactly neutralized as above. 1 cc. of 10 per cent $(NH_4)_2SO_4$ and 1.5 cc. $\frac{N}{10}$ NaOH are added and the solution is made up to the 10 cc. mark on the tube. Two samples of 10 cc. each of 1.5 per cent neutralized $AgNO_3$ are measured into 25 cc. glass-stoppered graduated flasks and the standard and test solutions in the test-tubes are added through a funnel with the stem drawn out so that the 10 cc. are delivered in about fifteen seconds. The liquid in the flasks is gently rotated while the phosphate solution is being run in, after which the test-tubes are rinsed out with small amounts of distilled water, and the rinse water is run in through the funnel. Finally the liquid in the flask is brought up to the mark by rinsing the funnel with distilled water, the whole well mixed, and readings are made in the nephelometer. Chlorides must of course be rigidly excluded—especially after the acid digestion—which is accomplished by using chlorine-free reagents and by the liberal use of good distilled water.

For the comparison, the two nephelometer tubes, after being rinsed with the solutions, are filled to the same height (the meniscus slightly above the edge of the dark strip) and placed in the nephelometer with the standard tube always on the same side. The movable jacket on the standard tube is set at a convenient point (50 mm. in the original Richards' instrument, 30 mm. in the modified colorimeter to be described in the succeeding article) and comparisons are made by adjusting the jacket on the test solution until the images of the two solutions show equal illumination. Not less than five readings are taken, alternately from

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[10] The phosphate solution is prepared by dissolving 0.6942 gm. of monopotassium phosphate (0.5 gm. H_3PO_4) in one liter of water. This solution is diluted ten times for use and when so diluted 1 cc. contains 0.05 mg. H_3PO_4.

above and below, and the average is taken as the reading. The values are inversely proportional to the readings and calculations are made in the same way as in the colorimetric methods.

DISCUSSION OF THE PROCEDURE.

The Extraction.

The above simple procedure has been found to give a good extraction of the fatty substances from blood. Running the fresh blood (or serum or plasma) *slowly* into the great excess of solvent which is kept in constant motion causes the precipitation of the blood proteins in finely divided flocculent form—in excellent condition for extraction. The solvent combines the penetrating power of the alcohol with the greater solvent power of the ether. Under these conditions the short heating is adequate to extract all but the most difficultly extractable fatty material from the whole blood and, as will be shown later, to extract the lipoids completely from serum or plasma. Moreover, the above relatively gentle treatment is believed not to decompose the phosphatides appreciably. The extract may be used for the determination of fatty acids and cholesterol also.

Inorganic Phosphates.

Since inorganic phosphates are soluble in the alcohol-ether mixture in small quantities it was necessary to examine the possibility of contamination of the phosphatide extract with these substances. Taylor and Miller[11] have reported that serum contains only traces of inorganic phosphates, "not enough to give a clear qualitative test," thus confirming the investigation of Gürber[12] who made a similar finding on the basis of dialysis experiments. Greenwald[13] finds considerable "acid-soluble" phosphate in blood serum and thinks that the main source of this must be inorganic phosphate. In order to test out this point in its relation to our method the following experiments were made.

[11] A. E. Taylor and C. W. Miller, *loc. cit.*

[12] Gürber, A., *Verhandl. d. phys.-med. Gesellsch. z. Würzburg*, 1894–95, xxviii, 129.

[13] Greenwald, *Jour. Biol. Chem.*, 1915, xxi, 29.

1. Control with Known Phosphate Solution.—3 cc. (0.15 mg. H_3PO_4) of phosphate solution were mixed with 15 cc. of the alcohol-ether and 7 cc. of ether and washed by shaking one minute with 75 cc. of saturated ammonium sulphate. The alcohol-ether was separated and the phosphate remaining in it determined. Only a trace (estimated at 0.02 mg.) was left in the alcohol-ether, showing that this treatment was adequate to remove water-soluble phosphates.

2. 15 cc. of alcohol-ether blood extract (containing 0.187 mg. of combined phosphoric acid) and 7 cc. of ether were washed by shaking for one minute with 75 cc. of saturated ammonium sulphate as above. Practically all the phosphoric acid (0.180 mg.) was recovered from the alcohol-ether extract. The slightly lower value after the washing may well have been due to the washing out of some of the lecithin. Other experiments gave essentially the same results and the conclusion seems justified that no appreciable amount of water-soluble phosphates is extracted during the alcohol-ether treatment of the blood. If present in the blood they are carried out by the protein precipitate.

The Digestion.

The digestion is essentially that of the Neumann method, very much shortened owing to the small amount of fatty material to be dealt with and the relatively large amount of Neumann's mixture used. It has been found necessary to heat the digestion mixture for a few minutes after it has become colorless, in order to secure quantitative results. A total heating of fifteen minutes is found to be sufficient—a great contrast to the slow and tedious process of oxidation of fatty substances by the original Neumann procedure. The heating is carried on slowly at first, otherwise the nitric acid is driven off before the oxidation is completed. During the heating, and especially if the amount of material to be oxidized is small, some sort of combination appears to take place between a part of the nitric and phosphoric acids, which so changes the phosphoric acid that it no longer precipitates with the reagent. This fact was apparently known to Neumann,[14] for in his procedure he directs that after the digestion the acid mixture be diluted with water and boiled for five to ten minutes to get rid of the combined nitric acid. Greenwald[15] found that in using the Neumann method on blood it was not always possible to get a complete precipitation of phosphomolybdate and recommended a modification.

[14] Neumann, A., *Ztschr. f. physiol. Chem.*, 1902–03, xxxvii, 115.

[15] Greenwald, *Jour. Biol. Chem.*, 1913, xiv, 369.

The action of nitric acid may be demonstrated with pure phosphates. After heating with sulphuric and nitric acids as in the method only about two-thirds or less of the phosphate may be precipitated by the silver reagent. Heating with sulphuric acid alone does not produce this effect. It was found that the addition after the heating of a very little organic material such as cane-sugar removed the interfering substance and allowed complete precipitation. The amount needed is very small and the slight charring produced by its addition to the acid mixture should disappear promptly on raising to boiling. If too much is added a permanent brown color is produced which must be removed by a drop of HNO_3 and short boiling. Strangely enough the addition of HNO_3 at this time does not interfere with the precipitation.

The Precipitation.

Since the total light reflected from a given depth of suspension depends not only on the number of reflecting particles but also on their other properties such as size, density, etc., it is obvious that conditions should be secured in which the condition of the particles in the standard and the test solutions is the same. For this reason it has been found necessary to make the physical and chemical conditions—as regards concentration of reagents, salts, reaction of the solution, temperature, volume, etc.—as nearly the same in standard and test as is conveniently possible. The procedure as outlined above is believed to satisfy these requirements. The standard contains the same amount of sodium sulphate and ammonium sulphate, is adjusted to the same reaction and volume, and is added to the precipitating solution at the same rate as the test and both solutions are at room temperature. Running the phosphate solution into the precipitant has been found to give more constant results than the reverse process.

Readings may be made at once after mixing the solutions and the relation between the solutions does not appear to change in the course of a half hour.

The Reagents.

All reagents must be free of more than traces of chlorine. A blank determination with the reagents should give no cloudiness with the silver reagent when mixed and only a trace on standing.

The sodium hydrate is best made from sodium but a sufficiently pure sample of ordinary hydrate may sometimes be obtained. It should contain but little carbonate since this interferes with the neutralization. It is not difficult to get ammonium sulphate or sulphuric acid which are chlorine-free. The silver nitrate solution should be neutral. The ordinary salt is generally slightly acid and it is necessary in all cases after making up the solution, to add a drop or two of alkali, mix thoroughly, and let stand for a short time. After filtering off the excess of precipitate the solution is ready for use.

Chlorides present in the blood extract or in the acids used for ashing (or purposely added) are driven off during the digestion.

The Nephelometer.

The Richards type of nephelometer was used throughout. The preliminary work was done with the original form of the nephelometer,[16] the later part with one made by a simple modification of the Duboscq colorimeter which is described in the following paper. The superior optical equipment of the Duboscq colorimeter makes it possible to obtain more accurate readings with greater ease than in the original Richards instrument. Since the readings obtained from different solutions are not exactly proportional to the amount of phosphate present and since the differences between the observed and theoretical reading increase as the differences in phosphate content increase it is necessary to calibrate the instrument for different strengths of test solution and for different standards, although where the test solution does not differ from the standard by more than 25 per cent the corrections fall within the limit of error of the determination and no correction can be made.

Solutions very different from the standard are difficult to compare and readings below 18 mm. or about 45 mm. when the standard is at 30 mm. should not be attempted except for approximate values.

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[16] Richards, T. W., *Ztschr. f. anorg. Chem.*, 1895, viii, 269. Richards, T. W., and Wells, R. C., *Am. Chem. Jour.*, 1904, xxxi, 235.

Results.

Known phosphate solutions may be determined with an accuracy of about 2 per cent. Duplicate determinations on blood frequently agree as well as the above but the average for determinations of blood lecithin is about 5 per cent. The following results on phosphate determinations in various organic materials indicate the possibilities of the method.

	per cent	
Phosphates in urine.		
A sample of dilute day urine.		
By uranium titration	0.0372	H_3PO_4
By nephelometric method	0.0366	
	0.0380	
In casein.		
A sample of purified casein (Bosworth).		
By Neumann method	2.21	H_3PO_4
By nephelometric method	2.20	
	2.15	
Lecithin.		
a. A sample of impure egg "lecithin."		
Direct determination by Neumann's method	7.98	H_3PO_4
By nephelometric method	7.89	H_3PO_4
b. Purified egg "lecithin."		
By Neumann's method	13.1	H_3PO_4
By nephelometric method	12.9	
	13.3	
Egg yolk. Total phosphoric acid.		
By Neumann's method	1.66	
By nephelometric method	1.70	
	1.71	

"Lecithin" in blood.

A sufficient variety of determinations of lecithin in blood to warrant discussion has not yet been made but the values obtained are somewhat higher than ordinarily given for blood lecithin, especially for dog blood. The values obtained expressed as "lecithin" ($H_3PO_4 \times 8$) are:

	per cent
Dog I. Whole blood varied from	0.36–0.42
Dog II. Whole blood	0.4
Plasma	0.33
Normal human plasma, oxalated, Sample I	0.28
Sample II	0.21
Cat plasma, oxalated	0.18
Sheep blood, whole	0.22
Plasma	0.16
Calf plasma	0.12

A SIMPLE METHOD OF CONVERTING THE DUBOSCQ COLORIMETER INTO A NEPHELOMETER.[1]

By W. R. BLOOR.

(From the Laboratories of Biological Chemistry of the Harvard Medical School, Boston.)

(Received for publication, June 21, 1915.)

Nephelometry, the determination of the quantity of a substance by measurement of the density of the precipitate which it produces with a reagent, has come into considerable use in the last year or two because of the increasing demand for methods suitable for accurate determination of small amounts of material where other methods for micro determination are not available. Also, for the same reason, the demand for these methods is bound to increase as their possibilities become known. Two forms of nephelometer are in use at the present time, one the Richards[2] instrument, which was the original type, and the other an instrument devised by Kober[3] by modification of the Duboscq colorimeter.

The Richards nephelometer, because of its poor optical and mechanical equipment, is inconvenient and requires experience to use successfully. Moreover there is an objection to the unnecessary multiplication of instruments especially in a time of rapid development, when an instrument may be quickly superseded by improved types or by better devices for the same purpose.

The Kober instrument is an improvement on the Richards in that it renders available for nephelometry the excellent optical and mechanical equipment of the Duboscq colorimeter. Its disadvantage is the use of coated plungers dipping into the solutions.

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[1] This instrument was demonstrated to the Northeastern section of the American Chemical Society at Boston, December, 1914.

[2] Richards, T. W., *Ztschr. f. anorg. Chem.*, 1895, viii, 269. Richards, T. W., and Wells, R. C., *Am. Chem. Jour.*, 1904, xxxi, 235.

[3] Kober, P. A., *Jour. Biol. Chem.*, 1912–13, xiii, 485; *Jour. Am. Chem. Soc.*, 1913, xxxv, 1585.

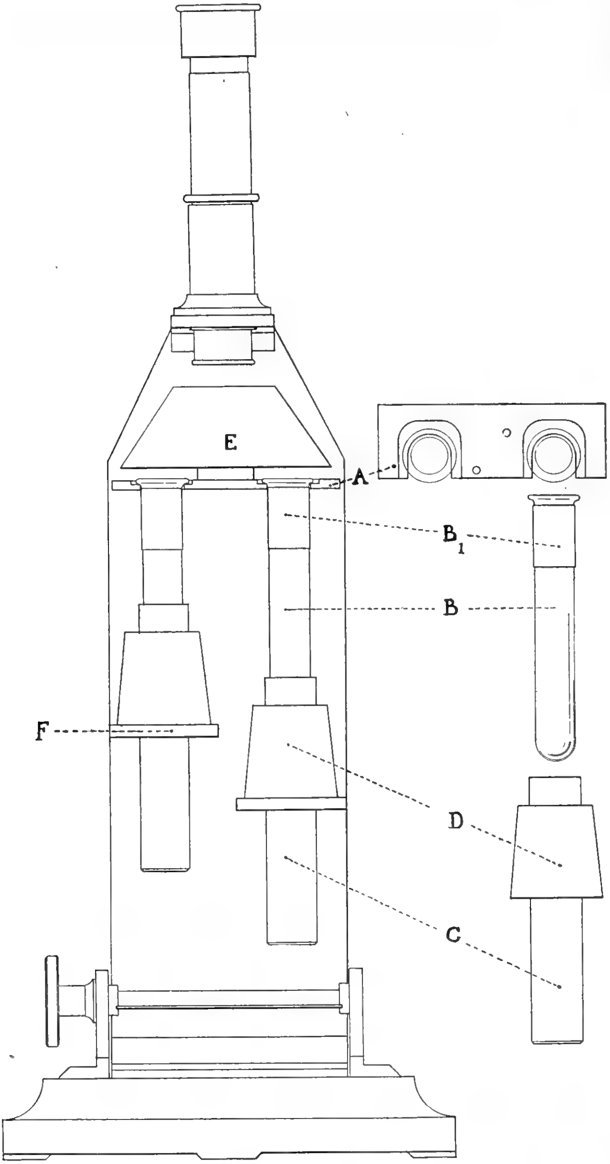

Chart I. The Duboscq colorimeter converted into a nephelometer, with the necessary extra parts.

Where the amount of substance to be determined is minute the presence of a foreign body like the plunger is always a menace because of the possible contamination with interfering substances from which it is difficult to keep the plungers free. The necessity of coating the plungers with something to exclude light rays increases the difficulty and provides a new source of trouble in that the coating is generally more or less soluble in some of the solutions used. Also from its liability to crack and scale it is apt to introduce unexpected errors. The objections mentioned above are emphasized when it is necessary to work with precipitants sensitive to substances like chlorides, which are present everywhere. The Richards type of instrument with sliding jackets does away with these sources of contamination and error, and the instrument described below is intended to combine the advantages of the Richards type of instrument with the superior mechanical equipment of the Duboscq colorimeter. It has been found to give satisfactory results even in the hands of beginners. The details of the instrument and the method of transformation from the colorimeter may be seen from the diagrams.

The brass plate carrying the colorimeter plungers is replaced by the plate A with two slots in which are supported the nephelometer tubes B with their flanges resting on the edges of the slots. The slots are so cut that the center lines of the tubes are exactly in line with the centers of the lower openings of the prism case E. If desired they may be countersunk to receive the flanges.

The colorimeter cups are replaced by the jackets C which project through the holes in the cup supports F and are supported on them by the collars D. They move when the cup supports move. The mirror is turned to the horizontal position so that it reflects no light; the light in the nephelometer comes from in front and not from below (see diagram, Figure II).

The nephelometer tubes are small test-tubes 100x15 mm., preferably made from the same sample of colorless glass tubing so that they are of exactly the same bore. The flanges at the top should be well made so that the tubes rest firmly and evenly in the slots. The glass should be as free as possible from imperfections or striations. After the tubes are made and fitted into place the jackets are moved up on each tube by means of the rack and pinion until the indicator on the scale is exactly at zero. Marks are made on each tube at the point reached by the top of the jacket and the portion of tube above that point is made opaque by a ring B_1 of black paper or paint. Tubes and jackets are then marked right and left and always used on the same side.

Since it is rare to find two tubes which when filled with the same solution give exactly the same readings it is necessary to take this fact into account and correct accordingly.

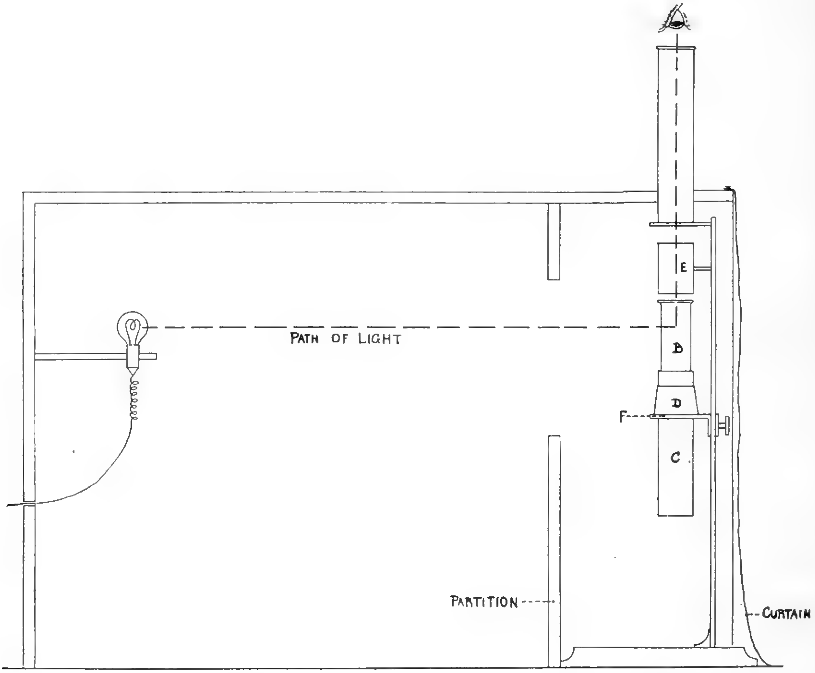

CHART II. The nephelometer in position in its box, showing its relation to the source of light.

The jackets C are made of tubing—metal or glass—a little larger than the tubes and about the same length (they should just clear the mirror when it is turned horizontal), closed at the bottom and made light tight by black paint or paper. The collars D supporting the jackets may be made of cork or more permanently of metal. A little cotton wool in the bottom of the jackets will prevent breakage if the tubes should fall into the jackets.

The openings in the prism case, particularly the lower ones, should be protected against accidental splashing by thin glass plates (thick cover slips) which are held in place by a little glue.

Artificial light is necessary and the lamp should be enclosed in a tight box into one end of which the nephelometer fits snugly. A partition extending part way up the box as shown in the diagram (Figure II) serves the double purpose of shutting off the light from the lower part of the instrument and of providing a stop against which the instrument is pushed, so that its distance from the light is kept constant. The box is conveniently made without a bottom and the end closed with a dark curtain after the nephelometer is pushed into place. The inside of the box should be painted black. A dark room is desirable but not necessary, as the instrument may be used satisfactorily in a room darkened by a dark shade or even in a dark corner of the laboratory.

The relations of the nephelometer and the light source may be seen in the diagram, Figure II. The lamp used is an ordinary 50 watt tungsten ('Mazda') supported by a bracket about 30 cm. from the nephelometer and at the height of the nephelometer tubes. The change from one instrument to the other can be made in one or two minutes, since it consists essentially only in unscrewing the brass plate carrying the plungers and screwing on the plate to carry the nephelometer tubes. The extra parts needed, plate, tubes, and jackets, are few and can be made if necessary from material at hand in any laboratory and by anyone with a slight degree of mechanical skill.[4]

The above description applies only to the later type of colorimeter where the cups move and the prisms are stationary. The changes required to convert the older type of instrument are more complicated and scarcely to be advised unless the instrument is to have fairly continuous use as a nephelometer. If the change is desired the nephelometer tubes are to be supported in the same way as above, but the jackets must be carried on special brackets which are made to replace the brackets carrying the plungers. The *nephelometer tubes must be stationary*, the jackets being the movable parts.

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[4] The extra parts necessary for the conversion of the colorimeter into the nephelometer may be obtained from the International Instrument Co. of Cambridge, Mass.

THE DETERMINATION OF IODINE IN THE PRESENCE OF ORGANIC MATTER.

By ROBERT B. KRAUSS.

(From the Henry Phipps Institute of the University of Pennsylvania, Philadelphia.)

(Received for publication, June 19, 1915.)

The quantitative determination of iodine in organic tissues, especially the iodine content of tuberculous tissues,[1] has occupied our attention in this laboratory for some time. Various methods have been proposed for such determination, those of Baumann,[2] Bourcet,[3] Hunter,[4] and Kendall[5] having attracted the most attention.

The Baumann method has been modified repeatedly and while it no doubt gives good results in experienced hands, the modifications indicate the dissatisfaction that has been felt with it. This method is not delicate enough for our particular purpose.

That of Bourcet requires large amounts of material (100 grams) and involves a subsequent liberation of the small amounts of iodine present. The first requirement it is seldom possible to fulfill, while the second is always a rather unsatisfactory procedure.

The method of Hunter has a serious drawback in the formation of oxychlorine compounds as pointed out by Foerster and Jorre[6] as well as Kendall.

Kendall's method has, in the past, in this laboratory given results that were generally satisfactory, but we have occasionally had cases in important series that gave blanks when the presence of iodine was certain, and on the other hand found traces which it seemed reasonable to believe were not present in the sample.

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[1] Lewis, P. A., and Krauss, R. B., *Jour. Biol. Chem.*, 1914, xviii, 313.

[2] Baumann, E., and Roos, E., *Ztschr. f. physiol. Chem.*, 1895–96, xxi, 489.

[3] Bourcet, P., *Compt. rend. Acad. d. sc.*, 1899, cxxviii, 1120.

[4] Hunter, A., *Jour. Biol. Chem.*, 1909–10, vii, 321.

[5] Kendall, E. C., *Jour. Am. Chem. Soc.*, 1912, xxxiv, 894.

[6] Foerster, F., and Jorre, F., *Jour. f. prakt. Chem.*, 1899, lix, 53.

To cover all contingencies it would become necessary to have a method filling the following requirements:

1. To be capable of detecting most minute traces of iodine.
2. To use a minimum of sample for the analysis.
3. To introduce no iodates or iodides.
4. To eliminate foreign active halogens as reagents.
5. To be rapid and of general application.

In the following pages I wish to present a method I have developed which seems to meet these requirements in greater measure than any of those which have been used previously.

The Palladium Iodide Colorimetric Method.

It is known that the precipitation of palladous iodide affords a method of separation of iodine from bromine and chlorine.

Palladous iodide is insoluble in water, alcohol, acetone, and ether; insoluble also in dilute hydrochloric and hydriodic acids. This method is based on the fact that palladous chloride when added in the cold to a dilute solution of iodide gives a deep brown color due to the formation of palladous iodide.

The value of iodine to which this color is due is approximated by comparison with a standard, and obtained absolutely by comparison in a Duboscq colorimeter. A change of color due to 0.01 mg. of iodine as iodide, is easily seen with the naked eye and the colorimeter gives a further range of 1 to 100.

The determinations are usually made in a dilution of 100 cc. This corresponds to 0.0001 mg. per 1 cc. It will therefore be seen that by working in small volumes such traces may be easily detected. The method of procedure follows.

Detailed Description of Method.

The amount of substance to be taken varies with the chemical properties of the compound, especially the iodine content. For thyroids running about 2 mg. per gram 0.5 gram of dry substance is sufficient. Larger amounts offer no disadvantage; the iodine is then determined in an aliquot portion.

The substance is mixed in a nickel crucible with about 8 grams of a mixture of 1 mol. each of pure anhydrous sodium carbonate, potassium carbonate, and potassium nitrate; and then covered with an additional 5 grams. The lid is placed on the crucible, leaving a small opening for the escape of waste gases.

The crucible is heated in a crucible muffle such as described by me in connection with the analysis of iodine trypan red dyes.[7] Such a one keeps the waste gases from the crucible but gives an even heat on the sides and the bottom. As soon as the contents of the crucible cease burning the heat may be increased until the edges of the mass just begin to melt, but not higher. By this time all carbon has disappeared and on removing the crucible and cooling, the contents are nearly always pure white. This requires about ten minutes. I have found that a moderately rapid burning is desirable, which is also the opinion of Hunter[8] and Oswald.[9] On cooling, water is added to the melt, the lid washed, and the crucible is placed on the hot plate. As soon as the contents have dissolved the liquid is carefully poured through a funnel into a 200 cc. Erlenmeyer flask (using larger flasks for larger melts), washing the crucible carefully with a fine-jet wash bottle. It is desirable here to keep down the volume of water as far as possible. In order to reduce any trace of iodate that may have been formed about 2 cc. of a 10 per cent solution of bisulphite are now added, and phosphoric acid (85 per cent) is carefully added to the inclined flask which is agitated between additions. A slight excess of acid should be used. A small corner of litmus paper is a convenient indicator. After standing for a few minutes, sufficient sodium carbonate is added from the tip of a spatula to make the solution alkaline.

An inclined funnel is placed in the neck of the flask to prevent splashing and the solution evaporated on a hot plate to the stage where it just remains liquid when cold.

On cooling, about 20 cc. of acetone are added from a wash bottle, washing the funnel and sides of the flask.

The flask is shaken until the salts form a semi-solid layer at the bottom. Absolute alcohol is added in 5 cc. portions with agitation and the extraction of the iodide aided by working the salts with a stirring rod, which is then used as an aid in decanting the acetone-alcohol mixture.

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[7] Krauss, R. B., *Jour. Am. Chem. Soc.*, 1914, xxxvi, 962.

[8] Hunter, *loc. cit.*

[9] Oswald, A., *Ztschr. f. physiol. Chem.*, 1899, xxvii, 31.

The use of acetone first seems to favor the complete extraction of the iodides.

By the time 40 cc. of solvents have been used the iodide is nearly always removed. Confirmation may be obtained by adding a drop or two of standard palladous chloride solution. If the solution remains colorless all the iodine has been extracted.

The solvent is decanted through a filter directly into a Nessler tube if a quantity of iodine below 1 mg. is involved; otherwise into a graduated flask, and diluted to definite volume with alcohol. In the latter case an aliquot portion is used for the estimation in the Nessler tube.

The following dilutions are best suited for accurate determinations.

Above 10 mg.	Dilute with water to 1 liter and determine iodide in an aliquot.
1 –10 mg.	Dilute to 500 cc. with water and determine iodide in an aliquot.
0.1 – 1 mg.	Filter directly into 100 cc. tubes.
0.001– 0.1 mg.	Evaporate if necessary and determine in 50 cc. tubes. In smaller volumes, 2 to 5 cc., it is possible to detect and estimate amounts of iodine as low as 0.0001 mg.

Dilute palladous chloride solution, 0.0005 gram per cc., acidulated slightly with hydrochloric acid, is added drop by drop, until the color no longer deepens. Of such a solution 2 cc. are sufficient to combine with about 1.5 mg. of iodine. Of the same solution 2 cc. diluted to 100 cc. give no perceptible color.

The standards are prepared as follows.

Standards.

A solution of pure potassium iodide containing 0.1 mg. per cc. is prepared by weighing out 1.31 grams of potassium iodide, making up to 1 liter, and diluting 100 cc. of this solution to 1 liter.

The requisite standard for any weight iodine, say 0.1 mg., is made up by measuring out 1 cc. of the standard iodine solution in a 1 cc. pipette calibrated to 0.01 cc. into a 100 cc. Nessler tube, diluting with 10 cc. each of alcohol and acetone and adding about 1 cc. of standard palladous chloride solution, then diluting with water to the mark. According to the weight of iodine present the color ranges from a light brown to a deep red-brown. The comparison is made after the tubes have stood at least five minutes.

Standards should be made up with approximately the same ratios of acetone, alcohol, and water as the unknown.

The colors develop best in more concentrated solutions; therefore the palladous chloride is added before diluting.

The unknown is compared with a series of standards; the nearest match is determined by inspection. A difference of 0.01 mg. of iodine is easily seen with the naked eye and is sufficient for ordinary work.

For better results the unknown and the nearest standard are compared in a Duboscq colorimeter which has a further range of 1 to 100.

I have devised an electrolytic procedure by means of which the palladous iodide precipitated in the colorimetric method is electrolyzed; the palladium and iodine are deposited on separate electrodes and weighed.

This forms a good check on a series of colorimetric determinations involving small weights of iodine per determination and gives good results on single determinations involving larger weights of iodine. It also gives accurate results when used for direct estimations.

The details of the electrolytic determination of iodine in organic matter will be reported shortly.

The following tables of analyses will give some information as to the results obtained by this method.

In Table I are given results obtained by fusing 0.5 gram of pure casein with the stated weight of iodine as potassium iodide, obtained by pipetting from a standard iodide solution; and finally determining the iodine by this method.

In Table II are given results of analyses on a commercial thyroid preparation marked 0.2 per cent iodine, giving a comparison between Kendall's method and the palladous iodide method. 0.5 gram samples were used and the results are expressed in mg. per gram.

Table III contains results of analyses on three samples of commercial thyroid preparations. Results are expressed in mg. of iodine per gram of sample.

TABLE I.

No.	Casein.	Iodine present.	Iodine found.	Dilution.
	gm.	*mg.*	*mg.*	*cc.*
1	0.5	——	——	10
2	0.5	——	——	10
3	0.5	0.001	0.001	10
4	0.5	0.005	0.005	10
5	0.5	0.010	0.009	50
6	0.5	0.050	0.051	50
7	0.5	0.100	0.100	50
8	0.5	0.500	0.499	100
9	0.5	1.000	0.999	100
10	0.5	1.500	1.498	100
11	0.5	2.000	1.998	100

TABLE II.

Thyroid, 0.2 per cent iodine.

Kendall.	Colorimetric.
mg.	*mg.*
2.07	2.028
1.91	2.020
1.77	2.000
2.36	2.040
1.92	2.018
2.01	2.020
2.14	2.015
2.24	2.031
2.12	2.040
1.98	2.008

Table IV contains characteristic analyses of organic iodine compounds. The colorimetric determinations were made in 100 cc. dilution. Of the iodide extract made up to 100 cc. in graduated flasks, 1 cc. was used for the determinations.

The writer is aware of the fact that the reaction between iodates and iodides as usually applied to the estimation of iodine may be combined with the colorimetric method explained above, thereby increasing the delicateness of both. Some of the advantages of the present colorimetric method are, however, lost thereby. Work on both this modification and the electrolytic estimation of iodine present in animal tissue is under way in this laboratory and will be reported shortly.

TABLE III.

No.	Weight taken.	Iodine found.
	gm.	*mg.*
A		
1	0.5	2.022
2	0.5	2.042
3	0.5	2.040
4	0.5	2.036
B		
1	0.5	1.888
2	0.5	1.896
3	0.5	1.896
C		
1	0.5	1.904
2	0.5	1.896
3	0.5	1.892
4	0.5	1.916
5	0.5	1.884

TABLE IV.

Substance.	Weight taken.	Iodine found.		Iodine calculated.
	gm.	*mg.*	*per cent*	*per cent*
Di-iodo-diphenyl......	0.1020	63.81	62.55	62.56
	0.1008	63.06		
Iodo-acetanilid........	0.1016	49.40	48.62	48.65
	0.2024	98.46	48.64	
Iodo-benzoic acid.....	0.2002	102.52	51.20	51.21
	0.2041	104.51	51.20	
	0.2011	102.98	51.20	

SUMMARY.

1. In a solution of iodides, the iodine present may be estimated directly by the palladous iodide colorimetric method.
2. The iodine present in animal tissues can be estimated after fusion by the same method.
3. No iodides, iodates, or halogens, usually the source of errors, are added in this method.

I am indebted to Dr. Paul A. Lewis for valuable suggestions and criticisms.

FURTHER OBSERVATIONS ON THE PRESENCE OF IODINE IN TUBERCULOUS TISSUES AND IN THE THYROID GLAND.

By PAUL A. LEWIS and ROBERT B. KRAUSS.

(From the Henry Phipps Institute of the University of Pennsylvania, Philadelphia.)

(Received for publication, June 19, 1915.)

In a previous paper[1] we gave the results of some analyses which showed that the tuberculous tissue of rabbits might contain appreciable amounts of iodine, even though iodine products had not been administered to the animals.

The points made in that paper may be repeated here by quoting the summary there given, as follows:

> Tuberculous tissue derived from animals to which no iodine preparation has been knowingly administered may contain amounts of iodine very appreciably higher than normal control tissue of the same animal. While it is quite probable that tuberculous tissue in animals treated with iodine products may store up iodine, this has been by no means clearly shown in any experiments so far reported. The highest figures for the tuberculous tissue of untreated animals in our experience may equal the highest figures of those reported by others as evidence for the localization in the tissue of iodine intentionally administered.

The analyses on which this first paper was based were done by the Kendall method. It was understood by us that this method was scarcely adequate to give perfect results with the small amounts of tissue available for study. Objections reached us from those who believed that the method was entirely inadequate and that consequently it was unlikely that our results represented the true situation.

Accordingly one of us (Krauss) has devoted considerable effort during the past winter to the development of more accurate and sensitive methods for the examination of tissues for iodine. This

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[1] Lewis, P. A., and Krauss, R. B., *Jour. Biol. Chem.*, 1914, xviii, 313.

study has been entirely successful and a method is now available which is competent to detect with certainty and measure with accuracy the merest traces of iodine.[2]

As in the older methods, the dried tissue is subjected to a process of incineration to convert the iodine into an inorganic form. Palladium chloride is added to an extract of the fusion mass and in the presence of iodine develops palladium iodide, which gives a characteristic color reaction. The intensity of the color developed is determined by colorimetry in comparison with standard solutions.

Using this method for the examination of the tissues for iodine we have reexamined the points at issue. Our work has been conducted as follows. Rabbits are inoculated on the cornea of one eye with a virulent culture of the tubercle bacillus of bovine type. This gives a rapidly progressing infection the characteristics of which we have previously described in detail.[3] At suitable intervals the animals are killed. The corneas are dissected free from the remainder of the eyes. The tissues are separately dried and analyzed for iodine. We have in this way for analysis, first, the tuberculous cornea; second, the normal cornea of the same animal; third, the remainder of both eyes; and fourth, any other normal tissue from the animal which it may be useful to examine. The thyroid gland has been included in this series in most instances. If the animals are allowed to live long enough of course the disease becomes generalized and tuberculous tissues from other parts of the body may also be examined.

The results of these experiments are presented in the following tables. The figures give the amount of iodine found in mg. per gram of dry tissue.

In Table I are collected the figures from the experiments which were carried through in exact accordance with the outline just given. The following points may be developed from these results.

The normal cornea frequently contains appreciable amounts of iodine. In animals in which this is the case tuberculous tissue arising on the opposite cornea also contains iodine, and this, so far as our results go, in relatively larger amounts. The remaining tissues of the eye usually contain no iodine. Certain exceptions

[2] Krauss, R. B., *Jour. Biol. Chem.*, 1915, xxii, 151.

[3] Lewis, P. A., and Montgomery, C. M., *Jour. Exper. Med.*, 1914, xx, 269.

to this appear in the results, Nos. 1180, 1054, 1194, 734. It is most probable that these exceptions are due to the fact that after several weeks the disease spreads from the cornea to the conjunctiva and gradually to the deeper structures of the eye. At this time the removal of the diseased tissue with the cornea is not complete. The proper procedure in these instances would be to examine the bulbar portions of the opposite eyes separately. This was done only once in our series, No. 734. In that case it appeared that the iodine in the bulbar portions occurred only in the infected eye.

It will be noticed that the amount of iodine in the tuberculous tissue or in the normal cornea bears no direct relation to thyroid iodine. The latter is high, low, or entirely absent in instances in which the iodine in the eye is about the same, Nos. 1187, 1205, 1194.

TABLE I.

Rabbit No.	Days lived.	Normal cornea.	Diseased cornea.	Thyroid.	Remainder of both eyes.
		mg.	*mg.*	*mg.*	*mg.*
1149	19	0.000	0.000		0.000
1152	19	0.3	5.50		0.000
1172	22	0.000	0.000	0.000	0.000
1180	26	0.000	0.000	0.000	0.086
1205	30	0.0876	0.1805	0.09	
1187	35	0.0826	0.1232	0.9301	0.000
1181	37	0.0960	0.1320	0.0476	0.000
1198	37	0.085	0.178		0.000
1054	37	0.0730	0.3384	0.0476	0.1178
1194	37	0.085	0.186	0.000	0.002
734	37	0.0620	0.2016	0.060	Left 0.025 Right 0.000

The results in Table II are intended to furnish additional evidence that tuberculous tissues may contain iodine. In these instances the disease of the eye had advanced so far that it was impossible to separate the tuberculous from the normal tissue. The whole eye was examined in comparison with the whole of the normal eye. These results agree in essentials with those presented in Table I. The amount of tissue examined is much larger and the relative difference in weight much smaller than in the first instances. This of course tends to equalize the figures for iodine as expressed in mg. per gram.

On the basis of our results as presented in Tables I and II as well as those of our previous paper it might have appeared possible that the tuberculosis of the cornea was a special case whose results would be without general application. Especially was this so in view of the fact that we have so often found iodine in the normal cornea, the fundamental tissue of the experiment. In Table III, therefore, we present a few analyses of the caseous lymph nodes of animals, four guinea pigs and one rabbit, suffering from more or less advanced generalized tuberculosis. The amount of iodine contained in this material is also far from negligible.

TABLE II.

Rabbit No.	Days lived.	Normal eye.	Diseased eye.	Thyroid.
		mg.	*mg.*	*mg.*
1182	56	0.000	0.030	0.222
1197	56	0.0312	0.0326	0.1390
1191	56	0.0100	0.0189	0.0700
1175	56	0.0070	0.0231	0.0159
1151	56	0.004	0.021	0.061

TABLE III.

Animals.	Material examined.	Iodine per gm.	Duration of disease.
		mg.	
Guinea pig 1437	Caseous nodes	0.000	Died in 1 mo.
" " 1434	" "	0.062	Killed in 1 mo.
" " 1312	" "	0.424	Killed in 1 mo.
" " 1403	" "	0.160	Killed in 5 weeks.
Rabbit 1151	" "	0.0132	Killed in 56 days.

The analysis of the thyroid gland requires some separate comment. In a recent review of work on the iodine content of the thyroid, Cameron[4] cites the work of various observers who have analyzed the glands from certain individuals with negative results. He emphasized the probability that iodine is always pres-

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[4] Cameron, A. T., *Jour. Biol. Chem.*, 1913–14, xvi, 465.

ent, but in amounts not detectable by the methods employed previously, making the following statement in italics. "No stress can be laid on any negative results so far published until they have been confirmed by an accurate method such as that of Hunter."

Our results indicate most clearly that iodine, if indeed it is always present, is at times not to be demonstrated by a method considerably more sensitive than any previously employed. Some of the earlier observers have believed that young animals were more apt to give negative results. The animals in our series were adults, several of them known to be more than one year old. We have examined the thyroids microscopically without being able to see the slightest difference between those containing iodine and those in which we could not detect this element.

We conclude from this work that iodine is frequently present in tuberculous tissue independently of any intentional administration of iodine-containing substances. The quantities found are such as to lessen the force of the conclusion of previous observers that the tuberculous tissue has an especial affinity for iodine when intentionally administered. This may of course be the case, but the evidence for it is so far insufficient. We have no evidence bearing on the nature of the iodine compounds in the tuberculous tissue or the source from which they are derived. The iodine is, however, independent of thyroid iodine in its quantitative relationships.

The thyroid gland of rabbits may at times be free from any appreciable amount of iodine.

THE QUESTION OF FAT ABSORPTION FROM THE MAMMALIAN STOMACH.[1]

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(Received for publication, June 22, 1915.)

CONTENTS.

INTRODUCTION.

Although it is generally assumed, in harmony with current views regarding the gastric functions and the chemical evidence offered by Klemperer and Scheurlen[2] that fat is not absorbed from the stomach, a number of investigators have recently published histological observations from which they conclude that ingested fat does pass through the stomach wall.

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[1] The experimental data in this paper are taken from the dissertation presented by E. J. Baumann for the degree of Ph.D., Yale University, 1915.

[2] Klemperer, G., and Scheurlen, E., *Ztschr. f. klin. Med.*, 1889, xv, 370.

Other means, particularly physiologicochemical procedures for attacking this problem, are available. With the hope of obtaining some decisive evidence, two methods, other than those previously employed, have been applied in the present research. One was the determination of the changes occurring in the fat content of the blood after introduction of fat into the stomach, which had been ligated at its pyloric end. The second consisted of tracing, by means of oil-soluble dyes, any fat absorbed under similar conditions. The data collected are in complete accord with the prevalent view and show conclusively that no active absorption of fat occurs from the ligated stomach.

HISTOLOGICAL STUDIES.

Historical.

Klemperer and Scheurlen conducted their investigations on dogs in which the stomachs were well washed out with water before the experiment. The intestine was ligated 1 to 2 cm. below the pylorus, a weighed quantity of the fatty material was introduced, and the cardiac end of the stomach was then tied off. At the end of a test period of three to six hours the stomach was excised and an analysis of the contents was made; it was possible to recover 99.5 per cent of the amount of triolein, oleic acid, or olive oil introduced, and from these results it was concluded that no fats or fatty acids were absorbed from the stomach.

The histological findings on this subject have been quite at variance with those of Klemperer and Scheurlen. The earliest observations were made by Kölliker[3] on kittens, young dogs, and mice as experimental animals. Sometimes only a few fat droplets were seen in the epithelial cells but usually considerable quantities were observed. He doubted whether any fat leaves the stomach through its walls, for macroscopic examination of the lymphatics of that organ never showed them to be filled and white.

Cunéo and Delamare[4] were unable to find any granules stained by osmic acid in the gastric mucosa after feeding fat. This observation is in conflict with a number of both earlier and later histological studies.

Kischensky[5] observed fat globules in the gastric mucosa after feeding milk, olein, and oleic acid to kittens fourteen hours to four months old. Identical results were obtained whether he used osmic acid, Flemming's solution, or scarlet red for staining his sections. In similar studies Wuttig[6]

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[3] von Kölliker, A., *Physikal.-med. Gesellsch. in Würzburg*, 1857, vii, 174.

[4] Cunéo and Delamare, G., *Compt. rend. Soc. de Biol.*, 1900, lii, 428.

[5] Kischensky, D., *Beitr. z. path. Anat.*, 1902, xxxii, 197.

[6] Wuttig, H., *ibid.*, 1905, xxxvii, 378.

observed that after the ingestion of fat the fundus as well as the pyloric region of the stomach contained fine droplets of fat in the epithelium and larger ones in the cells lying more deeply. In some sections, he could find few or no particles, while others were plentifully filled with them. Lamb,[7] using Weigert's method of myelin staining, found fat globules plentiful in the gastric mucosae of suckling kittens. Weiss[8] also found particles tinted by fat stains in the epithelium of the stomach of kittens and puppies, their frequency increasing from the fundus to the pylorus. He believes that the mucosa loses its power of "fat absorption" early in life, the fundus experiencing this loss first.

The conclusion of Weiss has been opposed by Greene and Skaer.[9] These investigators have reported histological evidence which indicates that considerable fat is absorbed from the stomachs of old as well as young mammals, though they admit that the process is much less active in the former. Using alkaline alcoholic solutions of scarlet red as a stain, they have shown that the gastric epithelia of rats, cats, and dogs contain a large number of stained particles after ingestion of fat. The number of these droplets bears a proportional relationship to the length of time that fat has been in the stomach. First the border of the cells becomes filled with small droplets; gradually an increase in the number and size of the particles occurs, until finally the cells are abundantly filled with them, a maximum loading being observed six to fifteen hours after feeding. The assumption that these observations represent a true absorption is based upon the constancy of this cycle. As fat leaves the stomach, the free borders of the cells lose their fat particles first, and thereupon those parts farther away from the lumen become free from fat particles. These findings were compared with others obtained from animals that fasted for twenty-four hours. Greene and Skaer also noted a similar cycle in the cells of the deeper gastric glands, but here the alternations ran their course much more slowly, and were not as extreme.

From the histological findings of many investigators it thus appears that an increase in the amount of fat in the gastric mucosa and submucosa results when fat is ingested, and furthermore, the appearance strongly resembles the microscopic picture obtained during intestinal fat absorption. On the other hand, the few physiological data on the subject fail to confirm the histological observations. This apparent contradiction needs to be elucidated.

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[7] Lamb, F. W., *Jour. Physiol.*, 1910, xl, p. xxiii.

[8] Weiss, O., *Arch. f. d. ges. Physiol.*, 1912, cxliv, 540.

[9] Greene, C. W., and Skaer, W. F., *Am. Jour. Physiol.*, 1911–12, xxix, p. xxxvii; 1913, xxxii, 358.

Experimental.

As a check upon the methods employed in our physiological experiments the stomachs were excised in a number of cases and portions examined microscopically, mainly with the idea of determining whether, under the conditions of the experiments to be described below, histological results could be obtained similar to those reported by Greene and Skaer and others whose work has just been described. Cats and dogs were used as experimental animals.

The details of the conditions of the various experiments may be obtained from the illustrative protocols given in the appendix. The animals, which had their last meal eighteen to forty-five hours before the experiment, were anesthetized with ether or chloroform, after which the pylorus was ligated and fat introduced into the stomach. Anesthesia was maintained during the entire test periods.

The fats used were cream, a peanut oil emulsion,[10] and in one or two experiments, olive oil. After the animals were killed, pieces of the stomach from the pyloric and fundic regions were cut out and fixed in strong Flemming's solution, stained with hematoxylin and differentiated for a few seconds. In a few instances pieces were fixed in formalin, sectioned with a freezing microtome, and stained with Sudan III. The sections were compared with those obtained from animals that had fasted for twenty-four hours.

In a general way the histological pictures were similar to those described by Greene and Skaer and others. The sections stained with Sudan III showed the presence of more fat particles than those in which Flemming's solution was used. In unfed animals very few or no fat globules were observed. No constant inequalities in the amount of fat found in the pyloric and fundic regions could be determined. In some animals there seemed to be more fat particles in one region, while in others a reversed condition existed.[11]

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[10] This unusually permanent emulsion was supplied by Fairchild Brothers and Foster, and was stated to have the following composition: peanut oil, 45 per cent; lecithin, 5 per cent; water, 50 per cent.

[11] In cases where the pylorus has not been ligated, observers have usually reported a greater number of fat globules in the pyloric area than in the

In a considerable number of sections practically no more fat was observed in the fed animals than in the control experiments. These negative results were particularly frequent in the earlier experiments. The cause of this undoubtedly was that pieces of stomach were cut from the part lying uppermost. The animals were kept on their backs during the entire experiment and consequently it frequently happened that no fat was in intimate contact with the ventral part of the stomach. But even when pieces which had been selected from the dorsal region were examined, many sections were found to be practically devoid of any fat; the distribution was by no means uniform. Young animals had more fat in their mucosae than old ones.

It should be noted that in Greene and Skaer's experiments there was no surgical interference. In spite of the operative procedures in our work no great difference in the histological appearance resulted.

INFLUENCE OF INTRODUCTION OF FAT ON BLOOD FAT CONTENT.

Fat Absorption from Stomach.

That an alimentary lipemia results when fat is absorbed has been demonstrated in many ways. The most satisfactory evidence has been furnished by Lattes[12] and Terroine[13] using the Kumagawa-Suto method, and Bloor[14] with his nephelometric procedure. They have been able to show rises of 12 to 120 per cent above the initial values in the fat content of blood, during alimentary lipemia.

If fat is absorbed from the stomach, a similar increase in blood fat may be expected to ensue; if no gastric absorption results, the level of blood fat should remain constant.

Method.—The following procedure was used to determine whether any fat left the stomach through its walls. Dogs were

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fundic. Greene and Skaer ('13) believe this to be due to the fact that the crypts of the cardiac region are small while those of the pyloric are larger and funnel-shaped, so that here all parts may more easily come in contact with the semi-fluid digested mass.

[12] Lattes, L., *Arch. f. exper. Path. u. Pharmakol.*, 1911, lxvi, 132.

[13] Terroine, É. F., *Jour. de physiol. et de path. gén.*, 1914, xvi, 212, 386, and 408.

[14] Bloor, W. R., *Jour. Biol. Chem.*, 1914, xvii, 377; xix, 1.

anesthetized with alcohol and chloroform; cats with urethane followed by chloroform. A laparotomy was performed and the pylorus was gently ligated with a strip of cheese-cloth.[15] About 50 cc. of peanut oil emulsion warmed to 40°C. were given by sound, after which the wound was sutured. Throughout the experiments the animals were kept under light anesthesia, care being observed to prevent cooling; otherwise, conditions as nearly normal as possible were maintained. The operations were completed in ten to fifteen minutes. The experiments were continued over periods varying from six to twelve hours. Samples of blood were taken from a marginal ear vein before the animals were anesthetized, and at intervals during the experiment, by simply cutting the vessel with a razor and allowing the blood to flow directly into a weighed graduated flask containing an alcohol-ether mixture. In a few experiments on cats where blood could not readily be obtained from the ear veins, it was drawn with a syringe from a femoral vein. Results obtained by both methods of procuring blood samples agreed closely.

Fat was determined by Bloor's method. Oleic acid (Kahlbaum) was employed as a standard instead of triolein, the former being more easily obtained in a pure condition. The error of the method is 1 to 5 per cent.

Choice of Anesthetic.—Bloor made a study of the effect of anesthetics upon blood fat. From his results it appears that morphine has little or no effect while alcohol causes a slight gradual rise of less than 0.1 per cent, and ether a very marked increase. Chloroform caused a slight fall of 0.05 to 0.08 per cent during three hours, but a subsequent rise was observed on the following two days. Chloroform, therefore, seems to be the most desirable for the purpose of these experiments, for blood fat is less affected by it than by any of the other anesthetics.

The results of seven experiments, three on dogs and four on cats, performed in the manner just described are summarized in Table I. Six of them are represented graphically in Chart I. A typical protocol (I) will be found in the appendix.

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[15] When cord was used inflammation resulted, probably because of interference with the circulation. To avoid this disturbance, advantage was taken of the fact that anesthesia usually inhibits peristalsis, so that it was only necessary to use broad strips of cheese-cloth gently drawn, to prevent passage of fat into the intestine.

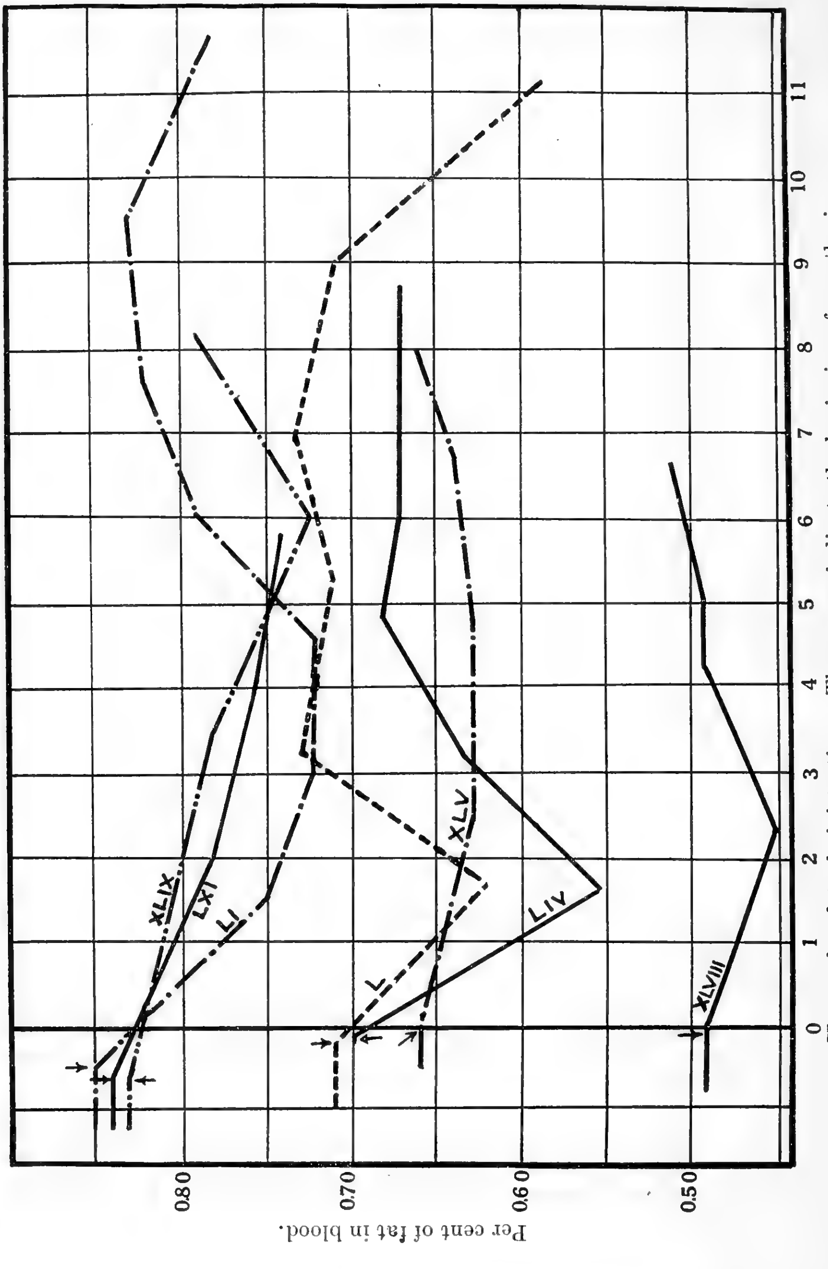
Discussion and Conclusions.—From these summaries and curves, it will be observed that invariably a slight but distinct fall in the level of blood fat occurred after the administration of fat, quite comparable to the results obtained by Bloor in his experiments on chloroform narcosis, though the decrease in our trials was usually a little more pronounced. The minimum level of blood fat was reached in three hours with cats, and in five hours with dogs, after which a gradual rise, back to the normal or nearly so, resulted. Experiment LIV was perhaps the most satisfactory of

TABLE I.

Summary.

Content of Fat in Blood after Introduction of Fat Into Ligated Stomach.

Animal.	Cat.		Cat.		Cat.		Cat.	
Experiment.	XLVIII.		L.		LIV.		LXI.	
	Time after introduction of fat.	Blood fat.	Time after introduction of fat.	Blood fat.	Time after introduction of fat.	Blood fat.	Time after introduction of fat.	Blood fat.
	hrs.	*per cent*	*hrs.*	*per cent*	*hrs.*	*per cent*	*hrs.*	*per cent*
Before experiment..		0.49		0.71		0.70		0.89
	¼	0.475	1¼	0.62	1½	0.56	2	0.78
	2¼	0.45	4¼	0.73	3	0.63		
	4¼	0.49	5¼	0.71	4¾	0.68	4	0.76
	5	0.49	7	0.73	6¼	0.67	5¾	0.74
	6¾	0.51	9	0.71	8¾	0.67		
			11¼	0.58				
Animal.	Dog.		Dog.		Dog.			
Experiment.	XLIX.		LI.		IX.			
Before experiment..		0.83		0.85		1.00		
	3½	0.78	1½	0.75	1¾	0.85		
	6	0.72	3	0.72	3¾	0.77		
	8¼	0.79	4½	0.72	5¼	0.73		
			6	0.79	7¼	0.76		
			7½	0.82	9¼	0.85		
			9½	0.83				
			11¾	0.78				



Hours after fat administration. The arrows indicate the beginning of anesthesia.

CHART I. Fat in the stomach; pylorus ligated.

this series for no operation was performed. In this case it was intended to study absorption from the alimentary canal under chloroform anesthesia, and fat was administered by sound just after the animal had been anesthetized. Peristalsis seemed to be inhibited during the narcosis, for the autopsy showed that no fat had entered the duodenum.

No evidence that even the slightest fat absorption took place in any one of these experiments can be found in the data on blood fat. From Experiment LIV it would appear that the surgical procedure used does not necessarily interfere with the processes occurring in the stomachs of cats and dogs under chloroform narcosis. To show that the operative measures did not influence the blood fat content, this point was subjected to further verification in the following two experiments.

A cat and a dog were anesthetized in the usual manner and the pylori were ligated. The wounds were then sutured and blood fat determinations made at intervals. A slight lowering of fat content resulted, similar to but not as pronounced as that which ensued when fat was put into the stomach. These protocols (II) are given in the appendix.[16]

Under normal conditions Terroine and others have found that the fat content of blood remains practically constant over long periods of time. This was confirmed in the experiments, one carried on for two days on a dog, and the other for eight hours on a cat. The average deviations were 1 and 2 per cent respectively, and these are well within the limits of error of the method of analysis.

Fat Absorption from Intestine.

As a further check upon the method employed, four experiments, three on cats and one on a dog, were performed to show that under the identical conditions prevailing, alimentary lipemias, similar to those obtained normally, could be produced when fat was injected into the intestine.

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[16] In the case of the cat, for some cause which cannot be explained, a sudden rise of about 0.2 per cent occurred after this fall was noted. Such an increase was never again observed even when fat was introduced into the stomach.

Method.—The general plan was as follows: Animals were anesthetized in the same way as in all the earlier experiments, the pylorus was ligated, and a fat emulsion introduced into the intestine. The animals were kept under exactly the same conditions as in the stomach experiments.

The data are summarized in Table II and illustrated graphically in Chart II. A typical protocol (III) is given in the appendix.

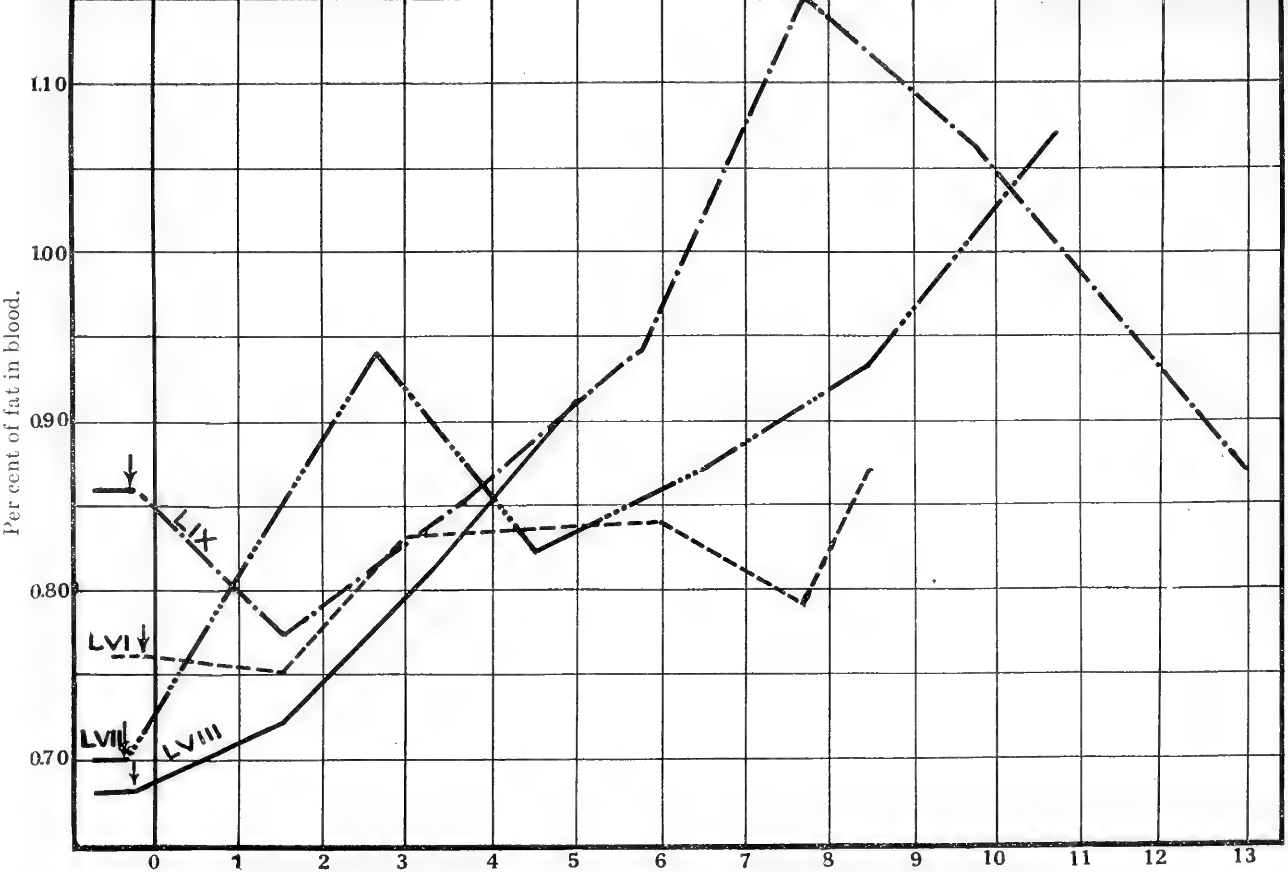
TABLE II.

Summary.

Content of Fat in the Blood during Absorption of Fat from the Intestine.

Animal.	Cat.		Cat.	
Experiment.	LVII.		LVI.	
	Time after introduction of fat.	Blood fat.	Time after introduction of fat.	Blood fat.
	hrs.	*per cent*	*hrs.*	*per cent*
Before experiment....		0.70		0.76
	2½	0.94	1½	0.75
	4½	0.82	3	0.83
	6½	0.87	4½	0.84
	8½	0.93	6	0.79
	10½	1.07	7¾	0.87
Animal.	Cat.		Dog.	
Experiment.	LVIII.		LIX.	
Before experiment....		0.68		0.86
	1½	0.72	1½	0.77
	3¼	0.81	3¾	0.85
	5	0.91	5¾	0.94
			7¾	1.15
			9¾	1.06
			13	0.87

Discussion.—From these data, it may be observed that the initial fall in the level of blood fat, observed when peanut oil emulsion was introduced into the stomach, was more or less completely counterbalanced by the increase in the amount of fat due to absorption. However, in Experiment LIX, the only one in which a dog was used, a decrease of almost 0.1 per cent occurred.



Hours after fat administration. The arrows indicate the beginning of anesthesia.

CHART II. Fat in intestine; pylorus ligated.

This difference in the behavior of the dog as compared with the three cats may be accounted for if the rate and degree of the initial fall be taken into consideration. When fat was introduced into the stomachs of dogs, a gradual lowering of blood fat ensued, extending over four to five hours, and amounting to from 0.11 to 0.27 per cent; while with cats the decrease varying from 0.09 to 0.14 per cent was not as great and the minimum was reached in three hours. When the fifth hour had elapsed in the case of the dog, a distinct rise in blood fat manifested itself, while with cats this phenomenon occurred in three hours.

The rises in the content of blood fat were 15, 35, and 67 per cent respectively above the normal values. The lipemias observed by Bloor were as high as 100 to 120 per cent in a few experiments, while Terroine's increases in blood fat during absorption varied from 15 to 90 per cent. The lipemias in our experiments were not as marked as the maxima obtained under normal conditions by the investigators just cited. One would hardly expect as marked rises in the fat content of blood in the writers' experiments, for peristalsis was interfered with as well as the mechanism which normally activates the pancreas. However, lipase was added in one case and bile in most of the others, to promote absorption.

The Stomach as an Organ of Absorption.

To show that the ligated stomach may still function as an organ of absorption under the conditions like those where fat was introduced into it, a similar experiment was performed in which a 10 per cent solution of sodium iodide was added to the fat which was administered. In all other respects precisely the same procedure was used. Estimations of fat and iodine were made on samples of blood at intervals, and the amount of iodine in the urine was also determined. For iodine determinations Seidell's[17] method was used.

Blum and Grützner[18] have shown that detectable quantities of iodine do not occur in the blood; therefore, the entire amount present in the blood and urine in the above experiment may be taken as the minimal quantity absorbed, inasmuch as some iodide

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[17] Seidell, A., *Jour. Biol. Chem.*, 1911–12, x, 95.

[18] Blum, F., and Grützner, R., *Ztschr. f. physiol. Chem.*, 1914, xci, 451.

is likely to be deposited in the tissues. After three hours 0.95 gram of iodine was found in the blood, and 0.01 gram in the urine; the combined quantities are equivalent to 1.14 grams of sodium iodide or 46 per cent of the amount introduced. Considerable amounts of sodium iodide were therefore absorbed. On the other hand, no evidence of the slightest fat absorption could be adduced from the data obtained. They resembled those already cited. The different behavior of the stomach toward sodium iodide and fat as an organ of absorption is strikingly shown on Chart III. The protocol (IV) is given in the appendix.

Hanzlik[19] found that when aqueous solutions of sodium iodide were injected into the stomachs of cats, the amount absorbed in a half hour varied from 26 to 77 per cent. The writers' experiment was carried on over a longer period than were the experiments of Hanzlik, but in a general way there is a close agreement between the results obtained, especially if a relation is assumed to hold true for sodium iodide similar to that which Salzman found for alcohol. He pointed out that fat-like substances, such as cream and emulsified egg yolk, lessened the amount of absorption of alcohol from the stomach. Hanzlik and Collins[20] discovered the same phenomenon to hold true for the intestine.[21]

General Discussion and Conclusions.

There are two factors which come into play in experiments of the type just reported: (1) absorption, and (2) deposition and utilization of fats. It is possible that the absorption may be so slow that the rate of disappearance of fat from the blood would equal it, in which case no rise in blood fat would result. That any considerable quantity of fat could be absorbed is very unlikely,

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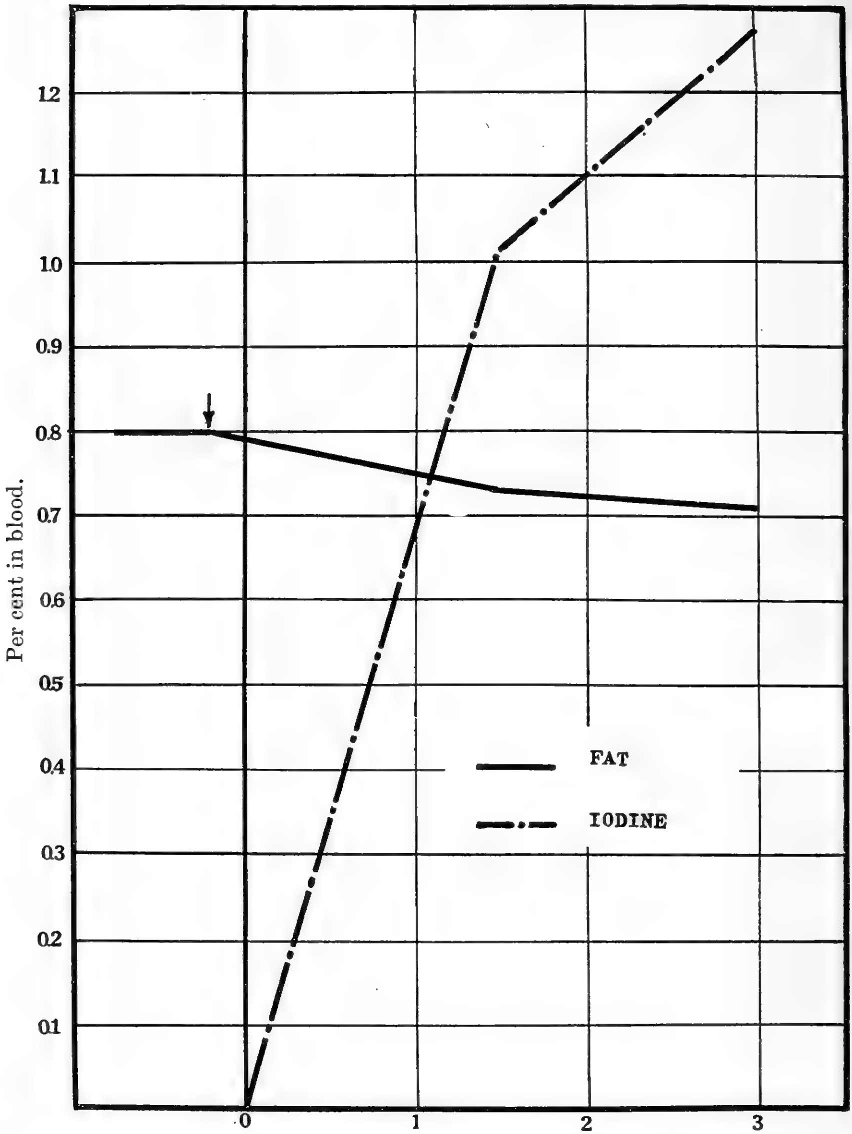
[19] Hanzlik, P. J., *Jour. Pharmacol. and Exper. Therap.*, 1911–12, iii, 387.

[20] Hanzlik, P. J., and Collins, R. J., *ibid.*, 1913–14, v, 185.

[21] In this connection an experiment was made in which an alcoholic fat solution was introduced into the ligated stomach of a cat. It was thought that possibly alcohol might aid in the absorption of fat as it does with some salts, alkaloids, sugars, etc. Ordinarily a fall in blood fat of 0.05 to 0.15 per cent ensued in three or four hours. In this experiment the blood fat remained practically constant. Additional data are necessary to determine whether or not the alcohol had any influence upon absorption.



Hours after administration. The arrow indicates beginning of anesthesia.

CHART III. Fat and sodium iodide in stomach; pylorus ligated.

for fat leaves the blood slowly and ought, therefore, to become evident. Ordinarily the marked lipemias which occur during absorption extend over six or eight hours. Bloor found that fat emulsions injected into the blood disappear very slowly, and the same phenomenon has been observed by Rabbeno;[22] they agree that the level of blood fat reaches its normal value in not less than seven hours.

Despite the fact that amino-acids leave the blood stream quite rapidly, Folin and Lyman[23] have shown that considerable rises in the non-protein nitrogen of the blood occur after injecting glycocoll, alanine, etc., into ligated stomachs of cats. The amount absorbed in this way ordinarily cannot be great, for Abderhalden, London, and Prym[24] recovered almost all of the amino-acids administered *per os* from a duodenal fistula.

From these considerations, one must incline to the belief that no noteworthy quantities of fat leave the stomach through its walls. If absorption does occur without producing a detectable rise in blood fat, because deposition and utilization take place as quickly as fat leaves the lumen, the rate must be exceedingly slow and the amount absorbed must be minimal at best.

FAT ABSORPTION STUDIED WITH THE AID OF FAT-SOLUBLE DYES.

Experiments on the Stomach.

The failure of fat to be absorbed from the stomach was further verified by the use of oil-soluble dyes. Fat can readily be traced by means of such stains as Sudan III and Alkanna red, and this method has been used for the study of fat absorption by a number of investigators.[25] From what we know of the intestinal absorption of fat, it seems likely that if any passes through the gastric mucosa it would proceed by way of the lymph channels. The lymphatics with which the stomach is richly sup-

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[22] Rabbeno, A., *Chem. Abstr.*, 1915, ix, 477.

[23] Folin, O., and Lyman, H., *Jour. Biol. Chem.*, 1912, xii, 259.

[24] Abderhalden, E., Prym, O., and London, E. S., *Ztschr. f. physiol. Chem.*, 1907, liii, 326.

[25] Some of the literature on this subject is reviewed by Mendel ('09), and Mendel and Daniels ('12); see footnotes 27 and 30.

plied empty into the thoracic duct (Martin[26]). After collecting the lymph, it can readily be learned whether or not any fat is absorbed through this channel, by extracting the fluid with ether. If absorption occurs by way of the blood stream, any Sudan III which would be carried with the fat would probably be wholly or partly excreted into the bile. By collecting the bile, this point can be determined.

Three experiments of a preliminary nature were performed, in which a laparotomy was made, the pylorus ligated, and peanut oil emulsion stained with Sudan III introduced into the stomach. The wound was then sewed up and the animal kept under chloroform anesthesia. About 15 or 20 cc. of blood were drawn at the end of the experiment and after defibrination the desiccated residue was extracted with ether and the extract evaporated to dryness to determine whether or not any Sudan III was present. The durations of the test periods were nine, twelve, and thirteen hours respectively. In no instance could any stain be found in the dried residue of the ether extract.

The results indicate that under the conditions of the experiments, absolutely no absorption of stained fat took place by way of either the blood or lymph streams. It was pointed out above that the height of fat absorption occurred normally in six hours; whereas under chloroform anesthesia the maximum fat content of blood is reached in eight hours. The experiments were presumably, therefore, carried on over periods sufficiently long to afford opportunity for absorption.

It could be urged that if absorption were very slow the dye might be excreted in the bile without being detected in the general circulation. If absorption occurs by way of the lymph channel, one should be able to discover any Sudan III that would be carried along with it, inasmuch as so little as 0.00001 gram can be detected (Mendel and Daniels[27]). If the blood stream were the path of absorption, however, it is quite probable that all of the dye might be immediately reexcreted into the bile, the latter being a better solvent for the Sudan III than is fat.

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[26] Martin, P., Anatomie der Haustiere, Stuttgart, 2nd edition, 1911.

[27] Mendel, L. B., and Daniels, A. L., *Jour. Biol. Chem.*, 1912–13, xiii, 71.

To answer these possible objections (and also gain additional data to confirm the results obtained thus far), a number of other procedures was planned to test each possible path of absorption separately.

Experimental.—Cannulas were inserted into the thoracic and common bile ducts of dogs and cats, and absorption was studied under these conditions. As before, stained fat was introduced into the ligated stomach. At intervals samples of 10 to 20 cc. of lymph were collected and dried with anhydrous sodium sulphate. Bile samples taken at intervals were dried in the same way after precipitating the bile pigments with barium hydroxide solution. At the end of the experiment, 20 cc. or more of blood were defibrinated and dried also. The desiccated masses were extracted with ether and the extracts evaporated to dryness to determine whether any Sudan III was present.

TABLE III.

Summary of Data of Experiments with Sudan III Stained Fat in Ligated Stomach.

Animal.	Duration of experiment.	Sudan III in			
		Bile secreted.	Bile in gall bladder.	Lymph.	Blood.
	hrs.				
Cat 20	6	—	—	—	—
" 25	7	—	—	—	—
" 34	12	—	—	—	—
" 39	5	—	—	—	—
Dog 1	5	—	—	—	—
" 3	9½	—	—	—	—
" 5	5½	Trace?	—	—	—
" 6	5	—			

Eight experiments—four on cats and four on dogs—were performed in the manner outlined above. The duration of the test periods varied from five to twelve hours. Typical protocols (V) are given in the appendix. Results are summarized in Table III.

In some cases, especially in old cats, the thoracic duct was merely ligated. Any absorption occurring under such conditions must proceed through the blood stream. In none of these trials was Sudan III found in the bile or blood.

In this connection mention may be made of an experiment reported by Mendel (1909).[30] A cat was fed with fat stained with Sudan III and four and a quarter hours later a cannula was placed in the thoracic duct. No dye was found in the ether extract of the lymph. Autopsy showed that the fat had not entered the intestine and had probably remained in the stomach for about five hours.

Discussion.—If any fat were absorbed by way of the lymph stream, all the pigment going with it would be obtained in a relatively small volume. If absorbed by way of the blood, the greater part if not all of the Sudan III leaving the stomach ought to be found in the bile. Accordingly, even an insignificant amount of stained fat that left the stomach ought to be detected.

From the data obtained it will be observed that in no instance was any stain found in the lymph; this agrees with the isolated result of Mendel, already referred to. Mendel's experiment is interesting because, for some reason, the pylorus remained closed without any operative interference, and the conditions of the present experiments were thus simulated without opening the abdomen. The writers were never able to find Sudan III in the blood or in bile from the gall bladder, except in Experiment XXXVI; nor was any ever detected in the bile collected during the test periods. In the one case cited a trace of dye was found in the residue of the ether extract of the bile. This unexpected finding is exceptional. It may have been an experimental error, or the stain may have been due to previously ingested carotin, which not infrequently shows a deep orange color. Since the pigment was only very small in amount, this isolated result is not regarded as significant.

It is possible, of course, that amounts of Sudan III smaller than are capable of being detected by this method might have been absorbed, but such quantities are of no importance. This method is not open to the objection which pertains in the study of blood fat, namely, that absorption may equal the rate of deposition and utilization; for here all absorbed stain should be found unchanged in the lymph or be in a comparatively small volume in the bile.

Conclusions.—These experiments show: (1) that no fat was absorbed through the lymph channels, since no dye could be extracted from the lymph; (2) that no absorption occurred by way of the portal circulation, because Sudan III was never found in the blood or bile. As far as this method allows, it is thus well established that no fat leaves the lumen of the stomach through its walls, since no dye could be detected in either of the circulating fluids. A further verification of these findings is found in the fact that the lymph never became turbid during an experiment as it regularly does after intestinal absorption of fat; in a number of instances the lymph which was milky at the beginning of an experiment became clear in the course of a few hours.

Experiments on the Intestine.

It may be contended that the conditions of the experiments were abnormal; this objection is one difficult to answer in a completely satisfactory manner. We know as a result of the work of many investigators—Folin and Lyman, Sollman, Hanzlik, and Pilcher,[28] and others, for example—that absorption of other substances from the stomach can take place under circumstances similar to those prevailing in these experiments.

Absorption of stained fat from the intestine will, however, occur under experimental conditions identical with those which existed in the cases where fat was placed in the stomach.

Method.—In one experiment, fat was introduced in the stomach and allowed to go through the alimentary canal; in two others fat containing some desiccated ox bile in solution was injected into the intestine after ligating the pylorus, a glycerol extract of pancreas being added to the peanut oil emulsion in one case. Temporary cannulas were placed in the thoracic and common bile ducts. The protocol (VI) of a typical experiment is given in the appendix.

Results.—When fat was allowed to pass through the alimentary canal, results typical of those reported by Pflüger,[29] Mendel,[30] and others were obtained. The lymph and bile were quite pink. In the two experiments (one on a cat, the other on a dog) in which

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[28] Sollmann, T., Hanzlik, P. J., and Pilcher, J. D., *Jour. Pharmacol. and Exper. Therap.*, 1909–10, i, 409.

[29] Pflüger, E., *Arch. f. d. ges. Physiol.*, 1900, lxxxi, 375.

[30] Mendel, L. B., *Am. Jour. Physiol.*, 1909, xxiv, 493.

fat was introduced into the intestine *after ligating the pylorus,* Sudan III was readily detected in the lymph also.

In view of the ready absorption of fat from the intestine under operative procedures comparable with those which pertained in the negative gastric experiments, it is unlikely that the abnormal conditions will account for the failure of fat absorption from the ligated stomach.

RÉSUMÉ.

Although there are histological indications that fat absorption may occur in the stomach (Kischensky, Wuttig, Greene and Skaer, and others), the lymphatics of this organ have never been observed to assume the same appearance that the lacteals of the intestine have during fat absorption. Klemperer and Scheurlen were able to recover 99.5 per cent of the fat introduced into a ligated stomach, after a test period of six hours. These physiological findings are the only ones reported on this subject until 1909 when Mendel, in a research in which intestinal fat absorption was studied, incidentally noted a single instance when the pylorus did not allow stained fat given by sound to pass into the intestine. The lymph from the thoracic duct showed no Sudan III to be present, though when stained fat was present in the intestine the lymph was always pink. As far as an isolated experiment can, this demonstrates that no fat is absorbed normally from the stomach in four and a quarter hours.

In the writers' work, no trace of fat absorption from the stomach could be demonstrated (1) by a study of blood fat after introduction of fat into the ligated stomach, for here no rise in the fat content of blood could be obtained; or (2) by tracing absorbed fat with the aid of Sudan III under similar conditions. The absorption of fat from the intestine was readily demonstrated under exactly the same experimental procedures.

However, sections of the stomach which had been in contact with fat showed fat droplets in cells similar in a general way to the findings of many investigators who have studied this problem. From these considerations it appears that histologically demonstrable fat gets into the tissue of the stomach, but none passes through the submucosa and beyond, into the blood or lymph streams. How can these apparently contradictory data be reconciled?

We may assume that the cell is either partly lipoidal in nature or that it possesses a greater solubility for fatty substances than does water. Such an hypothesis is not at all unreasonable; in fact, it is believed by many physiologists that in mammals the cell exteriors are partly lipoidal. There is some experimental evidence which would tend to show that either one of these conditions assumed in the premise exists. Katzenellenbogen,[31] in a study on the absorption of various polyhydric alcohols from perfused loops of intestine, showed that the rapidity of absorption is proportional to the lipoid solubility of the alcohol in question. In a comparison of mannitol, erythrol, and glycerol, the last left the intestinal lumen most rapidly, while mannitol was the most tardy. It is not unlikely that the same relation may hold true for the cells of the stomach.

Granting the fact that the gastric mucosa has a greater solubility for fats than has the gastric juice, the fatty substances would naturally pass from the place of lower to that of higher solubility. Once within the cells, the matter of transport becomes intelligible, and the histological observations of Greene and Skaer and others can be explained.

The absorption and transport are apparently nil or exceedingly slow and small in amount; for with methods that are fairly delicate no detectable quantities of fat could be shown to be absorbed either by way of the blood or lymph streams.

APPENDIX.

I. Selected Typical Protocols.

Protocol I.

Fat in the Stomach. Pylorus Ligated.

Experiment LI.—Dog 9. Nearly full grown, 5.3 kg. Last meal consisting of chicken bones forty-three hours before the experiment.

At 9.40 a.m. 18 cc. of 95 per cent alcohol diluted to 20 per cent were administered by sound. At 10.15 the animal was anesthetized with chloroform; at 10.30 the abdomen was opened aseptically, the pylorus gently ligated, and the wound sewed up. 50 to 60 cc. of peanut oil emulsion were

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[31] Katzenellenbogen, M., *Arch. f. d. ges. Physiol.*, 1906, cxiv, 522.

given by sound at 10.50. Blood samples were taken at intervals noted below and the results are tabulated. The dog died at 10.45 p.m.; the last sample was taken from the heart.

Time	9.30	12.20	2.00	3.30	5.00	6.30	8.30	10.45
Fat, *per cent*	0.85	0.75	0.72	0.72	0.79	0.82	0.83	0.78

Autopsy: No fat entered the intestine.

Protocol II.

Effect of Operative Procedures and Chloroform Anesthesia.

Experiment XLV.—Dog 8. Male, 7.8 kg. Last meal, twenty-four hours before the experiment, consisting of chicken bones and meat.

At 9.40 25 cc. of 95 per cent alcohol diluted to 20 per cent were administered by sound. At 10.20 the animal was anesthetized with chloroform. A laparotomy was aseptically performed at 10.15, and the pylorus gently ligated with a strip of cheese-cloth, after which the abdomen was sewed up. Samples of blood were taken from a marginal ear vein before the experiment, and at the intervals noted below. At 1 p.m. the animal vomited a few bones and some meat. The abdomen was opened at 5 p.m. and the ligature removed. A good recovery was made.

Time	9.30	12.30	2.50	4.50	6.00
Fat, *per cent*	0.66	0.63	0.63	0.64	0.66

Experiment LII.—Cat 50. Female, 3.3 kg. Last meal twenty-four hours previous consisting of cooked meat.

At 9.10 a.m. 1.5 gm. urethane were injected subcutaneously. Anesthesia at 10.00 with chloroform. Laparotomy aseptically performed at 10.15, and the pylorus ligated at 10.20. The wound was then sewed up. Blood fat determinations were made at the intervals noted below.

Time	9.20	12.00	2.00	4.00	6.00	8.00
Fat, *per cent*	0.54	0.53	0.54	0.71	0.73	0.71

Protocol III.

Fat in the Intestine. Pylorus Ligated.

Experiment LIX.—Dog 10. Female, just mature, 11.6 kg. Last meal twenty-two hours before the experiment. 36 cc. of 95 per cent alcohol diluted to 20 per cent, administered by sound at 9.30 a.m.

Operation at 10.05. The pylorus was ligated and 60 cc. of the oil emulsion plus 5 gm. of desiccated ox bile dissolved in 10 cc. of water were injected into the intestine at body temperature, at 10.15. The animal vomited; the ligature was still firm. The abdomen was sewed up at 10.25 and 60 cc. of the same mixture were given by sound. The results of the blood fat determinations are given below.

Time	9.25	11.45	2.00	4.00	6.00	8.00	11.15
Fat, *per cent*	0.86	0.77	0.85	0.94	1.15	1.06	0.87

Autopsy: A very little bleeding into the abdominal cavity occurred. The fat seemed to be almost completely absorbed, only a clear aqueous solution remaining in the intestinal lumen.

Protocol IV.

Sodium Iodide and Fat in the Stomach. Pylorus Ligated.

Experiment LV.—Kitten 54. Male, 1.5 kg. Last meal eighteen hours before the experiment.

At 10.00 a.m. 0.1 gm. of urethane were injected subcutaneously. The animal was anesthetized with chloroform at 10.40. The pylorus was ligated and the abdomen sewed up. 50 cc. of oil emulsion containing 2.5 gm. of sodium iodide in solution were given by sound at 10.55. The animal died at 2.00 p.m. The last samples of blood were taken from the heart. The bladder was excised and 11 mg. of iodine were found in the urine. The results of the blood analyses follow.

Time	10.00	12.30	2.00
Fat, *per cent*	0.80	0.73	0.71
Iodine, *per cent*		1.01	1.27

Autopsy: The stomach was well filled but the intestine was empty.

Protocol V.

Stained Fat in Ligated Stomach. A Study of Blood, Bile, and Lymph.

Experiment XXXI.—Cat 34. Male, 3.3 kg. Last meal twenty-four hours before the operation.

At 9.15, 1.5 gm. urethane were injected subcutaneously. The operation was begun at 9.50. A cannula was inserted in the thoracic duct at 10.50, and another in the common bile duct at 11.05. The pylorus was ligated and 50 cc. of stained oil emulsion warmed to 40°C. were injected into the stomach at 11.15. The wound was then sutured.

Both lymph and bile flowed rather slowly but continuously throughout the experiment. At the beginning, the lymph was milky, but it became clear at about 4 p.m. Lymph from 11.15 to 4.00 amounted to 5 cc.; bile for same period, 3 cc. Between 4.00 and 11.15 p.m. lymph and bile amounting to 12 cc. and 4 cc. respectively, were collected, the latter including a few drops of bile from the bladder. At 11.15 the animal was bled to death and 25 cc. of defibrinated blood were dried down as well as the lymph and bile. In no case was any Sudan III found in the ether extracts.

Autopsy: Intestine empty.

Experiment V.—Dog 1. Female, 16 kg. 60 cc. of 95 per cent alcohol diluted to 20 per cent were given by sound at 11.20. Vomited about 50 cc. of it.

Operation at 12.00. Cannula in the thoracic duct at 12.30. The pylorus was ligated and 50 cc. of stained cream were injected into the stomach at 12.40. The lymph flowed well but was bloody. The animal was kept under ether anesthesia during the experiment. It died at 5.30 p.m.

Samples of 15 cc. of blood were taken from the left femoral artery at 1.30 and 3 p.m. and a similar quantity was obtained from the heart at 5.40. The lymph obtained between 12.40 and 2.00 was 15 cc.; between 2.00 and 3.00, 20 cc.; between 3 and 4, 15 cc., and between 4.20 and 5.30, 15 cc. None of the lymph or blood samples showed the presence of any Sudan III nor did 10 cc. of the 23 cc. of bile in the gall bladder contain any.

Autopsy: A little bleeding occurred from a superficial vessel into the abdominal cavity. The pylorus was slightly inflamed.

Experiment XXXIII.—Dog 3. Female, just mature, 17.5 kg. Last meal twenty-four hours before the experiment. At 9.45 a.m. 40 cc. of 95 per cent alcohol diluted to 20 per cent were administered by sound.

Operation at 10.30. Cannula inserted into the thoracic duct at 11.30, and another into the common bile duct at 12.00. The pylorus was ligated and 50 cc. of stained oil emulsion were introduced into the stomach at 12.10. 2 gm. of ox bile dissolved in water, were injected into the intestine. The lymph and bile flowed very rapidly at first but slowed up considerably. At the beginning of the experiment the lymph was slightly turbid but at 2.30 it was quite clear and slightly bloody. 10 cc. of bile collected between 12.10 and 4.00 were taken as a sample and 25 cc. of the 145 cc. of lymph collected during the same period. 30 cc. of 100 cc. of lymph collected between 4.00 and 8.50 and 10 cc. of 50 cc. of bile collected during the same period were dried as well as 25 cc. of defibrinated blood, which were drawn at 8.50, when the animal was killed by bleeding. In no case was any pink residue obtained in any of the ether extracts of these fluids.

Autopsy: Intestine was empty.

Protocol VI.

Stained Fat in the Intestine. Pylorus Ligated.

Experiment XLII.—Dog 7. Female, 17 kg. Last meal twenty hours before the experiment. At 9.00 a.m. 50 cc. of 90 per cent alcohol diluted to 20 per cent were administered by sound.

The dog was anesthetized with ether at 9.35. The thoracic duct was dissected out, and a laparotomy performed; the pylorus was ligated at 10.00 and 50 cc. of stained oil emulsion were injected into the intestine. A lymph cannula was inserted at 10.30. The lymph collected between 11.30 and 1.30 contained no Sudan III. The ether extract of 2 cc. of lymph obtained at 5.15 was pink and contained a considerable quantity of the dye.

Autopsy: Slight inflammation about the pylorus.

II. Can Sudan III Dissolved in Alcohol be Absorbed from the Alimentary Tract?

The question of absorption of alcoholic solutions of Sudan III from the stomach and intestine arose incidentally in the work on fat absorption. When cream was used it was sometimes stained by adding a little saturated solution of Sudan III, dissolved in 95 per cent alcohol. It seemed desirable to determine whether or not any absorption of the dye could occur in this way independently of the fat. The stain is not very soluble in alcohol, especially in 20 to 50 per cent solutions of the latter.

From the Stomach.

In all of these experiments cats were used. Stained alcohol was injected into the ligated stomach and cannulas were placed in the common bile ducts. In one experiment the lymph was also collected.

The results are summarized in Table IV.

TABLE IV.

Summary.

Stained Alcohol in Ligated Stomach.

Experiment No.	Duration of experiment.	Strength of alcohol.	Sudan III in		
			Bile.	Blood.	Lymph.
	hrs.	*per cent*			
VII	6	20	+	–	
IX	5	20	–	–	
X	5¼	20	–	–	–
XI	6½	20	–	–	
XIII	6½	20	–	–	
XXII	5	50	–	–	

Probably no Sudan III is absorbed from 20 or 50 per cent alcohol solutions. The single positive result in Experiment VII stands alone, for in all other cases no stain was found in the blood or bile, nor was any found in the lymph in the one experiment cited.

From the Intestine.

Seven experiments were performed in which alcoholic Sudan III solutions were injected into loops of intestine which had been washed free of bile, with saline. Cannulas were inserted into the common bile ducts. In two cases the thoracic ducts were ligated.

The results of the experiments are summarized in Table V.

TABLE V.

Summary.

Experiment No.	Duration of experiment.	Strength of alcohol.	Sudan III in			Remarks.
			Bile.	Bladder.	Blood.	
	hrs.	*per cent*				
XXIII...	3	20	–	–	–	
XXV...	3	20	–	–	–	
XXXII...	6	20	–	–	–	
XXVI...	2½	20	–	–	–	
XXXVII...	5	50	–	–	–	
XXVII...	3	20	–	–	–	Thoracic duct ligated.
XXXIV...	5	20	–	–	–	Thoracic duct ligated.

In no instance was any evidence of absorption of Sudan III obtained. In one experiment not recorded above, in which the loop of intestine was not washed out, Sudan III was found in the bile, but not in the lymph collected for five and one-half hours after injection. The dye thus found was probably absorbed in solution in some bile present in the intestine.

A NOTE ON THE RATE OF METABOLISM OF PROTEINS AND AMINO-ACIDS.

By N. W. JANNEY.

(*From the Chemical Laboratory of the Montefiore Home and Hospital for Chronic Invalids, New York.*)

(Received for publication, July 1, 1915.)

An interesting study on the rate of metabolism of amino-acids has recently been reported by Csonka from Lusk's laboratory.[1] In feeding experiments with fasting phlorhizinized dogs, it was shown that the nitrogen and glucose originating from glycine and alanine were practically entirely excreted within nine hours after ingestion. As in these experiments both nitrogenous and non-nitrogenous metabolic products of amino-acids can be studied, the data obtained are of more value than those of similarly resulting studies previously made on this subject, where only the nitrogen could be observed.[2] Other amino-acids may require, however, considerably longer periods of time for their metabolism when fed to fasting phlorhizinized dogs.[3] To enlarge upon several explanations which may be offered for this does not serve the purpose of the present communication. It suffices here to emphasize that the metabolism of the simpler amino-acids requires under optimal conditions a period of about nine hours' duration.

In further experiments by Csonka glucose fed in like manner was found to require nearly the same length of time for its excretion as the extra sugar of equivalent quantities of glycine and alanine. From this observation the conclusion is drawn that the entire series of metabolic changes leading to the synthesis of glucose from amino-acids and its excretion occupied a period of time but slightly in excess of that necessary for the simple absorption and elimination of the corresponding amount of ingested glucose.

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[1] Csonka, F. A., *Jour. Biol. Chem.*, 1915, xx, 539.

[2] Levene, P. A., and Meyer, G. M., *Am. Jour. Physiol.*, 1909–10, xxv, 214. Bostock, G. D., *Biochem. Jour.*, 1911, vi, 48.

[3] See protocols of H. D. Dakin's experiments, *Jour. Biol. Chem.*, 1913, xiv, 321.

In the meantime it was discovered that the author in studying the rate of metabolism of proteins[4] had made use of precisely the same conditions in feeding pure proteins to fasting phlorhizinized dogs as were employed in the amino-acid and glucose experiments of Csonka. The results of the two series, though obtained quite independently of each other, were found to be entirely comparable as to amounts of both extra nitrogen and extra glucose produced. By mutual agreement they were, therefore, prepared on the same principle and reported schematically on the same scale (see articles).

In the present communication the results of both experimental series are directly compared to each other by means of the subjoined charts. The average results of five protein experiments, casein (2), serum albumin, gliadin, and edestin are represented as composite nitrogen and glucose curves. The combined results of feeding glycine and alanine are given in like manner. For the sake of clearness nitrogen is recorded on a separate schema. Marked similarity is noted in the extra glucose curves obtained in all cases, whether as determined from ingested glucose, amino-acids, or proteins. The same holds true in the case of the nitrogen. Indeed the variations observed are scarcely greater than could be accounted for by experimental error.

A clear demonstration is thus offered that the rate of protein metabolism may be practically identical with that of amino-acids. The time required by the organism to effect digestion of the complex protein molecule into amino-acids and the metabolism of the latter is but little longer than that occupied by the simple process of absorption and elimination of glucose. The metabolic changes here involved include hydrolysis of protein into complex and simpler polypeptides, their breakdown into amino-acids, absorption of these products, deamination, synthesis of glucose and urea, elimination of glucose and the nitrogenous end-products of protein metabolism.

These experiments serve to demonstrate the relative rapidity characteristic of metabolic reactions and emphasize the consequent difficulties attending the study of any particular step in intermediary metabolism.

The author desires to acknowledge his indebtedness to Professor Lusk for permission to use the data described above.

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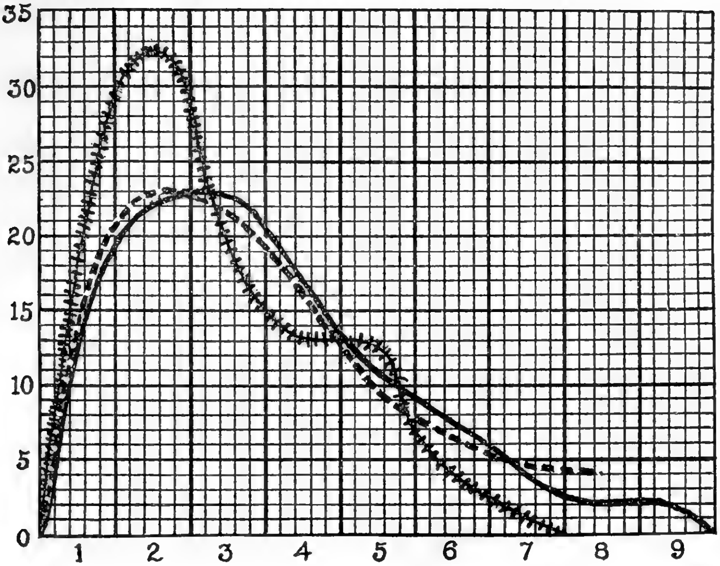
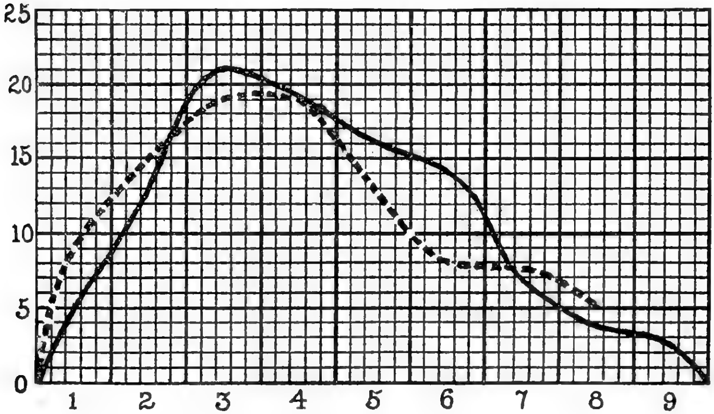
[4] Janney, N. W., *Jour. Biol. Chem.*, 1915, xx, 321.



——— Protein extra glucose.
- - - - - - - - Amino-acid extra glucose.
++++ Glucose extra glucose.

Chart I. Extra glucose excretion following ingestion of proteins, amino-acids, and glucose. In the curves height represents glucose and nitrogen hourly percentage excretion; length, hourly periods.



——— Protein extra nitrogen.
- - - - - - - - Amino-acid extra nitrogen.

Chart II. Extra nitrogen excretion following ingestion of proteins and amino-acids. Same experiments as in Chart I.

THE QUANTITATIVE DETERMINATION OF THE TOTAL PROTEIN AND NON-PROTEIN SUBSTANCES OF MUSCLE.

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The study of muscle from the standpoint of quantitative analysis has as yet received too little attention. Even the elementary composition of this organ cannot be regarded as very accurately established as the purity of the material examined is questionable. Attempts have also been made to ascertain the relative amounts of substances isolated from variously prepared muscle extracts but there exists no very exact knowledge of the percentage composition of muscle as a whole.[1] One of the chief causes of this uncertainty lies in the lack of an exact procedure for the separation and determination of the muscle proteins and the remaining constituents of muscle. The description of a method devised for this purpose is the subject of the present article.

The principle employed is the removal of the non-protein substances by means of extraction. Past experience has, however, made it doubtful whether a complete separation of the non-protein from the protein constituents of muscle can be effected by this means. On the other hand, the rather meager existing knowledge of the properties of muscle proteins precludes a departure from the extraction procedure. In spite of these difficulties it is believed, however, that the method described below will be found, if not rapid, to be sufficiently accurate for all general requirements.

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[1] For literature see König, J., Chemie der menschlichen Nahrungs- und Genussmittel, Berlin, 4th edition, 1903. von Fürth, O., and Schwartz, C., *Biochem. Ztschr.*, 1911, xxx, 413. Hammarsten, O., Lehrbuch der physiologischen Chemie, Wiesbaden, 8th edition, 1914, 539 and 565.

Aside from its value in the quantitative determination of muscle extractives, the procedure described is useful whenever it becomes desirable to ascertain the exact amount of protein present in muscle.[2]

Extraction of Muscle.

It has been observed by various workers that dried muscle can not be freed from non-protein substances even by prolonged extraction with ether. If, however, small amounts of the fresh organ be properly prepared, all but a minute quantity of the non-protein substances can be fairly easily removed by extracting successively with water, alcohol, and ether.

In the following control experiments the previous preparation and the extractions were carried out as described in the detailed account of the method (see below). The degree of completeness of the aqueous extraction was studied by reextracting in like manner a second and third time with additional water. The extracts so obtained were acidulated and evaporated to dryness on the water bath. But 1 to 3 mg. of solid substance were obtained as residues in each case. These residues on being dissolved in a few cc. of water failed to respond to the usual tests for the presence of protein. They evidently consisted of fat. To promote complete coagulation a little sodium chloride is added before treating with water. Other control experiments have demonstrated that the protein residue is free of this salt when the extraction is carried out as detailed. The material is then extracted in a Soxhlet apparatus twelve hours with 95 per cent alcohol. In addition extraction is continued in like manner six hours with absolute alcohol and a final six hours with ether. The results of an examination of the absolute alcohol and ether extracts for solids appear in the following table.

It is evident that the previous extraction with 95 per cent alcohol had removed all the alcohol-ether-soluble substances in nearly all cases. It is not safe, however, as the table demonstrates, to omit routinely the treatment with absolute alcohol and ether. The protein residues obtained in this way by extraction with

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[2] The proposed method has also been employed with fair results for organs other than muscle. This application has not as yet been extensively studied.

TABLE I.

Extraction of Fresh Muscle.

Species.	Material taken.	Absolute alcohol residue.	Ether residue.
	gm.	*mg.*	*mg.*
Dog	9.948	5.2	0
Dog	9.828	0	0
Dog	9.860	0	0
Ox	11.932	3.4	8.2
Ox*	14.789	228.8	11.7
Chicken	9.482	0	0
Fish	10.871	0	0
Rabbit	10.093	0	0
Cat	9.838	0	0

* This material contained large amounts of tough connective tissue and fat.

water, 95 per cent alcohol, absolute alcohol, and ether, were again extracted for varying periods of time with alcohol followed by ether. These extracts failed on examination to yield any solid residue whatever. In making such control examinations, it is necessary to employ extraction apparatus with ground glass parts and to make sure of the absence of dissolved substances in the solvents previous to their employment for extraction. As a further control of the completeness of extraction, the protein residues were subjected to various examinations (see below).

Method in Detail.

The fresh muscle is quickly freed from all visible fat and connective tissue, passed through a meat grinder, and thoroughly mixed. About 10 grams are weighed by difference from a weighing glass provided with a ground glass lid into a beaker glass of about 400 cc. capacity. After the addition of 100 cc. of distilled water and 5 cc. of a 10 per cent sodium chloride solution, the contents of the beaker are heated with stirring to 90°. In a few minutes' time at this temperature all turbidity in the liquid usually disappears. Coagulation can, however, be completed if necessary by the drop by drop addition of a maximum of twenty drops of $\frac{N}{2}$ acetic acid. Boiling and excess ot acid should be avoided. The coagulated material is allowed to settle and the hot supernatant liquid decanted through an ordinary round filter of 12.5 cm. diameter, which has previously been extracted with alcohol and ether, dried, and weighed. The contents of the beaker are further extracted in like manner with 300 cc. of hot water added in three portions. The protein material is then brought quantitatively on the filter and washed twice with water. A thin pellicle of fat which sometimes forms on the side of the beaker may be disregarded. If the coagulation has been properly carried out, the extract should filter readily, and be water-clear or at most show but a faint opalescence.

The moist filter is now carefully folded about the protein material, which is inserted in an extraction hull and extracted in an ordinary Soxhlet apparatus twelve hours with 95 per cent alcohol. The 95 per cent alcohol is then replaced by absolute alcohol and extraction continued six hours. A six hour ether extraction follows in like manner, without removal of the material from the apparatus. Care must be taken that the filter paper projects beyond the upper level attainable by the solvent which must then completely surround the protein. After completion of the extraction the filter with its contents is removed from the apparatus, dried to constant weight at 110° in a weighing glass provided with a ground glass lid, and the previously ascertained weight of the filter deducted. For greater accuracy 1 per cent of the weight of anhydrous protein may be subtracted in order to allow for the small residuum of fat and ash contained in it. Duplicate analyses usually agree to within 0.3 per cent. The same limit of error holds true for nitrogen determinations made in the protein preparations so obtained.

The total amount of non-protein substances when desired may be calculated by deducting the weight of the protein from that of the total solids contained in the fresh muscle. Total solids are determined as follows. From the sample glass, about 1 gram of the meat is weighed by difference into a small weighing glass, provided likewise with a ground glass cover. Drying is carried out in vacuum at room temperature for twenty-four hours, then at 55° to 65°.

Muscle Proteins.

The protein material from various analyses made as just described was examined to determine its purity. In appearance it resembled, except for pigmentation in some cases, pure preparations of isolated proteins. All preparations were partly soluble in $\frac{N}{20}$ sodium hydroxide solution. The presence of carbohydrate was tested for by the delicate Molisch reaction, fat determined according to the saponification method of Gephart and Csonka,[3] nitrogen according to Kjeldahl. The results of this examination appear in Table II.

TABLE II.

Muscle Proteins.

Proteins.	Amount present in muscle.		Nitrogen.*	Ash.**	Fat.**	Molisch reaction.
	Fresh.	Anhydrous.				
	per cent	*per cent*	*per cent*	*per cent*	*per cent*	
Dog	17.0	66.5	16.4	0.1	1.3	Negative
Ox	16.8	75.9	16.5	0.4	0.7	Negative
Chicken	17.8	69.9	16.7	0.2	0.5	Trace
Fish (halibut)	16.2	81.8	16.5	0.3	0.5	Negative
Rabbit	15.4	66.7	16.4	0.1	0.8	Negative

* Calculated for anhydrous, ash- and fat-free material.

** Calculated for anhydrous material.

It may be stated in general that the purity of the preparations of the muscle proteins corresponds rather favorably to that of analytically pure isolated proteins examined in like manner for the sake of control. This holds true entirely for carbohydrate and ash present and in most cases for fat. Apparently a small amount of fat escapes extraction, even when this is continued until all soluble substances are removed as in the present instance. With regard to the nitrogen, we have carried out nearly forty analyses in such muscle protein preparations from the dog, ox, chicken, fish, rabbit, and man. The variations in the results of these analyses are but little greater than what might be expected for the same number of preparations of pure isolated proteins. Nitro-

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[3] Gephart, F. C., and Csonka, F. A., *Jour. Biol. Chem.*, 1914, xix, 521.

gen calculated ash-free varied from 16.2 to 16.7 per cent regardless of species. In view of the fact that the material cannot be regarded as entirely homogeneous, these results may be considered to coincide rather closely with each other. Osborne and coworkers have obtained slightly lower nitrogen values in muscle proteins prepared in a similar way.[4] The ash values were somewhat higher.

Previously reported analyses of muscle, hydrolyses with amino-acid determinations, as well as the nitrogen estimations in carefully purified material just described indicate that *the general composition of muscle protein is very probably nearly the same for all species of higher animals.* This similarity of composition is also emphasized by the observation that the metabolism of muscle proteins from different animals yields glucose in nearly the same amount in all cases studied.[5]

Non-Protein Constituents of Muscle.

In order to become more conversant with the amounts of these substances, relative to the entire muscle and to solvents employed in their separation, the extracts from a series of analyses were studied with this in view. The aqueous and alcoholic portions were acidulated, evaporated to dryness on the water bath, rendered anhydrous, and weighed. The weight of these residues plus that of the protein obtained as the end-product is compared to the weight of dry muscle taken for analysis in Table III. Nitrogen balances calculated in a like manner from the nitrogen contained in the extractives and in the protein residue appear also. Evi-

TABLE III.

Balance of Solids and Nitrogen.

	Dog.	Ox.	Chicken.	Fish (halibut).
	per cent recovered	*per cent recovered*	*per cent recovered*	*per cent recovered*
Solids	99.7	100.6	100.0	99.8
Nitrogen	99.9	100.9	98.7	98.8

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[4] Osborne, T. B., and Heyl, F. W., *Am. Jour. Physiol.*, 1908, xxii, 433. Osborne, T. B., and Jones, D. B., *ibid.*, 1909, xxiv, 437.

[5] See next succeeding article.

dently it is possible to collect the extract substances without very appreciable loss. Data concerning these non-protein muscle substances have been calculated from these results and appear in Tables IV and V. The examination of the muscle proteins appearing in Table II was made from material in the same analyses as were used in the succeeding tables. It is to be borne in mind that the information here given refers to particular analyses and is not to be considered as representing average results for each species.

TABLE IV.

Non-Protein Substances in 100 Grams of Muscle.

	Fresh muscle.		Anhydrous muscle.	
	Amount of extractives.	Extract nitrogen.	Amount of extractives.	Extract nitrogen.
	gm.	*gm.*	*gm.*	*gm.*
Dog	8.4	0.47	33.1	1.83
Ox	5.5	0.37	24.7	1.67
Chicken	7.7	0.45	30.1	1.86
Fish (halibut)	3.6	0.35	18.0	1.71

TABLE V.

Non-Protein Muscle Substances (100 Grams).

	Soluble in water.	Soluble in alcohol.
	gm.	*gm.*
Dog	42.7	57.3
Ox	65.7	34.3
Chicken	56.3	43.7
Fish (halibut)	64.0	36.0

THE METABOLIC RELATIONSHIP OF THE PROTEINS TO GLUCOSE.

II. GLUCOSE FORMATION FROM BODY PROTEINS.

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(Received for publication, July 1, 1915.)

Evidence has been advanced that glucose is to be regarded as a normal intermediary product of protein metabolism. In a previous communication it was shown that the amounts of glucose yielded in the organism by various ingested proteins could be definitely determined.[1] The present article deals with glucose originating in the metabolism of body proteins. As previous calculations of the amount of protein which the organism is able to convert into sugar have been based on the relative amounts of glucose and nitrogen appearing in the urine of fasting diabetic animals, *i.e.*, the *G: N ratio*, a study of this value is consequently included.

The fully phlorhizinized fasting dog excretes glucose and nitrogen, according to Lusk, in the ratio of 3.67: 1. The G: N ratio 3.67: 1 has been generally accepted. Elsewhere, however, Lusk has used a slightly higher factor, namely, 3.7: 1. Making use of fasting phlorhizin periods with ratios 3.00 to 4.00 only, the authors have also calculated the same ratio from fifty-one observations of this author's experiments (Stiles and Lusk).

In recent years, however, there have been reported more extensive series of observations on fully phlorhizinized dogs.[2] The average G: N ratios from the fasting twelve or twenty-four hour periods of these experiments have been collected in the following

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[1] Janney, N. W., *Jour. Biol. Chem.*, 1915, xx, 321. (See also for literature.)

[2] Ringer, A. I., Dakin, H. D., Benedict, S. R., and Osterberg, E., Janney, N. W., Csonka, F. A., *ibid.*, 1912–15, xii-xx, etc.

table. Only ratios between 3.00 and 4.00 are here considered. Standard analytical methods were employed in all cases.

Average Glucose: Nitrogen Ratio.

Author.	G : N.	No. of periods.
Ringer	3.49	82
Dakin	3.45	42
Benedict	3.48	15
Csonka (Lusk)	3.46	4
Janney	3.35	84
Average G: N ratio	3.43	227

In practically all these experiments 1 gram or more of phlorhizin was injected daily according to Coolen, the modern technique. In such experiments a somewhat lower G: N ratio has been observed at times, during the night periods, and may be ascribed to failure of excretion of all the glucose in the organism at that time. As it has been shown by Lusk that under these circumstances the residual glucose is excreted after the next phlorhizin injection with corresponding elevation of the G: N ratio of the next succeeding period, the size of the mean ratio would not be affected. We would not, therefore, ascribe the low average G: N ratios of the table to inadequacies in the technique employed.[3]

The value *3.43: 1* taken from the table represents, it is believed, the average urinary G: N ratio for the fasting phlorhizinized dog more accurately than do the higher ratios previously reported. The urinary G: N ratio cannot, however, be regarded as a very exact basis for estimation of the amount of glucose derivable from body protein. The following consideration makes this plain. Total nitrogen of the urine contains only about 95.3 per cent of nitrogen derived from glucogenetic proteins, as about 4.7 per cent of the total nitrogen of fasting phlorhizinized dogs originates

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[3] Feces are usually entirely absent in such experiments. The nitrogen of starvation feces can be disregarded in calculations of the G: N ratio as it is of alimentary origin. Mendel, L. B., and Fine, M. S., *Jour. Biol. Chem.*, 1912, xi, 5.

from creatine, creatinine, and purines.[4] These substances in all probability yield no glucose in the organism, as meat extractives, in which they are present in considerable amount, have been shown to be non-glucogenetic.[5] The ratio 3.43: 1 corrected for this source of error becomes 3.60: 1. The ratio 3.60: 1 may then be regarded as corresponding more exactly to the relation existing between the nitrogen of glucogenetic body proteins of the dog broken down in starvation, and glucose of the same origin.

The G: N ratio just described is calculated from the *total* urinary nitrogen and glucose yielded by all the organ proteins undergoing decomposition in the fasting phlorhizinized animal. The muscle proteins undoubtedly occur, however, in more abundant amounts than do any other proteins in the body and it can be properly surmised that most of the urinary nitrogen and glucose of fasting animals originates in muscle proteins. The size of the ratio existing between the glucose and nitrogen yielded by the breakdown of *body* protein is, therefore, in all probability largely determined by the glucose and nitrogen yielded by *muscle* proteins. It has been demonstrated elsewhere that isolated proteins produce, according to their composition, various amounts of glucose on ingestion by phlorhizinized dogs. If the protein glucose: nitrogen ratio 3.60: 1 established by the authors for the dog be correct, dog muscle proteins should, therefore, be found under the same circumstances to yield an amount of glucose which would bear to the nitrogen of these proteins about the same relation as 3.60: 1.

A purified preparation of the total proteins contained in dog muscle, and fresh dog muscle containing a known amount of total protein, was accordingly fed to fasting phlorhizinized dogs and the metabolic glucose derived from the protein calculated. As the average result of seven experiments, 56.34 grams of glucose per 100 grams of protein containing 16.13 per cent of protein ni-

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[4] Total creatine nitrogen was calculated from the tables of Wolf, C. G. L., and Osterberg, E., *Am. Jour. Physiol.*, 1914, xxviii, 71. Benedict, S. R., and Osterberg, E., *Jour. Biol. Chem.*, 1914, xviii, 195. For purine nitrogen the value 0.10 gm. was accepted. (Wiechowski, W., in Neubauer-Huppert's Analyse des Harns, Wiesbaden, 11th edition, 1913, 912.)

[5] Csonka, *Jour. Biol. Chem.*, 1915, xx, 539.

trogen were recorded. The G: N ratio obtained from these values is 3.49: 1, which corresponds quite closely to the average urinary G: N ratio 3.43: 1, calculated by the authors. As the nucleic acid contained in the muscle protein is very probably not glucogenetic, nucleic acid nitrogen estimated as about 0.34 gram per 100 grams of muscle protein has accordingly been deducted from the total nitrogen contained, 16.13 − 0.34 = 15.79 grams. Inasmuch as 100 grams of muscle proteins yielded 56.34 grams of glucose in the organism, the corrected ratio *3.57: 1* represents more exactly the relation between the nitrogen and glucose arising in the metabolism of the muscle proteins. The ratio 3.57: 1 corresponds nearly to the corrected urinary G: N ratio 3.60: 1 (see above). *From the urinary ratio 3.60: 1 and 6.25, the general protein factor, 57.5 per cent of body protein, is calculated to be convertible into glucose. The amount of muscle proteins, the most abundant of all body proteins, converted into glucose in actual feeding experiments, is 56.3 per cent. The close correspondence of these values is striking.* Furthermore, the amounts of glucose arising in the metabolism of three other body proteins have been determined as follows: serum albumin 55 per cent, gelatin 65 per cent, fibrin 53 per cent, average 57.7 per cent.[6] From this it may be inferred that, though various amounts of glucose are yielded by various body proteins occurring in relatively lesser amounts in the organism, yet their average glucose yield will probably be found to correspond likewise rather closely to that of total body proteins or of muscle proteins. Lusk has calculated that 58.7 per cent or 60 per cent of body proteins of the fasting phlorhizinized dog are convertible into glucose. The close correspondence of these ratios to those established in the present paper is due to the fact that the higher urinary ratios, respectively 3.67: 1 and 3.70: 1, were employed and no allowance was made for nitrogen arising from non-glucogenetic substances (see above). If this correction be made, the protein glucose ratio 3.9: 1 is obtained, from which 62 per cent of protein (factor 6.25) would be convertible into glucose. In a similar way Cremer has indeed calculated 68 per cent of glucose using the ratio 3.7: 1. *It is believed that the investigation described above offers an adequate explanation based on direct experi-*

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[6] Janney, *loc. cit.*

mental evidence for the size of the average urinary G: N ratio in the phlorizin diabetic dog. That all the glucose and no more arising in the breakdown of body protein appears in the urine is made evident.

No explanation is as yet afforded, however, for unusually high or low G: N ratios. The latter are occasionally met with in dogs and are the rule in other animals. For the study of this question the rabbit was selected. The G: N ratio in phlorhizinized rabbits averages about 2.8: 1. Feeding experiments were accordingly carried out and the glucose derivable from the metabolism of *rabbit* muscle proteins was determined in the same manner as previously described for dog muscle.

It was found that an average of *60 per cent* of rabbit muscle protein is convertible into glucose. The corresponding G: N ratio is 3.8: 1, making due allowance for nucleic acid nitrogen (see above). *Though the urinary G: N ratio for the rabbit is 2.8: 1, the chief bulk of its body protein produces even more glucose than that of the dog with a urinary G: N ratio of 3.43: 1. Evidently the calculation based on the urinary ratio 2.8: 1, that a maximum of 45 per cent of glucose is yielded by the proteins of the rabbit in metabolism, is to be considered erroneous.*

For the very high ratios (over 4.0: 1) rarely met with in phlorhizin diabetic dogs, no adequate explanation has hitherto been offered. According to the authors' analyses, about 13 per cent of the total nitrogen in fresh muscle is present in extract substances. The amount of nitrogenous extractives in muscle is known to vary considerably. It is possible that when these substances are present in small quantities, this may account for the high urinary G: N ratios.

In view of the above mentioned data a distinction must evidently be made between the urinary G: N ratio and the G: N ratio calculated from glucose and nitrogen arising in the metabolism of body proteins. The latter value has been shown to be about 3.6: 1 for the dog and 3.8: 1 for the rabbit. There is also reason to believe that values could be obtained in a similar way for other species of higher animals which would show no great deviation from those just mentioned. In case of the ox and chicken, muscle protein hydrolyses with amino-acid determinations have been made.[7]

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[7] Osborne, T. B., and Heyl, F. W., *Am. Jour. Physiol.*, 1908, xxii, 433. Osborne, T. B., and Jones, D. B., *ibid.*, 1909, xxiv, 437.

From these data it has been calculated that ox muscle proteins undergoing metabolism in the organism can be expected to yield approximately 57.5 per cent glucose, corresponding to the G: N ratio 3.6: 1, and chicken 54.5 per cent glucose, G: N ratio 3.5: 1.[8] The fact that no very obvious change has been observed in the urinary G: N ratio of the phlorhizinized dog when beef has been ingested makes it also probable that similar amounts of glucose are yielded by the body proteins of the ox. From the urinary ratio 3.6: 1 reported for man in severe diabetes mellitus and phlorhizin diabetes,[9] it may be inferred that the amount of metabolic glucose yielded by the body proteins of the human species will be found not to deviate very widely from the values just described.[10] As reported analyses of muscle proteins of various higher animals[11] show a close correspondence to each other, it may be inferred that their general composition is also very similar. Consequently the amount of glucose formed in the metabolism of these substances would probably be found to exhibit but slight variations in each case.

Accepting that the muscle proteins and probably the total body proteins of higher animals yield in their breakdown in the organism amounts of glucose and nitrogen corresponding to about the average ratio 3.6: 1 (57.5 per cent glucose), what explanation can be offered for the occurrence of the much lower urinary G: N ratio, approximately 2.8: 1? This lower ratio is the rule in phlorhizin diabetic animals (rabbit, cat, goat, exceptionally dogs). From it has been calculated that 47 per cent of protein is convertible into glucose.[12] That this value for the dog and rabbit is very considerably less than the maximal amount of sugar, which can originate in the breakdown of the body proteins of these animals, the experiments described in this article demonstrate. In the case of the lower ratio obviously but two causal alternatives

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[8] For mode of calculation employed, see Janney, *loc. cit.*

[9] Mandel, A. R., and Lusk, G., *Jour. Am. Med. Assn.*, 1904, xliii, 241. Foster, N. B., *Deutsch. Arch. f. klin. Med.*, 1913, cx, 501. Benedict, S. R., and Lewis, R. C., *Proc. Soc. Exper. Biol. and Med.*, 1913–14, xi, 134.

[10] Experiments on this subject are being carried out. The results so far obtained nearly coincide with those reported for the dog and rabbit.

[11] See immediately preceding article.

[12] Others have previously calculated 45 per cent for this value. The value 47 per cent is based on the G: N ratio 2.8: 1, corrected for creatine, creatinine, and purine nitrogen as above.

can be possible. Either *less* glucose in proportion to nitrogen set free is formed from protein or *all* the glucose formed is not excreted in the urine.

With regard to the first explanation two possibilities are apparent. Either nitrogen in this case is set free from protein in considerably larger relative amounts than are carbon, hydrogen, and oxygen, or, if protein is broken down as usual, the ability to synthesize glucose must be lessened. Both these views are difficult of acceptance. It seems very unlikely that the above mentioned atypical breakdown of protein is possible as it would depend on extensive splitting off of amino-groups or even cyclically combined nitrogen without the freeing of the corresponding carbon, oxygen, hydrogen chains, which yield glucose. Again if the power to synthesize the usual amount of glucose derivable from protein be lost, it is reasonable to suppose that animals exhibiting the low G: N ratio would likewise be unable to synthesize the full amount of glucose possible of formation in the metabolism of *ingested* proteins. This is not the case, as sugar is produced from food proteins in like quantity in experiments showing either high or low ratios.[13]

In pancreas and phlorhizin diabetes exhibiting the G: N ratio 2.8: 1 it seems probable, then, that though the usual amount of glucose is formed from protein, it fails of complete excretion. In explanation it has been presumed that dextrose may exist in the organism in two or more colloidal combinations (Loewi, and Mandel and Lusk). Differences in combustibility of these colloids have been held to account for the different G: N ratios of diabetes. This hypothesis lacks experimental confirmation. The further suggestion of Lusk that the varying ability of amino-acids of body proteins to yield glucose can be responsible is not substantiated by the experiments reported in this series of articles.

A full discussion of this important question lies beyond the scope of the present article. The authors' views may, however, be stated in abstract as follows. Though glucose cannot be fully oxidized to oxygen and carbon dioxide in complete diabetes characterized by the urinary G: N ratio 2.8: 1, there is certain acceptable evidence that it can be utilized, necessarily in some other manner, by the tissues of such diabetic animals. Glucose, at

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[13] Janney, *loc. cit.*

least in any considerable quantity, is not stored as glycogen under these circumstances. Repair and maintenance of organ proteins can probably be accomplished synthetically from the intermediary products of carbohydrate metabolism and nitrogen.[14] It seems possible, therefore, that a portion of the glucose formed from the products of protein breakdown, may escape elimination from the body in this case, because it is synthetically utilized within the organism. In phlorhizin diabetes, exhibiting the higher G:N ratio, another condition may prevail. Here both the power of complete oxidation of glucose and its synthetic utilization may be lost. The maximal amount of this carbohydrate formed by the metabolism of body proteins consequently appears in the urine.

Further studies on this subject as well as the medical applications of the data of this series of articles and additional experiments are reserved for future communications.

SUMMARY.

The average *urinary* G:N ratio in the fasting phlorhizin diabetic dog is found to be 3.4: 1, rather than 3.67: 1 (Lusk).

The body proteins of the dog collectively yield in metabolism about 57.5 per cent of glucose corresponding to the *protein* G:N ratio 3.6:1; body proteins of the rabbit about 60 per cent glucose, *protein* G:N ratio 3.8: 1.

Body proteins of the other higher animals, including man, very probably yield nearly the same amounts of metabolic glucose as the dog and rabbit.

The calculated yield of 45 per cent glucose, as a maximum from body protein, based on the urinary G:N ratio 2.8: 1 in phlorhizin and pancreas diabetes, is incorrect.

EXPERIMENTAL.

The same experimental plan was employed as in a related investigation to which reference may be made.[15] Here it was shown that proteins, if proper precautions be observed in feeding, are broken down, absorbed, and the resulting nitrogen and glucose

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[14] Janney, *loc. cit.*

[15] Janney, *loc. cit.*

excreted completely within nine hours after ingestion. In the present experiments muscle proteins were fed as such or as fresh muscle containing corresponding amounts of protein per kilogram of dog's weight.[16] The nitrogen of meat fed even in large amounts to the dog is 100 per cent utilized.[17] No feces whatever were excreted during the experiments here reported. That absorption and elimination of the metabolic products formed from the ingested material were entirely complete, can therefore scarcely be doubted. Reducing substances, presumably carbohydrates and glycogen were estimated in the meat fed, and the minimal amounts found deducted from the extra glucose. The corrected values appear in the protocols. Muscle extractives do not yield very appreciable amounts of glucose in the phlorhizinized animal.[18] The present experiments corroborate this finding.

The fresh finely divided muscle was carefully freed from visible connective tissue, passed through the meat grinder, and thoroughly mixed. Nitrogen and glucose were determined as previously described, glycogen was determined according to Pflüger. Reducing substances were estimated by means of the following procedure, which was carried out with quantitative precautions. The muscle with a like quantity of water was coagulated by heating and filtered. The proteins were completely removed from the filtrate according to Schenck, the mercury precipitated by hydrogen sulphide, the excess of which was driven off by an air current. The reducing substances were determined in the final solution by the Pavy-Fehling method. Reliable results can be obtained in this manner.

The total protein was determined according to a method described in the paper immediately preceding. By application of this method to larger quantities the dog muscle protein for feeding was prepared from the same meat, from fasting phlorhizinized dogs, which was used in the remaining experiments. The purity of the protein preparation (nitrogen = 16.3 per cent) was controlled by subjecting a sample of the original fresh muscle from which it was obtained to the analytical procedure and determin-

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[16] The protein feeding experiments were carried out by Mr. Csonka in the laboratory of Dr. James P. McKelvey, Pittsburgh.

[17] Mendel and Fine, *loc. cit.*

[18] Csonka, *loc. cit.*

ing the nitrogen in the pure protein so obtained (see table). All calculations involving the muscle proteins are based on values obtained for anhydrous material.

Analysis of Meat Used in Feeding Experiments, Grams per 100 Grams.

	Total solids.	Nitrogen.	Reducing substances.	Glycogen.	Total protein.	Nitrogen per 100 gm. protein.
Dog...........	25.5	3.20	0.56	0.03	17.00	16.1
Rabbit (first four experiments)....	25.3	3.51	0.90	0.01	18.92	16.4
Rabbit (last three experiments)....	22.0	3.26	0.32	0.33	16.56	16.1

Dog Muscle and Protein Feeding Experiments.

(Periods 24 hours.)

Muscle fed.	Protein fed.	Nitro-gen fed.	Weight of dog.	Nitro-gen.	Glu-cose.	G : N.	Extra glucose.	
							Amount	In terms of protein fed.
gm.	*gm.*	*gm.*	*kg.*	*gm.*	*gm.*		*gm.*	*per cent*
			9.6	6.88	24.37	3.54		
	28.60	4.60		8.73	30.71	3.52	16.27	56.29
				6.42	22.22	3.46		
				5.79	21.41	3.70		
				5.81	19.44	3.35		
	27.38	4.41	9.1	7.92	27.40	3.46	14.95	54.64
				5.37	19.49	3.62		
				16.29	60.50	3.71		
	23.29	3.75	14.6	16.87	60.90	3.59	12.33	52.93
	22.72	3.66	14.1	15.53	57.24	3.69	13.22	58.29
				11.98	44.49	3.71		
				8.74	30.82	3.54		
140.7.......	23.92	4.60	9.2	10.83	36.32	3.36	14.64	61.21
				8.31	26.32	3.17		
127.0.......	21.59	4.25	8.5	8.98	27.22	3.03	11.76	54.46
				6.03	18.42	3.05		
				11.52	35.03	3.04		
182.0.......	30.94	5.95	11.9	13.09	40.61	3.03	17.47	56.46
				10.44	32.79	3.14		

Rabbit Muscle Feeding Experiments.

(Periods 12 hours.)

Muscle fed.	Protein fed.	Nitrogen fed.	Weight of dog.	Nitrogen.	Glucose.	G : N.	Extra glucose.	
							Amount	In terms of protein fed.
gm.	*gm.*	*gm.*	*kg.*	*gm.*	*gm.*		*gm.*	*per cent*
				8.41	30.44	3.62		
				8.48	29.85	3.52		
168.1.......	31.80	5.90	11.8	11.80	40.21	3.41	18.23	57.3
				7.27	24.85	3.42		
162.4.......	30.72	5.70	11.4	10.77	36.70	3.41	17.95	58.4
				6.36	21.60	3.40		
159.5.......	30.18	5.60	11.2	10.33	36.21	3.50	19.35	64.1
				5.97	18.60	3.12		
				5.77	18.27	3.16		
				6.86	22.22	3.24		
123.9.......	23.45	4.35	8.7	9.14	29.27	3.20	12.60	53.7
				6.31	19.80	3.14		
				6.13	20.60	3.37		
				5.62	17.24	3.07		
				5.05	15.44	3.06		
122.7.......	20.31	4.00	8.0	7.13	22.99	3.22	12.57	61.9
				4.39	12.77	2.91		
				4.20	13.33	3.17		
				3.90	10.87	2.79		
111.9.......	18.53	3.65	7.5	5.90	18.43	3.12	11.32	61.1
				3.20	9.22	2.88		
105.9.......	17.54	3.45	7.1	5.51	18.18	3.30	11.18	63.7
				3.08	10.05	3.25		

THE RÔLE OF THE DIGESTIVE GLANDS IN THE EXCRETION OF ENDOGENOUS URIC ACID.

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A number of hypotheses has been suggested to account for the origin of the uric acid of the urine. Among them the theories of Burian and Schur[1] and of Mareš[2] respectively claim more attention and consideration today than do any of the others. The theory of Burian and Schur was promulgated in 1900, Sivén[3] announcing a similar view almost simultaneously. Mareš' hypothesis was suggested in 1887, but because of the attention claimed by Horbaczewski's[4] leucocytosis theory, advanced in 1889 (according to which urinary uric acid was regarded as an expression of the extent of the disintegration of the white blood cells), the work of Mareš did not become current until after this latter view had been abandoned. A fresh impetus was given to Mareš' theory in 1910 when it was modified so as to be brought into accord with developments which had arisen since it was first formulated.

According to Burian and Schur the uric acid of the urine arises from two sources: the food and the tissues. That arising from the former is designated the exogenous uric acid; from the latter the endogenous portion is derived. The endogenous uric acid is supposed to be constant in amount for each individual, the exogenous varying between wide limits, due to the presence of different quantities of uric acid precursors in the food.

Materials free from uric acid precursors were presumed to have no effect whatever on the uric acid excretion; but regarding this point there is much difference of opinion. If they do exert an influence, then the endogenous portion also may be a variable quantity, since it is then reasonable;

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[1] Burian, R., and Schur, H., *Arch. f. d. ges. Physiol.*, 1900, lxxx, 241. 1901, lxxxvii, 239. Burian, R., *Ztschr. f. physiol. Chem.*, 1904–05, xliii, 532.

[2] Mareš, F., *Arch. slav. de biol.*, iii, 207, through *Centralbl. f. d. med. Wissensch.*, 1888, xxvi, 2; *Arch. f. d. ges. Physiol.*, 1910, cxxxiv, 59.

[3] Sivén, V. O., *Skandin. Arch. f. Physiol.*, 1901, xi, 123; 1906, xviii, 177; *Arch. f. d. ges. Physiol.*, 1912, cxlv, 283.

[4] Horbaczewski, J., *Monatsh. f. Chem.*, 1888–89, x, 624.

to believe that different foodstuffs or different quantities of the same foodstuff may result in the excretion of unequal amounts of uric acid. Most of the investigations of a decade or two ago were offered in support of Burian and Schur's theory, but the experimental procedures of the period were scarcely suitable to solve the problem.

Burian believed the muscles to be of primary importance as the source of the endogenous uric acid. Sivén's repetition of one phase of Burian's work, however, failed entirely to confirm this view; and at present there are no really conclusive data showing an increased uric acid output after muscular exercise.[5]

Mareš' present theory differs from the view just stated in that the endogenous uric acid is not thought to be a constant quantity, even for the same individual, except when the diet is uniform. According to Mareš foodstuffs can exert an influence on the amount of uric acid excreted and this is due to their action in stimulating the digestive glands to activity.

In 1905 Folin[6] submitted evidence opposing Burian and Schur's theory. He found that on his starch and cream diet the uric acid output may sink to about one-half its value on a purine-free milk and egg diet.

Mendel and Brown,[7] while studying the rate of uric acid elimination in man, noted slight increases in the uric acid output following the ingestion of purine-free meals. The results were not offered at the time as a confirmation of Mareš' theory, but they may be so interpreted.

Mareš' work was extended in his laboratory in 1911 by Smetánka[8] who concluded that purine-free foods including carbohydrate may exert an influence on the uric acid output.

Lambling and Dubois[9] and Maurel[10] have recently published results which seem confirmatory of Mareš' theory. The increases in the uric acid excretion noted by them after the ingestion of milk and eggs respectively are so remarkable as to make a repetition of their experiments desirable.

The augmenting effect of a high-protein, purine-free diet on the uric acid output observed by Taylor and Rose[11] is additional evidence in favor of Mareš' theory. These authors suggest that the effect may also be due to the exaggerated nuclein metabolism provoked by the high protein intake.

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[5] Raiziss, Dubin, and Ringer's single experiment regarding this point need not be accepted as final until it is repeated and more unequivocal data are presented than are given in their paper. See footnote 24.

[6] Folin, O., *Am. Jour. Physiol.*, 1905, xiii, 66.

[7] Mendel, L. B., and Brown, E. W., *Jour. Am. Med. Assn.*, 1907, xlix, 896.

[8] Smetánka, F., *Arch. f. d. ges. Physiol.*, 1911, cxxxviii, 217.

[9] Lambling, E., and Dubois, F., *Compt. rend. Soc. de biol.*, 1914, lxxvi, 614.

[10] Maurel, E., *Compt. rend. Soc. de biol.*, 1914, lxxvii, 190.

[11] Taylor, A. E., and Rose, W. C., *Jour. Biol. Chem.*, 1914, xviii, 519.

Hypotheses differing from those of Burian and Schur and Mareš have been suggested by recent investigators—Plimmer, Dick, and Lieb,[12] Weintraud,[13] Graham and Poulton,[14] and Berg.[15] Plimmer, Dick, and Lieb, because they failed to find any quantitative relationship between the food purines and those of the urine, advanced the idea that the food contains toxic substances which call forth an increase in the number of leucocytes. The metabolism of these cells is regarded by them as the essential cause of the increased uric acid output. Weintraud inclines to the belief that all food must become part of the living body before it can appear in the metabolic end-products. According to him, therefore, all uric acid is in this sense endogenous. Graham and Poulton have suggested that endogenous uric acid is formed in part by synthesis from protein and carbohydrate. Their data do not bear out the hypothesis and, indeed, are so conflicting as to make any interpretation difficult or impossible. Berg supposes that the variations in the uric acid excretion result directly from altering the reaction of the blood and thereby the products of metabolism also.

EXPERIMENTAL PART.[16]

In the present investigation an effort has been made to determine the source of the endogenous uric acid. The direction of the work had already been indicated by the results obtained in this laboratory by Mendel and Brown. They showed that a rise in the uric acid output may occur after the ingestion of food. When the food was purine-free the rise was small; it was, of course, large when the diet was not purine-free. These facts made it appear that Mareš' view, according to which endogenous uric acid arises from glandular activity, might be substantiated. It does not follow that the theory needs to account for the total endogenous output; it may serve to explain at least a part of it.

Plan of the Investigation.

The general plan of the investigation was to study the hourly outputs of uric acid in man under definite conditions. To this

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[12] Plimmer, R. H. A., Dick, M., and Lieb, C. C., *Jour. Physiol.*, 1909–10, xxxix, 98.

[13] Weintraud, W., *Verhandl. d. deutsch. Kong. f. Med.*, XXXth Congress, Wiesbaden, 1913.

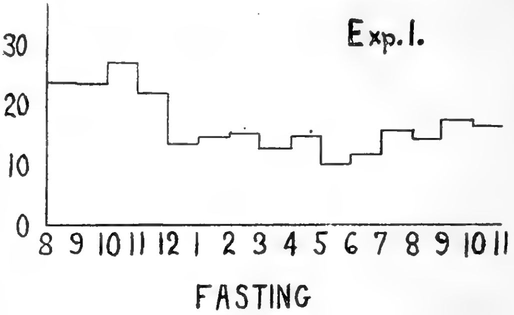
[14] Graham, G., and Poulton, E. P., *Quart. Jour. Med.*, 1913–14, vii, 13.

[15] Berg, R., *München. med. Wchnschr.*, 1914, lxi, 1275.

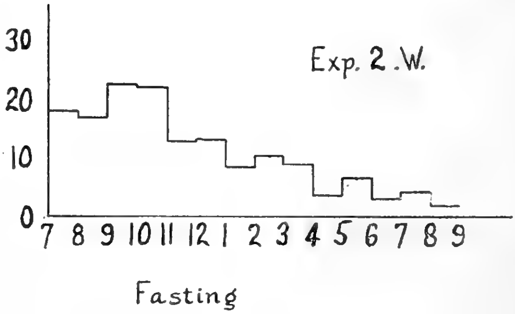
[16] The data presented are taken from the dissertation of R. L. Stehle, presented for the degree of Ph.D., Yale University, 1915.

end observations were made of the effects of agents assumed to act on the digestive functions, *viz.*: nutrients individually and in groups; various types of laxatives alone or with agar-agar; the

HR.	VOL.	mg.U.A.
8-9	32	23.4
9-10	62	23.3
10-11	165	27.0
11-12	109	21.7
12-1	18	13.5
1-2	16	14.7
2-3	17	15.2
3-4	15	12.9
4-5	20	14.7
5-6	14	10.0
6-7	13	11.8
7-8	13	15.8
8-9	17	14.1
9-10	49	17.6
10-11	29	16.7



HR.	VOL.	mg.U.A.
7-8	36	18.0
8-9	28	16.9
9-10	27	22.5
10-11	67	22.0
11-12	37	12.9
12-1	99	13.0
1-2	17	8.4
2-3	26	10.1
3-4	86	8.9
4-5	15	3.4
5-6	33	6.2
6-7	38	2.8
7-8	17	3.9
8-9	11	1.7



drugs atropine, pilocarpine, and alcohol. In this way it was thought likely that a determination of the influence of glandular activity, as well as a differentiation between glandular and muscular activity in relation to the digestive processes, would become possible.

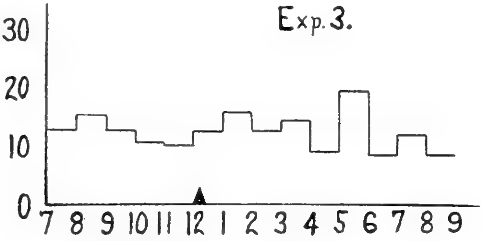
The routine of the individual experiments was as follows: The hourly outputs of urine were collected from 7 a.m. to about 9 p.m. or later. After 6 p.m. or earlier on the day preceding the experimental day nothing was eaten. In the few instances in which anything was eaten at that hour the food was such as is ordinarily considered purine-free: eggs, bread, butter, milk; but in the great majority of experiments the subject fasted from noon on the day previous to that on which the actual observations were made. The details of each experimental day are given in the protocols. The activity of the subject was approximately the same in each case and consisted of the usual laboratory routine.

Uric acid was determined by the Folin-Shaffer method. Where the hourly samples were less than 50 cc. they were diluted to that volume; where they were considerably larger (more than 90 cc.) they were concentrated on a water bath after the addition of a small amount of acetic acid. The latter process was seldom necessary.

Experiments 1 and 2, Fasting.[17]—The output of uric acid during fasting is shown in the accompanying charts.

It will be observed that the values are highest during the forenoon, sinking to a low level thereafter. An experiment similar to

HR.	VOL.	mg U.A.
7-8	20	12.9
8-9	33	15.2
9-10	45	12.9
10-11	27	10.7
11-12	21	10.1
12-1	21	12.4
1-2	71	16.0
2-3	70	12.5
3-4	21	14.6
4-5	19	9.
5-6	39	19.1
6-7	16	8.4
7-8	19	11.8
8-9	13	8.4



12M. 220g. sucrose

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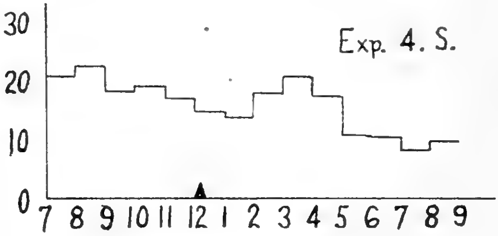
[17] A number of the experiments reported were carried out at the Battle Creek Sanitarium through the courtesy of Dr. J. H. Kellogg. In the charts the abscissae represent hours and the ordinates mg. of uric acid.

these carried through twenty-four consecutive hours showed a maximum output during the late morning followed by a slow decrease until very early the following morning and then a rise to the maximum again. The form of the graph varies for different fasting individuals.

Influence of Foodstuffs.

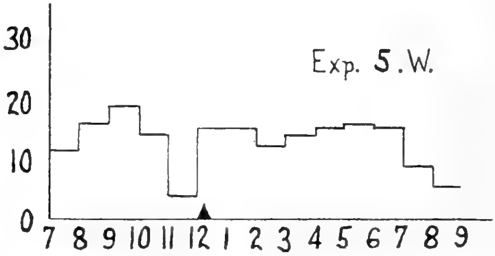
Experiments 3, 4, 5, and 6, Carbohydrate Feeding.—The object of this series was to determine the effect of the ingestion of carbo-

HR.	VOL.	mg.U.A.
7-8	23	20.8
8-9	42	22.5
9-10	120	18.3
10-11	88	19.0
11-12	172	17.1
12-1	66	14.9
1-2	180	13.8
2-3	44	18.0
3-4	48	20.8
4-5	148	17.4
5-6	290	10.7
6-7	174	10.5
7-8	73	8.2
8-9	176	9.8



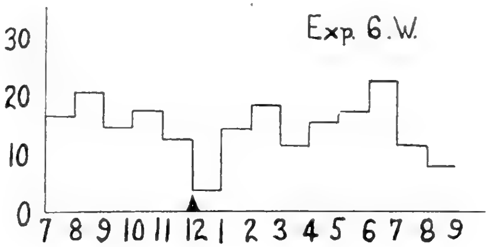
12M. 218g. sucrose

HR.	VOL.	mg.U.A.
7-8	22	11.8
8-9	36	16.3
9-10	50	19.2
10-11	42	14.6
11-12	33	3.9
12-1	298	15.4
1-2		15.4
2-3	42	12.4
3-4	31	14.1
4-5	37	15.8
5-6	43	16.3
6-7	43	15.8
7-8	25	9.0
8-9	17	5.6



12M. 220g. sucrose

HR.	VOL.	mg.U.A.
7-8	119	16.8
8-9	67	20.7
9-10	35	14.6
10-11	67	17.6
11-12	28	12.9
12-1	30	3.9
1-2	37	14.6
2-3	33	17.5
3-4	23	11.3
4-5	31	15.2
5-6	45	17.5
6-7	54	22.4
7-8	21	11.8
8-9	16	7.9

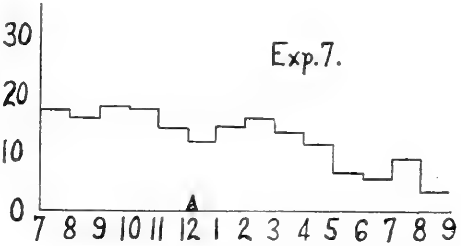


12M. 220g. sucrose

hydrate apart from protein and fat. Sucrose was fed. A minimum quantity of water only was allowed in order to prevent a subsequent diuresis which would necessitate a concentration of the urine samples. The effect is not clear cut. In Experiments 3 and 4 the increase in uric acid is small, but in 5 and 6 it appears to be more pronounced when compared with the output of the same subject during fasting (see Experiment 2).

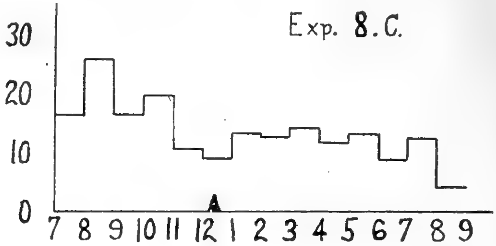
Experiments 7 and 8, Fat Feeding.—The influence exerted by fat, taken alone, seems to be nil. This result is not wholly in ac-

HR.	VOL.	mg.U.A.
7-8	34	17.5
8-9	61	15.9
9-10	44	18.0
10-11	73	17.5
11-12	21	14.1
12-1	16	11.8
1-2	24	14.6
2-3	47	15.8
3-4	76	13.5
4-5	27	11.3
5-6	16	6.8
6-7	17	5.6
7-8	21	9.0
8-9	117	3.3



12M. 105g. butter

HR.	VOL.	mg. U.A.
7-8	18	16.3
8-9	25	25.9
9-10	20	16.3
10-11	24	19.7
11-12	15	10.7
12-1	12	9.0
1-2	18	13.2
2-3	10	12.7
3-4	15	14.1
4-5	12	11.8
5-6	13	13.2
6-7	10	9.0
7-8	22	12.7
8-9	5	4.2

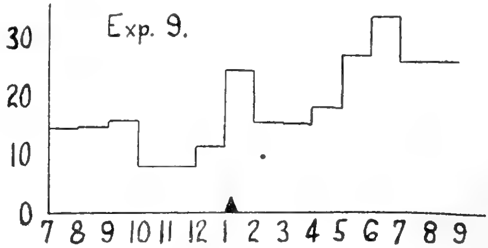


12 M. 104 g. unsalted butter

cord with the findings of Herrmann[18] and of Horbaczewski and Kaněra[19] who found a decreased uric acid output after fat ingestion. Their conclusions were based on determinations of daily outputs.

Experiment 9, Protein Feeding.—This series scarcely calls for detailed comment. The augmenting effect of the protein is unmistakable. The calorific value of the ingested gluten is consid-

HR.	VOL.	mg. U.A.
7-8	15	14.1
8-9	16	14.6
9-10	16	15.8
10-11	15	7.9
11-12		7.9
12-1	10	11.3
1-2	340	24.6
2-3	85	15.1
3-4	29	15.2
4-5	27	18.0
5-6	38	27.0
6-7	52	33.2
7-8	106	25.8
8-9		25.8



1 P.M. 130 g. gluten

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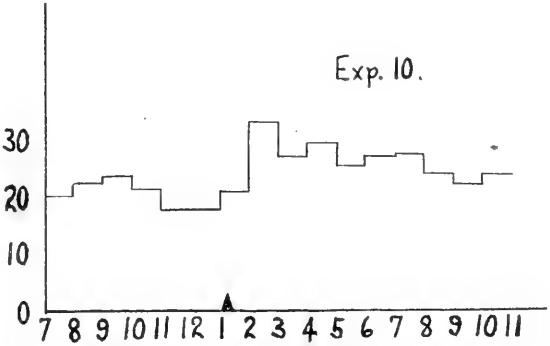
[18] Herrmann, A., *Deutsch. Arch. f. klin. Med.*, 1888, xliii, 273.

[19] Horbaczewski, J., and Kaněra, F., *ibid.*, 1886, vii, 105.

erably lower than the values of the carbohydrate and fat in the preceding experiments. This is due to the fact that the taste of the gluten was very disagreeable so that 130 grams represented the maximum amount which the subject could ingest.

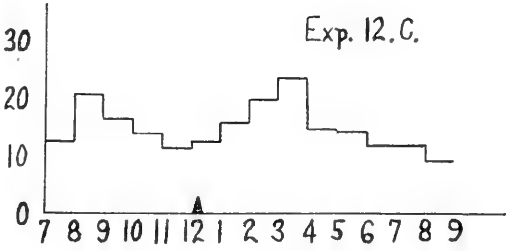
HR.	VOL	mg.U.A.
7-8	20	20.0
8-9		22.2
9-10	21	23.4
10-11	15	21.1
11-12	11	17.6
12-1	12	17.6
1-2	14	20.5
2-3	30	32.8
3-4	18	26.9
4-5	22	29.3
5-6	18	25.2
6-7	22	27.0
7-8	21	27.5
8-9	17	24.0
9-10	19	22.3
10-11	23	24.0



1 P.M. { 75 g. butter
154 g. sucrose

614 fat cal.
600 cbh. cal

HR.	VOL.	mg.U.A.
7-8	21	12.4
8-9	44	20.3
9-10	31	16.4
10-11	11	14.0
11-12	12	11.3
12-1	15	12.4
1-2	20	15.8
2-3	25	19.7
3-4	26	23.1
4-5	16	14.6
5-6	18	14.1
6-7	18	11.8
7-8	16	11.8
8-9	9	9.0



12 M. { 154 g. sucrose
60 g. butter

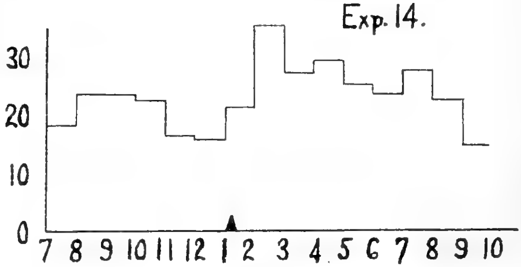
Experiments 10 and 12, Fat and Carbohydrate Feeding.—Although no increase in the uric acid output was found when fat was taken alone and only a very slight increase when carbohydrate was taken in this way, yet ingestion of the two together seems to produce an unmistakable effect. The accompanying charts make this plain.

Experiments 13 and 14, Protein and Carbohydrate, Protein and Fat.—When protein is taken together with either fat or carbohydrate the rise in the uric acid output is marked, as would be expected from Experiment 10 in which protein alone was employed.

HR.	VOL.	mg.U.A.
8-9	56	24.9
9-10	64	34.5
10-11	33	25.8
11-12	24	19.9
12-1	27	21.1
1-2	57	27.8
2-3	47	36.9
3-4	29	34.0
4-5	31	34.6
5-6	43	34.0
6-7	51	25.9
7-8	65	31.1
8-9	65	29.7
9-10	44	24.6

1 P.M. { 89g. casein, 92 egg white } 457 prot. cal.
{ 90 banana, 130 sucrose } 600 cbh. "

HR.	VOL.	mg.U.A.
7-8	11	18.2
8-9	16	23.4
9-10	15	23.4
10-11	17	22.3
11-12	12	16.4
12-1	10	15.8
1-2	13	21.1
2-3	20	35.2
3-4	26	27.0
4-5	23	29.3
5-6	31	25.2
6-7	28	23.4
7-8	37	27.5
8-9	35	22.3
9-10	22	14.7



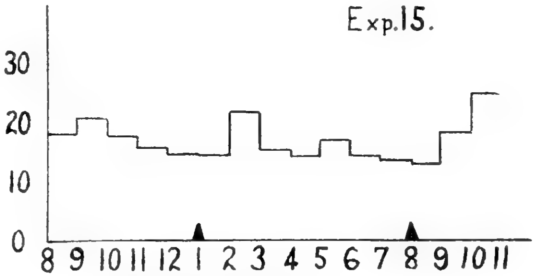
1 P.M. 265g. Swiss cheese { 856 fat cal., 300 prot. cal, 14 cbh. cal.

Influence of Laxatives.

The action of laxatives of several types on the excretion of uric acid was investigated in order to determine whether or not the digestive tract proper could, through their agency, be caused to augment the uric acid output.

Experiment 15, Epsom Salt.—Epsom salt is supposed to accomplish its purging effect without involving specific muscular or glandular activities. This laxative produced no significant effect on the endogenous uric acid elimination. Near the close of the experiment the purine-free food indicated was eaten in order to determine its effect at so late an hour. An immediate increase in the uric acid output occurred.

HR.	VOL.	mg. U.A.
8-9	13	18.0
9-10	22	20.5
10-11	17	17.6
11-12	14	15.8
12-1	13	14.5
1-2	10	14.2
2-3	15	21.5
3-4	9	15.1
4-5	12	14.1
5-6	13	17.0
6-7	14	14.1
7-8	13	13.5
8-9	13	12.9
9-10	17	18.2
10-11	20	24.6



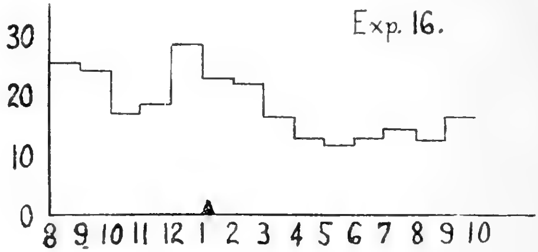
1 P.M. 25g. $MgSO_4$

8 P.M. { 2 eggs, 12g. butter, 50g. cheese, 95g. bread } = 150 prot. cal., 350 fat cal., 213 cbh. cal.

Experiments 16 and 17, Castor Oil.—The only action of castor oil in small amounts is to increase peristalsis. The large amounts employed in these experiments produce an effect similar to that of Epsom salt in addition to the stimulation of peristalsis. The uric acid excretion appears to be unaffected in Experiment 16. In Experiment 17, previous to the ingestion of the laxative, agar-agar was taken to augment any effect on the uric acid excretion by increasing the work of the intestine. The graph indicates the absence of any increase.

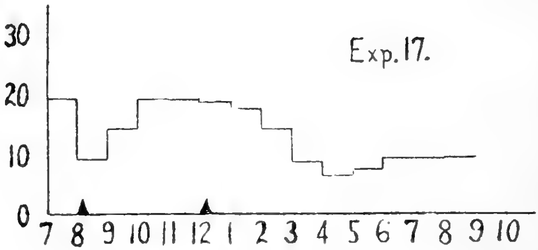
This result is not in accord with several experiments of Abl,[20] also carried out to determine the effect of castor oil on uric acid excretion. His conclusions were based on daily, not hourly, outputs. The increases obtained by him were attributed to the action of the oil in causing the intestine to secrete. Other laxatives—cascara sagrada, senna, croton oil, etc.—were also found by Abl to increase the uric acid output.

HR.	VOL.	mg. U.A.
8-9	18	25.2
9-10	15	24.0
10-11	14	17.0
11-12	21	18.7
12-1	44	28.1
1-2	35	22.5
2-3	20	21.9
3-4	13	16.4
4-5	11	12.9
5-6	15	11.7
6-7	12	12.9
7-8	17	14.1
8-9	14	12.3
9-10	17	16.4



1 P.M. 25 c.c. Castor Oil

HR.	VOL.	mg. U.A.
7-8	35	19.3
8-9	146	9.4
9-10	110	14.2
10-11	118	19.3
11-12	113	19.2
12-1	80	18.8
1-2	28	17.6
2-3	43	14.1
3-4	13	8.8
4-5	13	6.4
5-6	8	7.6
6-7	28	9.1
7-8		9.1
8-9	8	9.4



8 A.M. 25 g. agar-agar
12 M. 27 c.c. castor oil

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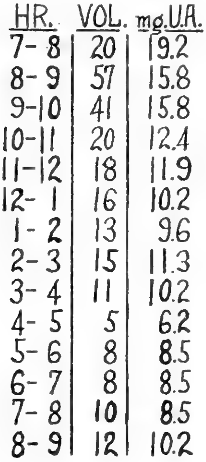
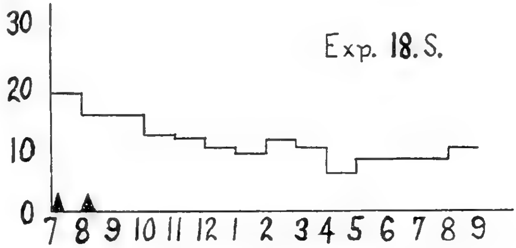
[20] Abl, R., *Arch. f. exper. Path. u. Pharmakol.*, 1913, lxxiv, 119.

Experiment 18, Phenolphthalein.—The mode of action of this drug has not been as satisfactorily explained as has that of the two preceding laxatives. According to Meyer and Gottlieb[21] the large intestine is the place of attack, the effect being increased peristalsis; but it is reported by Hydrick[22] that phenolphthalein acts on the intestine in general, producing both irritation and peristalsis. In this experiment, as in No. 18, agar-agar ingestion again preceded the taking of the laxative. As before the effect on uric acid excretion was nil.



HR.	VOL.	mg.U.A.
7-8	20	19.2
8-9	57	15.8
9-10	41	15.8
10-11	20	12.4
11-12	18	11.9
12-1	16	10.2
1-2	13	9.6
2-3	15	11.3
3-4	11	10.2
4-5	5	6.2
5-6	8	8.5
6-7	8	8.5
7-8	10	8.5
8-9	12	10.2



7 A.M. .2 g. phenolphthalein
8 A.M. 15 g. agar-agar

Influence of Other Agents.

Experiments 19, 20, and 21, Pilocarpine and Atropine.—These two drugs have opposite actions on the secretory apparatus of the alimentary tract, pilocarpine stimulating it to activity and atropine exerting an inhibiting action. If the uric acid output is influenced by the degree of glandular activity, the administration of these drugs should in the case of pilocarpine increase and in the case of atropine decrease the amount of uric acid excreted. Mareš had already demonstrated the increasing effect of the former. The present pilocarpine experiment confirms his result. Two

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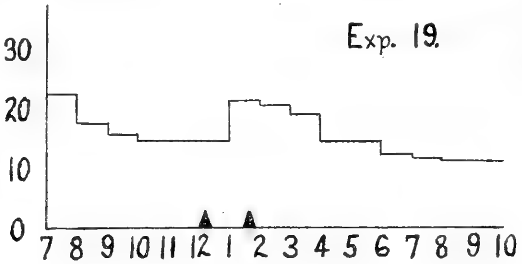
[21] Meyer, H., and Gottlieb, R., Die experimentelle Pharmakologie, Vienna, 1910.

[22] Hydrick, J. L., *Jour. Biol. Chem.*, 1914, xvii, p. xxxvi.

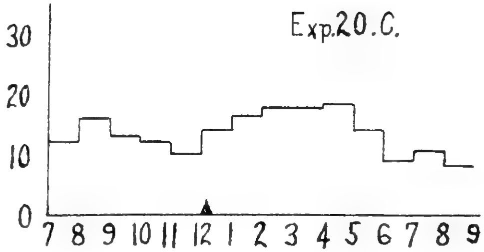
series were necessary to determine the effect of atropine—one a control in which an increase in the uric acid excretion above the normal fasting values was induced by the foodstuffs indicated, and another in which the effect of atropine on this increase was determined. Experiment 21, in which atropine was taken previously to the food stated, indicates plainly the absence of the augmented output found in Experiment 20 when no atropine was taken.

HR.	VOL.	mg. U.A.
7- 8	28	22.3
8- 9	24	17.6
9-10	20	15.8
10-11	18	14.7
11-12	15	14.7
12- 1	18	14.7
1- 2	40	21.1
2- 3	60	20.3
3- 4	81	19.0
4- 5	36	14.4
5- 6		14.4
6- 7	17	12.3
7- 8	14	11.7
8- 9	25	11.1
9-10		11.1



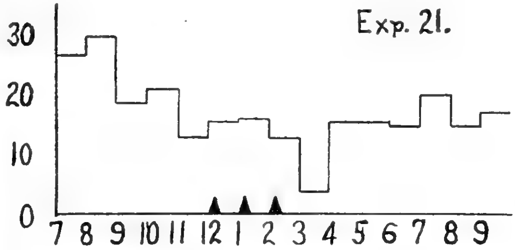
12 M. }
1.20 P.M. } 15mg. pilocarpine

HR.	VOL.	mg. U.A.
7- 8	18	12.4
8- 9	23	16.3
9-10	14	13.5
10-11	13	12.4
11-12	13	10.2
12- 1	17	14.1
1- 2	20	16.9
2- 3	22	18.0
3- 4	23	18.0
4- 5	24	18.6
5- 6	20	14.1
6- 7	15	9.0
7- 8	23	10.7
8- 9	17	8.4



12 M. { 2 egg sandwiches
1 glass milk

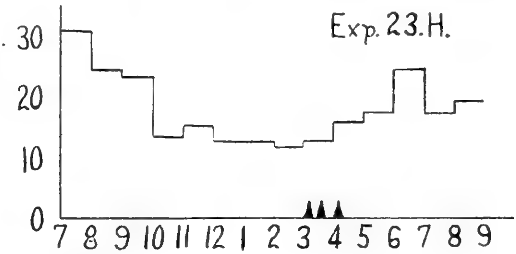
HR.	VOL.	mg. U.A.
7-8	31	26.4
8-9	40	29.5
9-10	24	18.6
10-11	18	20.5
11-12	13	12.9
12-1	16	15.2
1-2	16	15.8
2-3	18	12.9
3-4	108	3.8
4-5	28	15.2
5-6	16	15.2
6-7	20	14.7
7-8	33	19.9
8-9	30	14.7
9-10	31	17.0



12M. 3mg. atropine
1 P.M. 2mg. "
2P.M. 2 egg sandwiches
1 glass milk

Experiments 23 and 24, Alcohol.—In 1904 Beebe,[23] in this laboratory, found that after the intake of alcohol with a meat-containing diet the uric acid output was increased beyond what was excreted when no alcohol was taken. It appeared desirable, therefore, to determine its action with purine-free food. In the first experiment the normal excretion under the influence of the food

HR.	VOL.	mg. U.A.
7-8	96	31.1
8-9	96	24.6
9-10	49	23.5
10-11	57	13.4
11-12	46	15.2
12-1	38	12.9
1-2	58	12.9
2-3	146	11.7
3-4	47	12.9
4-5	280	16.0
5-6	200	17.6
6-7	98	24.6
7-8	26	17.6
8-9	27	19.3



3P.M. 1 egg sandwich
3.30P.M. " "
4.P.M. " "

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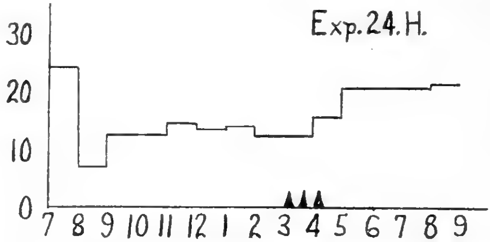
[23] Beebe, S. P., *Am. Jour. Physiol.*, 1904–05, xii, 13.

material indicated was determined, after which a similar series with the addition of alcohol to the food was carried out. As the graphs show, there was no noticeable effect.

HR.	VOL.	mg.U.A.
7-8	32	24.0
8-9	20	7.2
9-10	28	12.8
10-11		12.8
11-12	15	14.6
12-1	15	13.9
1-2	14	14.2
2-3	44	12.6
3-4		12.6
4-5	50	15.7
5-6	270	20.3
6-7		20.3
7-8	54	20.3
8-9	29	21.0



3 P.M. 1 egg sandwich + 60 c.c. 50% alc.
3.30 P.M. " " 30 "
4 P.M. " " " "

DISCUSSION.

The data presented offer no obstacle to the assumption that *a portion, at least, of the endogenous uric acid may originate from the activity of the alimentary secretory apparatus.* This conclusion follows from the increased output noticeable after various food stimuli and after the administration of pilocarpine which is known to stimulate the digestive glands. It is especially noticeable after the ingestion of protein, the most important of the succagogic factors. Atropine, which inhibits secretory activity, was found to suppress the uric acid output normally noted to follow the ingestion of the foodstuffs indicated. The passage of indigestible, bulky agar-agar through the digestive tract did not cause the effect which would be expected if the mere mechanical work of the intestinal musculature were a factor in uric acid production.

It might be objected that the increase in the uric acid excretion noted in most of the experiments was due to a coexistent diuresis. While it is true that the two phenomena do parallel each other to some extent, an inspection of the charts will show that in some cases the relation is an inverse one. Experiments 13 and 14 illustrate this feature.

In the paper of Raiziss, Dubin, and Ringer[24] rather low endogenous uric acid values are reported. They were obtained by employing a low-protein vegetable diet. When this was replaced by a starch and cream diet the values became still lower. Their data lead the authors to the conclusions that (1) real endogenous uric acid values can only be obtained on a strictly purine-free diet (such as starch and cream with no vegetables); (2) that the figures are much smaller than has hitherto been supposed; and (3) that different individuals excrete practically the same amounts of uric acid. The first of these conclusions must be interpreted in the light of the data above reported from which it appears that carbohydrates may exert some influence on the excretion of uric acid. The writers' data in this respect are in accord with those of Smetánka. It is apparent now that the uric acid output, aside from the portion arising from precursors in the food depends, to some extent at least, upon the degree to which the digestive glands are called into activity. The very low values obtained by Raiziss, Dubin, and Ringer may be due to the absence from the dietary of protein which has been shown to exert the greatest influence of any of the food principles upon uric acid excretion. The output of uric acid on a vegetable diet is also smaller than most of the endogenous values heretofore reported and may likewise be attributed to its low protein content. As regards the constancy in amount for individuals studied, this is only true with exceptions. To say that the variations are not extreme would express the facts better. It must be remembered that, as a rule, the make-up of the dietaries is also not subject to extreme variations.

The results of some of the experiments are not as clear cut as could be desired and some may even seem difficult of interpretation; but it is believed that the evidence as a whole lends some support to the hypothesis of Mareš.

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[24] Raiziss, G. W., Dubin, H., and Ringer, A. I., *Jour. Biol. Chem.*, 1914, xix, 473.

A MICRO-REFRACTOMETRIC METHOD OF DETERMINING THE PERCENTAGES OF GLOBULIN AND ALBUMIN IN VERY SMALL QUANTITIES OF BLOOD SERUM.

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(Received for publication, June 29, 1915.)

The principle of the method is essentially similar, on a reduced scale and with consequent necessary modifications of technique, to that of the refractometric method of serum analysis previously employed by the author and collaborators.[1] It differs from the refractometric method of serum protein determination employed by Reiss[2] in that four different quantities are determined; namely, the albumins, globulins, total proteins, and non-proteins, while Reiss' method yields only an approximate estimate of the total proteins. A little over 1.5 cc. of serum is ample for the determination of these four quantities, while if necessary as little as 0.5 cc. of serum may be employed.

The method consists of three separate estimations which are performed as follows.

a. The Estimation of the Refractivity of the Non-Protein Constituents.

Glass tubes 25 cm. long, having an inside diameter of 5 mm. and walls about 1 mm. thick, are sealed at one end. It is well to blow gently into the tube while the sealed end is still soft, thus

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[1] Robertson, T. B., *Jour. Biol. Chem.*, 1912, xi, 179; 1912–13, xiii, 325. Woolsey, J. H., *ibid.*, 1913, xiv, 433. Wells, C. E., *ibid.*, 1913, xv, 37. Buck, L. W., *Jour. Pharmacol. and Exper. Therap.*, 1913–14, v, 553. Thompson, W. B., *Jour. Biol. Chem.*, 1914, xx, 1. Briggs, R. S., *ibid.*, 1914, xx, 7.

[2] Reiss, E., *Beitr. z. chem. Phys. u. Path.*, 1904, iv, 150; *Arch. f. exper. Path. u. Pharmakol.*, 1904, li, 18; *Jahrb. f. Kinderh.*, 1909, lxx, 174.

making the contour of the bottom of the tube hemispherical and diminishing the tendency to crack on cooling. Into one of these tubes, which has been carefully cleaned and dried, is now introduced exactly 1 cc. of serum, with the aid of an ordinary bacteriological pipette which delivers 1 cc. and reads to 0.01 cc. It is convenient to draw out the tip of the pipette so as to enable a somewhat greater length to be introduced into the tube while delivering the serum, thus avoiding wetting the upper part of the tube and the formation of air bubbles, which prevent the serum from flowing to the bottom of the tube. The pipette is now washed out with distilled water followed by $\frac{N}{25}$ acetic acid solution, which may be made up with sufficient accuracy by diluting 4 cc. of glacial acetic acid to 1750 cc. With the same pipette exactly 1 cc. of $\frac{N}{25}$ acetic acid solution is delivered into the tube, as before avoiding wetting the upper part of the tube. A short piece of platinum or silver wire about 1.5 mm. in diameter is now dropped into the tube and the upper end is sealed off in a flame, taking care not to heat the contents. After it has cooled the tube is shaken. The piece of wire which is thus shaken up and down in the tube brings about in a short time complete admixture of the serum and the acetic acid solution. The tube is now placed in a beaker of cold water of such a depth as to immerse the top of the contained column of liquid a few millimeters below the water. It is well to rest the bottom of the tube upon a wad of cotton to avoid cracking by bumping during the subsequent boiling. The water is now slowly heated to boiling and allowed to boil energetically for from one to two minutes. The tube is then removed from the boiling water and allowed to cool to room temperature.[3]

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[3] The exact length of time during which the tube is immersed in *boiling* water is immaterial provided it exceeds one minute and does not exceed five minutes. The concentration of acetic acid employed (an equal volume of $\frac{N}{25}$) brings about complete coagulation on boiling without appreciable formation of soluble acid-albuminates. Lower concentrations (such as an equal volume of $\frac{N}{50}$ acetic acid) do not bring about complete coagulation during the periods of boiling mentioned above, and hence yield values for the "non-proteins" which are too high. Concentrations greater than $\frac{N}{20}$ (*i.e.*, addition of an equal volume of $\frac{N}{10}$) bring about gelatinization on boiling instead of coagulation and the values for the "non-proteins" are too high owing to the formation of soluble acid-albuminates.

When cool the tube is broken open a little above the surface of its contents and the coagulum is broken up. This is best accomplished with the aid of a platinum wire about 0.6–0.7 mm. in diameter and provided with several slight bends; this is inserted into the tube and the upper end twirled rapidly between the thumb and forefinger. The fluid and the coagulum are now separated by centrifugalization, the fluid is withdrawn by the aid of a dry, clean pipette, and the refractive indices of the fluid and of $\frac{N}{50}$ acetic acid solution are determined simultaneously. The difference between these two refractive indices, multiplied by 2, represents the refractivity of the non-protein constituents of the serum. By determining the refractive indices of the fluid and of $\frac{N}{50}$ acetic acid *simultaneously* the necessity for regulation of the temperature at which the readings are made is obviated, for although the absolute values of the refractive indices are affected by temperature, the difference between them is independent of the temperature at which it is determined.

The values for the refractivity of the non-protein constituents of blood serum, as determined by this method, are invariably higher than the average value of 0.00157 assumed by me in my older method. They approximate very closely to the average value of 0.00224 obtained by Reiss, who employed a similar method of determination. That these higher values are not attributable to partial hydrolysis of the proteins with the production of soluble substances is shown by the fact that increasing the period of boiling, or doubling the concentration of acid does not lead to any increase in the values obtained. It appears probable that there are substances in serum which are precipitated together with the proteins by alcohol but are not coagulable by heat. These would be reckoned as "albumins" in the older method and as "non-proteins" in the new.

The refractive index of a 1 per cent solution of NaCl is 0.00160 greater than that of distilled water. The refractivities of 1 per cent solutions of other inorganic salts and of glucose and urea are of very similar magnitude. To within a sufficient degree of accuracy, therefore, the percentage of non-protein substances in the serum may be estimated by dividing their refractivity by the factor 0.00160.

For the determination of the refractive index I employ a Pulfrich refractometer which reads the angle of total reflection to within one minute. A sodium flame is used as the source of light. When it is desired to make a number of successive determinations the cup of the refractometer should be carefully dried with absorbent cotton and lens paper before a new sample of fluid is introduced.

b. The Estimation of the Refractivity of the Albumins.

Glass tubes 9–10 cm. long are prepared, having an inside diameter of 5 mm. and one end closed. Tubes which have been employed in the estimation of the non-protein constituents, after having been cleaned and dried, may be cut down to the proper length and utilized for this purpose. Short pieces of glass tubing sealed at one end are employed as stoppers, the sealed end being pressed against the open end of the longer tubes and held in position by short pieces of rubber tubing. Into one of these tubes is introduced, with the aid of a pipette similar to that described above, 0.5 cc. of a saturated solution of ammonium sulphate (prepared by dissolving an excess of ammonium sulphate in hot water and allowing the excess to crystallize out on cooling). With another pipette which has been found, by weighing, to deliver the same amounts as the first, we now introduce 0.5 cc. of serum, drop in a piece of platinum wire, affix the stopper, and shake thoroughly with as little delay as possible. It is necessary to introduce the ammonium sulphate solution first, as otherwise, being of greater specific gravity than the serum, it sinks through the serum, portions of which are thus exposed for some time to ammonium sulphate of higher concentration than $\frac{1}{2}$ saturated. This leads to a precipitation of albumins which do not readily redissolve and the results obtained are erroneous and irregular. If the ammonium sulphate is introduced first the serum floats on the top of it and energetic shaking brings about almost immediate admixture of the serum and the reagent. It is well, while shaking, to hold the thumb against the bottom of the tube, thus diminishing the danger of cracking the tube by the impacts of the heavy piece of wire.

The tube, with the stopper still affixed, is now centrifuged. The precipitate soon settles and sufficient supernatant fluid may be drawn off to fill the lower end of a bacteriological pipette and at least 0.25 cc. over. This quantity of the supernatant fluid is delivered into another clean, dry tube of the type employed in the precipitation, the pipette is washed out with distilled water, and then employed to deliver 0.25 cc. of distilled water into the tube. A piece of platinum wire is dropped in, the stopper affixed, and the contents are shaken. We now determine the refractive index of this fluid[4] and that of $\frac{1}{4}$ saturated ammonium sulphate solution.[5] The difference between these refractive indices represents one-fourth of the combined refractivities of the albumins and of the non-protein constituents. Multiplying by 4, therefore, and subtracting the refractivity of the non-protein constituents, we have the refractivity of the *albumins*. Dividing this by 0.00177 we obtain the percentage of albumin in the serum.

c. *The Estimation of the Refractivity of the Globulins.*

We determine the refractive index of the serum and that of distilled water. Subtracting from the difference the known refractivity of the non-proteins and the known refractivity of the albumins we obtain the refractivity of the *globulins*. Dividing this by 0.00229 we obtain the percentage of globulins in the serum. Adding together the percentages of albumins and globulins we obtain the percentage of total proteins.

The following are illustrative determinations, employing ox serum obtained by whipping and centrifuging freshly drawn blood:

Refractive index of the fluid obtained after acidifying with acetic acid and boiling (three determinations)	1.33024
Refractive index of $\frac{N}{50}$ acetic acid	1.32902
Difference × 2	0.00244

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[4] The fluid may be emptied into the cup of the refractometer by removing the stopper, inverting the tube, and gently warming the sealed end, free from fluid, by means of a lighted match.

[5] This may be prepared, and kept as a stock solution, by mixing equal volumes of saturated ammonium sulphate solution and distilled water and adding to this mixture an equal volume of distilled water.

Estimated concentration of non-protein substances $= \frac{244}{160} = 1.5$ per cent[6]

Refractive index of the fluid obtained after precipitation of the globulins and subsequent dilution:

1st determination.............................. 1.35189
2nd " 1.35172
3rd " 1.35181
4th " 1.35189

Refractive index of $\frac{1}{4}$ saturated ammonium sulphate............ 1.34932

Differences × 4

1st determination.............................. 0.01028
2nd " 0.00960
3rd " 0.00996
4th " 0.01028

Refractivity of the albumins

1st determination.................. $0.01028-0.00244=0.00784$
2nd " .... $0.00960-0.00244=0.00716$
3rd " .... $0.00996-0.00244=0.00752$
4th " .... $0.01028-0.00244=0.00784$

Average.......................................0.00759

Concentration of albumins

1st determination $= \frac{784}{177} = 4.4$ per cent
2nd " $= \frac{716}{177} = 4.1$ "
3rd " $= \frac{752}{177} = 4.3$ "
4th " $= \frac{784}{177} = 4.4$ "

Average.............. $= 4.3$ "

Refractive index of the serum.................................. 1.34686
Refractive index of distilled water............................ 1.32887

Difference.. 0.01799
Refractivity of the proteins $=0.01799-0.00244=0.01555$
Refractivity of the globulins $=0.01555-0.00759=0.00796$
Concentration of globulins $= \frac{796}{229} = 3.5$ per cent
Concentration of total proteins $=3.5+4.3=7.8$

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[6] Direct analyses by Abderhalden (*Ztschr. f. physiol. Chem.*, 1897, xxiii, 521) yield the average result of 1.4 per cent of non-protein substances in ox blood serum.

Two determinations by the older refractometric method, employing the above determined value of the refractivity of the non-proteins, yielded identical results; namely,

Albumins	4.3 per cent
Globulins	3.5 "
Total proteins	7.8 "

Since the percentage of total proteins is dependent upon the dilution of the blood and is therefore highly variable, while the relative proportions of globulin and albumin are much more constant[7] it is convenient to express these in percentages of the total proteins. Thus in the above result, taking the total proteins as 100, we find that the globulins formed 45 per cent and the albumins 55 per cent of the total proteins. The average of four determinations reported by Hammarsten,[8] who employed the direct method of estimation in large quantities of serum, yields the result: globulins 42 per cent and albumins 58 per cent of the total proteins.[9]

The manipulations involved in the determination are simple and when proficiency has been attained can be carried out with considerable rapidity. Six or eight determinations may be made simultaneously and with practice this number of determinations may be made in less than three hours, a considerable part of which time is consumed by centrifugalizations. As regards accuracy, the most probable error is that of one minute in reading the angle of total reflection. This will lead to an error of ± 0.2 per cent in the estimation of the percentage of albumins, ± 0.15 per cent in the estimation of the percentage of globulins, and ± 0.1 per cent in the estimation of the percentage of non-proteins.

[7] Robertson, T. B., *Jour. Biol. Chem.*, 1912–13, xiii, 325.

[8] Hammarsten, O., *Arch. f. d. ges. Physiol.*, 1878, xvii, 413.

[9] Robertson, T. B., *Jour. Biol. Chem.*, 1912–13, xiii, 325.

PROTEIN MINIMA FOR MAINTENANCE.[1]

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In a recent paper[2] we have discussed the comparative nutritive value of certain proteins in growth and the question of the protein minimum. Attention was directed chiefly to the *growth* of albino rats on foods containing different proportions of the same protein, or the same proportion of different proteins. In the present paper we give the results of a preliminary study undertaken in order to learn whether differences in the relative efficiency of these several proteins in promoting growth would be apparent when they were supplied in quantities sufficient for *maintenance* alone.

In our previous paper we furnished data showing that when the percentage of protein in the food was reduced, a proportion was finally reached where growth either entirely ceased, or a decline in body weight occurred. This method of determining the proper proportion of protein in a diet designed for maintenance or for growth has long been used by investigators of animal nutrition to discover the most economical proportion of protein in the practical feeding of domestic animals. Experiments of this kind have been planned to furnish a sufficient supply of calories in the non-protein part of the ration and reduce the proportion of protein by adding food substances low in protein, or by diminishing the proportion of those rich in protein. All of

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

[2] Osborne, T. B., and Mendel, L. B., *Jour. Biol. Chem.*, 1915, xx, 351.

this has involved changes in so many factors with every alteration in the food mixture that the results have value only in respect to the particular mixture used for the animal in question, and are, therefore, to a very large extent empirical. Furthermore, possible differences in the relative efficiency of different proteins were formerly never considered in making such experiments; and the possibility of effecting economies in the use of the many available foodstuffs by so combining them that any nutritive deficiencies of the proteins of one food might be supplemented by proper combinations with another has heretofore generally escaped notice. Our previous papers have shown the favorable results obtained by feeding certain proportions and combinations of proteins to *growing* rats. We now have the results of similar experiments in relation to the *maintenance* of these animals of different sex, age, and size.

The ideal condition for the prosecution of such an investigation would consist in feeding a single protein as a part of a ration so liberal in all of the other essential food substances, both organic and inorganic, as to avert the need of more than a minimum of protein for the energy requirement on the one hand; and so restricted in respect to the content of protein on the other hand as to make the supply inadequate for any tissue construction or storage, yet sufficient to prevent loss of body weight. If these conditions could be fulfilled, an animal ought to remain in nutritive equilibrium, neither gaining nor losing weight from day to day. The protein minimum could thus be ascertained for each protein in individuals of any age, size, or sex in terms of some suitable unit of comparison, such as body weight or body surface.

Numerous difficulties preventing the ideal execution of the plan are at once suggested. Metabolism and, accordingly, the nutritive requirements are affected by a number of factors involving size, the effect of muscular activity, the influence of ingested foods, and the relative proportions of the different nutrients. Even temperament and other features incident to good health may need to be taken into account.

The recent discussions of a proper unit of comparison[3] indicate the insuperable obstacles to the establishment of a satisfactory

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[3] Compare Benedict, F. G., *Jour. Biol. Chem.*, 1915, xx, 263.

unit of measurement which shall serve as a reliable index of basal metabolism. We have therefore been compelled to adopt the unit of body weight as the only convenient standard for our animals, with the full recognition that although body weight plays an important part, there is no absolutely fixed relation between it and the total heat production. We also recognize that body composition, *i.e.*, the relative proportion of inert storage substance and active protoplasmic tissue, may vary greatly under a variety of circumstances where the body weight is the same. For the present we find no better plan than to use the weight as an index of the mass of metabolizing tissue.

The energy requirement of an animal must be satisfied, whatever the composition and intake of the food may be. In many cases the appetites of the subjects are evidently an excellent guide to their physiological needs. Sometimes, however, the quantities of food eaten appear to be out of all proportion to the actual energy or protein requirement. We have already discussed the range of variation in the food intake of growing rats on comparable diets and pointed out some features connected with it.[4]

In the present initial studies on the protein minima for the maintenance of rats, rations of a suitable sort have varied essentially only in respect to their percentage content of the nitrogenous foodstuffs. Our purpose was to learn first what the instinct of the rat would lead the animal to eat when supplied *ad libitum* with foods containing different proportions of various proteins. All of these foods, when sufficiently rich in the proteins in question, had been demonstrated to be adequate, not only for prolonged maintenance, but, with the exception of that containing gliadin, also for complete normal growth.[5] We have kept rats to old age on several of these mixtures and in the case of the edestin food have already raised four successive generations without offering any other ration.

The energy requirement of animals living under the conditions which we have maintained may perhaps be approached in a statistical way by recording the actual food consumption of a large number of rats on diets containing an abundance of added

[4] Osborne and Mendel, *Jour. Biol. Chem.*, 1915, xx, 364.

[5] For a catalogue of the results see Osborne and Mendel, *Jour. Biol. Chem.*, 1915, xx, 361.

protein (18 per cent) in food mixtures of the general composition indicated on page 245. Data thus obtained are summarized in Table A.

TABLE A.

Food Intake of Rats Maintained on 18 Per Cent Protein Foods.

Males.

Average body weight.	Average food intake per week.	Food intake per week per gm. of rat.	Protein intake* per week per gm. of rat.	Range of food intakes per week.	Number of observations.
gm.	*gm.*	*mg.*	*mg.*	*gm.*	
350-390	90	245	41.0	89-90	3
300-350	87	268	43.9	81-97	17
250-300	75	279	44.5	58-97	37
200-250	64	286	44.8	48-89	54
175-200	61	321	50.9	48-78	17
150-175	55	323	50.9	40-75	12
125-150	47	325	51.2	38-55	16
100-125	38	329	51.5	31-50	13
75-100	38	413	64.8	31-53	10
50- 75	29	451	70.0	23-37	18
30- 50	25	571	85.5	18-31	17

Females.

200-240	66	316	50.4	58-77	22
175-200	61	321	51.4	50-77	40
150-175	55	332	52.8	47-65	56
125-150	48	343	54.6	35-60	39
100-125	42	360	57.4	34-52	24
75-100	38	397	61.9	32-48	16
50- 75	30	471	72.5	25-41	21
30- 50	25	550	83.0	18-31	9

* No account is taken in this table of the small quantity of nitrogenous compounds in the "protein-free milk" present in these rations.

The results show the intake of animals in *maintenance*, in trials in which the variations in body weight did not exceed 5 to 10 grams during periods of not less than three weeks. Of course, these furnish no evidence as to the *minimal* protein need of the animals. They are rather intended to show the amounts of total food, *i.e.*, calories, ingested under such circumstances by rats eating *ad* *libitum* rations comparatively *rich* in protein. It will be noted that the food consumption per gram of rat decreased, as might be expected, with increase in weight of the animals. The statement of the number of rats for which the data have been collected in each case gives some indication of the degree to which the individual figures are representative averages. The records here collected may be of value to other investigators in this field, aside from their bearing on the protein problem.

Our subsequent procedure has consisted in feeding *ad libitum* mixtures of the sort indicated below, containing less protein, but with sucrose and starch used to replace it. As an illustration of a typical food mixture the following is cited:

	Per cent
Protein	9
Starch	30
Lard	7
Butter fat	18
Sucrose	8
"Protein-free milk"	28

Such foods, in which carbohydrate and protein may be interchanged without undue alterations in the energy value of the mixtures, furnish about five calories per gram of substance. Obviously a nutritive decline on a modification of this diet may be due to the *failure of the animals to eat enough food* to meet their energy requirement, quite as well as to *insufficiency of protein,* when this is reduced in amount. Varying degrees of muscular activity will call for unlike quantities of food to meet the energy requirement. We have not yet been able to make special allowance for this variable; but in view of the fact that our rats are confined in small cages where vigorous exercise is practically impossible, probably in the majority of cases the activity of the animals has not differed greatly. We have had to be content to counterbalance any inequalities due to unlike demands of muscular activity by observations on sufficiently large numbers of animals to offset the individual extremes.

When the composition of a liberal food mixture which suffices for maintenance is altered by reducing the protein content, noteworthy changes in body weight and in food consumption usually do not ensue until the protein has been decidedly decreased. For example, rations with 18 per cent, 15 per cent, or 12 per cent of protein may produce little alteration in the level at which body weight is maintained. Only when the proportion of protein becomes lower does the fall in weight manifest itself. This is frequently attended by a decline in food intake. When the proportion of protein in the diet is reduced to comparatively low limits there not infrequently appears to be a tendency on the part of the animals to eat much more liberally of the food poor in protein and thus make up the percentage deficiency of the ration by increased intake. Accordingly it will sometimes happen that a rat which has been maintained on a diet containing, let us say, 9 per cent of a definite protein will be equally maintained on a comparable ration containing 6 or 7 per cent of the same mixture, by eating somewhat more liberally thereof. There seems to be little difference in the outcome according to whether the proportion of protein is reduced suddenly or gradually, so long as it does not fall below the maintenance quota.

From the standpoint of the method thus far developed, with its inevitable limitations in respect to the energy factor, successful maintenance alone is significant, and then only where there is a reasonable abundance of non-protein food. It has not infrequently happened that the rats would fail to eat a portion sufficient for their energy needs. Obvious cases of calorie underfeeding have been excluded from the results recorded in this paper. A study of hundreds of figures obtained in the way outlined has enabled us to select a limited number of experiments for purposes of comparison. They represent selected positive data. The range of variation in the individual trials is not inconsiderable, and does not take into account extremes of size with accelerated or decreased tendency to grow, or other possible incidental causes of unlike food requirement. By successively altering the protein content of the food we have attempted to find levels of absolute protein intake (abundance of total calories presumably being ingested in each case) at which there will result: (1) exact maintenance of body weight; (2) slight decline; or (3) slight gain (where adult size had not yet been reached). In this way it seems possible to approximate in a statistical way the limits which determine the condition of maintenance in so far as the protein is concerned. In young animals it is not sufficient to decrease the protein intake to a point where growth just ceases; for there appears to be a considerable range below this at which growth fails, yet maintenance is still possible.

Without a demonstration of the level at which some decline ensues, observations on the maintenance minima are not complete. The lower limit—the minimum—can therefore be determined only by reaching a plane of protein intake which is just insufficient. A very slight increase or decrease in the intake of protein at this point should promptly restore maintenance or induce decline.

In Tables A, B, C, D, F, and H the per cent of protein is given in terms of the air dry preparation. The protein intakes are calculated from the nitrogen content of the food by means of the appropriate factor for each protein. In Table E the percentage of protein and also the protein intake were calculated from the nitrogen of the food.

The figures reported in the tabular summaries B, C, D, E, F are selected from experimental periods which meet these conditions. Periods of decline were succeeded by those of recovery and *vice versa.* Before finally considering a decline in body weight as due to the protein deficiencies of the diet we have tested the animal with a ration more liberal in the same protein to make certain that it could be maintained on a suitable dietary and was not merely declining because of unsuspected disease. Thus it sometimes occurs that a rat will continually lose weight on a diet supposedly liberal in all respects. Such animals are not infrequently found to be suffering from pulmonary infections, hair balls in the stomach, tumors, etc., the discovery of which explains the unexpected results. All data unsatisfactory in this respect have been excluded from the selected figures presented in this paper. The chart in the appendix will illustrate, in the case of a few animals, the type of trials from which the statistics were collected.

The tabular summaries, B, C, D, E, F, on pages 248 to 251, indicate quite unmistakably, we believe, that rats fed upon rations comparable in their make-up in every way except with respect to the content of protein can be maintained in body weight with a smaller intake of lactalbumin than of any other protein here reported. A résumé of the range of protein minima for maintenance found in the individual experiments is given in Table G.

TABLE B.

Protein Minimum for Maintenance.

Lactalbumin Food.

Males.

Rat.	Body weight.	Lactalbumin content of diet.	Food intake per week per gm. of rat.	Lactalbumin intake per week per gm. of rat.	Gain (+) or loss (−) of body weight during period.	Period of observation.
	gm.	*per cent*	*mg.*	*mg.*	*gm.*	*days*
2111	317	7	217	12.6	+2	14
1823	297	5	303	12.5	−3	98
2123	284	7	232	13.4	±0	21
2338	278	5	296	12.2	+5	70
1868	272	4	335	11.1	−1	42
2032	270	5	254	10.5	+3	49
2345	254	3.5	319	9.2	−2	70
2212	253	5	305	12.6	±0	35
2209	228	6	288	14.2	−3	14
2356	227	4	280	10.4	−2	35
2360	214	5	252	10.4	−1	42
2541	202	7	241	14.5	−3	56
2348	183	4	366	12.1	+1	28
2542	160	4	292	9.6	±0	70
2586	129	4	324	10.7	+12	28
2203	120	2.5	352	7.3	−1	49
2201	95	2.5	361	7.4	+3	35
2185	78	2	435	7.2	+8	154
2202	76	2	408	6.7	+8	56
2292	72	2	481	7.9	±0	175
2180	66	2	557	9.2	±0	63
2293	60	2.5	483	9.9	−1	28

Females.

Rat.	Body weight.	Lactalbumin content of diet.	Food intake per week per gm. of rat.	Lactalbumin intake per week per gm. of rat.	Gain (+) or loss (−) of body weight during period.	Period of observation.
2112	202	4	287	9.5	−3	28
1957	189	4	329	10.8	−5	35
1923	188	5	372	15.4	−2	28
2210	176	4	255	8.4	−1	35
1765	167	4.5	365	13.5	−1	21
2321	159	4	331	10.9	−4	21
2112	88	2	417	6.9	−1	42
2104	87	2	445	7.4	−3	35

TABLE C.

Protein Minimum for Maintenance.

Casein Food.

Males.

Rat.	Body weight.	Casein content of diet.	Food intake per week per gm. of rat.	Casein intake per week per gm. of rat.	Gain (+) or loss (−) of body weight during period.	Period of observation.
	gm.	*per cent*	*mg.*	*mg.*	*gm.*	*days*
2336	352	7	321	20.2	+7	42
1875	314	7	337	21.2	+1	28
1934	283	6	282	15.2	±0	28
2130	276	7	276	17.4	−3	28
2327	272	5	310	13.9	−2	21
2051	264	6	264	14.3	−1	35
2196	263	7	295	18.6	±0	56
2026	247	7	284	17.9	+4	56
1931	239	9	291	23.6	±0	35
1914	238	7	304	19.2	+2	28
2523	238	7	307	19.3	−3	21
2322	233	9	328	26.6	−3	56
2244	203	7	344	21.7	−5	28
2017	196	7	288	18.1	−4	28
2334	186	5	404	18.1	−1	56
2576	185	9	351	28.4	+7	28
2245	160	7	353	22.2	−6	28
2549	141	5	338	15.2	±0	28
2617	89	4.5	368	14.9	+2	35

[illegible] 7.0

Females.

1958	206	6	347	18.7	+3	56
1792	205	6	341	18.4	+1	14
1935	187	5	312	14.0	−3	28
1767	186	5	321	14.4	−2	21
1924	166	7	338	21.3	+6	21
1992	160	7	318	20.0	−4	21
2418	152	9	306	24.8	−4	49
2116	98	4.5	360	14.5	−3	35
2339	94	5	400	18.0	−1	42
2437	60	5	425	19.1	−2	14
2118	51	4.5	552	22.4	−4	14

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TABLE D.

Protein Minimum for Maintenance.

Edėstin Food.

Males.

Rat.	Body weight.	Edestin content of diet.	Food intake per week per gm. of rat.	Edestin intake per week per gm. of rat.	Gain (+) or loss (—) of body weight during period.	Period of observation.
	gm.	*per cent*	*mg.*	*mg.*	*gm.*	*days*
2329	366	6	271	15.1	−1	42
1864	341	9	264	22.1	+2	14
2315	336	7	250	16.3	−4	21
1675	294	5	252	11.7	±0	49
1930	286	7	293	19.1	+9	21
2302	282	6	288	16.0	−3	21
2316	276	5	321	14.9	+2	35
2376	247	7	365	23.8	+4	56
2404	244	6	310	17.3	+1	35
1976	244	9	247	20.7	±0	28
1931	237	7	297	19.3	−2	35
1817	214	7	317	20.6	−3	14
2410	111	6	373	21.0	−3	21
Females.						
2003	225	6	265	14.8	−3	21
2113	218	5	325	15.1	+6	42
2298	215	6	310	17.3	±0	28
2299	215	6	349	19.5	+7	28
1767	204	7	334	21.8	±0	21
2029	196	6	328	18.3	+3	21
1774	162	4.5	395	16.6	+2	63
2221	162	7	323	21.0	−1	14
2548	124	5	363	16.9	±0	42
2113	118	4.5	393	16.5	±0	63
2553	98	7	336	21.9	+2	35
2114	93	4.5	395	16.5	+3	28

TABLE E.

Protein Minimum for Maintenance.

Milk Proteins.

Males.

Rat.	Body weight.	Milk protein content of diet.	Food intake per week per gm. of rat.	Milk protein intake per week per gm. of rat.	Gain (+) or loss (—) of body weight during period.	Period of observation.
	gm.	*per cent*	*mg.*	*mg.*	*gm.*	*days*
2464	329	7.4	259	19.2	±0	42
1877	316	4.9	302	14.9	−2	35
1884	253	7.4	299	22.1	±0	70
2500	229	4.9	302	14.9	±0	28
2120	214	4.9	306	15.1	−4	21
2411	185	4.0	340	13.4	−1	28
Females.						
1871	170	4.9	351	17.3	+1	42
2422	156	4.9	378	18.7	±0	35
2414	137	6.2	342	21.1	±0	63

TABLE F.

Protein Minimum for Maintenance.

Gliadin Food.

Males.

Rat.	Body weight.	Gliadin content of diet.	Food intake per week per gm. of rat.	Gliadin intake per week per gm. of rat.	Gain (+) or loss (—) of body weight during period.	Period of observation.
	gm.	*per cent*	*mg.*	*mg.*	*gm.*	*days*
1882	293	12	266	27.0	−3	35
2275	285	12	241	24.4	+1	21
1860	267	12	268	27.2	±0	42
1878	266	9	308	23.4	±0	35
Females.						
1937	187	10.5	304	27.0	+6	42
1830	179	6.0	375	19.0	+1	105
2435	92	7.0	369	21.8	−1	21

TABLE G.

Range of Protein Minima for Maintenance in Individual Experiments.

Protein added to the ration.	Size of rats.	Food intake per week per gm. of rat.	Protein intake per week per gm. of rat.
	gm.	*mg.*	*mg.*
Lactalbumin.	Males.		
	60–100	361–557	6.7– 9.9
	100–200	292–366	7.3–12.1
	200–250	241–288	10.4–14.5
	250–300	232–335	9.2–13.4
	300	217	12.6
	Females.		
	80–100	417–445	6.9– 7.4
	100–200	255–372	8.4–15.4
Casein.	Males.		
	80–100	368	14.9
	100–200	288–404	15.2–28.4
	200–250	284–344	17.9–26.6
	250–300	264–310	13.9–18.6
	300–350	321–337	20.2–21.2
	Females.		
	50–100	360–552	14.5–22.4
	100–200	306–338	14.0–24.8
	200–250	341–347	18.4–18.7
Edestin.	Males.		
	100–200	373	21.0
	200–250	247–365	17.3–23.8
	250–300	252–321	11.7–19.1
	300–400	250–271	15.1–22.1
	Females.		
	90–100	336–395	16.5–21.9
	100–200	323–395	16.5–21.0
	200–250	265–349	14.8–21.8
Milk proteins.	Males.		
	100–200	340	13.4
	200–300	283–302	14.9–22.1
	300–350	259–302	14.9–19.2
	Females.		
	100–200	342–378	17.3–21.1
Gliadin.	Males.		
	250–300	241–308	23.4–27.2
	Females.		
	90–200	304–375	19.0–27.0

Ranges of 7 to 15 milligrams of lactalbumin per gram of rat per week represent minima lower than those found for casein, edestin, milk proteins, or gliadin, not to mention the impossibility of maintenance with zein. With the pronounced individual variations in the total food intake exhibited in our experience it is almost impossible to make precise comparisons of rats of the same size on precisely the same intake of protein. It is an obvious shortcoming of this method of procedure that the energy intake is not controlled, so that when it is large there may be a relatively greater protein-sparing effect where the non-protein nutrients are ingested in undue amounts. The selected figures recorded in our summaries scarcely give any justification for the assumption that greatly increased energy intake will explain the lower values at which maintenance was secured with the lactalbumin food. It will be noted further that whereas maintenance could be secured very frequently with a lactalbumin content of less than 5 per cent in the diet, this was rarely the case with the other proteins. With all the foods, however, very small rats, naturally eating larger quantities of food per unit of body weight, often attained maintenance (without growth) on lower protein intakes than sufficed for rats of larger size, *e.g.*, above 125 grams.

We have pointed out in our earlier communications that the "protein-free milk" which forms a part of the rations described is not entirely free from milk protein. It contains not more than 0.7 per cent of nitrogen, of which only a part at most is in the form of protein. Assuming that all of this nitrogen is equivalent in value to protein—a premise for which there is no experimental evidence—100 grams of the food mixtures used by us would contain 1.2 grams of such protein, besides the protein intentionally supplied. The hypothetical addition of protein would amount to 3.6 milligrams in a food intake of 300 milligrams per week per gram of rat. The increment would apply to all of the diets in essentially like amounts. Even if it were assumed that the rats on lactalbumin food eat twice as much of their ration, the increased intake of hypothetical protein in this extreme case would hardly suffice to equalize the protein minima of the lactalbumin-fed rats with those on the other proteins, when animals of comparable sizes are contrasted. The superiority of the lactalbumin in these trials, therefore, cannot be charged solely to protein derived from the so called "protein-free milk."[6] Speaking broadly, we have not been able to accomplish with a protein intake of 12 to 15 milligrams per gram of rat in the case of either edestin or casein, the degree of maintenance which is usually attained by feeding lactalbumin. For edestin and casein a correspondingly efficient intake seems to be about 20 milligrams in the form of our particular mixtures. It is too early to make thoroughly satisfactory

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[6] Referring to older papers by us, McCollum and Davis (*Jour. Biol. Chem.*, 1915, xx, 647) have contended that we have assumed too high a figure for the plane of intake of protein which will serve to maintain young rats in body weight. What we did (Osborne and Mendel, *Ztschr. f. physiol. Chem.*, 1912, lxxx, 341) was to compare the growth of rats on food mixtures containing different percentages of protein. When the lower limit of 7 to 9 per cent was reached, maintenance ensued but growth ceased. We failed to realize the importance of calculating and comparing the *actual protein consumption* of the animals in the earlier experiments in addition to contrasting the results of varying the *percentage* of protein in the food mixture. The food intakes and other data are actually reported in our charts so that they are available for comparison. In some of the cases it appears likely that the failure to grow better, and therefore the semblance of maintenance without growth, was due to insufficient total food intake, *i.e.*, calories. Even more significant is the fact that these early experiments were conducted with food mixtures on which it was subsequently shown that all rats sooner or later decline unless some adjuvant such as butter fat, egg fat, or cod liver oil is furnished (Osborne and Mendel, *Jour. Biol. Chem.*, 1913, xv, 311; 1913–14, xvi, 423; 1914, xvii, 401; 1915, xx, 379). We have therefore never included the results from the earlier trials with manifestly incomplete food mixtures in comparisons with our later experiments; and all attempts to do so seem to us unfair in the light of the literature of the subject. When McCollum and Davis attempt to defend the thesis that the lowest plane of protein for maintenance is, let us say, 3 per cent *of the food mixture* (in the case of a distinct ration prepared by them) one may question whether they are justified in making wide-spread comparisons on this basis. There is no guarantee whatever that rats will eat the same total quantity of unlike food mixtures which contain the same content of protein. The actual intake must be measured. Not until we can finally say that animals require a stated weight of protein per unit of body weight to secure maintenance in the presence of an abundance of energy-yielding food shall we be in a position to make absolutely conclusive comparisons. Incidentally it may be pointed out that we have recently published figures on the maintenance of rats with diets containing low percentages of protein quite in harmony with the low percentage figures of McCollum and Davis on a different type of ration (Osborne and Mendel, *Jour. Biol. Chem.*, 1915, xx, 351).

comparisons with the other proteins except to note that, comparatively, more gliadin is required. That this is not due to defective utilization in the alimentary tract in the case of any of these proteins has been demonstrated by us directly by analyses of the feces in those instances where a relatively high protein intake was found inadequate for perfect maintenance. The mixed proteins of cow's milk, as represented in our "milk food,"[7] containing casein together with some lactalbumin, approach casein and edestin in value for maintenance, possibly being somewhat more efficient as exemplified by a very slightly lower maintenance minimum figure. The higher figures for gliadin are in accord with its known exceptional character in respect to the yield of the various amino-acids characteristic of proteins. Gliadin yields a small amount only of lysine,[8] and 22 per cent of its nitrogen as glutaminic acid and 25 per cent as ammonia. What value the latter has in nutrition we do not yet know, but it is difficult to believe that it can be utilized for the synthesis of new protein in any degree comparable with that of the amino-acids. The apparent need in maintenance of greater quantities of gliadin than of most other proteins may be to a certain extent due to this relatively large proportion of nitrogen which it yields as ammonia.

The experiments described in this paper show how rats will actually respond to the particular diets offered, but they fail to give as accordant results as are necessary to reveal differences between the different proteins except in rare cases. This is chiefly due to the wide variations in the amount of food eaten, not only by different rats, but by the same individual at different times.

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[7] This consisted of milk powder, starch, and lard, the milk powder being in part replaced by starch, butter fat, and "protein-free milk" when it was desired to alter the content of protein, *e.g.:*

	15 per cent protein. *gm.*	6 per cent protein. *gm.*
Milk powder	60	25
Starch	12	33
Lard	28	11
Butter fat		14
"Protein-free milk"		17
	100	100

[8] Osborne, T. B., Van Slyke, D. D., Leavenworth, C. S., and Vinograd, M., *Jour. Biol. Chem.*, 1915, xxii, 259.

We are now engaged in a system of limited feeding which promises to solve the problem. In these trials a *limited* weighed amount of food was supplied daily and was eaten completely. The quantity furnished was that on which the rats were at the time growing normally when the food contained an abundant proportion of protein. We were thus assured that the animals received a sufficient supply of calories. Comparison was made between the different proteins by reducing the percentage until growth ceased, or a gradual decline in body weight occurred. The food and protein intakes were so adjusted as to be the same for the body weight of each animal in comparable experiments. The results of a few preliminary experiments are given in Table H. These data confirm those already obtained with the same proteins fed *ad libitum*.

TABLE H.

Rat.	Duration of experiment.	Initial body weight.	Final body weight.	Change in body weight + or −.	Food intake.*		Protein intake.		Protein content of food.
					Absolute total.	Per gm. of rat per week.	Absolute total.	Per gm. of rat per week.	
	days	*gm.*	*gm.*	*gm.*	*gm.*	*mg.*	*gm.*	*mg.*	
2645♂	42	169	170	+ 1	310.8	298–304	12.8	12.3–12.5	5% lactalbumin.
2646♂	42	169	170	+ 1	310.8	294–304	12.8	12.1–12.5	5% "
2645♂	42	170	165	− 5	310.8	307–312	10.3	10.1–10.3	4% "
2646♂	42	170	160	−10	310.8	306–322	10.3	10.1–10.6	4% "
2733♂	42	153	134	−19	277.2	305–340	12.5	13.7–15.3	5% casein.
2732♀	42	121	103	−18	218.4	306–354	9.8	13.8–15.9	5% "
2729♀	42	127	126	− 1	258.9	338–344	11.7	15.2–15.5	5% "

* The composition of the foods was as follows:

	Lactalbumin.		Casein.
	per cent	*per cent*	*per cent*
Protein	4	5	5
"Protein-free milk"	28	28	28
Butter fat	18	18	18
Lard	8	7	7
Starch	32	32	35
Sucrose	10	10	7

It will be noted that the total calories, ash, "protein-free milk" factors, etc., are nearly alike in their relation to the body weight.

The different proteins are, with a few exceptions, not so widely divergent in their general amino-acid make-up as to lead us to expect *wide* differences of protein minimum requirement, aside from a few striking examples. In the case of growth with its greater need of protein units, the divergencies of the proteins may manifest themselves more conspicuously than in mere maintenance. The apparently greater efficiency of lactalbumin, in contrast with the other proteins recorded above, is in harmony with the observations of the apparent economy of this protein as a supplement to deficient rations.[9]

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[9] Osborne and Mendel, *Jour. Biol. Chem.*, 1914, xvii, 325; 1914, xviii, 1.

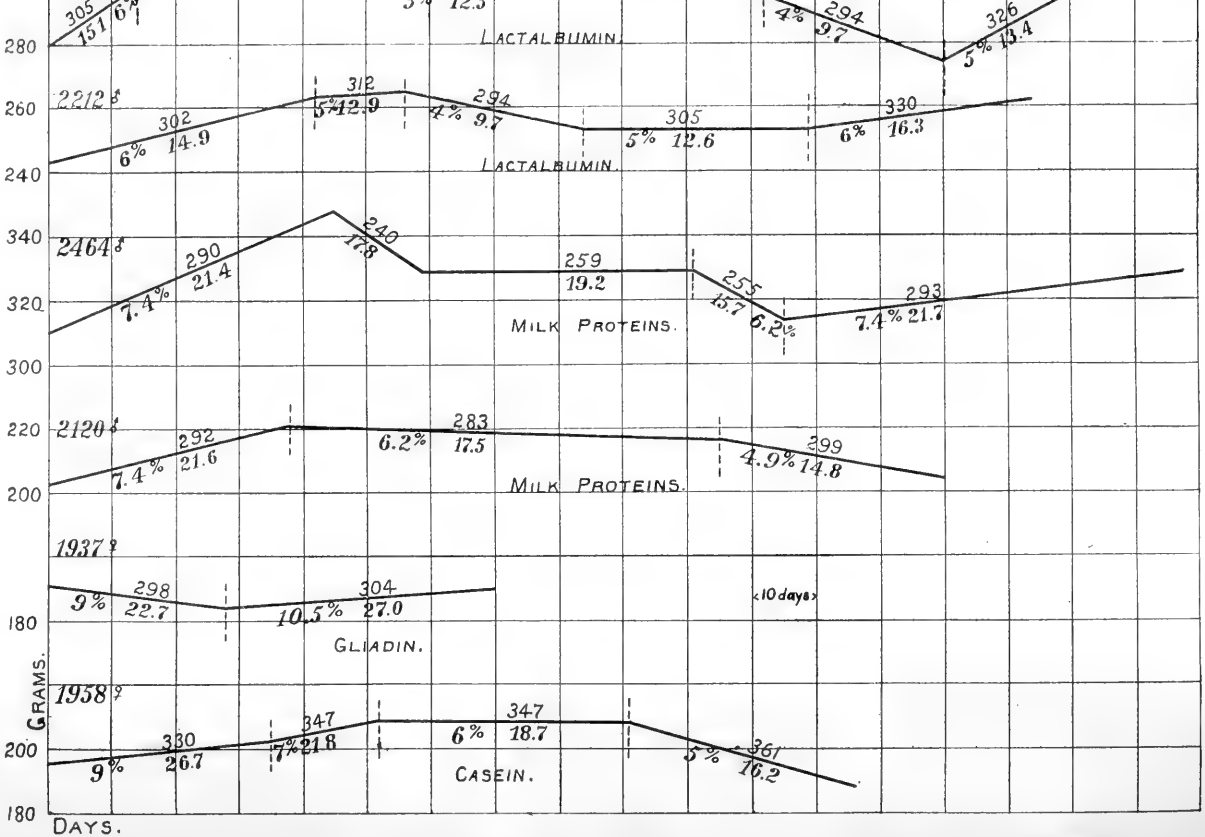

Chart illustrating changes of body weight in typical experiments involving successful or unsuccessful maintenance or increment of body weight on various absolute intakes of different proteins. The nature and percentage of the protein added to the diet, together with the total food intake (above the line) and the protein intake (below the line) expressed in mg. per week per gm. of rat, are indicated on the curves.

SOME PRODUCTS OF HYDROLYSIS OF GLIADIN, LACTALBUMIN, AND THE PROTEIN OF THE RICE KERNEL.[1]

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To settle questions concerning the quantity of some amino-acids yielded by several proteins which form important constituents of human food we have cooperated in the following analyses in order to eliminate, as far as possible, uncertainties due to inexperience in the use of methods, and also those due to working with samples of different origin. We have also paid especial attention to the determination of lysine, in order to gain further information in respect to the differences frequently encountered between the results of the direct isolation of this amino-acid as picrate and its indirect estimation by the Van Slyke method.[2]

The Amount of Lysine in Gliadin.

The discovery of a small quantity of lysine among the products of hydrolysis of gliadin has some important bearings. In the first place it raises the question of the individuality of gliadin, for the amount of lysine isolated was so small that it seemed likely that it was derived from some contaminating protein. A rigid fractionation of 500 grams of this preparation of gliadin yielded extreme fractions from which similar small quantities of lysine were obtained, thus giving no evidence of the presence of any other protein.[3]

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[1] A portion of the expenses of this investigation was also shared by the Carnegie Institution of Washington, D. C.

[2] Compare Van Slyke, D. D., *Jour. Biol. Chem.*, 1913–14, xvi, 531.

[3] Osborne, T. B., and Leavenworth, C. S., *Jour. Biol. Chem.*, 1913, xiv, 481.

That gliadin is in fact a definite substance and not a mixture of two or more proteins has recently been asserted by Gróh and Friedl[4] who have compared several of the physical properties of successive fractions of carefully purified gliadin, and conclude that there is only one alcohol-soluble protein in wheat gluten.

In the second place the results of an analysis by Van Slyke,[5] made by his indirect method, indicated a much larger quantity of lysine than that isolated as picrate, and consequently raised the question of the limits of accuracy of the determinations of lysine made by this method, as well as those by Kossel's method. The great importance of gliadin as a constituent of human food, as well as its extensive use in nutrition experiments, from the results of which conclusions frequently have been drawn on the assumption that it yields no lysine, makes it important to know, as accurately as possible, its true content of the lysine complex, as well as of its other constituents. We have therefore made four new analyses by the Van Slyke method, the closely agreeing results of which, corrected for the solubility of the bases, can now be compared with those obtained by other methods.

Gliadin. Van Slyke Method.

	Per cent of total nitrogen. Uncorrected.				Corrected for solubility of the bases.				Average of corrected results.
	I*	II*	III**	IV**	I	II	III	IV	
Ammonia N..	24.27	24.68	24.67	24.83					24.61
Humin N.....	0.83	0.51	0.50	0.48					0.58
Cystine N....	0.28	0.30	0.38	0.38	0.70	0.72	0.90	0.90	0.80
Arginine N...	5.11	4.96	4.65	4.79	5.62	5.47	5.29	5.43	5.45
Histidine N..	2.84	2.93	2.43	2.57	3.44	3.59	3.19	3.33	3.39
Lysine N.....	1.14	1.28	1.26	1.30	1.22	1.36	1.36	1.40	1.33
Amino N of filtrate.....			52.70	53.10			51.65	52.25	51.95
Non-amino N of filtrate..			12.07	11.08			11.10	10.30	10.70
Total.......			98.66	98.53					98.81

* Total nitrogen = 0.624 gm.
** Total nitrogen = 0.501 gm.

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[4] Gróh, J., and Friedl, G., *Biochem. Ztschr.*, 1914, lxvi, 154.

[5] Van Slyke, *Jour. Biol. Chem.*, 1911–12, x, 15.

Determinations of the basic amino-acids in this same preparation of gliadin, and also in two samples obtained from it by fractional precipitation from alcohol, gave the following results:[6]

Gliadin. Former Results by Kossel's Method.

Grams from 100 gm. of gliadin.

		Arginine.	Histidine.	Lysine.
This sample	I	2.67	1.63	0.16
	II	2.78	1.49	
Fraction A	I	2.48	1.43	0.15
	II	2.43	1.44	
Fraction B	I	2.92	1.49	0.07
	II	2.90	1.48	

An earlier determination of arginine in the gliadin hydrolyzed by Osborne and Clapp[7] gave 3.16 per cent. Kossel and Kutscher[8] found in three different fractions of the alcohol-soluble protein of wheat 3.05, 2.75, and 3.13 per cent of arginine and 1.20 and 1.53 per cent of histidine.

These determinations agree as closely as can be expected when the difficulties involved in the execution of Kossel's complicated method are taken into consideration. The following figures facilitate a comparison of the average of the above results with those obtained by the Van Slyke method.

	Grams amino-acid from 100 gm. of gliadin by the methods of: Kossel. Former analyses.	Van Slyke.* Present analyses.
Arginine	2.70	2.97
Histidine	1.49	2.19
Lysine	0.13	1.21

* Calculated for 17.5 per cent of nitrogen in the gliadin.

That the figures for arginine and histidine by the Van Slyke method are somewhat higher is to be expected, for slight losses in carrying out the Kossel method are almost inevitable. We can consider, therefore, that the proportions of these two amino-acids are now fixed within reasonably satisfactory limits. The

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[6] Osborne and Leavenworth, *loc. cit.*

[7] Osborne, T. B., and Clapp, S. H., *Am. Jour. Physiol.*, 1906–07, xvii, 231.

[8] Kossel, A., and Kutscher, F., *Ztschr. f. physiol. Chem.*, 1900–01, xxxi, 165.

difference among the several results for lysine is relatively great, and we have therefore subjected it to further investigation.

Since the amount of lysine picrate previously obtained from this sample of gliadin was lower than the amount corresponding to either Van Slyke's lysine nitrogen or the free amino nitrogen (see latter part of this paper), new determinations were made of the basic nitrogen precipitated by phosphotungstic acid according to the modified Hausmann method, and also of lysine by Kossel's method. The new direct determination of lysine was made as nearly as possible under the conditions of Hausmann's method for determining basic nitrogen, with the modification that losses which might occur on decomposing the phosphotungstate precipitate were avoided by using the amyl alcohol-ether method.[9]

100 gm. of the air dry gliadin, equal to 95.28 gm. ash- and moisture-free, were boiled with 1000 cc. of 20 per cent hydrochloric acid for twenty-four hours, the solution was concentrated under diminished pressure until most of the hydrochloric acid was removed, and boiled with an excess of magnesium oxide until free from ammonia. The solution was then filtered and concentrated to 1 liter. Two portions of 10 cc. each were diluted to 100 cc. and the nitrogen precipitable by phosphotungstic acid was determined by the modified Hausmann method. We thus found in each portion 0.0096 gm. of basic nitrogen, equal to 1 per cent of the gliadin, or exactly the same amount as in the determinations previously made with 1 gm. portions.

The remainder of the solution was diluted to 10 liters, 300 cc. of concentrated sulphuric acid, and then 3000 cc. of a 20 per cent phosphotungstic acid solution, containing 5 per cent of sulphuric acid, were added. After standing over night the precipitate was filtered out and washed with a 5 per cent solution of phosphotungstic acid. The precipitate was then suspended in about 500 cc. of water, to which about 10 cc. of 30 per cent sulphuric acid were added, and shaken out with a liberal quantity of a mixture of equal parts of amyl alcohol and ether. A clear solution of phosphotungstic acid in the organic solvent formed at once, the decomposition being effected with great ease. The aqueous solution was drawn off and the amyl alcohol-ether layer was washed three times with distilled water. The aqueous solution was then shaken three times more with fresh amyl alcohol-ether mixture, and the latter was each time washed with water to avoid loss.

The solution of the bases was brought to a volume of 1 liter, and nitrogen was determined in two portions of 10 cc. each, 0.86 and 0.86 mg. being found. This is equal to 0.88 gm., calculated back to the whole of the original solution which had previously been found to contain 0.96 gm. of

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[9] Van Slyke, *Jour. Biol. Chem.*, 1915, xxii, 281.

nitrogen precipitable with phosphotungstic acid. The loss by decomposition of the precipitate was 0.08 gm., or 8.3 per cent of the basic nitrogen. How this loss occurred is not clear.

Arginine and histidine were removed from this solution by baryta and silver nitrate, as in the Kossel method, and the lysine by precipitating with phosphotungstic acid. The lysine phosphotungstate was decomposed by the amyl alcohol-ether process as just described. After removing the sulphuric acid with an excess of baryta and the latter by carbonic acid, the solution of the lysine carbonate was made up to 1000 cc. and nitrogen determined in two portions of 25 cc. each, 2.6 and 2.4 mg. being found, equal to 0.1000 gm. of nitrogen, or 0.5210 gm. of lysine, in the whole solution. Correcting this for the solubility of lysine phosphotungstate in the 15 liters from which it was precipitated gives 0.7085 gm. of lysine, equal to 0.76 per cent of the gliadin. After evaporating to a syrup and diluting with a little alcohol, an alcoholic solution containing just enough picric acid to convert this quantity of lysine into the picrate was added. A characteristic yellow precipitate formed at once, which was filtered off the next day, and when dried weighed 0.9746 gm., equal to 0.3794 gm. of lysine.

Since lysine phosphotungstate is soluble to the extent of 12.5 mg. of lysine per liter, and the volume of the solutions from which this had been precipitated was 15 liters, we must add 0.1875 gm. to the lysine found, making the total 0.5669 gm., equal to 0.64 per cent of the ash- and moisture-free gliadin. The purity of the substance weighed was shown by recrystallizing from water whereby 0.8390 gm. of pure, crystallized lysine picrate was recovered. Adding to this 0.0756 gm. for the solubility of lysine picrate in the 14 cc. of filtrate gives a total of 0.9146 gm. equal to 94 per cent of the substance weighed. The nitrogen in the solution from which the 0.9746 gm. of lysine picrate had been obtained was equivalent to 0.5210 gm. of lysine, equal to 0.1416 gm. more lysine than was isolated as picrate. The filtrate from the 0.9746 gm. of substance was evaporated to dryness, during which process a very small quantity of yellow substance, similar in appearance to lysine picrate, separated. The residue was extracted with alcohol, and the insoluble matter filtered out, washed with alcohol, and dissolved in water. The solution when evaporated to dryness left a residue which weighed only 6 mg. The alcoholic solution was also evaporated, and left a residue weighing 0.3450 gm. Since 0.7764 gm. of picric acid had been added, of which 0.5952 gm. separated with the lysine picrate, 0.1812 gm. of picric acid was therefore left in this filtrate. Deducting this from the weight of the residue left on evaporating the filtrate we have 0.1638 gm. of substance. The solution from which the lysine had been precipitated contained 0.1000 gm. of nitrogen, and the precipitated lysine 0.0728 gm., leaving 0.0272 gm. of nitrogen, equal to 16.4 per cent of the residue, providing there was no loss on decomposing the second phosphotungstate precipitate. If all of this residue was lysine it would be equal to only 0.17 per cent of the gliadin which shows that the loss of lysine in precipitating as picrate was, at the most, small.

The amount of lysine which we have thus found, 0.64 per cent, is much more than the 0.16 per cent obtained in the earlier analyses, but considerably less than the 1.21 per cent calculated from the partition of nitrogen in the Van Slyke analysis.

Since the conditions of precipitation of the phosphotungstates in the Hausmann method are quite different from those in the Van Slyke, we repeated the determination of lysine in gliadin, under the conditions of the latter method, in order to determine whether or not the differences here encountered may thus be explained.

100 gm. of the same sample of gliadin, equal to 93.38 gm. ash- and moisture-free, were hydrolyzed as before. After removing the ammonia 400 cc. of concentrated hydrochloric acid were added and the solution was made up to 4 liters. After heating to 95°, a boiling solution of 320 gm. of phosphotungstic acid, dissolved in 1350 cc. of water was added, and the solution allowed to stand for forty-eight hours, during which time the phosphotungstates crystallized out. The precipitate was sucked out and washed with an ice cold 2.5 per cent solution of phosphotungstic acid in 0.8 normal hydrochloric acid. The precipitate was decomposed by the amyl alcohol-ether method. The sulphuric acid used in the decomposition was removed with baryta, and the arginine and histidine were removed with silver nitrate and baryta from a volume of 400 cc. Barium and silver were removed by sulphuric acid and hydrogen sulphide, and the solutions made up to 1000 cc. after adding 30 cc. of concentrated sulphuric acid.

The lysine was then precipitated from the solution heated to 95° by adding a boiling solution of 40 gm. of phosphotungstic acid dissolved in 200 cc. of 5 per cent, by volume, sulphuric acid. The lysine phosphotungstate was decomposed with baryta in the usual way and, when freed from baryta with carbonic acid, the solution was made up to 1000 cc. and nitrogen determined in two portions of 25 cc., 0.0038 gm. being found in each, equal to 0.1520 gm. nitrogen or 0.7920 gm. lysine in the entire solution. After concentrating the remaining 950 cc. of solution to a thin syrup, alcohol was added until a turbidity formed and then an alcoholic solution containing 1.18 gm. of picric acid. After standing over night the lysine picrate was filtered out, washed with alcohol, and dried at 100°. It weighed 1.23 gm., equal to 0.4788 gm. lysine. Since the first phosphotungstic acid precipitate was thrown down from 5350 cc. and the second from 1200 cc., this figure, when corrected for the solubility of lysine phosphotungstate, namely 0.0125 gm. lysine per liter, is equivalent to 0.5607 gm. of lysine in the original solution, or 0.63 per cent of the gliadin.

This agrees exactly with the result obtained by precipitating the phosphotungstates under the conditions of Hausmann's modified method, namely 0.64 per cent. It also shows that no greater loss occurred on decomposing the lysine phosphotungstate with baryta than with amyl alcohol and ether.

The filtrate from the lysine picrate was evaporated and the residue extracted with alcohol to remove excess of picric acid. The substance insoluble in alcohol was dissolved in water and every effort made to isolate lysine picrate from it, but none could be obtained. We have thus shown that gliadin contains at least 0.64 per cent of lysine and have reduced the difference between the amount found by direct determination as picrate by the Kossel method and that indirectly to 0.57 per cent. Since the results obtained by the Kossel method are unquestionably somewhat too low and those by the Van Slyke method may be somewhat too high this difference between the two methods is probably no greater than we should expect. The amount of lysine actually yielded by this protein is probably not far from the average of these two results, namely, 0.92 per cent of the gliadin.

The Amide Nitrogen of Gliadin.

The closely agreeing figures for nitrogen as ammonia in the Van Slyke analyses are of interest inasmuch as they confirm the already large number of determinations previously made on this, as well as many other preparations. In a paper[10] published some time ago attention was directed to the fact that proteins which yield much nitrogen as ammonia also yield much glutaminic acid, and it was suggested that this nitrogen might be in amide union with one of the carboxyl groups of the glutaminic and aspartic acids. The amount of ammonia required for such a union was calculated, and for most proteins found to correspond closely with that determined by analysis. Among the few for which a notable difference was found was gliadin. New determinations have shown a higher yield of glutaminic acid[11] which, together with the aspartic acid, corresponds closely with the quantity required for this assumed union with the ammonia actually yielded by gliadin, namely 5.12 per cent.

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[10] Osborne, T. B., Leavenworth, C. S., and Brautlecht, C. A., *Am. Jour. Physiol.*, 1908–09, xxiii, 180.

[11] Osborne, T. B., and Guest, H. H., *Jour. Biol. Chem.*, 1911, ix, 425.

If, as is probable,[12] the amount of aspartic acid found is about one-half of that present, the calculated amount of ammonia, if this were in amide union, would be 5.18 per cent. The agreement between the results of analysis (5.12 per cent) and this calculation is striking, and suggests that the amount of glutaminic acid which has been obtained from glutenin, hordein, and zein may likewise be less than that actually yielded by these proteins.

The Amount of Lysine in Lactalbumin.

The only analysis of the products of hydrolysis of this protein is that published by Abderhalden and Přibram.[13] Since the feeding experiments with lactalbumin by Osborne and Mendel[14] have shown that a much smaller quantity of this protein supplements the nutritive deficiencies of the lysine-free zein than does any one of numerous other proteins, it seemed probable that it would be found to yield relatively large amounts of this amino-acid. This expectation has been realized, as the following figures show.

The preparation of lactalbumin employed for these analyses was a part of a large quantity that was used for the feeding experiments just referred to, and was made in the following manner: Nearly all the fat was separated from fresh cow's milk by centrifugation, and the casein was removed by adding very dilute hydrochloric acid until the precipitation was complete. Most of the casein was strained out on cheese-cloth and the solution then filtered through paper pulp. A perfectly clear filtrate with only a faint trace of opalescence was thus obtained. The lactalbumin was coagulated by heating rapidly to boiling, and at once filtered out and freed from chlorides and lactose by grinding with water in a "Nixtamal" mill, and then washing with boiling water. The coagulum was next digested six times with strong alcohol, whereby all but an insignificant amount of substance soluble therein was removed. This preparation, when dried in the air at room temperature, weighed 1775 gm. and contained 8.17 per cent moisture, 0.66 per cent ash, and, calculated ash- and moisture-free, 15.51, 15.47, and 15.49 per cent of nitrogen (Kjeldahl).

In regard to the chemical individuality of this preparation we can only say that it represents a mixture of all of the coagulable

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[12] Compare Osborne, T. B., and Jones, D. B., *Am. Jour. Physiol.*, 1910, xxvi, 305.

[13] Abderhalden, E., and Přibram, H., *Ztschr. f. physiol. Chem.*, 1907, li, 409.

[14] Osborne, T. B., and Mendel, L. B., *Jour. Biol. Chem.*, 1914, xviii, 1.

proteins present in the milk serum after removing casein. No attempt was made to separate these from one another as we wished to know the amino-acid make-up of the coagulum thus prepared for use in feeding experiments.

The analysis by the Van Slyke method gave the following results.

Lactalbumin. Van Slyke Analysis.

Per cent of total nitrogen.

	Uncorrected.			Corrected for solubility of bases.***			Average of corrected results.
	I*	II**	III**	I	II	III	
Ammonia N	8.26	8.84	8.60				
Humin N absorbed by lime	2.11	2.15	2.69				
Humin N in amyl alcohol extract		0.47	0.51				
Cystine N	0.56	0.40	0.30	1.39	1.31	1.21	1.30
Arginine N	6.61	6.23	6.20	7.35	7.14	7.11	7.20
Histidine N	3.74	3.47	2.94	4.65	4.80	4.27	4.57
Lysine N	12.19	11.91	12.17	12.27	12.09	12.35	12.24
Amino N of filtrate	63.3	62.8	62.3	62.1	61.0	61.5	62.0
Non-amino N of filtrate	3.65	3.5	3.3	2.5	1.8	1.6	2.0
Total	100.45	99.77	98.91				

* Total nitrogen = 0.556 gm.

** Total nitrogen = 0.571 gm.

*** The corrections for II and III are double the usual amounts, because the bases were recrystallized from a volume of solution equal to that in which they were precipitated.

Determination I was made exactly as indicated in the original description of the method, except for the improvements in manipulation outlined in the next succeeding article. Since the result of this determination for lysine was higher than that of the Kossel determination, it was thought that some mono-amino-acids might have been occluded in the phosphotungstate precipitate of the bases, and failed to be entirely dislodged by the washing. Consequently in Determinations II and III the phosphotungstates, after being washed in the usual manner, were purified by recrystallization.

For this they were suspended in water, and dissolved by the addition of just sufficient sodium hydrate. The solution was then acidified, heated, and the bases were reprecipitated by addition of 15 cc. of concentrated hydrochloric acid and 5 gm. of phosphotungstic acid in each determination, the volume of the final mixture being 200 cc., as in the case of the first precipitation. The recrystallized precipitate of the bases was finally washed seven times with suction, and decomposed with amyl alcohol, ether, and acid, as described in the following paper. All the coloring matter which accompanied the bases was extracted by the amyl alcohol and ether. The nitrogen taken up by the organic solvents was determined by driving off the latter with vacuum distillation, taking the residue up in water, and treating by the Kjeldahl method. This is given in the table as "humin N in amyl alcohol extract."

That when the bases are thoroughly washed recrystallization is unnecessary is indicated by the fact that this process did not affect the figure for lysine, and that the amount of nitrogen and amino nitrogen in the mother liquors from the recrystallization corresponded only to the amount calculated from the solubility of the bases. The total nitrogen in the filtrate and washings from the second precipitations in Determinations II and III was, in each case 0.0087 gram, the amino nitrogen 0.0036 gram. These amounts are added to the total figures for the filtrate, uncorrected, but deducted in the corrected figures.

The good agreement of Analysis I with II and III indicates that the phosphotungstate precipitate in all three analyses was quite pure. Otherwise the recrystallization used in II and III would have changed its composition.

In order to obtain further evidence as to the purity of the phosphotungstate precipitate, *i.e.*, whether it contained only lysine, arginine, histidine, and cystine, we have endeavored to exclude possible contamination by tryptophane, the only known amino-acid which, under the conditions of the analysis, could possibly be precipitated with the bases.

In the original paper on the hydrolysis method it was shown that tryptophane when boiled with hydrochloric acid yields a product which, in not too dilute solution, can be partially precipitated by phosphotungstic acid. The solubility of this phosphotungstate is, however, so much greater than that of the hexone base phosphotungstates, that tryptophane in order to contaminate the latter would have to be present in larger amounts than it occurs in any protein we have previously analyzed (the non-amino nitrogen of the mono-amino fraction in no case exceeded the minimum proline + oxyproline nitrogen sufficiently to make possible the presence of more than a few per cent of tryptophane). An indication of the amount of tryptophane which would have to be present in lactalbumin in order to contaminate the base precipitate is indicated by the following experiment.

0.200 gm. of tryptophane, equivalent to 5.5 per cent of the lactalbumin hydrolyzed in Analyses II and III, was boiled twenty-four hours with 50 cc. of 20 per cent hydrochloric acid. The solution was concentrated on the water bath and then treated like the hydrolysate in the Van Slyke protein analysis. Relatively little ammonia and humin nitrogen (0.6 and 1.0 mg.) was found. The filtrate from the humin was brought to 50 cc., with the addition of 2 cc. of concentrated hydrochloric acid and 4 gm. of phosphotungstic acid, the concentrations of these acids being those ordinarily used in precipitating the bases. When the solution stood over night a considerable amount of crystalline phosphotungstate separated, as in the former experiments of Van Slyke and of Gortner, where 38.7 per cent of the nitrogen was precipitated.

That the precipitation is dependent on the concentration of the tryptophane solution, however, is shown by the following:

150 cc. of a solution containing 3.5 per cent of HCl and 2.5 per cent of phosphotungstic acid were added to the above mixture after the phosphotungstate had crystallized, and the crystals were dissolved by warming. The solution was then allowed to stand several days, the dilution being that usually used in the Van Slyke protein hydrolysis. A perceptible precipitate formed, but it was slight, and contained only 1.8 mg. of nitrogen.

If this amount of tryptophane nitrogen had been present in the precipitate of the lactalbumin bases only one-fourth or 0.45 mg. of nitrogen, equivalent to 0.08 per cent of the total, would have been calculated as lysine, the base on which the Kossel and Van Slyke hydrolyses gave the most widely deviating figures, namely, 10.5 and 12.2 per cent of the total nitrogen. As the precipitate from the lactalbumin was recrystallized at equal dilution, however, at least twice as much tryptophane, or an amount equal to 11 per cent of the lactalbumin used, could have been present in the protein without appreciably affecting the lysine figures.

In order to ascertain whether there can be more than this amount of tryptophane in lactalbumin we have utilized Gortner and Blish's[15] discovery that 86 per cent of tryptophane can be converted into humin by adding glucose to the hydrochloric acid solution in which the protein is hydrolyzed. The following experiment confirms Gortner on this point.

0.200 gm. of tryptophane and 1 gm. of glucose were boiled twenty-four hours with 50 cc. of 20 per cent hydrochloric acid. The solution, unlike a tryptophane-HCl solution without glucose, blackened during the boiling. Thereafter it was treated like the hydrolysate in the Van Slyke protein analysis, with the following results.

	N mg.	Per cent of the N.
Ammonia	0.69	2.5
Humin	23.74	86.5
Phosphotungstate precipitate	1.16*	4.2
Filtrate (by difference)	1.87	6.8
	27.46	

* This precipitate was brown, flocculent, of low specific gravity. It appeared more like humin that had escaped precipitation with calcium hydrate than like the amino-acid phosphotungstates, which are crystalline and heavy.

In the lactalbumin analysis 7 grams of albumin and 7 grams of glucose were boiled with 200 cc. of 20 per cent hydrochloric acid. The hydrolysis was continued forty-six hours, as a preliminary experiment indicated that the glucose retards somewhat the rate of hydrolysis. The hydrolyzed solution was brought to 250 cc., and 100 cc. containing 0.3948 gram of nitrogen were taken for analysis.

Lactalbumin IV. Hydrolyzed in the Presence of Glucose.

	Per cent of total nitrogen.*	
	Uncorrected.	Corrected for solubility of the bases.
Ammonia	8.37	
Humin	3.70	
Cystine	0.39	1.05
Arginine	7.29	8.10
Histidine	2.26	3.22
Lysine	12.42	12.54
Amino N of filtrate	60.10	58.79
Non-amino N of filtrate	4.82	3.58
Total	99.35	

* Total nitrogen = 0.395 gm.

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[15] Gortner, R. A., and Blish, M. J., *Jour. Am. Chem. Soc.*, 1915, xxxvii, 1630.

The chief effect of the glucose is to increase the humin nitrogen from 2.1–2.7 up to 3.7 per cent. The total nitrogen of the bases is not reduced at all, the percentage, corrected for solubility, being 25.66 with glucose, compared with 24.94 to 25.91 without glucose. Nor are the proportions of amino to non-amino nitrogen in the bases significantly affected. (The lower histidine figure in Analysis IV is due to the high result in the arginine determination, not to differences in either the total, or the amino N of the bases.)

The above results show in two ways that tryptophane could not have interfered with the figures for the bases.

1. There was not enough tryptophane present. Making the apparently justified assumption that the 3.7 per cent of humin nitrogen represented 86 per cent of the tryptophane nitrogen, the maximum amount of tryptophane nitrogen present would be 4.3 per cent of the total, equivalent to 4.85 per cent of tryptophane in the protein. The experiment with tryptophane reported on a preceding page indicates that over twice as much would have had to be present in order to contaminate the bases appreciably.

2. Removing the tryptophane by changing it into humin did not affect the figures for the bases.

The distribution of nitrogen according to the modified Hausmann[16] method was determined with the following results.

Hausmann Analysis of Lactalbumin.

	Per cent of the lactalbumin.		
	I.	II.	Average
Ammonia N	1.31	1.33	1.32
Humin N	0.55	0.53	0.54
Basic N	3.62	3.59	3.61
Non-basic N	10.01	10.04	10.02
Total N	15.49		

If we compare the corresponding data from the Van Slyke hydrolysis we obtain figures which agree well:

	Per cent of the lactalbumin.	
	Van Slyke.	Hausmann.
Ammonia N	1.31	1.32
Humin N	0.36	0.54
Basic N	3.44	3.61
Non-basic N	10.38	10.02

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[16] Osborne, T. B., and Harris, I. F., *Jour. Am. Chem. Soc.*, 1903, xxv, 323.

Comparing the percentages of these bases, as determined directly by the Kossel method, with those calculated from the results of the Van Slyke method we have in per cent of the lactalbumin:

	Per cent of the lactalbumin. Kossel.		
	I.	II.	Van Slyke.
Arginine	3.01	3.00	3.47
Histidine	1.53	1.52	2.61
Lysine	8.06	8.10	9.89

The agreement between these analyses for arginine is satisfactory, but for histidine and lysine the difference is too great.

Since for the hydrolysis by the Kossel method sulphuric acid was used, it appeared possible that the decomposition of the lactalbumin might not be as complete as with hydrochloric acid, which was used for the Van Slyke analysis. Furthermore, as suggested by Van Slyke,[17] losses also might have been caused by adsorption during the process of decomposing the voluminous phosphotungstate precipitates with baryta. We therefore made new determinations of lysine, making the precipitations with phosphotungstic acid as nearly as possible under the conditions of the Van Slyke method. Although the yield of lysine was increased to 8.8 per cent, calculated on the unrecrystallized picrate, or 8.45 per cent, calculated from the recrystallized product the figure could not be raised to the level (9.9 per cent) estimated by the Van Slyke method.

In making this analysis 50 gm. of the same preparation of the air dry lactalbumin, equal to 45.45 gm. ash- and moisture-free, were boiled with hydrochloric acid for twenty-four hours, and ammonia was removed by heating with magnesia. The solution was then divided into equal parts, the second phosphotungstate precipitate of the lysine from Part I being decomposed with baryta, and that from Part II by shaking with dilute sulphuric acid and a mixture of amyl alcohol and ether.

The final solutions containing the lysine sulphate were made up to 1000 cc. each. In two portions of 25 cc. of Solution I we found 0.0102 and 0.0100 gm. nitrogen, and of Solution II 0.0102 and 0.0102 gm., equal to 0.4080 gm. in each of the total solutions. These results show that in this case, at least, there was no loss of lysine through adsorption by the voluminous barium phosphotungstate. Since the phosphotungstates were precipitated

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[17] Van Slyke, *Jour. Biol. Chem.*, 1913–14, xvi, 531.

from a volume of 2642 cc., the nitrogen found should be increased by 0.0066 gm. on account of the solubility of lysine phosphotungstate, equal to 0.0025 gm. nitrogen per liter, which makes the corrected lysine nitrogen 0.4146 gm., equal to 2.1393 gm. of lysine, or 9.60 per cent of the lactalbumin, as against 9.89 per cent estimated by the Van Slyke method.

To determine how much of this nitrogen could be isolated as lysine picrate the remaining 950 cc. of Solutions I and II were treated with picric acid and the precipitates dried at 100°. These weighed 4.71 and 4.69 gm., equivalent to 8.82 and 8.78 per cent of lysine respectively, after correcting for solubility of the phosphotungstate precipitates and for the portions previously removed for nitrogen estimations. The precipitated lysine picrate was recrystallized from water and 4.44 and 4.41 gm. were obtained. Adding 0.0810 gm. for the solubility of the picrate in the 15 cc. of mother liquor gives 4.52 and 4.49 gm., equal to 95 per cent of the precipitated lysine picrate recovered analytically pure.

The corrected percentage of the recrystallized picrate, 8.47 and 8.42, is comparable with that obtained in the earlier analyses, namely, 8.06 and 8.10. This slightly higher result may be due to a more complete hydrolysis by hydrochloric acid than by sulphuric acid, or to a more complete decomposition of the first phosphotungstic precipitate of the arginine, histidine, and lysine by the amyl alcohol-ether method, compared with the baryta method. The latter assumption is improbable, however, because the second precipitate of lysine phosphotungstate, although decomposed with baryta, yielded as much lysine as the first.

We have thus been able to isolate lysine picrate in a pure crystalline condition equal to 8.47 per cent or to 8.82 per cent in the form of the amorphous precipitate, which was at least 95 per cent pure and probably much purer. The solution from which this lysine picrate was obtained contained nitrogen equal to 9.6 per cent of lysine.

Since a very careful examination of the filtrates from the lysine picrate failed to yield even traces of this salt, the picric acid was removed by acidifying the concentrated solutions with hydrochloric acid, filtering out the greater part of the picric acid which crystallized out, and removing the remainder by shaking with ether. To the suitably concentrated solutions phosphotungstic acid was added, whereupon relatively considerable precipitates formed at once. These were decomposed by hydrochloric acid and amyl alcohol and ether, and the solution was evaporated to remove excess of acid. From one of these solutions an attempt was made to obtain more lysine picrate, but none whatever could be secured. In view of the not inconsiderable amount of nitrogen unaccounted for, it seems highly probable that lysine picrate could have been thus obtained had this nitrogen belonged to lysine.

The other solution was freed from chlorine with silver carbonate, silver was removed from the filtrate from the silver chloride by hydrogen sulphide, and the solution was evaporated to small volume. This was very strongly alkaline to litmus, became turbid on adding alcohol, and after standing, yielded a crystalline precipitate. Owing to the small amount of the substance contained in this solution it was impossible to determine its nature.

It is evident that besides lysine these solutions contained some other substance or substances precipitated with the lysine phosphotungstate, and that this fact may explain in part the higher result for lysine in lactalbumin given by the Van Slyke method. On the other hand, the amount of cystine which was precipitated by phosphotungstic acid in the Van Slyke analysis, and therefore would be expected in the lysine fraction here, would account for all of this undetermined nitrogen, and leave the deficit to be explained only by the unavoidable losses of manipulation.

The Protein of Polished Rice.

Six hundred grams of commercial polished rice were washed with water and then allowed to soak over night at room temperature. The softened grains were next ground to a thin paste in a special mill, together with 2 liters of water, which included that in which they had been soaked. Two liters of 0.2 per cent sodium hydroxide solution were added, the insoluble matter was allowed to settle, the solution syphoned off, and the residue centrifuged. The entire extract thus obtained was filtered perfectly clear through a pulp filter and precipitated with dilute acetic acid, added until the protein separated sharply. After the precipitate had settled the solution was drawn off and replaced by alcohol equal to three times the volume of the solution which remained. This was done to remove any alcohol-soluble protein that might be present, and which could best be extracted from the finely divided precipitate before it was rendered more compact by collecting on a filter. After standing in this dilute alcohol for two days the precipitate was boiled out twice with 500 cc. of 80 per cent, by volume, alcohol, and dehydrated with absolute alcohol. The air dry preparation weighed 25 grams, equal to 4.17

per cent of the polished rice, and contained 7.98 per cent moisture, 0.78 per cent ash, and 15.20 and 15.29 per cent nitrogen, equal to 16.68 per cent calculated for the ash- and moisture-free substance.

The alcoholic extracts were concentrated and found to contain a very little protein, similar to gliadin from wheat in its solubility. The amount was too little to enable us to learn anything about it, and we were also unable to determine whether it was an original constituent of the seed.

The preparation of the rice protein, which is equal in amount to somewhat more than one-half of the protein of the polished rice, represents the glutelin of the seed which Rosenheim and Kajiura have named oryzenin.[18] This protein has been hydrolyzed by Suzuki, Yoshimura, and Fuji[19] who identified arginine, histidine, and lysine among its decomposition products, but failed to make satisfactory quantitative determinations of these amino-acids. We therefore thought it would be of interest to analyze our preparation by the Van Slyke method, and have done so with the following results.

Rice Protein.

	Per cent of total nitrogen.*				Grams amino-acid per 100 gm. of protein. **
	I.	II.	Average.	Average corrected for solubility of bases.	
Ammonia N	11.23	11.43	11.33		
Humin N	1.46	1.72	1.59		
Cystine N	0.41	0.50	0.46	0.88	1.26
Arginine N	16.85	17.50	17.17	17.69	9.15
Histidine N	4.54	4.99	4.77	5.39	3.32
Lysine N	4.81	4.82	4.82	4.90	4.26
Amino N of filtrate	52.95	53.00	52.98	52.13	
Non-amino N of filtrate	6.05	6.10	6.08	5.28	
Total	98.30	100.16			

* Total nitrogen = 0.611 gm.

** Calculated on the assumption that the ash-free protein contains 16.68 per cent of nitrogen.

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[18] Rosenheim, O., and Kajiura, S., *Jour. Physiol.*, 1907–08, xxxvi, pp. liv-lv.

[19] Suzuki, U., Yoshimura, K., and Fuji, S., *Jour. College of Agriculture, Tokyo Imperial University*, 1909, i, 77.

In the following table the amount of basic substances yielded by oryzenin, which probably constitutes nearly all of the protein of the endosperm of rice, is compared with that yielded by the proteins of the endosperm of the seeds of wheat and maize. The figures for wheat are calculated on the assumption that wheat gluten, which contains nearly all of the protein of the endosperm of the seed, consists of equal parts of gliadin and glutenin; those for maize on the assumption that corn gluten, which represents the protein of the endosperm of the maize kernel, consists of two parts of zein and one of maize glutelin.

Basic Substances Yielded by the Proteins of the Endosperm of the Seeds in per cent of the Protein.

	Wheat.	Maize.	Rice.
Arginine	3.86	3.39	9.15
Histidine	2.02	1.55	3.32
Lysine	1.58	0.97	4.26
Ammonia	4.62	3.13	2.22
Percentage of assimilable nitrogen*	40	29	88

* The figures for assimilable nitrogen are taken from the work done in Rubner's laboratory by Karl Thomas.[20] The different proteins were taken in moderate amounts after a period on a protein-free diet, when the body was presumably in a condition to retain and assimilate as much of the absorbed protein as the amino-acid content rendered possible. The percentage of absorbed nitrogen incorporated into, or at least retained by, the body tissues, was calculated, with the results given above. It is rather striking that the figures for the utilizability of these proteins correspond so closely with the lysine which they yield.

From these figures it is evident that the protein of the rice kernel does not show the marked differences which the other cereal proteins show in respect to their amino-acid make-up when compared with the majority of food proteins, and particularly with most tissue proteins. It is, therefore, not improbable that the nutritional requirements may be satisfied by a smaller quantity of this protein than of those of wheat or maize kernels. These facts are in harmony with the extensive use of rice as food for men despite its low content in protein; and furthermore with the biological utilizability of the respective proteins as determined by Thomas.

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[20] Thomas, K., *Arch. f. Anat. u. Physiol., Physiol. Abt.*, 1909, 219.

Free Amino Nitrogen of the Proteins.

Van Slyke and Birchard have determined in a series of proteins the amount of nitrogen which reacts as free NH_2 with nitrous acid.[21] In all the native proteins analyzed by them, except a gliadin preparation of doubtful purity, the free amino nitrogen was found equal to one-half the lysine nitrogen, as determined by Van Slyke's nitrogen distribution method. This relationship, in conjunction with the fact that the free amino nitrogen of the native proteins reacts with nitrous acid at the slower rate characteristic of the ω-NH_2 group of lysine as compared with the faster acting α-NH_2 groups of the other amino-acids, led to the conclusion that the ω-NH_2 group of the lysine is free in the native proteins, and is the only free amino group therein, all the α-NH_2 groups of the constituent amino-acids being bound in peptide linkings.

Recently Hartley[22] has added further evidence in support of this conclusion. He finds that in the albumin and the globulins of horse and ox serum the free NH_2 is equal to half the lysine nitrogen.

In order to find whether the 2:1 ratio between lysine nitrogen and free amino nitrogen holds for lactalbumin and rice protein, and for gliadin, we have determined the free amino nitrogen in all three proteins and compared the results with those for lysine.

0.75 gm. of each protein (air dried) was dissolved and diluted to 25 cc. 5 cc. portions were taken for Kjeldahl determinations, and 2 cc. for amino nitrogen by the nitrous acid method.[23] The solvents used were different. The gliadin was dissolved in 2.5 cc. of glacial acetic acid plus 1 cc. of water, and the clear solution diluted up to 25 cc. The lactalbumin was dissolved in 5 cc. of cold 10 per cent sodium carbonate, and the turbid solution diluted to 25 cc. The rice protein was taken up with sufficient 4 per cent potassium carbonate to make the volume 25 cc. Blanks were run in duplicate in each case with acetic acid or carbonate of similar concentration, the volume of gas obtained from the blank being subtracted from that obtained in the analysis. The results are given in the following table:

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[21] Van Slyke, D. D., and Birchard, F. J., *Jour. Biol. Chem.*, 1913–14, xvi, 539. The evidence showing that nitrous acid does not hydrolyze the proteins under the conditions of the determination is given in this paper.

[22] Hartley, P., *Biochem. Jour.*, 1914, viii, 544.

[23] Van Slyke, The Gasometric Determination of Aliphatic Amino Nitrogen in Minute Quantities, *Jour. Biol. Chem.*, 1913–14, xvi, 121.

Free Amino Nitrogen of the Proteins Compared with the Lysine Nitrogen

Protein.	Total N in 2 cc. solution.	N gas from 2 cc.	Pressure.	Temperature.	Amino N in 2 cc. solution.	Per cent of total N as amino N.	Per cent of total N = ½ the lysine N as determined by method of:	
							Van Slyke.	Kossel.
	mg.	*cc.*	*mm.*	*°C.*	*mg.*			
		1.07						
Lactalbumin..	9.21	1.05	759	25	0.588	6.49	6.10	5.45
Average....		1.06						
Rice protein..	7.92	0.30	751	19	0.172	2.17	2.00	—
Average....		0.30						
Wheat		0.11						
gliadin*....	9.40	0.10	762	25	0.058	0.62	0.66	0.32
Average....		0.105						

* Repetition with more material gave the same result. A solution three times as concentrated gave 0.31 cc. of nitrogen gas at 26°, 760 mm., equivalent to 0.61 per cent of the total nitrogen.

It is evident from the above table that the ratio $\frac{\text{lysine N}}{\text{free NH}_2} = \frac{2}{1}$ holds for all three proteins when the lysine determinations by the Van Slyke method are taken. The lysine nitrogen figures for lactalbumin and gliadin by Kossel's method run somewhat lower, but parallel.

SUMMARY.

1. From gliadin an amount of analytically pure lysine picrate was isolated equivalent to a lysine content of 0.64 per cent of the ash-free protein, correction for the solubility of lysine phosphotungstate being made. The amount obtained is several times that reported by previous authors (0 to 0.16 per cent), from which figures gliadin has heretofore been regarded as practically lysine-free.

The lysine content estimated from the partition of nitrogen by the Van Slyke method was still higher, 1.21 per cent.

As the results by the picrate method are based on the weight of pure substance actually isolated, they are minimum figures. The nitrogen partition results, on the other hand, may be taken as maximum figures; for they represent all the basic amino nitrogen not in the form of arginine, histidine, and cystine. It appears probable, therefore, that the true lysine content of gliadin lies between the results by the two methods, and may be stated as 0.93 ± 0.28 per cent.

Expressed in the same manner, the histidine content is 1.84 ± 0.35 per cent, the arginine 2.84 ± 0.14 per cent, the upper limit in each case giving the results by the Van Slyke method, the lower that by Kossel's.

In the case of lysine especial efforts were made to eliminate all sources of loss which might account for the lower results by Kossel's picrate method as compared with the distribution method; but, as indicated above, without raising the results by the former method to the level of the latter. The lower results by Kossel's method were not due to hydrolysis with sulphuric acid instead of hydrochloric, or to precipitation of the phosphotungstates under conditions different from those employed in the Van Slyke method, or to loss in decomposition of the lysine phosphotungstate with baryta.

2. The amount of nitrogen yielded by the gliadin as ammonia corresponds closely with that required for amide union with one carboxyl group of the larger amount of glutaminic and aspartic acids recently obtained from gliadin. This fact is added evidence that the nitrogen yielded as ammonia by acid hydrolysis exists in the protein molecule in acid-amide form as in asparagine and glutamine.

3. In all three proteins, as in the series analyzed by Van Slyke and Birchard and by Hartley, the free amino nitrogen determinable by the nitrous acid method was found equal to one-half the amount of lysine nitrogen determined by Van Slyke's method. As in all cases Kossel's picrate method gave somewhat lower, though parallel, results for lysine, the lysine determined as the picrate was less than that calculated from the free amino nitrogen of the proteins, although the deviation was not great. The approximate constancy of the ratio, free NH_2 : lysine NH_2 = 1:2, indicates that one of the two NH_2 groups of lysine exists free in the protein molecule, the free group being probably the ω-NH_2 group.

4. The hexone base content of lactalbumin was found to be the following, the upper limit in each case representing the results by Van Slyke's method, the lower that by Kossel's: lysine, 9.16 ±

0.68 per cent; histidine, 2.06 ± 0.54 per cent; arginine, 3.23 ± 0.23 per cent.

As in the case of gliadin, it was found impossible to isolate an amount of lysine picrate corresponding to the entire content indicated by the nitrogen distribution method. The difference between the two sets of results could not be reduced below 1.3 per cent, despite exceptional precautions to eliminate error in both methods. Both agreed, however, in showing that lactalbumin is exceptionally rich in lysine. This fact is particularly interesting in view of the ability of lactalbumin, shown by Osborne and Mendel, to stimulate the growth of rats when used to supplement a ration low in lysine.

5. The partition of nitrogen among the products of hydrolysis of oryzenin, the chief protein of the endosperm of rice, was determined by the Van Slyke method. Compared with the endosperm proteins of wheat or maize, the protein of rice yields relatively much of each of the basic amino-acids, arginine, histidine, and lysine, and comparatively little ammonia and non-amino nitrogen. In its general amino-acid make-up it more nearly resembles the majority of the proteins of animal tissues than do the proteins of maize or wheat. This may explain the extensive use of rice as an almost exclusive diet in spite of its low protein content.

IMPROVEMENTS IN THE METHOD FOR ANALYSIS OF PROTEINS BY DETERMINATION OF THE CHEMICAL GROUPS CHARACTERISTIC OF THE DIFFERENT AMINO-ACIDS.

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(From the Hospital of the Rockefeller Institute for Medical Research.)

(Received for publication, July 12, 1915.)

Although the principle of this method for determining the products of protein hydrolysis remains as described in the original article in this Journal,[1] the technique has been so modified at a number of points that the liability to error has been diminished and the ease and sureness of the manipulations increased. Brief mention of these points therefore appears justified.

In *washing the phosphotungstic precipitate of the bases* it is possible, with a steady and moderately strong suction, to dispense with the special form of plaited filter described in the original paper, and utilize a two-inch Buchner funnel with a flat circle of hardened filter paper pressed over the perforations in the usual manner.[2] The washing is carried out without at any time releasing the suction. Successive portions of 10 to 15 cc. of the washing solution are poured onto the well packed precipitate, which is stirred up with each portion by means of a flat-tipped rod, so that all parts of the precipitate are reached. If a steady suction is maintained all the time, this can be done without danger of loosening the filter from the floor of the funnel, and is a considerably neater and easier process than that originally described. It can not be utilized with a strong central vacuum, as this sucks the washing solution through before it can be stirred up with the precipitate. Nor can it be used with a weak pump, as the filter paper will not

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[1] Van Slyke, D. D., *Jour. Biol. Chem.*, 1911–12, x, 15.

[2] This simplification was introduced by Dr. Percival Hartley, of the Lister Institute, during the course of his analyses of serum proteins (*Biochem. Jour.*, 1914, viii, 541).

be held strongly enough to the floor of the Buchner funnel to prevent its detachment during the stirring. With a good aspirator pump of the ordinary type, however, this technique is preferable to the original, because the area of filter paper that must be quantitatively washed is much smaller.

The *washing solution should be cooled to 0°* before it is poured onto the precipitate. It has been found that the solubility of the phosphotungstates of the hexone bases is only about one-fourth as great at 0° as it is at room temperature. Consequently when the washing solution is used at or near 0° the danger of dissolving appreciable amounts of the precipitate is reduced to a minimum.

The above suggests the obvious desirability of not only washing, but precipitating the bases at 0°. We can not yet say that this is a safe procedure, however. The phosphotungstates of some of the mono-amino-acids, such as alanine, phenylalanine, and proline, are fairly insoluble at 0°, and it is possible that an attempt to precipitate the bases in a solution cooled to this temperature might result in some cases in the precipitation of such acids of the mono-amino fraction.

The number of washings necessary in each case to free the phosphotungstate precipitate completely from the mother liquors is determined by *testing the successive washings* for calcium. For this a 10 per cent sodium hydrate solution containing some sodium oxalate is used. The specific gravity of this solution is so high that the drops of washing solution tested can be floated in a layer on top, and the sensitiveness of the test made much more delicate than when the portion of washing liquid to be tested is mixed with several volumes of oxalate solution. For the test, 1 or 2 cc. of the alkaline oxalate solution are placed in a test-tube of 8 or 10 mm. diameter, and 2 or 3 drops of the washings from the stem of the Buchner funnel are allowed to run down the side of the tube and form a layer on top of the solution. The tube is now gently shaken until just enough alkali has mixed with the upper layer to turn the latter alkaline. The tube is viewed against a dark background, but with plenty of light passing through the solution itself. If no cloud is visible in the upper layer, the tube is allowed to stand a few minutes. If no evidence of a precipitate can then be seen, the washing is complete.

The fact that under these conditions the washing out of the mono-amino fraction is really complete, is shown by the results of recrystallization of the basic phosphotungstates (see lactalbumin analysis in the preceding paper, p. 267, for example). The mother liquors from the recrystallized bases were found to contain amino and non-amino nitrogen only in the amounts to be expected from the solubility of the bases. If in any analysis, however, there should be reason to doubt the purity of the phosphotungstate precipitate, or the efficiency of the washing, the precipitate may be *recrystallized* in the manner described in the report of the lactalbumin analysis.

For *decomposition of the basic phosphotungstates* (removal of the phosphotungstic acid and putting the bases into solution again) we have found quite a different procedure to be preferable, as a rule, to the use of barium originally recommended. Winterstein[3] showed that when a precipitate of basic phosphotungstates was suspended in dilute hydrochloric acid and shaken with ether the precipitate went into solution, the greater part of the phosphotungstic acid being extracted from the aqueous mixture by the ether. By shaking with repeated portions of ether, all the phosphotungstic acid could be removed, and concentration of the water solution yielded as a residue the pure hydrochlorides of the bases. Our experience indicated that with this method the complete removal of phosphotungstic acid, such as is necessary before the cystine sulphur can be determined, is, though possible, extremely difficult. Jacobs some time ago discovered, however, that amyl alcohol has a surprising affinity for phosphotungstic acid, and extracts it with great readiness from water solution.[4] We find that a mixture of equal parts of ether and amyl alcohol serves perfectly for decomposing the phosphotungstic precipitate of the hexone bases. It is preferable to amyl alcohol alone because of the lighter specific gravity of the ether mixture, and it appears to extract phosphotungstic acid from water as readily and completely as does amyl alcohol. The following procedure is utilized.

The basic precipitate from the 3 or 4 grams of protein ordinarily used in an analysis is removed from the filter by a spatula and

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[3] Winterstein, E., *Ztschr. f. physiol. Chem.*, 1901–02, xxxiv, 153.

[4] Jacobs, W. A., *Jour. Biol. Chem.*, 1912, xii, 429.

washing, and transferred to a half-liter separatory funnel, using 200 or 300 cc. of water to effect the transfer. Five or 10 cc. of concentrated hydrochloric acid are added, and the mixture is shaken with the 1:1 amyl alcohol-ether, using so much of the latter that it all floats in a layer above the water after the precipitate has gone into solution. Usually about 100 cc. of the ether-amyl alcohol suffice, and one or two minutes' shaking results in complete solution of the precipitate. If too little of the ether-amyl alcohol has been used, some of it will, after taking up phosphotungstic acid, sink as an oil below the aqueous phase. In case this happens, more of the extracting mixture is added, until all floats in one layer at the top.

In some cases the aqueous and ether-amyl alcohol layers do not separate readily with a clean boundary between them. This effect is due to the presence of a slight amount of humin which may have escaped previous adsorption by calcium hydrate. In this case the unadsorbed humin is carried down with the basic phosphotungstates, and fouls the solution when their precipitate is decomposed as above described. In order to clear the solution up, it is all, without separation of the aqueous and ether-amyl alcohol layers, passed through a Buchner funnel with suction. The clear filtrate readily separates into two layers. The aqueous layer is extracted with three more successive portions of ether-amyl alcohol, using each time a volume of the mixture equal to about one-fourth the volume of the water solution. Finally the combined amyl alcohol-ether extracts are shaken out once with water, to remove traces of bases that might have been carried into the extract; this portion of water is then shaken once or twice with fresh amyl alcohol-ether, and combined with the main solution of the bases. The latter should be free of phosphotungstic acid, as demonstrated by the absence of a precipitate when a few drops are added to a saturated solution of barium hydrate in a small test-tube. The solution of the bases is now concentrated to dryness in vacuum in order to drive off the free hydrochloric acid, and the residue is washed into a 50 cc. flask.

The above procedure is much quicker than the decomposition with sodium hydrate and barium chloride originally described, involves the use of smaller volumes of solution, and finally gives a solution of the bases that contains no barium. This is of great advantage in the subsequent arginine determination, where the presence of barium is likely to cause disagreeable bumping.

The *determination of amino nitrogen in the bases* can now, with the micro-amino apparatus, be performed with only 1 or 2 cc. of the total 50 cc. It is consequently desirable to use the micro-apparatus and perform duplicate determinations of amino nitrogen, rather than use the larger amino apparatus with 10 cc. of solution, and be limited to a single determination.

The *total nitrogen of the bases*, as determined in the arginine residue, can also be determined advantageously in duplicate. The alkaline solution, left after decomposition of the arginine and removal of the ammonia formed, is diluted up to 100 or 200 cc. and divided into halves. Each half of this solution is then Kjeldahled separately, using 20 cc. of sulphuric acid for the digestion.

In conclusion it may be well to call attention again to the fact that the method was designed for use only with proteins not accompanied by other classes of substances, particularly nitrogenous substances, which would obviously falsify the interpretation of the results unless the behavior of the non-protein substances were so accurately known that corrections could be made.

Reference to the accompanying paper by Osborne, Van Slyke, Leavenworth, and Vinograd will show that with the above changes in technique the agreement between duplicate determinations is much closer than that claimed for the method in its original description.[5]

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[5] *Humin nitrogen as a measure of tryptophane.* Gortner and Blish (*Jour. Am. Chem. Soc.*, 1915, xxxvii, 1630) have recently shown that if tryptophane and glucose are boiled together in 20 per cent hydrochloric acid under the conditions used for the protein hydrolysis, 86 per cent of the tryptophane is converted into humin. We have repeated the experiment with the same result. It appears highly probable that if an amount of glucose equal to that of the protein be added to the protein-acid mixture before hydrolysis, the humin nitrogen divided by 0.86 will approximately indicate the maximum amount of tryptophane. Gortner and Blish have shown that the action of glucose increases the humin yield of zein and gliadin, and we have found the same for lactalbumin. It may be well to mention that it appeared necessary to boil the lactalbumin in the presence of the glucose 48 hours, instead of 24, in order to attain complete hydrolysis.

AN OXALIC ACID PRODUCING PENICILLIUM.[1]

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(Received for publication, July 22, 1915.)

Oxalic acid formation by fungi has received the attention of several investigators since de Bary[2] observed that the mycelium of *Sclerotinia sclerotiorum* became incrusted with an oxalate in its older stages. The most elaborate study of the problem was made by Wehmer.[3] His results were published in a series of twenty-three papers. Tabulated data showing oxalic acid production under various conditions were given for *Aspergillus niger* and *Penicillium glaucum*. No other molds were considered in detail.

Wehmer reviewed his own work and also that of other investigators up to 1906 in his chapter on the chemical action of Aspergillaceae contributed to Lafar's Handbuch der technischen Mykologie.[4] More recent literature contains several casual references to citric acid production in small quantities by various fungi but records no systematic study of the problem.

Wehmer states that the only acids produced by either *Aspergillus* or *Penicillium* in notable quantities are oxalic and citric. He concludes that investigators who have reported lactic, formic, and acetic acids did not work with pure cultures. Oxalic acid fermentation in its true sense, the production of free oxalic acid, according to Wehmer has been observed only in cultures of *Aspergillus niger*. *Aspergillus glaucus*, *Penicillium glaucum*, *Botrytis cinerea*, *Sclerotinia sclerotiorum*, and *Rhizopus nigricans* produce oxalic acid only in traces and only as the media is maintained close to the neutral point.

Wehmer studied the various conditions which influence oxalic acid production. He found it was favored by low temperature, abundant carbohydrate food supply, the presence of an insoluble calcium salt as calcium phosphate or carbonate, and the presence of substances from which the mold can produce basic material such as organic nitrogenous compounds and salts of organic acids.

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[1] Published by permission of the Secretary of Agriculture.

[2] de Bary, A., *Bot. Ztg.*, 1886, xliv, 401.

[3] Wehmer, C., *ibid.*, 1891, xlix, 233.

[4] Lafar, F., Handb. d. technischen Mykologie, Jena, 2nd edition, 1905-07, iv, 242.

We have studied the oxalic acid production of twenty-three species of *Penicillium*. Many of these occur on the foodstuffs of man and of domestic animals under such conditions that oxalates might be produced. With the exception of the one species with which this paper is chiefly concerned it is doubtful if the quantity of oxalates formed would in any case be large enough to become a significant factor in food chemistry.

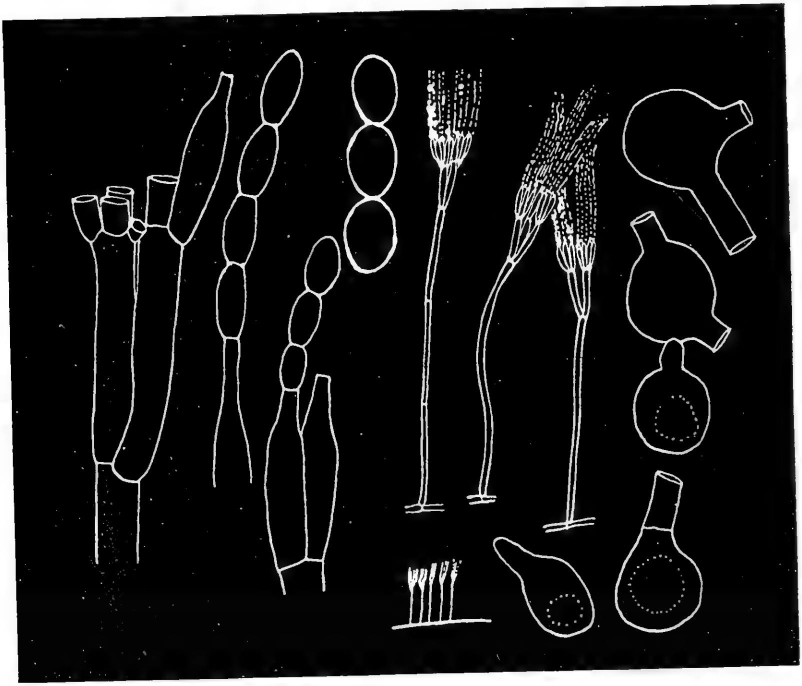

FIG. 1.

The three most important facts demonstrated are: (1) Oxalic acid is produced by many species of *Penicillium*. As a common product of their metabolism it may be likened to lactic acid among bacteria. (2) Oxalic acid is a transient and not an end-product of metabolism. (3) An undescribed *Penicillium* for which we propose the name *Penicillium oxalicum* should be added to the list of true oxalic acid ferments. A technical description of this organism with drawings is therefore given.

Description of Penicillium Oxalicum.

Colonies on Czapek's agar solution near ivy-green (Ridgeway XXXI) velvety, spreading widely with surface growth conidiophores only; reverse of colony pale yellow; agar uncolored or only slightly colored; conidiophores up to 200 μ long by 3.3 to 5.4 μ in diameter enlarged to 5 μ at apex; conidial fructification consisting of a single verticil of two to three branches 15 to 20 x 3.5 μ, appressed, bearing verticils of conidiiferous cells 10 to 14 x 2.5 to 3.5 μ in small parallel whorls which bear conidial chains in close columns which form visible masses of conidia easily broken free from the surface of the colony by tapping or jarring the glass; conidia at first cylindrical then elliptical from 2 x 3μ, 2 x 4 μ, 2 x 5 μ, up to 3.5 x 5 μ persistently elliptical, remaining in chains in alcoholic mounts swelling to 7 to 10μ in diameter and germinating by one or sometimes two tubes. No odor discernible.

Gelatin without sugar slowly liquefied more or less, with alkaline reaction. Produced rhombic crystals in prune gelatin. Grew richly at 37°C.

Shows a morphology of conidial apparatus resembling *P. italicum*, Wehmer, but differs widely from that species in appearance and habits. First found by T. P. Haslam in cultures from corn in Kansas. Later isolated from moldy corn and corn meal from many localities including Connecticut, Maryland, Virginia, and Illinois. It also occurs in soil cultures but evidently if it is a soil organism it is so well adapted to grow upon corn that it becomes a very common component of the flora of moldy corn and corn products.

Methods of Culture and Analysis.

The media employed were after the general formula of Czapek. The magnesium sulphate and potassium chloride were reduced because the magnesium tends to precipitate out the phosphate and the sulphate and chloride radicals add to the difficulties of separating the organic acids. Two media were used, one of which will be designated 3 per cent cane-sugar and the other 5 per cent cane-sugar. The inorganic salts used in each are shown in the following table.

	3 per cent cane-sugar.		5 per cent cane-sugar.	
Sodium nitrate	1.0	gm. per 1000 cc.	3.0	gm. per 1000 cc.
Potassium nitrate	1.0	"	0.0	"
Potassium dihydrogen phosphate	0.5	"	1.0	"
Magnesium sulphate	0.01	"	0.25	"
Potassium chloride	0.01	"	0.25	"
Ferrous sulphate	0.01	"	0.01	"

Where the 3 per cent cane-sugar medium was used the mold was inoculated into 125 cc. of medium and 100 cc. of filtrate were analyzed. In all other cases the medium was filtered, the mycelium thoroughly washed with hot water, and the wash water added to the filtered medium. In no case did the mycelium after thorough washing yield a further quantity of oxalates even when digested with hydrochloric acid. The filtrate was then made alkaline with ammonia, heated almost to boiling, and a little magnesia mixture added. In this magnesia mixture chlorides were replaced by nitrates. This removes the excess of phosphates and a considerable quantity of pigment and other substances which would interfere with the purification of the oxalate. The oxalate was then precipitated as the calcium salt, dissolved in dilute hydrochloric acid, and reprecipitated. The twice precipitated oxalate was then dissolved in dilute sulphuric acid and estimated by titration with potassium permanganate. The purity of the calcium oxalate obtained by this procedure was established for nearly every mold studied by estimating the calcium oxide by direct ignition of the oxalate. When a calcium salt had been added to the medium the entire culture was thoroughly digested with warm hydrochloric acid before filtration and the removal of phosphates by magnesia mixture had to be omitted.

Examination of these data shows that *P. oxalicum*, culture number 103, under all conditions investigated far exceeded all others as a producer of oxalic acid. *P. italicum* which resembles *P. oxalicum* in morphology and also numbers 28 and 2467 which belong to the *Citromyces* group, produced very considerable amounts of oxalic acid when grown on media containing calcium carbonate. Wehmer[5] states that some of his *Citromyces* cultures formed oxalates as a secondary reaction on the citrate first formed, when cultivated in the presence of an excess of calcium carbonate.

Two cultures of *P. oxalicum* designated by numbers 103 and 3563.8 which had been derived from different sources were selected for further study. These were grown at 20°C. on 5 per cent cane-sugar media, and also on 10 and 20 per cent cane-sugar media in which sodium nitrate and potassium dihydrogen phosphate were proportionately increased. The other inorganic salts

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[5] Wehmer, *Chem.-Ztg.*, 1909, xxxiii, 1281.

TABLE I.

Oxalic Acid Produced per 100 cc. of Medium, Expressed in $\frac{N}{10}$ *cc.*

Penicillium.*	Culture No.	3 per cent cane-sugar. 21 days at 25°.	3 per cent cane-sugar. 28 days at 25°.	5 per cent cane-sugar. 28 days at 25°.	5 per cent cane-sugar + $CaCO_3$. 28 days at 25°.	5 per cent cane-sugar. 14 days at 20°.	Per cent of CaO in calcium oxalate. Theory, 38.39.
Pinophilum	1	0.0	0.0	0.0	0.0	0.0	
Brevicaule	2	0.35	0.0	0.0	0.8	0.0	
Camemberti	5	11.6	21.7	31.0	52.1	22.0	38.42
Granulatum	9	11.7	15.1	31.6	32.3	21.4	38.41
Italicum	10	21.5	22.2	44.9	118.4	21.3	38.41
Luteum	11	17.5	12.5		15.6		38.32
Expansum	14	14.1	12.4	31.9	24.4	10.9	38.46
Citrinum	15	0.0	0.0	4.6	0.0	0.0	
Purpurogenum	17	0.0	0.0	0.0	0.0	0.0	
Roqueforti	18	21.0	26.2	30.4	48.8	11.6	38.51
Duclauxi	20	0.0	0.0	0.0	5.3	0.0	
Commune	23	14.6	19.0	32.9	15.5	16.8	
Chrysogenum	26	14.4	7.6				38.45
Stoloniferum	27	12.2	8.4	36.0	20.4	20.4	38.38
Divaricatum	34	0.0	0.0	0.0	0.0	0.0	
Spinulosum	45	0.0	0.0	0.0	0.0	0.0	
Notatum	102	0.0	0.0		38.1	6.2	
Oxalicum	103	54.1	53.0	35.2	288.0	86.6	38.39
Variabile	3551	0.0	0.0		19.2	10.2	
Not named	3553	11.2	13.4	14.1	38.7	25.4	38.27
Citromyces group	63	10.2	20.2			10.0	38.49
" "	2467	16.0	16.7	24.0	148.6	14.4	38.30
" "	28	17.4	14.6	13.6	122.0		38.29

* Designation of cultures is the same as that used by Thom, Cultural Studies of Species of Penicillium, *U. S. Dept. of Agriculture, Bureau of Animal Industry, Bull. 118,* 1910.

were as given for the 5 per cent cane-sugar media. At various intervals the acidity of these cultures was determined by titration with phenolphthalein as indicator and the oxalate radical estimated by precipitation as calcium oxalate and titration with potassium permanganate. For comparison *Aspergillus niger*, the oxalic acid ferment *par excellence* was included. Numbers 4, 5, and 6 were gently rotated daily to increase diffusion throughout the media. It is difficult to accomplish this without submerging the mycelium, with resultant interference in continuous metabolism.

TABLE II.

Acidity per 100 cc. of Medium and Total Oxalate Radical, Expressed in $\frac{N}{10}$ cc.

	Per cent of cane-sugar.	5 days.		8 days.		12 days.		14 days.		21 days.		28 days.	
		Acidity.	Oxalate.	Acidity.	Oxalate.	Acidity.	Oxalate.	Acidity.	Oxalate.	Acidity.	Oxalate.	Acidity.	Oxalate.
1 *A. niger*	5	104.4	125.7	128.8	158.4	188.8	216.8	130.8	157.5	98.4	124.8	66.0	95.2
2 103.....	5	59.8	89.6	55.2	86.0	54.4	84.8	59.2	86.6	29.8	56.8	7.6	35.2
3 3563.8..	5	11.2	39.6	44.0	70.2	42.8	72.8	27.2	52.0	29.8	40.2	5.2	31.4
4 103.....	5	61.6	91.0	71.2	101.6	40.8	70.6	48.0	78.6				
5 103.....	10			67.5	117.1	97.5	151.4	79.0	132.8				
6 103....	20			72.0	119.0	106.5	179.3	124.0	195.8				

These results show that *P. oxalicum* though a less rapid producer of oxalic acid than *Aspergillus niger* is nevertheless a true oxalic acid ferment. It very quickly produces an acidity in media which under favorable conditions may reach 0.8 per cent free oxalic acid $(COOH.H_2O)_2$. The media from cultures of *P. oxalicum* are only slightly colored during the first eight to ten days and give a sharp end-point with phenolphthalein. Without the presence of an alkali carbonate to neutralize the acid formed, more than 1 per cent of oxalic acid accumulates as the free acid and as oxalates. Although the mold grows poorly and forms very few spores when grown upon media to which calcium carbonate is added, the yield of oxalic acid is greatly increased and may reach nearly 40 per cent of the weight of sugar employed. The results show clearly that oxalic acid is not an end-product but reaches a maximum point in eight to twelve days and then declines. If the media be agitated this maximum point is attained more quickly.

Penicillium oxalicum is very omniverous in habit. Its ability to produce oxalic acid from various sources of carbon was tested by growing it upon media made up exactly as the 5 per cent cane-sugar media except that the sugar was replaced by 5 per cent of the organic substance to be tested.

TABLE III.

Oxalic Acid Produced per 100 cc. on 5 per cent Medium, Expressed in $\frac{N}{10}$ cc.

Culture.	10 days at 20°.		15 days at 20°.	
	103.	3563.8.	103.	3563.8.
Glucose	105.6	78.0	87.8	41.6
Lactose	95.6	34.4	114.8	93.8
Potato starch	106.8	66.8	103.0	66.6
Glycerin	5.3	10.3	19.4	56.6
Peptone	89.3	58.8	117.6	80.4
Calcium lactate			15.2	8.6

Both cultures grew luxuriantly on all these media except glycerin and calcium lactate. The most striking cultural difference observed between these two cultures was their deportment on glycerin media. In all other cases 103 had showed itself more active than 3563.8, but on glycerin this was reversed. This was shown very plainly in the abundance of mycelium as well as in the actual production of oxalic acid. The ability of this organism to hydrolyze gelatinized starch is shown from the fact that it produced as much oxalic acid in ten days from starch as from a soluble sugar.

It has not been the purpose of the authors to indulge in any speculations concerning the mechanism of oxalic acid formation or its biological significance with regard to the metabolism of fungi. The chief interest of this paper lies in the fact that we have observed a *Penicillium* which forms oxalic acid as the chief product of its metabolism. This is an observation hitherto unrecorded in the literature of fungi.

THE HEXONE BASES OF MALIGNANT TUMORS.

By R. A. KOCHER.

(From the George Williams Hooper Foundation for Medical Research, University of California, San Francisco.)

(Received for publication, July 6, 1915.)

Interest in the hexone bases of malignant tumors has been stimulated by the recent work of Osborne and Mendel[1] who found a physiological relationship between certain members of this group and normal growth. In prolonged experiments with white rats these investigators showed that, if lysine was omitted from the diet of a growing animal, growth halted. If lysine was added to the diet, the animals began to grow again. On the other hand, in the case of fully grown animals lysine could be omitted from the diet without disturbing the nutritive balance. Thus they distinguished in the diet the factor of growth from that of maintenance. *Lysine is purely a growth-factor.* Experiments with arginine indicated that it had a similar function but as yet this has not been established experimentally.

No satisfactory explanation for the dependence of growth upon either of these amino-acids has been advanced. It seems obvious, however, that the body cannot synthesize these substances and must depend upon outside sources for them. Furthermore, there is a difference between a growing tissue and a fully developed one in its requirement for a given amino-acid, for the adult tissue contains some of this amino-acid; and the difference in requirement would seem to be quantitative.

It is also instructive that certain types of germinating tissue are rich in hexone bases. Spermatozoa are made up largely of a protamine or a histone, combined with nucleic acid. On analysis protamine yields large amounts of diamino-acids, particularly arginine which may constitute as much as 80 per cent of the

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[1] Osborne, T. B., and Mendel, L. B., *Jour. Biol. Chem.*, 1912, xii, 473; 1912–13, xiii, 233; 1914, xvii, 325.

protein molecule. In germinating seeds, also, arginine and lysine occur in abundance, and even free. These facts have led me to examine the quantitative relationship between these amino-acids in rapidly growing tissue, namely, human malignant tumors. Of pertinent interest is the work of Miss Slye[2] who showed in the case of a mouse tumor that heredity is an important factor. In other words the problem of cancer she believes is a problem of growth rather than of infection.

Chemical analyses of tumor tissue are recorded but they are more or less incomplete. Up to the present, strange to say, no one has made a quantitative determination of the hexone bases in tumors. This is especially remarkable since we have reliable quantitative methods for estimating these amino-acids.

Neuberg,[3] using the ester method of Emil Fischer in estimating certain mono-amino-acids, concluded that these were present in about the same proportion in cancer as in normal tissue. Bergell and Dörpinghaus[4] using the same method in the study of carcinoma and sarcoma considered that the proportions of alanine, glutamic acid, aspartic acid, and phenylalanine, were higher than in normal tissues; but the author of this method never claimed that it gave quantitative results.

Selecting a group of three amino-acids, *i.e.*, glycocoll, tyrosine, and glutamic acid, Abderhalden[5] concluded that the percentage of these in malignant tumors does not vary from normal. Bergell[6] using the N-distribution method of Hausmann, determined the total N of hexone bases in rat tumors, and concluded that this was much increased. However, this method is grossly inaccurate and conclusions cannot be drawn from such results.

EXPERIMENTAL DATA.

Material for this investigation was obtained through the courtesy of Drs. Rusk and Cooke of the Pathological Department

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[2] Slye, M., *Jour. Med. Research*, 1915, xxxii, 159.

[3] Neuberg, C., *Arb. a. d. Path. Inst. z. Berlin*, 1906, 593.

[4] Bergell, P., and Dörpinghaus, T., *Deutsch. med. Wchnschr.*, 1905, xxxi, 1426.

[5] Abderhalden, E., and Kautzsch, K., *Ztschr. f. physiol. Chem.*, 1910, lxvi, 69.

[6] Bergell, P., *Ztschr. f. Krebsforsch.*, 1907, vi, 204.

of the University of California. The specimens were obtained from four to twelve hours after death. Immediately the material was dissected free from all extraneous tissue, weighed, examined, and prepared for analysis. The mass was ground fine through a meat chopper and treated repeatedly with hot water to remove non-protein nitrogen. The water extract was concentrated *in vacuo* to a small volume and the protein in solution precipitated by the addition of several volumes of alcohol. This precipitate was added to the main bulk of protein which had been thrown down by heat. The united material was placed on the water bath, heated to dryness, ground to powder, and extracted repeatedly, in the cold, first with alcohol and later with ether. Then the dried material was weighed and found to represent from 16 to 18 per cent of the original weight. These values are shown in Table I. The dry powder was used for analysis.

Five cases were analyzed, as follows: (1) carcinoma of the larynx; (2) metastatic mass in the liver from a primary carcinoma of the stomach; (3) lymphosarcoma; (4) endothelioma; and (5) a liver metastasis from a primary carcinoma of the ileum. Notes bearing on the histories of the cases, as well as the gross anatomical and microscopic diagnoses, are given below.

TABLE I.

No.	Diagnosis.	Weight of fresh material.	Weight of dry substance.	Percentage of dry substance.
		gm.	*gm.*	
1	Primary carcinoma of larynx.........	126	21.5	17.0
2	Metastasis to liver from primary carcinoma of stomach	1350	230.5	17.4
3	Lymphosarcoma....	228	38.0	16.6
4	Endothelioma......	154	28.0	18.0
5	Liver metastasis from primary carcinoma of ileum...	408	67.0	16.4

Case 1.—Carcinoma of the larynx. Duration of symptoms, 9 months. Anatomical diagnosis: Recurrent carcinoma of the larynx with metastases in regional lymph nodes and invasion of the pharynx, palate, and esophagus. The growth in the neck on section was pale, showing areas of necrosis

and softening alternating with grey and cellular tissue. The regional lymph nodes showed replacement of the greater part of their substance by tumor tissue having a tendency to early central degeneration and softening.

Microscopic examination: Tumor is made up of branching strands of atypical cells evidently of epithelial origin. There are no typical "pearls." Mitotic figures and giant cells are common. Average sections consist of about three-fifths well preserved tumor cells and two-fifths loose edematous stroma containing various wandering cells.

Case 2.—Primary carcinoma of the stomach, with metastasis to the liver. Duration, 4 months. Anatomical diagnosis: Metastases were found in the liver, mesentery, omentum, diaphragm, and right lung. The liver, weight, 3950 gm., was almost all replaced by greyish white metastases which varied in size from a few mm. in diameter to 10 to 15 cm. The larger masses showed central broken down, semi-liquid material. The moderate sized ones showed slight necrosis. The smaller ones showed no gross evidence of degeneration.

Microscopic examination: The growth consisted of a typical carcinoma simplex with only a slight amount of necrosis in the smaller nodules. Mitotic figures are found but are not especially common. Tumor nodules consist of about two-thirds well preserved tumor cells and one-third stroma and other cells.

Case 3.—Sarcoma of the right kidney. Duration of symptoms, 4 months. Anatomical diagnosis: Metastases found in retro-peritoneal and mediastinal lymph nodes and liver. Lobar pneumonia, acute peritonitis. Right kidney, which was the seat of the tumor, showed in the upper half an extensive tumor growth. On the surface were central isolated and confluent opaque grey areas which are surrounded by grey and rather translucent tissue. The entire upper half of the kidney is infiltrated by a firm grey tumor which has involved the cortex more or less completely and much of the medulla.

On section, the tumor shows a pale grey translucent tissue with numerous larger and smaller opaque greyish yellow necroses. Retro-peritoneal lymph nodes are enlarged and matted together, from 1 to 4 cm. in diameter. On section pale grey areas of necrosis are seen. Lymph nodes along the aorta in the posterior mediastinum show similar metastases.

Microscopic examination: There are irregular areas of degeneration and necrosis around which are seen growing tumor cells. These cells are somewhat elongated and hexagonal, and have a considerable amount of pink-staining protoplasm. Cells appear singly or in small nests and are separated by fibrous stroma. They are invading the surrounding tissue. Occasional mitotic figures are found. The picture suggests a sarcoma rather than a hypernephroma. The analysis was made from gland metastases and such glands show about two-thirds of the section to be well preserved large plump cells. The other third consists of lymph strands and stroma.

Case 4.—Endothelioma of the kidney. Anatomical diagnosis: Specimen consists of kidney about four times the normal volume removed at operation. Superficially it is for the most part irregularly lobulated with low projections held beneath the tense capsule. The surface is pale and smooth except at one pole where there is a nodular mass covering an area about 3 x 5 cm. as if the tumor was projecting through the capsule.

These nodules vary from ochre yellow to brown with spots suggesting hemorrhage. From the pelvis a nodular cream-colored mass projects. On section of the mass the tumor tissue appears as a uniform soft white somewhat edematous growth. There are few vessels and connective tissue septa. The invaded kidney is in great part replaced by tumor tissue except a pale marginal zone of cortex in which it is almost impossible to make out the markings.

Microscopic examination: Shows the growth to be composed of large cells arranged in columns and circular masses with a delicate stroma for the most part, but also with broad tense connective tissue bands in places. In the stroma are thin-walled capillaries. The tumor cells show small rather deeply stained nuclei, a large vacuolated inner protoplasmic zone, and a thin line of cytoplasm at the periphery. Sections stained with Scharlach R show the presence of large amounts of fat. Tumor sections show approximately four-fifths well preserved tumor cells. The rest is made up of stroma and other cells.

Case 5.—Primary adeno-carcinoma of the ileum with metastasis to the liver. Duration of symptoms, 3 weeks. Anatomical diagnosis: Adenocarcinoma of the ileum with perforation. Metastatic growths in the liver along the bronchial, mesentery, and retro-peritoneal lymph nodes. Primary tumor was situated in the lower ileum. Its center had become necrotic and the bowel wall had perforated, leading to general peritonitis. The liver from which the material was taken weighed 3140 gm. and showed numerous pearl-grey metastatic nodules from 0.3 to 4 cm. in diameter. The larger nodules showed considerable central necrosis.

Microscopic examination: The picture is typical of a very rapidly growing undifferentiated cancer. The glandular arrangement is not in evidence in the metastatic nodules. Stroma is inconspicuous and the tumor cells make up about nine-tenths of the bulk of the nodules in the liver. The amount of hyaline necrosis in the tumor nodules cannot be estimated but the nuclear shadows can still be made out and there is little evidence of autolysis.

Determination of the Hexone Bases.

The method of Kossel and Kutscher[7] for the determination of the hexone bases has been modified by Osborne, Leavenworth,

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[7] Kossel, A., and Kutscher, F., *Ztschr. f. physiol. Chem.*, 1900–01, xxxi, 165. Kossel, A., and Pringle, H., *ibid.*, 1906, xlix, 318.

and Brautlecht,[8] also by Steudel,[9] and by Van Slyke.[10] In this work all these modifications were employed and the method followed was essentially that used by Van Slyke in his analysis of casein. The material was hydrolyzed with ten volumes of 20 per cent hydrochloric acid for a period of thirty-two to thirty-eight hours. The progress of the hydrolysis was followed until complete by determinations of the amino nitrogen with the Van Slyke apparatus. Lysine was isolated as the picrate, weighed, and its purity controlled by analysis of the amino nitrogen. Arginine and histidine were isolated as picrolonates according to the method of Steudel, and purity was controlled by melting point and analysis of the nitrogen. In all cases the values for the bases were calculated from the salts and not from the determination of the nitrogen in solution. The analytical data are given in Table II.

The results shown in the table indicate that in the malignant tumors studied the amount of hexone bases was fairly uniform. The arginine N expressed in per cent of the total nitrogen was between 11.63 and 13.65, an average of 12.42 per cent. The highest figures found, 12.8 and 13.65, were in Cases 2 and 5. These were liver metastases and consisted almost exclusively of carcinoma cells. In the other cases beside the tumor cells more or less connective tissue stroma was present. In Cases 3 and 4 approximately a third to a fifth of the tumor mass consisted of connective tissue.

The histidine N represents 3.88 to 5.58 per cent or an average of 4.86 per cent of the total N. The lysine N represents 9.89 to 12.65 per cent, an average of 11.23 per cent of the total N. It is noteworthy that if one of the members of this group is increased, the other two also are relatively more abundant. Perhaps these three substances are bound in some way as a unit in the protein molecule. The work of Wakeman[11] also favors this view.

The interpretation of the results of these analyses is facilitated by reference to similar analyses of normal tissues. Unfortu-

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[8] Osborne, T. B., Leavenworth, C. S., and Brautlecht, C. A., *Am. Jour. Physiol.*, 1908–09, xxiii, 183.

[9] Steudel, H., *Ztschr. f. physiol. Chem.*, 1902–03, xxxvii, 219; 1905, xliv, 157.

[10] Van Slyke, D. D., *Jour. Biol. Chem.*, 1913–14, xvi, 535.

[11] Wakeman, A. J., *ibid.*, 1908, iv, 119.

TABLE II.

No.	Diagnosis.	Weight of substance analyzed.	Length of hydrolysis.	Total N in hydrolyzed tissue.	Arginine picrolonate.	Histidine picrolonate.	Lysine picrate.	Nitrogen of the bases.				N of bases in percentage of total N.			
								Arginine.	Histidine.	Lysine.	Total base N.	Arginine.	Histidine.	Lysine.	Total base N.
		gm.	hrs.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	per cent	per cent	per cent	per cent
1	Carcinoma of larynx	20	34	2.1355	2.0195	0.8270	2.9352	0.2581	0.0829	0.2192	0.5602	12.06	3.88	10.25	26.20
2	Liver metastasis from primary carcinoma of stomach..	40	33	4.4906	4.4980	2.4743	7.6132	0.5750	0.2480	0.5682	1.3812	12.80	5.52	12.65	30.75
3	Lymphosarcoma .	36	38	4.1250	3.8562	1.9918	6.2654	0.4930	0.1996	0.4675	1.1601	11.95	4.84	11.32	28.14
4	Endothelioma.....	28	32	3.0652	2.7881	3.5562	4.0602	0.3565	0.1367	0.3030	0.7962	11.63	4.46	9.89	25.94
5	Liver metastasis from primary carcinoma of ileum....	40	35	5.595	5.9695	3.1190	9.0425	0.7642	0.3125	0.6752	1.7519	13.65	5.58	12.06	31.32
	Average											12.42	4.86	11.23	28.47

nately, however, few are at hand. A series of analyses of various organs including those of man was made by Wakeman who employed essentially the methods I have used. He found that the percentages of the hexone bases for a given tissue vary but slightly in different animals and not greatly in different organs of the same animal. For comparison with my results I reproduce in Table III the figures obtained by Wakeman.

In Table IV are given the percentages of the hexone bases in a few isolated proteins.

TABLE III.

Animal.	Organ.	Arginine N in percentage of total N.	Histidine N in percentage of total N.	Lysine N in percentage of total N.	Total base N in percentage.
*Adult man........	Normal liver	6.88	2.01	6.60	15.48
*Adult man........	Normal liver	5.61	1.76	5.54	12.91
*Dog..............	Normal liver	8.32	2.31	4.81	15.44
*Horse............	Normal liver	5.72	1.76	5.64	13.12
*Dog..............	Kidney	4.19	2.51	6.05	12.75
**Dog.............	Placenta at full term	4.3	0.30	3.5	8.1
**Ox..............	Muscle	7.5	1.8	7.6	16.9
Average for somatic tissue........		6.07	1.78	5.68	13.52
Average for tumor................		12.42	4.86	11.23	28.48

* Wakeman.

** Koelker and Slemons.

TABLE IV.*

Protein.	Arginine.	Histidine.	Lysine.	Total base N.
	per cent	*per cent*	*per cent*	*per cent*
** Egg albumin.......	10.2	2.7	4.3	17.2
** Gliadin (wheat)....	6.7	2.4	?	9.1
** Casein.............	8.0	4.0	8.6	20.6
** Lactalbumin (milk)	6.2	2.4	9.2	17.8

* These figures for nitrogen percentages are calculated from the amino-acid percentages by factors given by Van Slyke (*Ber. d. deutsch. chem. Gesellsch.*, 1911, xliv, 1690).

** Osborne and Mendel.

A comparison of the amounts of hexone bases in malignant tumors with the amounts in various normal adult tissues shows an increase in the former. Generally, the yields of arginine, lysine, and histidine obtained on hydrolysis of malignant tumors are over 100 per cent greater than those obtained on hydrolysis of normal somatic tissue. The quantities of these bases are also greater than those found in any isolated protein except the protamines and histones.

Attempts to isolate or to obtain qualitative tests for histone and protamine, using the fresh tumor tissues, were unsuccessful.

These results in conjunction with those of Osborne and Mendel may explain the action of lysine in promoting normal growth on the ground that this diamino-acid which cannot be synthesized in the body is an inherent requirement of the newly forming protein and is embodied in the structures of the latter molecule. If this is true for lysine, it seems probable that further experiments with arginine and histidine will show these bases to have a similar rôle.

It is pertinent that Sweet[12] and Rous[13] have recently demonstrated that when white rats having carcinoma are fed on a diet deficient in the hexone bases the tendency to metastasize is diminished. In some of their cases, however, the tumor grew as rapidly as ever, and in that event there was a correspondingly rapid emaciation of the body. This fact, it seems to me, may indicate that the somatic tissue was broken down to furnish these substances to the rapidly growing tumor cells.

Whether such high percentages of the hexone bases as we have found is a peculiarity of malignant tumors, or whether such an increase will be found in normal rapidly growing tissue is a problem now under investigation in this laboratory.

SUMMARY.

In five cases of malignant tumor the quantity of hexone bases was approximately double that found in normal tissue.

These results suggest a new approach to the study of the cancer problem, especially as it relates to metabolism.

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[12] Sweet, J. E., Corson-White, E. P., and Saxon, G. J., *ibid.*, 1913, xv, 181.

[13] Rous, P., *Bull. Johns Hopkins Hosp.*, 1915, xxvi, 146.

A COLORIMETRIC METHOD FOR THE DETERMINATION OF PHENOLS (AND PHENOL DERIVATIVES) IN URINE.

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(Received for publication, July 26, 1915.)

Since the appearance in 1882 of the pioneer researches of Baumann on the urinary phenols, a large number of investigations has been made on this subject. The method used for the quantitative determinations of these bodies has been almost invariably the iodometric titration variously modified and improved by Kossler and Penny,[1] by Neuberg,[2] Mooser,[3] and by Hensel.[4] At best the iodometric method has many disadvantages, being extremely time consuming and requiring the use of a comparatively large amount (500 cc.) of urine. Further, as will be shown below, if the directions given by the various investigators who have worked on the technique of this method be followed a considerable portion of the phenol contained in the urine may be lost.

The first step in the determination of phenols according to the methods of Kossler and Penny, Neuberg, Mooser, etc., consists in evaporating the faintly alkaline urine on a water bath to about one-fifth of its original volume. We have found that during this evaporation a considerable proportion of the phenol is oxidized, thus producing results much below the truth.

Experiments Showing the Loss of Phenol When Heated in Alkaline Solutions.

1. 25 cc. of phenol solution required 16.80 cc. $\frac{N}{10}$ iodine when titrated according to the iodometric method.

25 cc. of the above phenol solution were treated with 0.5 cc. of 10 per cent sodium carbonate and 100 cc. of water, after evaporation for one

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[1] Kossler, A., and Penny, E., *Ztschr. f. physiol. Chem.*, 1893, xvii, 139.

[2] Neuberg, C., *ibid.*, 1899, xxvii, 123.

[3] Mooser, W., *ibid.*, 1909, lxiii, 176.

[4] Hensel, M., *ibid.*, 1912, lxxviii, 373.

hour on the steam bath during which time it was reduced to a volume of 50 cc. It was found on titration to require only 16.00 cc. $\frac{N}{10}$ iodine.

2. 25 cc. of the same phenol solution when treated like the above except that one drop of 10 per cent sodium hydrate was used instead of sodium carbonate, required when titrated only 6.00 cc. $\frac{N}{10}$ iodine.

3. 25 cc. of a solution of paracresol required on titration 14.00 cc. of $\frac{N}{10}$ iodine.

25 cc. of the same solution when evaporated on the steam bath for one hour after the addition of one drop of 10 per cent NaOH and 100 cc. of water required 4.00 cc. of $\frac{N}{10}$ iodine.

Besides the uncertainty attending the preliminary evaporation in alkaline solution, the subsequent removal of the phenols from the urine by distillation with mineral acid, and the redistillation necessary to separate the phenols from acetone, formic acid, and other substances capable of reacting with iodine, make determination of phenols in urine a formidable task. By the use of the phosphotungstic phosphomolybdic reagent described by us[5] some three years ago we are now able to make a colorimetric phenol determination in urine in ten to fifteen minutes and with the use of not more than 10 or 15 cc. of ordinary urine.

Our method is as follows: 10 cc. of ordinary, or 20 of very dilute, urine are placed in a 50 cc. volumetric flask. To this is added an acid silver lactate solution[6] (from 2 to 20 cc.) until no more precipitate is obtained. A few drops of colloidal iron are then added, the flask is shaken, filled to the mark with distilled water, shaken again, and its contents are filtered. By means of this precipitation uric acid and traces of proteins, both of which also give a blue color with our reagent, are quantitatively removed. We have convinced ourselves by numerous experiments on a variety of phenols that these substances are not precipitated by the above procedure and may be quantitatively recovered in the filtrate. 25 cc. of the filtrate are transferred to a 50 cc. volumetric flask, and to it is added a sufficient quantity of saturated sodium chloride solution containing 10 cc. of strong hydrochloric acid per liter to precipitate all the silver. The flask is then filled to the mark and the contents are filtered.

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[5] Folin, O., and Denis, W., *Jour. Biol. Chem.*, 1912, xii, 239.

[6] This solution consists of a 3 per cent silver lactate solution in 3 per cent lactic acid.

To determine "free" (non-conjugated) phenols 20 cc. of this filtrate are placed in a 50 cc. flask and treated with 5 cc. of the phosphotungstic phosphomolybdic acid reagent[7] and 15 cc. of saturated sodium carbonate solution. After diluting to volume with lukewarm water (30–35°C.) and allowing to stand for twenty minutes the deep blue solution is read in a Duboscq colorimeter against a standard solution of phenol.

To determine total (free and conjugated) phenols 20 cc. of the same filtrate are transferred to a large test-tube; to this are added ten drops of concentrated hydrochloric acid, and the test-tube is covered with a small funnel. This mixture is heated rapidly to boiling over a free flame and is then placed in a boiling water bath (we usually use for this a tall beaker) for ten minutes. At the end of this time the tube is removed, cooled, and the contents are transferred to a 100 cc. volumetric flask. 10 cc. of the phosphotungstic phosphomolybdic reagent and 25 cc. of saturated sodium carbonate solution are now added, and after making up to volume and shaking, the solution is read (after twenty minutes) against a standard solution of phenol. As a standard we use a solution of pure phenol in $\frac{N}{100}$ HCl containing 1 mg. of the former substance in 10 cc. 5 cc. of this solution (equivalent to 0.5 mg. of phenol) when 10 cc. of saturated sodium carbonate solution are added and the whole is made up with water at about 30°C. to a volume of 100 cc., give, when set in the colorimeter at 20 mm., a convenient standard. As phenol is an exceedingly hygroscopic substance it is necessary to standardize the solution by means of the iodometric titration.

This titration is carried out as follows: Make a phenol solution in $\frac{N}{10}$ HCl containing 1 mg. of crystallized phenol per cc. Transfer 25 cc. of the phenol solution to a 250 cc. flask, add 50 cc. $\frac{N}{10}$ sodic hydrate, heat to 65°C., add 25 cc. $\frac{N}{10}$ iodine solution, stopper the flask, and let stand at room temperature thirty to forty minutes. Add 5 cc. of concentrated hydrochloric

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[7] This reagent is prepared by boiling together for two hours 100 gm. of sodium tungstate, 20 gm. of phosphomolybdic acid (or an equivalent amount of molybdic acid), 50 cc. of 85 per cent phosphoric acid, and 75 cc. of water. After the period of heating, cool, make up with water to a volume of 1 liter, and filter if necessary.

acid and titrate excess of iodine with $\frac{N}{10}$ sodium thiosulphate solution.[8] 1 cc. of $\frac{N}{10}$ iodine solution corresponds to 1.567 mg. of phenol. On the basis of the results dilute the phenol solution so that 10 cc. contain 1 mg. of phenol.

The above method as described is not directly applicable to the determination of phenols in blood, tissues, or feces. A modification suitable for work on this class of material will be published shortly.

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[8] Because of the red precipitate in the solution it is rather difficult to see the end-point of the titration. For those who have not had much experience it may be advisable to dilute the solution to a definite volume (after adding the hydrochloric acid), then to filter, and to titrate a portion of the filtrate as recommended by Sutton; with a little practise, however, the titration can be made without this procedure and we prefer not to use it.

THE EXCRETION OF FREE AND CONJUGATED PHENOLS AND PHENOL DERIVATIVES.

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The formation and significance of phenol and phenol derivatives in the animal metabolism was formerly a very important subject of biochemical investigation. The papers by Baumann in this field are still models of successful research as well as of simple and clear exposition.[1] At that time and in fact up till a short time ago carbolic acid was extensively used as an antiseptic by surgeons; and the dark colored "carbolic acid urines" were accordingly constantly available and constituted abundant evidence of the fact that externally applied phenol is absorbed. Similar urines were obtained when laboratory animals were poisoned by means of external applications of phenol. The absorbed phenol was at first supposed to be excreted with the urine either as alkaline salts (phenolates) or as free carbolic acid, but it was later shown in Hoppe-Seyler's laboratory that, although traces of phenolates or free phenol might at times occur, such traces were insignificant compared with the amounts of phenol present in the form of some combination which yielded free phenols only on hydrolysis with mineral acids.

Clinical or experimental "carbolic acid urines" were by no means the only urines which yielded a positive phenol reaction when distilled with mineral acids. In fact all urines gave more or less phenol though the urines of herbivorous animals were particularly conspicuous in this respect.

Baumann's work on the phenols was a development of his previous researches on the sulphates in urine. He had found that after the sul-

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[1] See particularly one of his papers, Baumann, E., ***Arch. f. Physiol.,*** 1876, xiii, 285.

phates in urine had been precipitated with barium chloride in the presence of acetic acid a second crop of barium sulphate was obtained on boiling the filtrates with hydrochloric acid. Baumann seems at once to have appreciated the possible significance of the fact that phenol and sulphuric acid were produced in urines under the same conditions although at that time (1875) the phenol sulphuric ester which he had in mind was a purely hypothetical product which had not yet been prepared. By analyses of different kinds of urine he soon found that quantitatively the sulphuric acid and the phenol liberated by treatment with mineral acids varied together and seemed to go hand in hand. The urines of horses and cattle which had previously been shown to be rich in phenols were also extraordinarily rich in organic or "conjugated" sulphates.

The indirect evidence thus obtained was soon followed by the isolation of the hypothetical phenol sulphuric acid compound. The product as it occurred in evaporated urine residues was soluble in dilute alcohol yet almost insoluble in absolute alcohol and on the basis of this fact Baumann by a series of extractions and fractional precipitations succeeded in obtaining from horse urine a potassium salt which could be split by hydrolysis into phenol and sulphuric acid and which by analysis was shown to be a salt of the stipulated phenol sulphuric ester $C_6H_5O_2SO_2OH$. Some time later Baumann prepared the product synthetically and thus positively proved its identity.[2] He isolated the same product from clinical and experimental "carbolic acid urines" and found that in such urines practically all the sulphates existed in the form of ethereal sulphates identical with or analogous to his phenol sulphuric ester.

In attempting to determine the location of the condensation process involved in the formation of phenol esters Baumann found that the conjugation took place with very great rapidity; within an hour after intravenous injections of phenols the free phenols disappeared from the system and were replaced by conjugated phenols. Since this rapid condensation occurred without the simultaneous treatment with sulphates it was *a priori* highly improbable that sulphates or the sulphuric acid formed in the metabolism could account for the rapid disappearance of the free phenols. In the liver, for example, Baumann found only extremely minute quantities of conjugated sulphates together with extraordinarily large amounts of conjugated phenols. He thus came on the track of another substance besides sulphuric acid which is capable of giving ester combinations with phenol. The other substance was of course glucoronic acid. With regard to this substance Baumann assumed, and the assumption was long regarded as correct, that normally it plays a secondary rôle; because normally the sulphuric acid and sulphates are present in excess, and because the phenols, as he believed, combine first of all with sulphuric acid, so that condensations with the glucoronic acid should occur only after the supply of sulphates had been exhausted. This view turned out to be

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[2] Baumann, *Ztschr. f. physiol. Chem.*, 1878–79, ii, 337.

incorrect, as was later shown by Neuberg, Salkowski, and others.[3] They were able to isolate glucoronic esters from urines containing an abundance of inorganic sulphates.

The origin of the phenol thus found in urine was at first more a matter of speculation than of actual knowledge. The fact that the urine of herbivorous animals contained so much more phenol than did the urines of dogs or of man suggested that the mother substances were derived from plant food and the presence in such materials of tannins pointed to these as the probable precursors. It was soon found, however, that in man the phenol excretion was not increased by vegetable food, but rather by food rich in proteins; and since all the common food proteins contain considerable tyrosine, this substance soon came to be looked upon as a precursor of at least a part of the phenol. Through the investigations of Brieger it was shown that phenol, as well as indol, is indeed formed in ordinary putrefaction of protein materials; and Brieger[4] found that the intestinal contents, and more specifically the contents of the large intestine, contained phenol. These observations, which were later verified and extended by Baumann and by Baumann and Brieger working together,[5] furnished satisfactory explanation as to the origin of the phenol found in the urine. The greater amounts of phenols found in the horse and the cow are probably not due to any specific precursors present in the food of these animals but to the fact that their intestinal tract is very long and their food comparatively indigestible, so that the phenol-forming bacteria exert a relatively greater destruction of the food material. Weyl,[6] one of Baumann's pupils, then succeeded in showing that putrefactive bacteria under conditions prevailing in the intestine, *i.e.*, in the absence of oxygen, produce large quantities of phenols from tyrosine. By feeding experiments with tyrosine it was found that the urinary phenols were increased (Brieger). By means of vigorous cathartics the phenols could be made to practically disappear from the urine, and, on the other hand, it was found that in cases of severe constipation or intestinal obstruction, whether clinical or experimental, the phenol excretion was much increased. In its broader outlines the history of the urinary phenol was thus cleared up in a relatively short time.

The whole subject was, however, by no means exhausted. Ordinary phenol represents but a small fraction of the total phenol products found in the urine.

The most important phenol quantitatively is in fact paracresol. In addition there is in urine some orthocresol and at least two dioxyl

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[3] Mayer, P., and Neuberg, C., *ibid.*, 1900, xxix, 256. Salkowski, E., *ibid.*, 1904, xlii, 224.

[4] Brieger, L., *ibid.*, 1878–79, ii, 241.

[5] Baumann and Brieger, *ibid.*, 1882–83, vii, 274. Baumann, *ibid.*, 1882–83, vii, 282.

[6] Weyl, T., *ibid.*, 1879, iii, 312.

benzenes, namely pyrocatechin and hydroquinone, as well as some aromatic oxy acids, particularly paraoxybenzoic acid.

The origin and occurrence of these products is, however, substantially the same as that of phenol. Weyl found more paracresol than phenol among the decomposition products of tyrosine. Baumann and Preusse[7] found that paracresol when fed to dogs is in part excreted as paracresol (ester) and in part is oxidized to paraoxybenzoic acid. Paraoxybenzoic acid, on the other hand, when fed to dogs or when acted on by putrefactive bacteria, lost carbonic acid and thus gave rise to phenol.[8] Phenol, paracresol, and paraoxybenzoic acid were thus definitely shown to be derived from tyrosine, a normal protein constituent.

$$\begin{array}{l}
\mathrm{OH\ C_6H_4\ \ CH_2\ CHNH_2\ COOH}, \text{ tyrosine} \\
\qquad\quad\ \downarrow \\
\mathrm{OH\ C_6H_4\ \ CH_2\ COOH}, \ p\text{-oxyphenyl acetic acid} \\
\qquad\quad\ \downarrow \\
\mathrm{OH\ C_6H_4\ \ CH_3}, \ p\text{-cresol} + \mathrm{CO_2} \\
\qquad\quad\ \downarrow \\
\mathrm{OH\ C_6\ H_4\ COOH}, \ p\text{-oxybenzoic acid} \\
\qquad\quad\ \downarrow \\
\mathrm{OH\ C_6\ H_5}, \text{ phenol} + \mathrm{CO_2}
\end{array}$$

Of the dioxybenzenes pyrocatechin is more abundant than is the hydroquinone; the existence of the latter in normal urine is in fact rather doubtful. After feeding phenol or benzene both diphenols have been obtained in quantities adequate for definite identification. Thus from feeding 100 gm. of benzene to a dog in the course of eight days Baumann obtained 0.56 gm. of pure hydroquinone.

The oxidation of benzene to phenol and to dioxybenzenes is from a biochemical standpoint very interesting as a definite illustration of the vigorous oxidations which can and do occur in the animal body. As a matter of fact the oxidation of benzene does not result wholly in such phenols. A large part, probably the larger part, of the benzene is oxidized to the straight chain compound muconic acid. This fact escaped Baumann altogether and was not discovered until a few years ago (1909) by Jaffé.

The oxidation of benzene to phenol and dioxybenzenes is in a sense different from the way the phenols originate from tyrosine in the intestinal tract. The latter is due to bacterial decomposition, the former to oxidations in the body tissues. Such oxidations doubtless play some rôle in the ordinary formations of phenol derivatives as for example in the formation of oxybenzoic acid from cresol or in the production of the dioxybenzenes from phenol.

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[7] Baumann, E., and Preusse, C., *ibid.*, 1879, iii, 156. Preusse, *ibid.*, 1881, v, 58.

[8] Baumann, E., and Herter, E., *ibid.*, 1877–78, i, 261.

The formation of phenol esters in the animal organism represents a most interesting illustration of the development of protective mechanisms by virtue of which poisonous or deleterious substances are converted into harmless compounds. Any such protective mechanism may or may not furnish completely adequate protection to the organism. In general biologists are inclined to assume that the protective processes are adequate, while clinicians are constantly considering established or possible deficiencies and generally believe in "auto-intoxications" as an important feature in the etiology of diseases. In respect of the phenols it has been tacitly assumed on the basis of Baumann's brilliant results that their condensation into harmless products is quantitative, and since Baumann's time this phase of the problem has received very little attention.

Our chief purpose in this paper is to show that the extent to which the phenols are conjugated into harmless compounds in the body must still be regarded as a distinctly open question.

We first encountered the phenols both in urine and in blood as the substances which interfere with the direct determination of uric acid by the colorimetric method. Since the conjugation of the phenols with sulphuric (or glucoronic) acid yields products in which the free hydroxyl group responsible for the color reaction with our phenol or uric acid reagent disappears, the results obtained certainly indicated that both in urine and in blood there are notable quantities of free phenol groups. Exactly how much of such materials is present, as well as their relation to the total phenol products, could not be determined until we had procured the necessary analytical technique. This technique as we have used it in the present investigation is described in the preceding paper.

As an interesting and possibly convincing proof that the phenols formed within the body are in fact eliminated as free phenols, we would cite our analytical figures (Table I) obtained from a leukemia patient who was treated with small quantities of benzene. From the very first day of the benzene treatment to the end of the ten day experiment there was an unmistakable increase in the elimination of the free as well as of the conjugated phenols. Indeed toward the end of the benzene treatment, as before this treatment was begun, the free phenols predominated. The diet was qualitatively the same throughout the experiment and consisted of bread and butter, milk, and eggs. The total nitrogen figures are cited to show that the diet is not responsible for the variations in the phenol excretion.

The reason why we attach considerable weight to the free phenol figures recorded in Table I lies in the fact that benzene is known to yield phenols and cannot yield aromatic oxy acids when oxidized within the body. This is important because in our present scheme of colorimetric phenol determination we determine the total and the total free phenol products without attempting to distinguish between the true phenols and the phenol acids. The latter seem to take comparatively small part in the conjugation processes and have long been known to occur chiefly in free form (or as salts) in the urine.

TABLE I.

*Free and Conjugated Phenols Produced from Benzene.**

Volume of urine.	Total N.	Phenols.			Amount of benzene given in 24 hrs.
		Total.	Free.	Conjugated.	
cc.	*gm.*	*gm.*	*per cent*	*per cent*	*gm.*
1120	11.8	0.30	56	44	0
1260	12.6	0.31	55	45	0
1300	13.0	0.53	31	69	1
1400	12.7	0.55	46	54	1
1500	10.9	0.75	42	58	1
1600	11.3	0.40	68	32	1
1420	10.9	0.78	60	40	1.66
1920	10.8	0.77	40	60	1.66
1325	9.2	0.83	55	45	1.66
1400	12.7	0.87	57	43	1.66

* The subject of this experiment, an early case of myelogenous leukemia, was a man 44 years old, weighing 56 kg.

Without reference to their pharmacological action the phenol acids, like the phenols proper, whether free or combined, are primarily products of intestinal putrefaction. As an index or measure of such putrefaction the phenol products determined by our scheme of analysis may fairly be considered at least as important as the indican determinations which heretofore have been used almost exclusively as measures of the intestinal putrefaction. The indican is derived from the tryptophane group of the decomposed protein—the phenols, as already referred to, come from the tyrosine group. Very little is known concerning the tryptophane contents of different protein materials, but there is no reason for believing that the tryptophane and the tyrosine content of proteins go hand in hand. Nor can we assume that the putrefactive bacteria attack these two amino-acids in any very definite proportion. As measures of intestinal putrefaction indican and the phenol products found in urine therefore do not necessarily vary together. That they do not do so was shown by Baumann. We have made a number of indican determinations (by the method of Myers and Fine) and we have reached the same conclusion (see tables).

TABLE II.

Phenol Excretion on High Protein Diets.

No.	Volume of urine.	Total N.	Phenols.				Indican.	Diagnosis and remarks.
			Free.	Total.	Free.	Conjugated.		
	cc.	*gm.*	*gm.*	*gm.*	*per cent*	*per cent*		
161	2500	31.2	0.33	0.43	77	23	0	Typhoid, 6th week.
93	1100	22.	0.30	0.36	83	17		Chorea, convalescent.
83	1560	20.	0.23	0.45	51	49	Trace	Pleurisy, convalescent.
84	1540	19.8	0.23	0.38	61	39	0	Pleurisy, convalescent.
Vt	1940	19.4	0.35	0.56	62	38		Pernicious anemia.
177	2440	19.5	0.24	0.39	62	38		Pneumonia, temperature 103°.
96	1140	18.2	0.35	0.64	55	45		Chronic infectious arthritis.
209	2000	16.8	0.28	0.40	70	30		Pneumonia, just after crisis.
204	1060	16.6	0.17	0.35	48	52		Pneumonia, temperature 103°.
181–3	1690	16.2	0.28	0.49	57	43		Pneumonia, convalescent.
172	1820	16.0	0.25	0.42	59	41		Pernicious anemia.
37	1100	16.0	0.25	0.39	61	39	Trace	Syphilis, gumma of liver.
174	1120	15.7	0.22	0.29	75	25		Pneumonia, temperature 104°.
19	2400	15.3	0.42	0.66	63	37	Trace	Arthritis, endocarditis.

Since our method of determining the phenols in urine does not involve a preliminary (necessarily imperfect) separation by extraction or distillation as has been the case in all the methods hitherto available it was to be expected that the figures obtained by our procedure would be higher than the phenol figures recorded in the literature. In point of facts our phenol figures are so different that it is hardly worth while to institute any comparisons. Moreover, since we in the course of the past year have made probably a larger number of phenol determinations (several hundred) than all the determinations previously recorded in the literature, we feel justified in simply classifying and recording a representative proportion of our own results.

TABLE III.

Phenol Excretion on Low Protein Diets.

No.	Volume of urine.	Total N.	Phenols. Total.	Phenols. Free.	Phenols. Conjugated.	Indican.	Diagnosis and remarks.
	cc.	*gm.*	*gm.*	*per cent*	*per cent*	*gm.*	
78	1160	4.0	0.34	87	13	0	Pleurisy, convalescent.
7	200	4.4	0.14	69	31	Trace	Carcinoma of stomach.
91	900	4.5	0.16	70	30	0	Chorea.
17	600	5.0	0.36	61	39	0.14	Gastric ulcer.
2	1200	5.1	0.20	72	28	0.16	Gastric ulcer.
163	460	5.1	0.16	71	29		Chronic infectious arthritis.
77	1290	5.7	0.34	87	13		Pleurisy, convalescent.
179	850	5.9	0.29	62	38		Cirrhosis of liver.
181–2	1320	6.2	0.25	64	36		Pneumonia, convalescent.
190	1160	6.6	0.14	63	37	0	Pneumonia, convalescent.
85	1900	6.7	0.26	66	34	0	Pleurisy, convalescent.
164	580	6.9	0.31	58	42		Chronic infectious arthritis.
4	800	7.1	0.22	61	39	0.1	Renal calculus.
21	1000	7.4	0.23	70	30	0	Pleurisy.
197	1100	7.5	0.24	63	37	0	Arthritis.
40	520	7.6	0.21	74	26	0.29	Cardiorenal disease.

Tables II and III contain results obtained on high and low protein diets respectively. The subjects investigated were all patients in the Massachusetts General Hospital. No attempt has been made to classify the subjects according to the nature of the diseases for which they were treated at the Hospital. Our (unrecorded) results cover very nearly every kind of clinical material occurring with any degree of frequency in a general hospital, but no evidence was obtained tending to show the existence of anything approaching a specifically abnormal production of phenols in connection with any one disease.

Our results obtained from adult hospital patients including the figures recorded in Tables II and III are somewhat vitiated by one rather serious defect. Because of the vigilance with which the bowels are kept open by means of enemata and cathartics in hospital patients the formation and excretion of phenols is doubtless reduced to an incalculable extent. The recorded figures do show nevertheless that the phenol excretion tends on the whole to be much higher on protein-rich diets and to fall on diets containing small amounts of protein. The differences would doubtless have been more striking except for the condition referred to above.

Concrete examples of the effect of the protein intake on the level of the phenol excretion are shown in the figures cited below.

Effect of Protein Feeding on the Phenol Excretion.

Total N.	Total Phenols.	
gm.	*gm.*	
7.6	0.32	Mr. M. convalescing from pneumonia.
7.2	0.25	
16.0	0.48	
15.0	0.49	
7.2	0.20	Mr. D. convalescing from pleurisy.
20.0	0.45	
4.5	0.16	Miss C. convalescing from chorea.
22.0	0.35	

0.3 to 0.5 gram of "total phenols" in a twenty-four hour quantity of human urine seems large in comparison with the phenol figures heretofore recorded in the literature. That such figures are not indicative of an abnormal phenol formation is shown however, by the figures recorded below which were obtained from perfectly normal healthy subjects.

Phenol Excretion in Normal Persons.

	Total N.	Phenols.	
		Free.	Total.
	gm.	*gm.*	*gm.*
Normal men			
1	11.2	0.29	0.48
2	12.0	0.27	0.39
3	14.0		0.38
Normal women			
1	9.6	0.22	0.33
2	11.1	0.17	0.29
Normal boy 11 years old	7.5	0.17	0.24
Normal boy 7 years old	5.4	0.116	0.18
Normal infant 3 months old	1.3	0.03	0.05

TABLE IV.

Phenol Excretion in Hospital Children and Infants.

No.	Volume of urine.	Total N.	Phenols.				Diagnosis and remarks.
			Free.	Total.	Free.	Conjugated.	
	cc.	*gm.*	*gm.*	*gm.*	*per cent*	*per cent*	
61	860	1.3	0.042	0.043	98	2	Malnutrition, 10 months old.
63	740	1.8	0.04	0.04	100	0	Malnutrition, 4 months old.
64	550	1.6	0.068	0.068	100	0	Malnutrition, 2 months old.
65	610	1.7	0.031	0.035	89	11	Malnutrition, 2 months old.
69	1150	1.5	0.050	0.052	96	4	Infectious diarrhea, 4 years old.
71	755	1.5	0.040	0.042	95	5	Malnutrition, 6 months old.
50	890	4.3	0.08	0.09	89	11	Still's disease, 10 years old.
60	600	1.3	0.03	0.05	70	30	Normal infant, 3 months old.
66	620	2.9	0.05	0.06	77	23	Rachitis, very early stage, 10 months old.
54	850	5.4	0.15	0.25	60	40	Normal boy, 11 years old.
90	1140	6.6	0.15	0.28	52	48	Chorea, 15 years old.

From infants and children we have obtained phenol figures which are remarkable when considered from the prevailing concept of the protective mechanism by means of which the phenols are supposed to be "detoxicated" by transformation into conjugated compounds. From the figures given in Table IV it will be seen that while some children show substantially the same ratio between the free and the conjugated phenols as we have found in adults, others, for one reason or another, reveal little or absolutely no conjugation, the whole of the phenols being excreted in free form (or as salts).

TABLE V.

Phenol Excretion in the Cat, Rabbit, Pig, Dog, Guinea Pig, and Goat.

Animal.	24 hr. urine.	Total N.	Phenols.				Food.
			Free.	Total.	Free.	Conjugated.	
	cc.	*gm.*	*gm.*	*gm.*	*per cent*	*per cent*	
Cat 1.........	150	1.9	0.012	0.031	39	61	Rice and cream.
“	80	3.5	0.017	0.042	40	60	Lean beef.
“	122	5.6	0.021	0.058	36	64	“ “
Cat 2.........	130	1.0	0.013	0.022	59	39	Rice and cream.
“	150	1.1	0.014	0.026	54	46	“ “ “
“	95	4.5	0.023	0.037	62	38	Lean beef.
Rabbit 1......	350	3.4	0.055	0.075	73	27	Oats and spinach.
“	150	4.3	0.055	0.062	89	11	“ “ “
“	440	5.6	0.057	0.077	74	26	“ “ “
“ 2......	330	3.9	0.065	0.100	65	35	“ “ “
Pig 1.........	3490	2.3	0.10	0.24	42	58	Starch.
“	2850	16.8	0.48	0.63	76	24	Wheat bran.
“ 2.........	1700	2.2	0.10	0.17	60	40	Starch.
“	2080	11.6	0.46	0.58	79	21	Wheat bran.
Dog 1.........	195	1.9	0.05	0.06	83	17	Bread and milk.
Guinea pig 1..	20	0.37	0.006	0.011	58	42	Oats.
“ ..	20	0.45	0.008	0.012	65	35	“
“ 2..	20	0.45	0.008	0.013	61	39	“
“ ..	13	0.33	0.008	0.012	66	34	“
Goat 1........	250	8.25	0.346	0.775	44	56	“

The distribution of the phenols between the free and the conjugated is virtually the same in the urine of other animals as in man, the free phenols representing from 30 to 90 per cent of the total. That this is so is shown by the phenol figures obtained from cats, rabbits, guinea pigs, pigs, and a goat—reported in Table V.

The variation in the total phenol excretion with variations in the level of the protein metabolism is most strikingly shown in the figures obtained from the pigs, though the same phenomenon is illustrated by the results obtained from the cats.

SUMMARY.

The excretion of total phenol products in the urine appears to be much greater than is indicated by the phenol figures heretofore recorded in the literature.

The phenols are by no means quantitatively converted into conjugated phenols. The detoxication process involved in such conjugations appears therefore to furnish only a partial protection against the toxic or deleterious effect of the phenol products formed by putrefaction in the intestinal canal.

In any given individual man or animal, the total phenol excretion tends to vary directly but not proportionally with the protein intake.

SOME OBSERVATIONS ON THE SELECTIVE ACTIVITY OF THE HUMAN KIDNEY.

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The kidney is manifestly an organ possessed of selective power in the sense that it excretes waste products and does not excrete (except in minute traces) materials which have food value. In the light of our present knowledge it seems probable that the kidney selection of the waste products may be subdivided into several more or less independent and more or less specific selections of specific waste products. Such independent specificity on the part of the kidney is certainly suggested by the specific functional deteriorations which are known to occur, as for example, the "salt retention" and the "nitrogen retention" types of nephritis and the uric acid retention characteristic of gout.

The occurrence of such retentions has been shown chiefly by means of blood analysis, for so far ordinary urine analysis has failed to throw much light on these more detailed features of kidney activity. It is possible, however, that if the urine passed by each kidney could be collected separately and quantitatively urine analysis might afford valuable additional information concerning the effect of disease on the specific selective function of each kidney in relation to the excretion of the different waste products. The chief practical obstacle in the way of such investigations is the difficulty of collecting separately, quantitatively, and simultaneously the urine passed by each kidney for reasonably long periods.

In the present paper we wish to record some analyses of "single kidney" urines. These urines fulfill the requirement of representing complete and simultaneous collections from each kidney, but unfortunately do not represent the best kind of clinical material from which we could hope to find evidence of specific deteriorations of the kidney functions. Our patients were obtained from the Genito-Urinary Department of the Massachusetts General Hospital. They had been operated on for malignant growths in the bladder, and were serviceable for our studies chiefly because the double ureterostomy performed by the surgeons rendered possible the separate collection of the urine from each kidney.

The analytical figures obtained would seem to merit publication: first, because practically no similar or analogous data have yet been recorded; and, secondly, because the figures incidentally serve to illustrate the effect of moderate clinical hydronephrosis and pyelitis on the excretory activity of the kidney. In each of the four patients studied one or both kidneys had been infected and had in addition been somewhat damaged (according to the surgeons' reports) by the pressure of the bladder tumor on the ureteral opening. Since the hydronephrosis resulting from such back pressure is believed by pathologists to injure the convoluted tubules rather than the glomeruli one might expect (on the basis of current physiological theories of kidney excretion) that the damage to the kidney, if sufficient to demonstrably reduce its function as a whole, should be far more striking in relation either to the "salt" or to the nitrogen (urea, creatinine, and uric acid) excretion. A more highly specific deterioration, as for example, with reference to the excretion of urea or uric acid alone is scarcely to be expected from such cases.[1]

Case I.—J. B., male, a Russian Jew, 53 years old, weight 140 lbs. Operated on Oct. 24, 1914, by Dr. O'Neil. At this time a double ureterostomy was done, the ureters being brought out through the lumbar muscles. As soon as the wound had healed it was possible to make quantitative collections of urine from each kidney by means of two cups, provided with outlet tubes, which were held tightly against the body by means of a rubber belt. In this patient prior to operation there had existed marked obstruction to the outflow of urine from the right ureter with resultant

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[1] The analytical methods used were:

Nitrogen, Folin-Farmer method (*Jour. Biol. Chem.*, 1911–12, xi, 493).
Creatinine, Folin's micro method (*ibid.*, 1914, xvii, 469).
Uric acid, Folin-Denis method (*ibid.*, 1913, xiv, 95).
Sodium chloride, Volhard method.
Phosphates, by the uranium acetate titration.

hydronephrosis of the right kidney. In this kidney a fairly active pyelonephritis had also existed, but at the time our first series of observations was made the infection seemed to have subsided. The left kidney was believed by the surgeon to be in normal condition. Between the end of Period 1 and the beginning of Period 2, an interval of one month, the patient suffered from an abscess which formed near the point of exit of the right ureter. The right kidney soon became badly infected. The effect of this infection is shown in the diminished relative power for the excretion of nitrogenous bodies shown by the right kidney during Period 2.

Besides the observations made of the excretion of the ordinary urinary constituents (recorded in the first table), we have also made at the end of the first period a study of the excretion of phenolsulphonephthalein, of levulose, and of hippuric acid.

The sulphonephthalein excretion when 6 mg. of the dye were injected subcutaneously amounted in two hours to 31 per cent for the right kidney and to 48 per cent for the left.

For the levulose test 150 gm. of the sugar were given before breakfast (7.30 a.m.). Urine was collected from each kidney at fifteen minute intervals; from the left kidney excretion began in about seventy-five minutes (9.45 a.m.); from the right in four hours (11.30). When 2 gm. of sodium benzoate were given at 7.30 a.m. there was excreted in the urine passed by the right kidney between 10.45 a.m. and 4.15 p.m. 0.26 gm. of hippuric acid, and in that from the left kidney 0.36 gm.; no free benzoic acid could be found in either specimen of urine.

Case II.—A. B., male, 60 years old. Diagnosis, carcinoma of bladder. On March 1 a double ureterostomy was done by Dr. G. G. Smith. The day before operation the phenolsulphonephthalein test gave the following results:

Left ureter, appearance time fourteen minutes. Amount excreted in one hour 35 per cent.

Right ureter, appearance time forty minutes. Amount excreted in one hour 15 per cent.

On account of lack of time we were unfortunately unable to repeat the phthalein test at the time our specimens of urine were collected. As in Case I the bladder tumor had caused a considerable obstruction to the outflow of urine from the right kidney with a resultant moderate hydronephrosis; a pyelitis of moderate severity also existed on the right side.

Case III.—M. C., male, 55 years old. Diagnosis, carcinoma at base of bladder in region of the right ureter, bilateral pyelonephrosis. On March 10 a bilateral lumbar ureterostomy was done by Dr. Hugh Cabot. Six days later it was possible to make quantitative collections of urine from each kidney.

Case IV.—E. V., female, 60 years old. Diagnosis, papilloma on left lateral bladder wall about the site of the left ureteral orifice. During the operation it was necessary to cut the left ureter during the excision of the tumor. Our collections were made on the fourth day after operation when the patient was still wearing a catheter in the left ureter and a second catheter in the urethra.

CASE I.

Urine from Left and Right Kidney (Hydronephrosis of the Right Kidney).

Date.	Time.	Volume of urine.		Nitrogen.		Creatinine.		Uric acid.		Sodium chloride.		Phosphate P_2O_5.	
Period 1.		Left.	Right.	Left.	Right.	Left.	Right.	Left.	Right.	Left.	Right.	Left.	Right.
Nov.		cc.	cc.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.
14	9.30–11.30	172	86	561	430	58	30	30	46	481	540	51	44
"	11.30– 1.30	135	57	380	333	55	38			406	361	85	74
"	1.30– 3.30	99	30	430	190	64	23			222	320	77	40
"	6– 9	142	125	580	350	69	52	39	39	730	840		
Period 2.													
Dec.													
16	10.50– 1.40	205	94	1022	360	130	30	67	21	300	220	200	10
"	1.40– 5	238	150	1265	520					420	400	100	21
17	10.15– 5.15	460	195	2160	500					1010	610	210	90

CASE II.

Urine from Left and Right Kidney (Hydronephrosis of the Right Kidney).

Date.	Time.	Volume of urine.		Nitrogen.		Creatinine.		Uric acid.		Sodium chloride.	
		Left.	Right.	Left.	Right.	Left.	Right.	Left.	Right.	Left.	Right.
March		cc.	cc.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.
11	9.20–11.20	77	45	691	90	70	8	50	12	200	150
"	11.20– 1.20	58	43	610	80	64	10	51	8	140	150
"	1.20– 3.20	57	40	612	80	66	10	50	8	133	150

CASE III.

Urine from Left and Right Kidney (Hydronephrosis of the Right Kidney).

Date.	Time.	Volume of urine.		Nitrogen.		Creatinine.		Uric acid.		Sodium chloride.		Phosphate P_2O_5.	
		Left.	Right.*	Left.	Right.	Left.	Right.	Left.	Right.	Left.	Right.	Left.	Right.
March		*cc.*	*cc.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*
16	8.50–10.50	60	0	540	0	70	0	19	0	200	0		
''	10.50–12.50	92	0	610	0	70	0	17	0	231	0		
''	12.50– 2.50	150	0	1482	0	140	0	50	0	300	0		
17	9–11	106	0	780	0	100	0	40	0	192	0		
''	11– 1	160	0	962	0	120	0	80	0	321	0		
''	1.30– 3.30	142	51	1131	200	121	53	60	8	252	200		
18	9–11	220	56	1210	280	111	55	70	14	500	641	57	30
''	11– 1	170	53	1022	260	100	52	68	42			75	19
''	1– 3	197	36	1380	191	120	54	98	21				

* During March 16 and the morning of March 17 the outflow of urine from the right kidney was completely cut off by a blood clot in the ureter caused by the introduction of a catheter.

CASE IV.

Urine from Left and Right Kidney (Hydronephrosis of the Left Kidney).

Date	Time.	Volume of urine.		Nitrogen.		Creatinine.		Uric acid.		Sodium chloride.		Phosphate P_2O_5.	
		Left.	Right.	Left.	Right.	Left.	Right.	Left.	Right.	Left.	Right.	Left.	Right.
March		*cc.*	*cc.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*
10	8.10–10.10	78	112	420	740	39	60	43	56	582	832	68	119
''	10.10–12.10	88	132	400	750	32	55	29	54	600	950	62	140
''	12.10– 2.10	70	136	361	811	32	63	28	62	540	1051	45	93
''	2.10– 4.10	86	144	450	862	34	62	30	57	322	1140	39	66
''	4.10– 6.10	100	130	501	800	34	61	33	45	580	1890	56	169
''	6.10– 8.10	112	210	480	1382	36	55	31	42	490	842	39	118

Below is given in tabular form the averages of our main analytical results obtained from Cases I, II, and IV. From these it would appear that the damage produced by moderate hydronephrosis complicated by pyelitis is not particularly uniform in character so far as the excretory function of the kidney is concerned. Since the chloride excretion has suffered practically no damage in the first two cases and has diminished no more than the nitrogen excretion in the fourth case it appears that the power of the kidney to excrete nitrogenous bodies is more easily or more frequently damaged than is the salt excretion by moderate hydronephrosis and pyelitis. Slight indications of more detailed selective injury are also present. The phosphate excretion, for example, does not correspond particularly closely to the chloride excretion. On account of the limited number of cases and figures we do not think, however, that any broad generalization is warranted. The excretion of creatinine and that of uric acid also do not vary together, the creatinine excretion resembling more nearly the level of the total nitrogen excretion, but here again it is quite possible that a larger number of results might lead to a different conclusion.

Average Ratio between the Excretion of the Normal and That of the Injured Kidney (the Excretion from the Normal Kidney Taken as 100).

Case.	Water.	Nitrogen.	Creatinine.	Uric acid.	Sodium chloride.	Phosphate.
I Period 1	66	66	60	125	111	77
Period 2	50	31			71	24
II	66	13	14	19	95	
IV	63	50	60	63	48	44

It is clear, however, that the figures obtained rather agree with the view that the excretion of urine by the kidney can be subdivided into an unknown number of different more or less independent excretions.

For the above cases we are indebted to Dr. Hugh Cabot who has kindly allowed us to make free use of this interesting material. We have also to thank Dr. E. G. Crabtree for his assistance in the management of these patients during the periods of observation.

A QUALITATIVE (REDUCTION) TEST FOR SUGAR IN NORMAL HUMAN URINE.

By OTTO FOLIN.

(From the Biochemical Laboratory of the Harvard Medical School, Boston.)

(Received for publication, July 26, 1915.)

The sensitiveness of the various alkaline copper solutions employed in testing for sugar depends probably most of all upon how little cuprous oxide they are capable of holding in solution. The merit of S. R. Benedict's qualitative test for sugar is due in large part to its small solvent effects on the cuprous oxide, although it possesses the additional advantage of being but slightly reduced by creatinine. The creatinine disturbs not only by its reducing power, but also by its property of holding relatively considerable traces of cuprous oxide in solution.

The fact that all human urines probably contain varying amounts of sugar, and that therefore no sharp or definite line of demarcation exists between normal and pathological urines with reference to their sugar contents, is a fact which is not adequately shown or recognized by the current qualitative "tests for sugar" in urine. The test described in this paper is proposed as a simple and instructive method for showing the presence of such normal traces of sugar.

The test depends, first, on the use of an uncommonly sensitive alkaline copper solution, and, secondly, on the fact that in the presence of copper alkaline picrate solutions are not reduced by sugar. The importance of the last named consideration lies in the fact that it permits the removal of the creatinine and other substances with picric acid before applying the reduction test.

The reagent is made up in two solutions:

A. 5 grams of crystallized copper sulphate are dissolved in 100 cc. of hot water and to the cooled solution are added 60–70 cc. of pure glycerin.

B. 125 grams of anhydrous potassium carbonate are dissolved in 400 cc. of water.

One part of the glycerin-copper solution (A) is mixed with two parts of potassium carbonate solution (B). Only small portions should be mixed at a time as the reagent (after mixing) does not keep but undergoes gradual reduction.

The test is made as follows: To about 10 cc. of urine in a test-tube or small flask add about 2 grams of picric acid and about 2 grams of good quality bone-black (Kahlbaum's or Merck's blood charcoal), shake for five minutes, and filter.

Concentrated urines, which give the most trouble in testing for sugar, contain from 3 to 5 mg. creatinine per cc. By the above procedure the creatinine content is reduced to practically nothing—at the most a few hundredths mg. per cc. being left in the filtrate. Bone-black has very strong adsorbing properties for the picrates of creatinine. By allowing the urine and picric acid to stand for a longer time (half an hour or over night) the addition of bone-black may be omitted if desired. The filtrate in that case will contain about 0.1 mg. per cc., a quantity too small to interfere with the test for sugar.

Add 1 or 2 cc. of the creatinine-free filtrate to about 10 cc. of the freshly mixed sugar reagent in a large test-tube (together with a pebble or two to prevent bumping) and boil *with constant shaking*[1] for one and one-half minutes. If the sugar present is considerable (above the normal variations), a typical reduction is obtained. If the trace of sugar is smaller, but still rather large, the whole solution will become turbid as in Benedict's test. If no such turbidity is produced and the boiling mixture remains clear, transfer it at once (*i.e.*, while still very hot) to a centrifuge tube and centrifuge for one to two minutes. Typical red cuprous oxide such as is obtained with pure sugar solutions will be found in the bottom of the centrifuge tube below the green crystalline potassium picrate which usually forms as the liquid cools.

Some copper reagents made as described above give a slight cuprous oxide sediment when boiled alone, *i.e.*, without any added sugar or urine. When that is the case the reagent must be boiled and centrifuged once before using it for the test.

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[1] The shaking is desirable to avoid bumping and is necessary to prevent superheating and consequent reduction of the reagent on the sides of the test-tube.

In the course of nearly one hundred tests made on the urines of as many different normal persons (students) a positive reduction was obtained in every case. In many instances the amount of cuprous oxide found in the bottom of the centrifuge tube was very large indeed. The amount of sugar present in normal human urine is therefore probably much greater than is indicated by the negative findings recorded on the basis of the clinical qualitative tests for sugar in current use.

ON *d*-LYXOHEXOSAMINIC ACID AND ON α-α_1-ANHYDROMUCIC ACID.

BY P. A. LEVENE AND F. B. LA FORGE.

(*From the Laboratories of the Rockefeller Institute for Medical Research.*)

(Received for publication, July 9, 1915.)

Theoretically there are possible as many α-amino hexoses as there are hexoses. In order to simplify the present argument the enantiomorphous isomerism of the entire molecule will not be taken into consideration. The hexoses will be discussed as if they belonged to one series, either *d* or *l*. Under such conditions the number of hexoses and of α-hexosamines is eight. For each hexosamine there is possible a corresponding α-hexosaminic acid, while the number of the possible α-α_1-anhydro-tetrahydroxyadipic acids is only six.

Of the eight possible α-hexosamines two are known. Of these one (glucosamine) was obtained from natural sources[1] and synthetically,[2] and the other (chondrosamine) was discovered recently by the present writers.[3] Of the eight possible hexosaminic acids four were known prior to the present work: glucosaminic acid (obtained from glucosamine and synthetically from arabinose),[4] chondrosaminic acid (obtained by us from chondrosamine), xylohexosaminic, and ribohexosaminic acids (obtained by us synthetically).[5]

There is a smaller gap in the series of possible α-α_1-anhydrotetrahydroxyadipic acids, since, prior to this publication, only one was missing of the six possible. The five known acids were: the

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[1] Ledderhose, G., *Ztschr. f. physiol. Chem.*, 1878–79, ii, 213; 1880, iv, 139.

[2] Fischer, E., and Leuchs, H., *Ber. d. deutsch. chem. Gesellsch.*, 1903, xxxvi, 24.

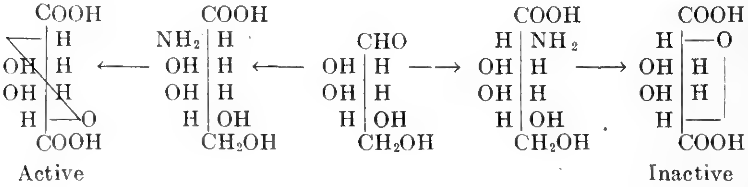
[3] Levene, P. A., and La Forge, F. B., *Jour. Biol. Chem.*, 1914, xviii, 123.

[4] Fischer, E., and Tiemann, F., *Ber. d. deutsch. chem. Gesellsch.*, 1894, xxvii, 138. Fischer and Leuchs, *ibid.*, 1902, xxxv, 3787.

[5] Levene and La Forge, *Jour. Biol. Chem.*, 1915, xx, 433; 1915, xxi, 351.

so called "isosaccharic acid," which was proven by the present writers to be α-α₁-anhydro-mannosaccharic; further, α-α₁-anhydro-saccharic, α-α₁-anhydro-idosaccharic, α-α₁-anhydro-allomucic, α-α₁-anhydro-talomucic acids.[5]

The present work deals with the preparation of one more hexosaminic acid, namely, *d*-lyxohexosaminic acid, and of α-α₁-anhydro-mucic acid. The amino-acid was obtained by the action of hydrocyanic acid on lyxosimine. The anhydro-tetrahydroxyadipic acid was obtained by deamination of the amino-acid and subsequent oxidation with nitric acid. The structure of the acid was established by the fact that it was found to be optically inactive. Of the two epimeric anhydro-tetrahydroxyadipic acids derived from *d*-lyxohexosaminic acid, one only, namely, the one corresponding to mucic, is optically inactive; the other is identical with anhydro-talomucic acid and has been obtained by us from chondrosamine.



Thus the series of anhydro-tetrahydroxyadipic acids is completed.

Referring to the preparation of the α-α₁-anhydro-mucic acid it must be emphasized that the amino-acid needs to be absolutely pure in order that it may yield a satisfactory quantity of the anhydro-mucic acid.

Reference may also be made to the mode of preparation of lyxose. Starting out from milk sugar it was found convenient to isolate first the galactose by an unpublished process originated in the Bureau of Chemistry, Washington, D. C. The galactonic acid was isolated directly as the calcium salt, which in turn was readily converted into the pentose by Ruff's method.[6]

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[6] Ruff, O., and Ollendorff, G., *Ber. d. deutsch. chem. Gesellsch.*, **1900**, xxxiii, 1798.

EXPERIMENTAL.

Preparation of Acid Calcium d-Galactonate.

1600 grams *d*-galactose were dissolved in four parts of water, 2000 grams bromine added and allowed to stand three days, with occasional shaking at room temperature. The solution was then concentrated in vacuum to about two-thirds of its volume, to remove the excess of bromine. The hydrobromic acid was removed with lead carbonate, the filtrate freed from lead with hydrogen sulphide and concentrated to about 3 liters in vacuum. It was then boiled with an excess of calcium carbonate for about three-quarters of an hour and filtered hot on a Buchner funnel. On cooling, the calcium salt crystallized out in an almost chemically pure condition. For the preparation of lyxose it was recrystallized once from hot water. Final yield: 1500 grams.

d-Lyxosimine.

100 grams crystalline finely ground *d*-lyxose were dissolved in 100 cc. dry methyl alcoholic ammonia saturated at about +10°. Crystallization of the imine began in a few minutes and was complete after about forty-eight hours. The product was chemically pure and needed only to be washed with a little methyl alcoholic ammonia and dried. The yield was quantitative; melting point, 142–143° (uncorrected).

0.1932 gm. of substance gave 12.6 cc. $\frac{N}{10}$ NH_3.

	Calculated for $C_5H_{11}NO_4$:	Found:
N	9.40	9.12

d-Lyxohexosaminic Acid.

32 grams of finely divided lyxosimine were covered with 32 cc. of water, 18 cc. of 80 per cent hydrocyanic acid added, and the suspension was warmed carefully to about 35° by immersion in warm water. Solution took place rapidly and soon spontaneous warming of the reaction mixture began. The temperature was controlled by a thermometer placed in the reaction flask and prevented from rising above 42°. When the tendency to warm

up had ceased, the solution was allowed to stand for about one-half hour at room temperature, cooled to 0°, mixed with 300 cc. cold concentrated hydrochloric acid, and saturated with hydrochloric acid gas.

Three portions, as above, were combined and worked up exactly as in the case of xylohexosaminic acid.[7] Care must be taken in handling the final concentrated aqueous solution. If too great an excess of methyl alcohol is added, it carries down gummy impurities, which are hard to remove from the crystals. Hence the alcohol should be added very carefully. From 95 grams imine the yield was 18 grams. It was recrystallized from 50 per cent methyl alcohol. *d*-Lyxohexosaminic acid darkens slowly above 180° and decomposes at about 215°.

0.1217 gm. of substance gave 15.3 cc. amino N, at 21.5°, 762 mm.
0.1036 gm. of substance gave 0.1382 gm. CO_2 and 0.0632 gm. H_2O.

	Calculated for $C_6H_{13}O_6N$:	Found:
N	7.18	7.12
C	36.92	36.54
H	6.66	6.85

The rotation of the substance in 2.5 per cent hydrochloric acid solution was as follows:

$$[\alpha]_D^{25^\circ}, \text{ after 15 minutes} = \frac{-0.25^\circ \times 2.1662}{1 \quad \times 0.1505} = -\ 3.58^\circ$$

$$[\alpha]_D^{25^\circ}, \text{ after 44 hours} = \frac{-1.44^\circ \times 2.1662}{1 \quad \times 0.1505} = -20.72^\circ$$

α-α_1-*Anhydro-mucic Acid.*

9 grams of lyxohexosaminic acid were dissolved in 100 cc. 3 per cent hydrochloric acid and 15 grams of silver nitrite added in portions over a period of about four hours. A few cc. of 10 per cent hydrochloric acid were then added and the reaction was allowed to proceed over night at room temperature. The silver chloride was filtered off and all but a slight excess of the hydrochloric acid removed from the filtrate with silver nitrate. The clear solution was concentrated *in vacuo* to about 25 cc., 30 cc. of concentrated nitric acid were added, and the solution was warmed in an Erlenmeyer flask over a small flame until a vigorous evolu-

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[7] Levene and La Forge, *Jour. Biol. Chem.*, 1915, xxi, 354.

tion of red fumes began. The flame was then removed and the reaction allowed to proceed with occasional warming. After twelve minutes the solution was poured into two glass dishes and evaporated to a thick syrup on the water bath. The contents of the dishes were then diluted with a little water, combined, and again concentrated. After evaporating once more with water, the syrup on cooling solidified to a mass of white crystals which appeared under the microscope as white oblong plates. After standing in the refrigerator for a time the mass was triturated with a few cc. of a mixture of dry acetone and ether and filtered on a Buchner funnel. The process was repeated once more to remove the adhering syrup. The crude product after drying weighed about 2 grams, although more crystallized from the acetone-ether mixture used for drying. For analysis it was recrystallized by dissolving in about one hundred parts of boiling dry acetone, filtering and concentrating the solution to about one-eighth of its volume. Anhydro-mucic acid crystallizes from acetone without crystal water in long prisms which melt at 203–204° (corrected).[8]

0.1040 gm. of substance required 10.7 cc. $\frac{N}{10}$ KOH; calculated, 10.8 cc.

0.1563 gm. of substance in 3 cc. H_2O showed no rotation with D-light in a 1 dm. tube, where a rotation of 0.02° would not have escaped detection.

0.1424 gm. of substance gave 0.1946 gm. CO_2 and 0.0534 gm. H_2O.

	Calculated for $C_6H_8O_7$:	Found:
C	37.50	37.27
H	4.20	4.17

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[8] Temperature given for anhydro-allomucic, 200–201°, also corrected.

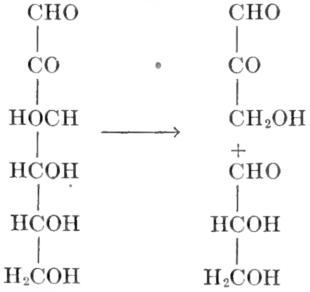
ON THE ACTION OF ASEPTIC TISSUE ON GLUCOSONE.

By P. A. LEVENE and G. M. MEYER.

(*From the Laboratories of the Rockefeller Institute for Medical Research.*)

(Received for publication, July 9, 1915.)

It is definitely established by the work of recent years that in the process of animal oxidation the hexose molecule suffers transformation into lactic acid, and that this phase is preceded by the transformation of one molecule of hexose into two of methyl glyoxal. In a previous communication the possible steps leading to this transformation were discussed.[1] It was shown in that publication that whenever any one hydrogen atom of the hexose molecule was substituted by a complex organic or mineral radicle the sugar was not affected by the tissue enzymes. In the present experiments it was aimed to test the action of the tissue enzymes on glucosone. Theoretically glucosone may dissociate into one molecule of glyceric aldehyde and one of hydroxymethyl glyoxal, and these in turn may be transformed into lactic (through methyl glyoxal) and pyruvic acids respectively.



$$\begin{array}{c} \mathrm{CHO} \\ | \\ \mathrm{CO} \\ | \\ \mathrm{HOCH} \\ | \\ \mathrm{HCOH} \\ | \\ \mathrm{HCOH} \\ | \\ \mathrm{H_2COH} \end{array} \longrightarrow \begin{array}{c} \mathrm{CHO} \\ | \\ \mathrm{CO} \\ | \\ \mathrm{CH_2OH} \\ + \\ \mathrm{CHO} \\ | \\ \mathrm{HCOH} \\ | \\ \mathrm{H_2COH} \end{array}$$

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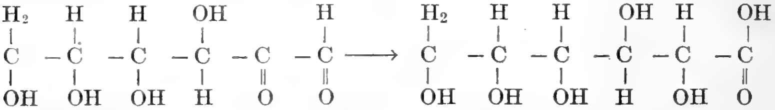
[1] Levene, P. A., and Meyer, G. M., *Jour. Biol. Chem.*, 1914, xviii, 469.

On the other hand, glucosone may be regarded as a substituted glyoxal, and by analogy with methyl glyoxal might have been expected to be transformed into a hexonic acid:[2]



$$\underset{\underset{OH}{|}}{\overset{\overset{H_2}{|}}{C}} - \underset{\underset{OH}{|}}{\overset{\overset{H}{|}}{C}} - \underset{\underset{OH}{|}}{\overset{\overset{H}{|}}{C}} - \underset{\underset{H}{|}}{\overset{\overset{OH}{|}}{C}} - \underset{\underset{O}{\|}}{C} - \underset{\underset{O}{\|}}{\overset{\overset{H}{|}}{C}} \longrightarrow \underset{\underset{OH}{|}}{\overset{\overset{H_2}{|}}{C}} - \underset{\underset{OH}{|}}{\overset{\overset{H}{|}}{C}} - \underset{\underset{OH}{|}}{\overset{\overset{H}{|}}{C}} - \underset{\underset{H}{|}}{\overset{\overset{OH}{|}}{C}} - \underset{\underset{OH}{|}}{\overset{\overset{H}{|}}{C}} - \underset{\underset{O}{\|}}{\overset{\overset{OH}{|}}{C}}$$

The experiments were performed under the same conditions as in the previous work. Aseptic kidney tissue was used as enzyme. In all experiments the glucosone solution remained unchanged. The reducing power for Fehling's and for permanganate solutions was not altered by the action of kidney tissue. Also the yield of phenyl osazone was apparently not affected by the experiment.

Thus the results of the present experiments are in harmony with those of last year. They show that only the unchanged hexose molecule is affected by the tissue enzyme, and that only the unchanged hexose undergoes a cleavage into two molecules, each containing a chain of three carbons. This in its turn may signify that the first step in the process of lactic acid formation from sugar consists in the dissociation of the hexose molecule into two of glyceric aldehyde. Perhaps this phase is the part of a special enzyme.

EXPERIMENTAL.

Tissues.—Kidneys were removed under aseptic conditions from exsanguinated rabbits.

Solutions.—For the preparation of glucosone 60 gm. of phenyl glucosazone in portions of 15 gm. were decomposed with 150 cc. concentrated HCl and the glucosone was isolated according to the method of Fischer.[3] The glucosone was obtained as a clear syrup. With phenyl hydrazine it formed an osazone in the cold. Approximately 3 gm. of this syrup were dissolved in 150 cc. sterile 1 per cent Henderson phosphate solution and passed through a sterile Berkefeld filter. This solution was divided into three portions of 50 cc. each. One rabbit kidney was cut into small pieces and added to each flask. Two of the flasks were allowed to stand at 37° for three hours; the other served as control.

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[2] Dakin, H. D., and Dudley, H. W., *Jour. Biol. Chem.*, 1913, xiv, 155. Levene and Meyer, *ibid.*, 1913, xiv, 151 and 551. Neuberg, C., *Biochem. Ztschr.*, 1913, xlix, 502.

[3] Fischer, E., *Ber. d. deutsch. chem. Gesellsch.*, 1889, xxii, 87.

Bacteriological Control.—Smears and cultures were made of all tissue mixtures before analysis and only those free from contamination were considered. The bacterial examinations were made by Dr. Martha Wollstein, to whom we desire to express our appreciation.

Methods of Analysis.—The diluted solutions were freed of proteins by small amounts of metaphosphoric acid. The presence of unchanged glucosone was noted in all solutions after standing at 37°.

Oxidation with Fehling's Solution.—The Lehmann-Maquenne method as described by Griesbach and Strassner[4] was employed.

Oxidation with Permanganate.—The total oxidation as described by Greifenhagen, König, and Scholl, and outlined in a previous communication, was also used.[5]

Oxidation with Fehling's Solution.

	Original solution.	$CuSO_4$.	$\frac{N}{10}$ $Na_2S_2O_3$.	$CuSO_4$ used.	Equivalent glucose.	Glucose.
	cc.	*cc.*	*cc.*	*cc.*	*mg.*	*per cent*
I Control..........	2	27.8	21.8	6.0	19.0	0.95
After 18 hrs......	2	27.8	21.8	6.0	19.0	0.95
II Control..........	4	27.8	17.5	10.3	33.2	0.83
After 18 hrs......	4	27.8	17.4	10.4	33.6	0.84
After 42 hrs......	4	27.8	17.3	10.4	33.6	0.84
III Control..........	4	27.8	17.3	10.5	34.0	0.85
After 18 hrs......	4	27.8	17.2	10.6	34.3	0.86
After 42 hrs......	4	27.8	17.3	10.5	34.0	0.85

Oxidation with Permanganate.

	Original solution.	$\frac{N}{5}$* $KMnO_4$	$\frac{N}{5}$ oxalic acid.	$\frac{N}{5}$ $KMnO_4$ used.	Glucosone.	Glucosone.
	cc.	*cc.*	*cc.*	*cc.*	*mg.*	*per cent*
I Control..........	2	41.00	22.6	18.4	30.1	1.51
After 18 hrs......	2	41.30	22.7	18.6	30.4	1.52
II Control..........	2	40.00	23.8	16.2	26.5	1.33
After 18 hrs......	2	41.25	24.5	16.6	27.1	1.35
After 42 hrs......	2	40.65	23.3	16.3	26.6	1.33
III Control..........	2	40.90	24.1	16.8	27.5	1.38
After 18 hrs......	2	41.00	23.9	17.1	27.9	1.39
After 42 hrs......	2	43.00	26.1	16.9	27.6	1.38

* 1 cc. $\frac{N}{5}$ $KMnO_4$ = 1.634 mg. glucosone.

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[4] Griesbach, W., and Strassner, H., *Ztschr. f. physiol. Chem.*, 1913, lxxxviii, 199.

[5] Levene and Meyer, *Jour. Biol. Chem.*, 1912, xii, 268.

ACIDITY OF UNDILUTED NORMAL GASTRIC JUICE FROM A CASE OF HUMAN GASTRIC FISTULA.

By M. L. MENTEN.

(From the Hull Laboratories of Biochemistry and of Physiology, University of Chicago.)

(Received for publication, July 8, 1915.)

Although acidity of gastric contents from several cases of human gastric fistula has been determined by the titration method, recent review of the literature (Boldyreff[1]) on this subject reveals no estimation of the acidity by the more accurate gas chain method. At the suggestion of Professor A. J. Carlson, who very kindly supplied me with samples of collected material, the hydrogen ion concentration of various samples of gastric juice from his gastric fistula subject, Mr. V., was measured. This case, on which chemical studies have already been reported by Professor Carlson,[2] because of a complete cicatricial stenosis of the esophagus, furnished gastric juice undiluted with saliva. In these studies, which are tabulated below, the method used was that outlined in detail by Michaelis,[3] and which, therefore, requires no description here. Where the amount of material permitted, the measurements were made in duplicate; if the quantity was insufficient for this, after the first measurement, the electrode tube was emptied of its hydrogen, refilled with a new supply, and a second reading made. In only one or two cases did the variation in duplicate samples amount to 1 millivolt. The terms for designating the different sorts of gastric juice are those employed by Professor Carlson in his previous reports of this case, *viz.*, "hunger" juice, where continuous secretion obtains; "appetite" juice, the secretion produced by the stimulation of the gustatory mechan-

[1] Boldyreff, W., *Quart. Jour. Exper. Physiol.*, 1915, viii, 1.

[2] Carlson, A. J., *Am. Jour. Physiol.*, 1915, xxxviii, 248.

[3] Michaelis, L., Die Wasserstoffionen Konzentration, Berlin, 1914.

ism; and "empty stomach fluid," that taken one hour after the stomach has been washed out with 100 cc. of water.

No.	Date.	P_H.	H.	Description.
	April			
1		1.09	$8.13, 10^{-2}$	Appetite (mixed, old), clear, transparent.
2		1.26	$5.50, 10^{-2}$	Appetite (mixed), S.E.,* very faintly opalescent.
3	6 12.00	1.11	$7.76, 10^{-2}$	Hunger, markedly opalescent.
4	“ 12.10	1.12	$7.59, 10^{-2}$	Appetite (slow secretion), markedly opalescent.
5	“ 12.20	1.00	$1.00, 10^{-1}$	Appetite, slightly opalescent.
6	“ 12.30	1.13	$7.49, 10^{-2}$	“ “ “
7	7	1.42	$3.80, 10^{-2}$	Empty stomach, opalescent with gastric mucin.
8	“	1.13	$7.40, 10^{-2}$	Hunger, opalescent with gastric mucin.
9	“	1.00	$1.00, 10^{-2}$	Appetite, faintly opalescent.
10	“	0.94	$1.15, 10^{-2}$	“ “ “
11	12	1.00	$1.00, 10^{-1}$	“ contains gastric mucin, greenish tinge.
12	“	1.08	$8.15, 10^{-2}$	Appetite, clear, transparent.
13	“	1.19	$6.45, 10^{-2}$	Hunger, greenish in color.
14	“	1.58	$2.63, 10^{-2}$	Empty stomach, slightly opalescent.
15	13	0.97	$1.07, 10^{-2}$	Appetite, bile tinged.
16	“	1.13	$7.41, 10^{-2}$	“ slightly opalescent.
17	“	1.01	$9.77, 10^{-2}$	Hunger, “ “
18	“	1.33	$4.68, 10^{-2}$	Empty stomach, opalescent with gastric mucin.
19	“ 12.00	1.45	$3.55, 10^{-2}$	Empty stomach, opalescent with gastric mucin.
20	“ 12.10	0.99	$1.00, 10^{-1}$	Hunger slightly opalescent.
21	“	1.06	$8.71, 10^{-2}$	“ greenish with much gastric mucin.
22	14	1.25	$5.62, 10^{-2}$	Hunger, opalescent with small amount of mucin.
23	“	0.97	$1.08, 10^{-1}$	Appetite, opalescent with small amount of mucin.
24	“	0.92	$1.19, 10^{-1}$	Appetite, opalescent with few shreds of gastric mucin.

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* This gastric juice was from Professor Carlson's second gastric fistula subject, Mr. S. E.

The results obtained were in very good agreement with those obtained in the same specimens by Professor Carlson by titration. It may also be noted that they correspond fairly closely to the figures obtained by Fraenckel[4] for acidity of gastric juice collected from Pawlow pouches in dogs and measured by the gas chain method.

Other reports of electrically measured human gastric juice, undiluted and after test meals, are those published by Michaelis and Davidsohn[5] and by Christiansen,[6] but as both of these series of measurements are on gastric juice collected from various pathological cases and show extreme variations in acidity, they need not be discussed here.

I wish again to acknowledge my indebtedness, and to express my sincere thanks to Professor Carlson for his kindness in supplying the material for the experiments outlined in this paper.

[4] Fraenckel, P., *Ztschr. f. exper. Path. u. Therap.*, 1905, i, 431.

[5] Michaelis, L., and Davidsohn, H., *ibid.*, 1910–11, viii, 398.

[6] Christiansen, J., *Biochem. Ztschr.*, 1912, xlvi, 24; *Deutsch. Arch. f. klin. Med.*, 1911, cii, 103 and 456.

A SPECTROSCOPIC EXAMINATION OF THE COLOR REACTIONS OF CERTAIN INDOL DERIVATIVES AND OF THE URINE OF DOGS AFTER THEIR ADMINISTRATION.

By ANNIE HOMER.

(From the Lister Institute, London, and the Departments of Biochemistry and Pathological Chemistry, University of Toronto.)

(Received for publication, July 7, 1915.)

A study of the literature of the subject of urinary pigments shows that the presence of a variety of chromogens has been detected in normal and pathological urines amongst which may be mentioned: urochrom, urobilin, urohematoporphyrin, urohematin, urocyanin, indirubin, indigotin, urorosein, uroerythrin, purpurin, skatol red, etc.

There is some difference of opinion as to whether the above mentioned substances represent separate pigments, or whether some of the color reactions were due to the presence of the same chromogen in an impure condition, or even to a mixture of two or more commonly occurring chromogens. It will be seen that there has been much discussion as to whether the indigotin of animals and of plants were one and the same substance; whether the indirubin of Rosin is not identical with the uroerythrin of Simon, with the urorubihematin of Harley, with the purpurin of Golding Bird, with the uromelanin of Thudichum, and with the urorosein of Sieber; whether the skatol red of Brieger and urorosein, uroerythrin, and purpurin are not the same substance; whether urobilin is a mixture of pigments, etc.

It is quite possible that a pigment noticed and named by one observer was in reality the same as that noted by another as a new substance under the special conditions of his experiments.

With a complex fluid such as urine in which there may be several chromogens present the question as to the possible identification of a pigment by its absorption spectrum is open to criticism unless precautions are taken to ensure the elimination of the other chromogens present in the fluid. A little experience with spectroscopic analysis shows that the position of the absorption bands of a substance in solution is often affected by the solvent and by the reaction of the solution. Moreover, if there are two or more substances in solution each of which has a characteristic absorption spectrum then the absorption bands of the one will be affected by the presence of the other in the solution and *vice versa.*

From considerations such as these it is obvious that, unless steps be taken to separate the pigment under observation from others in solution, varying results will be obtained and some already known chromogen will be described as an apparently new pigment precursor in the urine.

In many cases of the description and identification of pigments in the urine no exact measurements of the color reaction have been recorded; this in itself leads to confusion as neither the naked eye nor a spectroscope with the Fraunhoffer lines as the only scale indication can be used with any degree of accuracy. Moreover, many of the observations recorded have not been made under comparable conditions; this again leads to the production of contradictory results.

It was ascertained that there was a certain amount of fragmentary data as to the color reactions of some indol derivatives; in a few cases measurements had been made of the pigments obtained from the chromogens in the urine after the administration of certain indol substances and speculations as to the possibility of the precursors of certain pathological pigments being indol derivatives had been put forward.

As I was fortunate enough to have in my possession specimens of several indol compounds closely related to tryptophane, it was thought of interest and perhaps of service to make a systematic spectroscopic study of the color reactions of these compounds and of the urine of dogs to which these substances had been administered whether by mouth or by subcutaneous injection; such a study might demonstrate the connection between certain urinary pigments and indol derivatives.

It was also possible that a comparative study of the absorption spectra thus mapped out would furnish indirect qualitative evidence as to whether the absorption of tryptophane from the alimentary canal normally involves its preliminary decomposition by intestinal bacteria, the quantitative aspect of which problem is being carried out in conjunction with Dr. Hopkins of Cambridge, England.

The substances investigated were: indol, skatol, indolaldehyde, indolcarboxylic acid, indolacetic, indolaceturic, and indolpropionic acids, isatin, tryptophane, and some of the condensation products of tryptophane with aldehydo- and keto-compounds.

The method of procedure was as follows.

1. For the Substances Themselves.—Dilute aqueous solutions or suspensions of the substances were treated with concentrated hydrochloric acid and, if necessary, with a few drops of a dilute solution of an oxidizing agent such as potassium nitrite, ferric chloride, hydrogen peroxide, or calcium hypochlorite. In most cases it was necessary to warm the reacting liquids for the production of the color reaction; in some the reaction took place at room temperature. The concentration of acid required for the reactions varied considerably; in each case the temperature of the reaction and the degree of acidity were adjusted so as to ensure the production of the full color reaction and to make the absorption bands as pronounced and as sharp as possible.

In most cases the pigments were extracted with an organic solvent and the extract was well washed with dilute acid; in others it was found advisable to examine the acid solutions directly.

It was noticed with all these indol compounds that an excess of the oxidizing agent transformed the color of the solutions to a yellowish or brown shade; also that the pigments in solution in organic or inorganic solvents required the presence of acidic ions for their colors to be evident; alkalis converted the color of the solutions from purple or red to brown.

2. For the Urines under Investigation.—The samples of the urines of dogs to which the above mentioned substances had been administered by mouth or by subcutaneous injection were subjected to the treatment used for the substances themselves. It was necessary to add acid to the extent of one-half to one-third of the volume of urine taken, and it was noticed that a greater amount of oxidizing agent was required than for the aqueous solutions of the substances themselves; this was to be expected.

The dogs were put on a diet on which they were not excreting the chromogen of indican. The normal urine of every dog used was tested for the presence of the chromogens of indican, urobilin, and other pigments.

It was found that the dogs employed excreted no indoxyl compounds and no urobilin on diets of bread and milk. Even after the administration of comparatively large doses of some of the above mentioned indol compounds there was no urobilin detected in the urine.

In the case of pathological human urines it was found necessary to remove urobilin by means of lead acetate solution before proceeding to an examination for indol pigments. If indican were present, the urine, after treatment with nitrite or other oxidizing agent, was repeatedly extracted with chloroform to remove all traces of indigo pigments before attempting to make an examination of the amyl alcohol-soluble pigments. After the complete removal of the chloroform-soluble substances the urine was extracted with amyl alcohol and the extract well washed with dilute acid before being examined spectroscopically.

a. A Spectroscopic Examination of the Color Reactions of Various Indol Derivatives.

An examination of Table I shows that of the simple indol compounds all except indolpropionic acid gave characteristic absorption spectra; none of them gave any indication of the production of indigo by the oxidation methods employed.

Indolaldehyde gave a color reaction with acid alone; the addition of an oxidizing agent did not affect the position of the absorption bands. The position of these bands measured with the instruments at my disposal agreed with those given by Ellinger (Table III).

It will be seen that indolaldehyde, skatol, and tryptophane have an absorption band in common, *viz*: λ549–536. Indolacetic acid has a corresponding band but the position is slightly shifted (λ552–539).

Indolacetic acid, indolaceturic acid, and tryptophane show signs of a band λ505–485 which is the characteristic absorption band of the indolcarboxylic acid pigment. It looks as though in the oxidation of these substances there is a tendency to produce indolcarboxylic acid or some product in common with that formed from the latter substance.

b. A Spectroscopic Examination of the Urine of Dogs after the Administration of Various Indol Derivatives.

An examination of Table II shows that the administration of indol and indolaldehyde was followed by a vivid indican reaction which could be detected for three days after doses of 0.1 gram of these substances.

After the administration of skatol the dog urine exhibited a well marked color reaction and the absorption band agreed with that given by Brieger and by Porcher and Hervieux for skatol red (Table III). On the second day after the dose, whether given by mouth or subcutaneously, the urine gave a well marked indican reaction.

The administration of indolcarboxylic acid was followed by the elimination of the substance in the unchanged state, a fact evidenced by the color reaction of the urine.[1]

Laidlaw and Ewins[2] have shown that the administration of indolacetic acid is followed by the elimination of indolaceturic acid. The accompanying comparison (Tables I and II) of the color reactions of the urine after the administration of indolacetic acid with the color reactions of indolaceturic acid itself lends support to the quantitative experiments of these observers. It was noticed, however, that if the urines were allowed to become "stale" then the color reactions of the urine were those of indolacetic and not of indolaceturic acid.

Peculiar interest is attached to the chromogen present in the urine after the administration of indolpropionic acid. The acid itself does not give any characteristic color reaction with an oxidizing agent, but administration of the substance to a dog is followed by the elimination of a chromogen which is very sensitive to the merest trace of acid.

The color reaction was given by the urine on addition of acid and being allowed to stand at room temperature in contact with

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[1] The excreted indolcarboxylic acid was identified by qualitative and quantitative analyses (unpublished observations).

[2] Ewins, A., and Laidlaw, P. P., *Biochem. Jour.*, 1913, vii, 18.

air but the absorption spectrum exhibited under these conditions is not the same as that given by the urine after oxidation with nitrite or with ferric chloride and acid.

The production of a cherry-red color from the urine suggested the presence of the chromogen of urorosein, but the spectroscopic examination revealed the presence of a substance different from the chromogen of urorosein.

From Table II it will be seen that incomplete oxidation of the chromogen under consideration gives rise to the bands λ557–537 and λ521–496 whereas after complete oxidation three bands were measured, *viz.*, λ587–561, λ546–533, and λ499–485 of which the band λ499–485 was the best marked. The wave length of the third band suggests that of urobilin (Garrod and Hopkins λ505–485) and the question arose as to whether this band was an inherent property of the pigment or whether it indicated the presence of urobilin, a factor which might well be introduced on account of the reaction of the animal to this substance.[3]

It was found that the unknown chromogen like urobilin was precipitated by lead acetate solution and by saturation of the urine with ammonium sulphate. The precipitated chromogen (Garrod and Hopkins' method for the separation of urobilin was used) was carefully tested for the presence of urobilin; the ether-chloroform solution showed a faint, ill defined band at about λ490. An alcoholic solution of the chromogen made alkaline with ammonia and treated with zinc chloride did not give a fluorescent solution. It was obvious that there could not have been more than a trace of urobilin present in the urine.

The precipitated chromogen when treated with acid and an oxidizing agent was converted into the colored substance having the three well marked bands described above.

From these observations it is evident that the band λ499–485 is an inherent property of the pigment and is not due to the presence of urobilin.

The production of this pigment is of interest as it probably represents some profound change in the molecule of indolpropionic acid during its sojourn in the animal body. The chemical

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[3] The account of the chemical and physiological reaction of the animal body to this substance will be given in a later paper by Dr. Hopkins and the author.

aspect of this change is one of the problems which Dr. Hopkins and the writer of this paper are attempting to solve.

Administration of tryptophane, whether in small or large amounts, and also of its condensation products with formaldehyde, with glyoxylic acid, and with pyruvic acid, was not followed by the elimination of any corresponding chromogen in the urine; there was no indication of the chromogens of indican or of any other indol pigment, of urobilin, or of any unchanged tryptophane in the urine.

The spectroscopic evidence is in favor of the statement that normally the metabolism of tryptophane in the dog does not require the preliminary formation of the substances known to be the products of bacterial action on tryptophane. In the feeding experiments under consideration as much as 10 grams of tryptophane, both in the solid form and in solution were given in single doses to a dog of 23½ pounds' weight. In this way the organs of the animal were overloaded with the substance and the chances were that any of the above mentioned bacterial products, if formed from tryptophane normally, would be present under these conditions in sufficiently large amounts to be detected in the urine by their particular color reactions as it had been observed that after the administration of the various indol derivatives the color reaction, even in the case of small doses, could be detected in the urine excreted during the next thirty-six hours or more.

In certain pathological conditions or by the adoption of some special diet whereby the flora of the intestine is altered, the metabolism of tryptophane is often affected so that there is production of indol compounds leading to the elimination of the chromogens of indigo and of indol pigments in the urine.

The pigments of clinical interest in the urine which seem to have a close connection with indol compounds are *urorosein* and *indican*.

The urorosein reaction is characteristic of the urines of patients suffering from a variety of diseases such as chlorosis, pulmonary diseases, osteomalacia, nephritis, typhoid, carcinoma of the esophagus, ulcer of the stomach, anacidity, perityphlitis, and diabetes. The production of the chromogen of this pigment from such a variety of diseases is probably due to disturbances in the intestinal tract, but at the same time it must not be forgotten that the detection of a chromogen by the production of a rose-red color is somewhat unreliable unless the position of the absorption band is measured in each case. It has been my own experience with several pathological urines sent to me as exhibiting the urorosein reaction that, if the color reaction were carefully carried out so as to get the maximum effect, the spectroscopic examination revealed the presence of indican and not of urorosein.

Nencki and Sieber,[4] Rosin,[5] and Garrod and Hopkins[6] have made careful measurements of the absorption band exhibited by urorosein so that its detection, provided the investigator possesses a spectroscope fitted with a scale, should be a simple matter.

Herter[7] investigating the case of a child with a peculiar intestinal flora whose urine exhibited a marked urorosein reaction isolated indolacetic acid as the precursor of the pigment. But Laidlaw and Ewins[2] have shown that absorption of indolacetic acid from the intestine was followed by the elimination of indolaceturic acid in the urine. Noticing the color of the urine they suggested that indolaceturic acid was the chromogen of the pigment but they did not make measurements of the absorption band exhibited by the pigment derived from indolaceturic acid or by urorosein urines.

The work described in this paper had been started some time previous to the publication of Laidlaw and Ewins' paper; these observers very kindly furnished me with a colorless sample of crystalline indolaceturic acid and so enabled me to continue my spectroscopic work without having to isolate this substance from the urine of dogs to which I had administered indolacetic acid for other purposes.

It will be seen from the accompanying tables that indolacetic acid treated with an acid and an oxidizing agent gave a characteristic absorption band λ552–539; indolaceturic acid under the same conditions gave a well marked band λ579–539. Of these two the mean position for the indolaceturic pigment agrees with that given by Nencki, Rosin, and Garrod and Hopkins for urorosein urines.

Laidlaw and Ewins' observations on the fate of indolacetic acid in the organism together with the spectroscopic evidence adduced in this paper make it unlikely that indolacetic acid is the

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[4] Nencki, M., and Sieber, N., *Jour. f. prakt. Chem.*, 1882, cxxxiv, 333.

[5] Rosin, H. R., *Arch. f. path. Anat. u. Physiol.*, 1891, cxxiii, 519.

[6] Garrod, A. E., and Hopkins, G., *Jour. Physiol.*, 1896, xx, 112.

[7] Herter, C. A., *Jour. Biol. Chem.*, 1908, ccxxxix, 253.

chromogen of urorosein but at the same time the fact that Herter actually isolated indolacetic acid as the chromogen has to be accounted for.

In order to throw some light on the subject I attempted to isolate the chromogen from human urines exhibiting the urorosein reaction. There were many difficulties in the way, amongst others the fact that so many of the urines sent to me did not yield the urorosein but the indican reaction and that in many cases (especially of chlorosis) the reaction was so slight that there was little hope of isolating a crystalline product: a fraction of a milligram of the pure substance gives an intense color reaction.

There was sent to me a sample of urine from a patient at Guy's Hospital, London, suffering from achlorhydria gastrica. In the first sample sent there was indication of the presence of indolaceturic acid (absorption band λ573–540). The urine, evaporated to half its bulk *in vacuo,* was treated according to the directions given by Laidlaw and Ewins as follows: The urine after saturation with ammonium sulphate was acidified, extracted with ether, and the ethereal layer washed with ammonium sulphate solution and then with water. The acid was shaken out of the ether with sodium carbonate solution which was withdrawn, acidified, and again saturated with ammonium sulphate and extracted with ether. The ether extract was washed as before. After the removal of the ether by evaporation a small amount of a gummy oil was left. However, this would not form a picrate in the manner described by Laidlaw and Ewins.

The gummy residue gave an intense glyoxylic reaction and on treatment with acid and an oxidizing agent gave an intense color λ553–533 which proved to be that of indolacetic acid and not of indolaceturic acid and also not that of the original urine.

It was obvious that during the concentration and subsequent treatment the chromogen in the urine had been hydrolyzed to indolacetic acid.

Further samples were sent from the same case. However, a spectroscopic examination after the removal of urobilin revealed the presence of indolacetic and not indolaceturic acids. The urine was extracted as before and an oily magma of crystals was obtained; these crystals were identified as hippuric acid. The oily magma was therefore dissolved in 5 per cent sulphuric acid and precipitated with mercury reagent. After the decomposition of the mercury precipitate in the usual way the filtrate from the sulphide was freed from sulphuretted hydrogen and from acid, and extracted with ether. The ethereal solution was well washed and after the evaporation of the ether there remained a small amount of an oily substance which slowly began to crystallize. The crystalline residue was too small in amount to be recrystallized for melting point determinations, but spectroscopic examination of the crystals indicated that the substance was indolacetic acid.

In another case of urine supposed to be exhibiting the urorosein reaction it was observed that the absorption band was λ533–524; this corresponds to neither indolacetic nor indolaceturic acid pigments and was probably due to the presence of a mixture of pigments, although to the eye the color was that of urorosein.

Moreover, it was found that with samples of the urine of dogs after the administration of indolacetic acid to them, if the urorosein test were made with the freshly excreted urine the reactions were those of indolaceturic acid, but if the urine were allowed to become "stale" the color reaction changed to that of indolacetic acid. Further, if crystalline indolaceturic acid were added to normal urine which was then allowed to stand for some days, the color reaction changed to that of indolacetic acid.

From the work of Laidlaw and Ewins and from the evidence adduced in this paper it is obvious that the chromogen of urorosein is indolaceturic acid; from the above observations it is possible that on standing the indolaceturic acid urines may undergo bacterial decomposition with formation of indolacetic acid and glycine and, further, that even starting with a urine giving the reaction for indolaceturic acid, attempts to isolate the latter substance when present in moderately small amounts may fail on account of its hydrolysis into indolacetic acid.

From considerations such as these it is easy to understand the isolation of indolacetic acid and not of indolaceturic acid by Herter as the chromogen of urorosein. Had he measured the position of the absorption band of the pigment derived from indolacetic acid he would have seen that it did not agree with that given by Rosin and others for the absorption band of urorosein.

It is thus evident that the urorosein reaction is due to the presence of indolaceturic acid in the urine which (from the observations of Laidlaw and Ewins) has been formed from indolacetic acid absorbed from the intestine. Indolacetic acid may therefore be regarded as the precursor of the pigment urorosein in the metabolic functions of the body but indolaceturic acid is the actual chromogen of the pigment: the failure to realize this point may lead to confusion in the examination of urines with a "rose-red" color reaction.

In connection with a study of the urorosein reaction it seemed necessary to consider the question of the identity of *skatol red* and *urorosein.*

Brieger[8] isolated skatol from the products obtained by the distillation of feces and showed that after administration of this substance to a dog the urine contained a chromogen which on oxidation gave rise to a pigment which he called *skatol red.* The work of Brieger, Mester,[9] and others has shown that the chromogen of skatol red is skatoxyl sulphate. The only evidence of the occurrence of skatoxyl compounds in human urines is that furnished by Otto[10] who has shown that in the early stages of diabetes mellitus indoxyl compounds could be isolated from the urine whereas in the later phases of the disease, when gastric symptoms occurred, skatoxyl compounds were excreted.

In view of the more recent work on the color reactions of the urine of patients with disordered digestive functions and of the capacity of substances, even when analytically pure, to adsorb chromogenic materials this work of Otto calls for repetition, especially as he identified the compound isolated by him by analysis of the nitrogen and sulphuric acid constituents only; analysis of the carbon and hydrogen content was also necessary in a case in which a decision was being made between compounds whose composition and molecular weights so nearly approximated.

But whether skatoxyl compounds are to be found in human urines or not, the fact remains that skatol after administration by mouth or by subcutaneous injection is eliminated as skatoxyl sulphate, and that this substance on treatment with an acid and an oxidizing agent is transformed into a pigment having an absorption band λ577–550 (Brieger). The position of this band is the same as that observed for urorosein. Brieger considered it different from indirubin but the same as the pigments urorubin, urorosein, uroerythrin, and purpurin, a view shared by Porcher and Hervieux[11] but not held by Rössler[12] and Maillard.[13]

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[8] Brieger, L., *Ztschr. f. physiol. Chem.*, 1880, iv, 414.

[9] Mester, B., *ibid.*, 1888, xii, 130.

[10] Otto, J. G., *Arch. f. d. ges. Physiol.*, 1884, xxxiii, 607.

[11] Porcher, C., and Hervieux, C., *Compt. rend. Acad. d. sc.*, 1904, cxxxviii, 1725.

[12] Rössler, C., *Centralbl. f. inn. Med.*, 1901, xxii, 847.

[13] Maillard, L. C., *Ztschr. f. physiol. Chem.*, 1905, xlvi, 515.

Maillard considered that the skatol red of Brieger was not a pure pigment but was contaminated with indirubin and, further, that the skatoxyl eliminated in the urine does not account for the whole of the skatol injected; he suggests that some may have been converted into an indoxyl compound. Porcher and Hervieux were opposed to Maillard's views and considered that skatol becomes entirely converted into skatoxyl: they emphatically upheld their views as to the identity of skatol red and the above mentioned pigments.

The work of MacMunn and of Garrod has demonstrated that urorosein and uroerythrin are different substances (Table III).

From the work of Laidlaw and Ewins, of Herter, and from the evidence adduced in this research, it seems clear that the precursor of the urorosein pigment is indolaceturic acid. On the other hand it has been clearly demonstrated by Brieger, Otto, and Mester that the chromogen of skatol red is skatoxyl. But the evidence as to the occurrence of skatoxyl in human urines is by no means established whereas the urorosein reaction has been commonly noted and its connection with indolacetic and indolaceturic acids demonstrated.

It is thus obvious that under suitable conditions there can be isolated from the urine two distinct chromogens which produce the same color reaction. Some explanation of this phenomenon must be found.

It is possible that the same oxidation product may have been formed from these two chromogens, although the fact that the absorption spectrum of the indolacetic acid pigment is different from that of the indolaceturic acid pigment does not favor this hypothesis.

It is known that the character of an absorption spectrum depends on the atomic and molecular vibrations of the substance and that the actual position of the bands is influenced by the size, character, and weighting effect of the side chains of the ringed compounds: thus, the position of the band of the indolaceturic acid pigment is nearer the red end of the spectrum than that of the indolacetic acid pigment, and the latter nearer the red than the indolcarboxylic acid pigment. There is very little difference between the weighting of the molecules of indolaceturic acid and skatoxyl sulphate, and it is conceivable that the weighting of their respective oxidation products (which presumably belong to the same type of compound) is such that there is similarity of their absorption spectra both as regards character and position of absorption bands; but even so, until we know the type of reaction this color reaction involves it is with extreme diffidence that suggestions of this kind are brought forward.

At the same time it must be remembered that even though there is a certain similarity between the colors produced in the two cases absolute identity or a difference cannot be claimed until the colored substances themselves thus formed have been isolated and identified; for the present purposes such a course is not expedient.

However, an examination of the pigments formed from indolaceturic acid and from skatoxyl urines indicated that although at first the evidence seemed to point to their identity it was shown later that skatol red apparently consists of two pigments.

The two chromogens of the pigments whose identity was under consideration were not precipitated to any appreciable extent by the addition of lead acetate solution to the urine; they were also incompletely precipitated by saturation of the urine with ammonium sulphate.

The pigments were insoluble in chloroform, ether, and xylol but were readily soluble in ethyl and amyl alcohols.

The *indolaceturic pigment* is insoluble in chloroform to which has been added one-third its bulk of alcohol, but if the chromogen be dissolved in ethyl alcohol and oxidized with nitrite in the presence of the required amount of acid and then shaken with chloroform, the latter solvent takes up the pigment to a slight extent. The absorption band of the pigment in the chloroform-alcohol layer shows a shift in position (λ580–545). On washing the chloroform-alcohol extract with water the color is removed from the chloroform to the aqueous-alcohol layer. Reacidification of the liquids does not restore the color to the chloroform layer; this observation is contrary to Garrod and Hopkins' experience.[6]

On the other hand if a small amount of amyl alcohol were added to the chloroform the indolaceturic pigment was readily absorbed and could not be washed away with water. The solution showed a well marked band λ580–540.

The *skatol red* urines were treated with lead acetate solution and the excess of lead was removed. The clear filtrate was then oxidized in the usual way.

It was found that the skatol red pigment was insoluble in chloroform but on shaking the urine to which a small amount of ethyl alcohol had been added, there was a separation of the color into two component parts, the one soluble in the chloroform-ethyl alcohol layer as a bluish purple almost blue color and the other remaining in the aqueous acid layer as a rose-red color. After the removal of the chloroform-alcohol-soluble pigment the acid layer was extracted with amyl alcohol.

The bluish purple chloroform-alcohol-soluble pigment showed a diffuse band λ585–560 with sharp edges; the rose-red amyl alcohol extract showed a diffuse band λ560–530 and a band λ505–480.

Mixture of the chloroform-ethyl alcohol and the amyl alcohol extracts of the components of skatol red gave a band λ572–545 and in this case the band λ505–485 was hardly discernible.

It was found that an amyl alcohol-chloroform extract of the pigment obtained from the skatol red urines previously treated with lead acetate solution showed a slight shift of the band (λ575–545) but the character of the band was not that of the chloroform-ethyl-alcohol component (above): it was ill defined and diffuse. It is clear therefore that the latter extract contains a separate pigment and the position of the band λ585–560 can not be ascribed to the shifting effect of the chloroform on the position of the absorption band of skatol red.

It is evident therefore that the pigment known as skatol red can be resolved into two component parts; such a separation could not be accomplished with the indolaceturic pigment.

A further point of dissimilarity was the comparative stability of skatol red both in acid-aqueous and in acid-amyl-alcohol solutions whereas the characteristic color of urorosein urines and of the pigment from the isolated indolaceturic acid fades very rapidly, even from concentrated solutions in acid-amyl-alcohol.

Moreover, it was ascertained that after the administration of indolacetic acid to dogs there was no trace of indican in the urine. In the case of skatol given to a dog who was on a diet on which he was excreting no indoxyl compounds, no indican could be detected in the first day's urine but the chloroform extract was reddish purple in color and showed an absorption band λ535–560; this was probably the indirubin referred to by Maillard as contaminating Brieger's skatol red. On the second day the urine showed evidence of the formation of indoxyl compounds (positive indican reaction) and on this day the skatol red reaction was much less vivid than on the first day. Both the indican and skatol red reactions were faint on the third day.

It is possible that the bulk of the skatol is eliminated as skatoxyl sulphate while a small amount is converted into indoxyl sulphate. In the first day's urine, in the presence of excess of skatoxyl, the indoxyl combines with part of the skatoxyl to form a compound of the indirubin type while in the second day's urine, the skatoxyl compound being present in a much less amount, the indoxyl follows the usual path of indigotin formation.

From these considerations it is probable that skatol red and urorosein, although they possess similar absorption spectra are not identical. The experimental evidence shows that the skatol red of the urine is not a simple pigment but a mixture of pigments which, when present together, mutually affect each other's absorption so as to produce an absorption band corresponding to that of urorosein.

The indican reaction is exhibited by the urine of dogs excreted for several days following after the administration of indol and indolaldehyde. After the administration of skatol the indican reaction is not evidenced until the second day. In the case of indol and indolaldehyde the color reaction is very intense and persists for some time after their administration (Table II). The question as to the production of indican in the urine is one of interest in view of these observations.

The chromogen of indican is a sulpho- or gluco-compound of indoxyl and is to be found normally in the urine of herbivora and in pathological human urines. While Ellinger considers that the indoxyl compounds of the urine owe their origin solely to the bacterial production of indol from tryptophane in the intestine, Rosenfeld states that under certain conditions there appear, in the urine of rabbits, indoxyl compounds which can only have been produced from the breakdown of the tissues and not from the intestinal decomposition of tryptophane. Ellinger, criticizing Rosenfeld's experimental evidence,[14] states emphatically that the indican of the urine comes solely from indol formed in the intestine, a view also held by Salkowski, Underhill, Porcher, and others.[15]

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[14] Blumenthal, F., and Rosenfeld, F., *Charité-Ann.*, 1903. xxvii, 46. Ellinger, A., *Ztschr. f. physiol. Chem.*, 1903, xxxix, 44. Ellinger, A., and Gentzen, M., *Beitr. z. chem. Phys. u. Path.*, 1904, iv, 171. Also, Rosenfeld, F., *ibid.*, 1904, v, 83.

[15] Underhill, F. P., *Am. Jour. Physiol.*, 1903–04, x, p. xxvii. Salkowski, E., *Ztschr. f. physiol. Chem.*, 1904, xlii, 213.

The indican reaction seemed to be a feature of many of the pathological urines sent to me. In some cases the reaction was slight; in others, especially in the urine of patients suffering from pernicious anemia and cirrhosis of the liver, the reactions were most vivid.

The experimental data which I am slowly accumulating point to the liver as being the seat of the decomposition of tryptophane. It is quite feasible that in pathological cases such as those cited above, the presence of indoxyl compounds in the urine is not of necessity due to the bacterial disturbances in the intestinal tract but may owe its origin to the formation of indolaldehyde in the liver as a result of the failure of the disordered liver cells to deal normally with tryptophane.

SUMMARY.

A study of the absorption spectra of the colored substances obtained by the action of certain reagents on indol derivatives has been made and correlated with those derived from the chromogens eliminated in the urine of dogs to which these indol derivatives had been administered. The results show that:

1. Of the various indol derivatives administered, indol, skatol, indolaldehyde, indolcarboxylic and indolacetic acids undergo little change in their passage through the animal body. Apparently with indolpropionic acid there are more deep seated changes taking place.

2. Tryptophane under normal circumstances is apparently not broken down into any of the above mentioned substances before absorption from the alimentary canal.

3. The *urorosein* reaction is due to the presence of indolaceturic acid in the urine which has been produced from indolacetic acid in the intestine, the latter probably being formed as the result of bacterial action on tryptophane. Under some conditions the urorosein reaction is changed to that of indolacetic acid.

4. The pigment known as *skatol red* has the same absorption spectrum as urorosein and some observers consider them to be identical. The evidence adduced in this research shows that they are different. Skatol red consists of a mixture of two pigments which can be isolated by the use of suitable solvents; further, the

stability and solubility of the mixed pigments are different from those of urorosein.

5. The indican reaction is given markedly by the urine of dogs within a short time after the administration of indol and indolaldehyde and persists for some days. This color reaction is not given until the second day after the administration of skatol.

No indican could be detected in the urine after the administration of other indol derivatives.

6. The presence of indoxyl compounds in pathological human urines may be due to: (a) disorders of the liver whereby the liver cells may be incapable of dealing with tryptophane beyond the stage of indolaldehyde, or (b) the absorption from the intestinal tract of indol or of indolaldehyde produced from tryptophane by the action of intestinal bacteria.

TABLE I.

Spectroscopic Examination of the Color Reactions of Indol Derivatives.

Substance.	Treatment.	Solvent for pigment.	Absorption bands.
Indol	HCl and KNO_2	Amyl alcohol	λ528.5–490 (concentrated solution). λ528.5–505 (dilute solution).
Skatol	HCl and KNO_2	Amyl alcohol	λ546.5–533.5 and a diffuse ill defined band λ494–505.
		$CHCl_3$	λ546.5–533.5 and a diffuse ill defined band λ494–505.
Indolaldehyde	HCl (concentrated)	HCl aqueous	λ549–536.5 and a strong band λ495–480.
	HCl and KNO_2	HCl aqueous	λ549–533.5 and λ494.5–480.
		HCl aqueous	(Dilute solution) λ546–527.5 and λ494.5–475 (well marked).
	HCl and $FeCl_3$	Amyl alcohol	λ549–533.5 and λ499.5–475.
Indolcarboxylic acid	HCl and KNO_2	Amyl alcohol	λ505–484.
Indolacetic acid	HCl and KNO_2	HCl aqueous	λ552–539 (faint) and λ505–490 (faint).
		Amyl alcohol	λ557–539 and λ505–492.
	HCl and $FeCl_3$	Amyl alcohol	λ552–531 and λ505–492.
	HCl and KNO_2	Amyl alcohol	λ552–531 and λ505–492.
Indolaceturic acid	HCl and KNO_2	Amyl alcohol	λ579–539 (strong) and λ502–485 (very faint).
	HCl and H_2O_2	Amyl alcohol	λ579–539 and λ502–492.
	HCl and $FeCl_3$	Amyl alcohol	λ579–539 and λ502–492.
Tryptophane	Slow oxidation with $FeCl_3$ in presence of air and acid	$CHCl_3$	λ570-471 (faint).
	HCl and KNO_2	Amyl alcohol	Ill defined bands λ552–534 and λ505–485.
	HCl and $FeCl_3$	Amyl alcohol	λ549–531 and λ502–485.

TABLE I—*Concluded.*

Substance.	Treatment.	Solvent for pigment.	Absorption bands.
Condensation products of tryptophane with:			
1. formaldehyde	HCl (concentrated)	Acid aqueous	Glyoxylic color reaction.
	HCl and KNO_2	Amyl alcohol	Yellowish brown solutions.
	HCl and $FeCl_3$	Amyl alcohol	
	HCl and H_2O_2	Amyl alcohol and also $CHCl_3$	No bands.
2. glyoxylic acid	HCl and KNO_2	Amyl alcohol	Yellowish brown solutions.
	HCl and $FeCl_3$	Amyl alcohol	
	HCl and H_2O_2	Amyl alcohol and also $CHCl_3$	No bands.
3. pyruvic acid	HCl and KNO_2	Amyl alcohol	Yellowish brown solutions.
	HCl and $FeCl_3$	Amyl alcohol	
	HCl and H_2O_2	Amyl alcohol and also $CHCl_3$	No bands.
Isatin	HCl and KNO_2	Amyl alcohol	Brown solution. No bands.
	HCl and $CaOCl_2$	$CHCl_3$	No bands.

TABLE II.

*Spectroscopic Examination of the Urine of Dogs after the Administration of Various Indol Derivatives.**

Substance administered.	Treatment of urine.	Solvent for pigment.	Absorption bands.
Indol (by mouth)	HCl and $CaOCl_2$	CHCl	λ627–598 (indican).
(by injection)	HCl and $CaOCl_2$	CHCl	λ627–598 (indican).
	HCl and KNO_2	Amyl alcohol	No other pigment could be detected even three days after the dose.
Skatol† (by mouth and by injection)	HCl and $CaOCl_2$	$CHCl_3$	No indican first day.
	HCl and $FeCl_3$	$CHCl_3$	Purple color λ560–535 first day.
		$CHCl_3$	λ627–598 second day. Indican.
Skatol† (by mouth and by injection)	KNO_2 and HCl	Amyl alcohol	λ572–545. More dilute 565–546.
Component 1		*Chloroform-alcohol* solution: sharp band λ585–560; solution almost blue in color.	
Component 2		*Amyl alcohol* solution: diffuse and ill defined band λ560–530 and another λ505–480; this latter is not due to the presence of urobilin. Amyl alcohol solution almost rose-red in color.	
Chloroform-amyl alcohol solution of mixture of 1 and 2 shows band λ575–545.			
Indolaldehyde (by mouth)	HCl and $CaOCl_2$	$CHCl_3$	λ627–598 (well marked).
	HCl and $FeCl_3$	$CHCl_3$	λ627–598.
	HCl and KNO_2	Amyl alcohol after removal of pigments soluble in $CHCl_3$	None.
Indolcarboxylic acid (by mouth)	HCl and $CaOCl_2$	$CHCl_3$	None (no indican).
	HCl and $FeCl_3$	$CHCl_3$	None.
	HCl and KNO_2	Amyl alcohol	λ505–484.

TABLE II—*Continued.*

Substance administered.	Treatment of urine.	Solvent for pigment.	Absorption bands.
Indolacetic acid (by mouth and by injection)	HCl and $CaOCl_2$ HCl and $FeCl_3$	$CHCl_3$	None (no indican).
Fresh urine	HCl and KNO_2	Amyl alcohol	λ570–542 and λ505–490.
	HCl and H_2O_2	Amyl alcohol	λ570–542 and λ505–490.
	HCl and $FeCl_3$	Amyl alcohol	λ570–542 and λ505–490.
Stale urine	HCl and KNO_2 HCl and H_2O_2 HCl and $FeCl_3$	Amyl alcohol	λ550–533 and λ505–490.
Normal urine to which had been added:			
1. indolacetic acid	HCl and KNO_2	Amyl alcohol	λ555–533 and λ505–490.
2. indolaceturic acid	HCl and KNO_2	Amyl alcohol	λ570–542 and λ505–490.
3. indolaceturic acid, and allowed to stand for some time	HCl and KNO_2	Amyl alcohol	λ552–534 and λ505–490.
Indolpropionic acid (by mouth and by injection)	Acidified urine allowed to stand in contact with air	Amyl alcohol	λ521–496 (very marked) and λ557–537.
	Kynurenic acid precipitated from urine (Capaldi) colored red	Ether or ethyl alcohol extract of kynurenic acid precipitate	λ527–500.
	HCl and $CaOCl_2$ HCl and $FeCl_3$	$CHCl_3$	None except in one case when after injection on the third day there was a band λ625–598 (indican).
	HCl and KNO_2	Amyl alcohol	Three well marked bands. λ587–561, λ546–533, and λ499–485 (best marked).
	HCl and H_2O_2	Amyl alcohol	
	HCl and $FeCl_3$	Amyl alcohol	
Tryptophane (by mouth and by injection)	HCl and $CaOCl_2$	$CHCl_3$	None (no indican).
	HCl and $FeCl_3$	$CHCl_3$	None.
	HCl and KNO_2	Amyl alcohol	None; ill defined brown color.
	HCl and H_2O_2	Amyl alcohol	
	HCl and $FeCl_3$	Amyl alcohol	

TABLE II—*Concluded.*

Substance administered.	Treatment of urine.	Solvent for pigment.	Absorption bands.
Formaldehyde, glyoxylic acid, and pyruvic acid condensation products with tryptophane (by mouth)	HCl and $CaOCl_2$	$CHCl_3$	None.
	HCl and $FeCl_3$	Amyl alcohol	None; ill defined brown color.
	HCl and KNO_2		
Isatin (by mouth)	HCl and $CaOCl_2$	$CHCl_3$	None.
	HCl and KNO_2	Amyl alcohol	None; ill defined reddish brown color.

* Normal dog urine, with the diet chosen, gave negative tests for urobilin, indican, and other pigments with characteristic absorption spectra. Rabbit urine was taken and tested for indican and the band measured.

The absorption band of indican extended from λ624–598.

† The skatol pigment was fractionally extracted and was separated into two components, the one soluble in chloroform-alcohol and the other in amyl alcohol.

TABLE III a.

Absorption Bands of Pigments Derived from Indol Derivatives Previously Described by Other Observers.

Substance.	Observer.	Absorption bands.
Indolaldehyde (with HCl)	Ellinger	λ540–530 and λ490–480.
Indolacetic acid	Salkowski	λ550–530.

TABLE III b.

Absorption Bands of the Pigments Obtained from Reactions with Urine Previously Described by Other Observers.

Substance.	Observer.	Absorption bands.
Indican	Bouma De Negri	λ630–590. λ620–559.
Indirubin	Bouma De Negri Rosin	λ562–527. λ580–535. λ589–474 (maximum 578).
Uroerythrin	MacMunn Garrod	λ546–481. In presence of H_2SO_4 λ586–552; in presence of HCl λ608–517. In alcoholic solution λ546–520 and λ506–481.
Urohematoporphyrin	Garrod	In acid solution: λ597–587; λ576–570; λ557–541.
Urorosein	Garrod and Hopkins Rosin Nencki	λ561–540; in $CHCl_3$ λ582.5–550. λ538 (maximum). λ557 (maximum).
Skatol red	Brieger Porcher and Hervieux	λ577–550. λ577–550.

TABLE IV.

*Pigments Exhibited by a Few of the Pathological Urines Submitted for Investigation.**

Case.	Urobilin.	Indican.	Other pigments after treatment with KNO_2 and HCl.
Chlorosis 1	Yes	Yes	λ573–546 (urorosein).
2	Yes	Yes	None.
3	Yes	Very marked	Ill defined band λ524–504.
Pernicious anemia	Yes	Vivid reaction	None.
Pernicious anemia	Yes	Vivid reaction (no oxidizing reagent required)	None.
Cirrhosis of the liver	Yes	Vivid reaction	Ill defined band in the green.
Achlorhydrin gastrica 1	Yes	No	λ573-540 (urorosein).
An attempt to isolate the chromogen resulted in the separation of a substance having an absorption			λ552–533 (indolacetic pigment).
Achlorhydrin gastrica 2	Yes	No	λ552–533 (indolacetic pigment).
Achlorhydrin gastrica 2 Isolated crystalline residue			λ552–534 (indolacetic pigment).
Achlorhydrin gastrica 3	Yes	No	λ533–524 (? a mixture of pigments).

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* If the urines gave a marked urobilin reaction they were treated with lead acetate before being tested for the presence of indol pigments. If the urines contained the chromogen of indican, after oxidation with nitrite and acid, they were repeatedly extracted with chloroform to remove this pigment before being extracted with amyl alcohol.

A METHOD FOR THE ESTIMATION OF THE TRYPTOPHANE CONTENT OF PROTEINS, INVOLVING THE USE OF BARYTA AS A HYDROLYZING AGENT.

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INTRODUCTION.

A. Historical.

At the time that the work described in this paper was started there were very few data as to the amounts of tryptophane present in proteins, and for the lack of such data their tryptophane content was usually represented as positive or negative.

Hopkins and Cole,[1] the first investigators to isolate tryptophane from a protein digest, weighed the substance in the crystalline form and found that 1.5 per cent of tryptophane could be obtained from pure casein. Osborne and Harris[2] made use of Hopkins and Cole's glyoxylic reaction to find the relative amounts of tryptophane present in various vegetable proteins. Their observations were of a qualitative rather than a quantitative nature.

Levene and Rouiller[3] suggested that for the estimation of the tryptophane content of proteins, the digestion mixtures, made 5 per cent acid, be treated with mercury sulphate reagent; the mercury sulphate precipitate containing tryptophane be decomposed in the way described by Hopkins and Cole; and the tryptophane in solution be estimated by direct titration with bromine water in the presence of amyl alcohol. The disappearance of the characteristic purple color was taken by them as an indication of the end of the reaction. They published data with regard to the estimation of tryptophane in presence of tyrosine and cystine;

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[1] Hopkins, F. G., and Cole, S. W., *Jour. Physiol.*, 1901–02, xxvii, 418.

[2] Osborne, T. B., and Harris, I. F., *Ztschr. f. analyt. Chem.*, 1904, xliii, 376.

[3] Levene, P. A., and Rouiller, C. A., *Jour. Biol. Chem.*, 1906–07, ii, 481.

made suggestions as to the method of procedure should these two substances be present in the digest; and stated their intention of publishing data as to the tryptophane content of proteins; but nothing further has been published by them on this subject.

Fasal[4] precipitated the tryptophane in the protein digest by means of mercury sulphate reagent and after decomposition of the mercury sulphate precipitate, estimated the amount of tryptophane in the solution by means of Hopkins and Cole's glyoxylic reaction. Using the same method he also estimated the percentage of tryptophane in malignant tumors.[5] He published the following figures for the percentage of tryptophane in various proteins: Hammarsten's casein 0.65 per cent; crude lactalbumin 3.07 per cent; and edestin 0.38 per cent.

Sanders and May[6] subjected proteins to pancreatic digestion and then inoculated the digest with fecal bacteria and colorimetrically estimated the indol thus produced. Their method assumes that the tryptophane is completely converted into indol, but such is not necessarily the case; a certain proportion of skatol and other derivatives of indol will always be simultaneously formed.

Herzfeld[7] has taken the tryptic digest of proteins and has colorimetrically estimated the tryptophane present in the digest by means of *p*-dimethylaminobenzaldehyde. He compared the color produced by the interaction of this reagent and tryptophane with the color of a solution of ammoniacal copper sulphate previously expressed in terms of a solution of pure tryptophane similarly treated with *p*-dimethylaminobenzaldehyde.

The writer of this paper adopting Herzfeld's directions has investigated the color reactions of various indol derivatives with this reagent, and has ascertained that the production of a blue color is not specific to tryptophane. A similar blue color is produced by indolacetic, indolaceturic, and indolpropionic acids and by skatol: indolaldehyde and indolcarboxylic acid give a greenish blue color while indol under the same conditions gives a red color ultimately changing to purple. Under the conditions given by Herzfeld in another paper[8] the red color of indol with this reagent does not change.

Herzfeld has estimated the tryptophane content of about 20 proteins and amongst others he quotes: casein 0.51 per cent; edestin 0.58 per cent; lactalbumin 0.91 per cent; Witte's peptone 1.25 per cent.

It will be noticed that these numbers for casein are lower than those given by Fasal and by Hopkins and Cole. Herzfeld's low results may be due to the fact that he worked with small quantities of the respective proteins (0.1 gm.) and that he continued the digestion for twenty-four hours only, and further, that he made use of a color reaction not specific to

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[4] Fasal, H., *Biochem. Ztschr.*, 1912, xliv, 392.

[5] Fasal, *ibid.*, 1913, lv, 88.

[6] Sanders, J. A., and May, C. E., *Biochem. Bull.*, 1912–13, ii, 373.

[7] Herzfeld, E., *Biochem. Ztschr.*, 1913, lvi, 259.

[8] Herzfeld, *loc. cit.*, 82.

tryptophane. Hopkins and Cole showed that the maximum yield of tryptophane was obtained after seven to ten days' digestion, a fact recognized and appreciated by Plimmer and Eaves[9] in their estimation of tyrosine in protein digests. The result given by Fasal for casein is much lower than that given by Hopkins and Cole who actually isolated tryptophane in the crystalline form. It is a matter of common experience to obtain a 1 per cent yield of tryptophane from commercial casein. Fasal's numbers were based on colorimetric reactions involving the use of glyoxylic acid. From the author's observations on the factors affecting the color reaction in question[10] it is conceivable that the discrepancy between the figures quoted by Fasal and by Hopkins and Cole was due to the interference of other substances of an indol nature thrown down by the precipitating reagent.

Finally Elizabeth Kurchin,[11] using Herzfeld's method, has estimated the tryptophane content of normal and pathological kidneys.

B. Method Adopted in the Present Investigation.

It is a matter of common experience that tryptophane can not be isolated in any appreciable extent from the products formed during an acid hydrolysis of proteins and for this reason in most of the previously described methods the protein has been hydrolyzed by trypsin. As the use of trypsin is accompanied by many disadvantages it seemed worth while to ascertain whether any other hydrolyzing agent could be used with any degree of satisfaction: it was found that baryta answered the purpose.

In the method for the estimation of the tryptophane content of proteins which is advocated in this paper the protein is hydrolyzed with baryta water. After the removal of the baryta with sulphuric acid the protein digest, made 5 per cent acid with sulphuric acid, is precipitated with mercury sulphate reagent. The precipitate is decomposed and the filtrate from the mercury sulphide is freed from substances likely to absorb bromine. The tryptophane content of the final solution thus obtained is estimated by titration with solutions both of nascent and of molecular bromine previously standardized against solutions of pure recrystallized tryptophane.

The determinations of the percentage of tryptophane in casein by the method described in this paper were made in the spring

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[9] Plimmer, R. H. A., and Eaves, E. C., *Biochem. Jour.*, 1913, vii, 297.

[10] Homer, A., *ibid.*, 1913, vii, 101.

[11] Kurchin, E., *Biochem. Ztschr.*, 1914, lxv, 451.

and autumn of 1913; the publication of the results has been delayed as it was desired to ascertain whether indol derivatives likely to be formed in the digest would be precipitated by the mercury reagent, and if so to what extent their formation would affect the determinations. It was also desired to make various quantitative experiments on the action of acids and alkalis on tryptophane.

The estimation of the tryptophane content of casein by a method involving the use of baryta as the hydrolyzing agent, of mercury sulphate as the precipitant for the tryptophane liberated during the hydrolysis, and of bromine for the final estimation of the tryptophane in solution, therefore resolved itself into the following considerations:

I. The absorption of bromine by tryptophane in aqueous solution.

II. A study of the extent to which substances other than tryptophane are precipitated by mercury sulphate reagent and of the bromine capacity of the substances thus precipitated which are likely to appear in the final solution for estimation.

III. The estimation of the tryptophane content of the hydrolysis mixtures involving: a. The treatment of the alkaline digests so as finally to obtain for bromination a solution containing the tryptophane and as free as possible from other substances likely to absorb bromine. b. The estimation of the tryptophane content of the final solutions by a determination of the ratio of their absorption of bromine to that of a solution of pure recrystallized tryptophane under the same experimental conditions.

IV. The action of acids and alkalis on tryptophane.

Section I. The Estimation of the Bromine Absorption of Tryptophane in Aqueous Solution.

The following solutions were required for the estimations:

A. Solutions of tryptophane prepared by dissolving known weights of the substance in known volumes of water.

B. A saturated solution of bromine in water. This solution was standardized (every time used) against potassium iodide and a standard solution of thiosulphate.

C. Solutions of nascent bromine prepared by the interaction (in presence of acid) of aliquot parts of the following: a solution of sodium bromate containing 15.1 grams per liter and a solution of sodium bromide containing 51.5 grams per liter.

10 cc. of each of these solutions were measured into stoppered bottles and 5 cc. of concentrated hydrochloric acid were added. After standing for some time there was added an excess of a solution of potassium iodide, and the liberated iodine was titrated with a standard solution of thiosulphate. The bromine value of the mixture of bromide and bromate was thus made.

D. A standard solution of thiosulphate ($\frac{N}{10}$): a 10 per cent solution of potassium iodide, and a freshly prepared solution of starch.

Method of Procedure.—Accurately weighed amounts of tryptophane were dissolved in known volumes of distilled water and aliquot parts of the solution were measured into stoppered flasks and treated with a known excess of an aqueous solution of bromine, or with a known amount of nascent bromine liberated by the addition of 5 cc. of concentrated hydrochloric acid to the mixture of the previously measured volumes of sodium bromate, bromide, and tryptophane solutions. The flasks were tightly corked and left at room temperature for fixed periods of time. Excess of a 10 per cent solution of potassium iodide was then added, and the flasks were recorked and allowed to stand for at least fifteen minutes. The iodine, representing the excess of bromine, was estimated by titration with standard thiosulphate solution. The difference between the amount of bromine originally added to the solution and the unabsorbed bromine finally titrated represents the bromine absorbed by the substance.

In these determinations the use of a thermostat is to be recommended as it was found that the bromine absorption of solutions of pure tryptophane at room temperature during a given period of time was greater in summer than in winter seasons of the year (England); further, to ensure constant values for the absorption of bromine it is necessary to have considerable excess of bromine and to adhere rigidly to a definite time for the reaction to take place; a variation in the time of the reaction is accompanied by slight variations in the absorption of bromine by a given weight of the substance.

It has been stated by Plimmer and Eaves that under the conditions employed by them, one molecule of tryptophane absorbs about six atoms of bromine. My results (Table I) indicate a greater absorption; one molecule of tryptophane absorbs about eight atoms of bromine, although there are indications that its bromine capacity is higher still. In the initial stages there is rapid absorption of bromine but the rate of absorption soon falls off and, as would be expected, complete saturation does not take place for some time. It will also be seen that the bromine absorption with molecular bromine is slightly higher than with nascent bromine.

From these results it is obvious that the absorption of bromine by tryptophane in aqueous solution takes place in stages and that in the complete saturation of the substance with bromine the time and temperature of the reaction must be taken into account.

Section II. The Use of Mercury Sulphate Reagent as a Precipitant and the Extent to Which the Substances Other than Tryptophane Precipitated Therewith Will Absorb Bromine.

The mercury sulphate reagent[12] according to Hopkins and Cole precipitates tryptophane, cystine, tyrosine to a certain extent, polypeptides, and other incompletely hydrolyzed products. Of these, tryptophane, tyrosine, and polypeptides will absorb bromine: there is conflicting evidence as to whether cystine absorbs bromine or not. Levene and Rouiller[3] consider that it does, whereas Plimmer and Eaves,[9] in view of the work of Knoop[13] on the absorption of bromine by amino-acids in digests, do not regard cystine as a factor to be considered in the bromination of protein digests. However, cystine, although precipitated by the mercury reagent, can not interfere because it is destroyed by the alkaline hydrolysis. To make quite sure of this point 300 cc. of the solutions as finally prepared for estimation with bromine were treated with zinc and hydrochloric acid, and the reduced liquid was tested for the presence of cystine by means of the sodium nitroprusside and the ferric chloride color reactions. Negative results were obtained, thus showing that after a baryta hydrolysis

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[12] 10 per cent mercury sulphate in 5 per cent sulphuric acid.

[13] Knoop, F., *Beitr. z. chem. Phys. u. Path.*, 1908, xi, 356.

TABLE I.

The Absorption of Bromine by Tryptophane in Aqueous Solution.

Weight of tryptophane in volume of solution taken.	Time of reaction.	Weight of bromine added.	Weight of bromine residual.	Bromine absorbed by	
				1 gm.	1 gm. molecular weight
				of tryptophane.	
	min.	*gm.*	*gm.*	*gm.*	*gm.*
Mean results.					
Room temperature (summer).					
May					
0.0648	30	0.4901 (nascent)	0.2770	3.29	671
June					
0.0311	30	0.4884 (nascent)	0.3859	3.31	673
August					
0.0311	30	0.4884 (nascent)	0.3848	3.33	679
October					
0.0311	30	0.4884 (nascent)	0.3858	3.30	673
May					
0.0648	30	0.4130 (molecular)	0.1903	3.43	700
June					
0.0467	30	0.1935 (molecular)	0.0391	3.30	673
July					
0.0648	30	0.4136 (molecular)	0.1909	3.41	696
October					
0.0311	30	0.1290 (molecular)	0.0261	3.38	690
Room temperature (winter).					
0.02255	30	0.2301 (nascent)	0.1619	3.02	616
0.02255	45	0.2301 "	0.1588	3.16	645
0.02255	60	0.2301 "	0.1577	3.21	654
0.02255	2 hrs.	0.2301 "	0.1572	3.23	659
0.02255	4 hrs.	0.2301 "	0.1546	3.35	683
Temperature of reaction 17°C.					
0.0624	30	0.4602 "	0.2732	3.00	608
0.04992	2 hrs.	0.2301 "	0.0711	3.19	650
0.04721	4 hrs.	0.4683 "	0.3122	3.31	675
0.04721	7 hrs.	0.4683 "	0.3085	3.40	693
0.04721	8 hrs.	0.2301 "	0.0723	3.34	681
Temperature of reaction 17°C.					
0.0500	30	0.4720 "	0.3213	3.00	608
0.0500	1 hr.	0.4720 "	0.3136	3.17	647
0.0500	2 hrs.	0.4720 "	0.3116	3.21	655
0.0500	4 hrs.	0.4720 "	0.3037	3.36	685
0.0500	6 hrs.	0.4720 "	0.2985	3.47	708
0.0500	8 hrs.	0.4720 "	0.2990	3.46	706

of proteins and subsequent precipitation with mercury sulphate reagent there is no cystine in the precipitate.

It was found that solutions of indol, skatol, indolaldehyde, indolcarboxylic, indolacetic, indolpropionic acids, tryptophane, and the various additive compounds of tryptophane[14] when dissolved in 5 per cent sulphuric acid were all precipitated by mercury sulphate reagent. Further, it was found that all these compounds would absorb bromine under the conditions employed for the estimation of tryptophane.

In the case of tryptophane it has been shown (Table I) that the bromination is affected by conditions of time and temperature. The same is probably true for the absorption of bromine by other indol derivatives, but unfortunately, owing to scarcity of material, it was impossible to do similar time experiments with regard to their absorption of bromine: their bromine absorption after half an hour's reaction was investigated, as this was the time chosen for the bromination of the solutions obtained from the protein digests.

The solubility of indol, skatol, indolaldehyde, and indolcarboxylic acid in water is so slight that it was impossible to get reliable data as to their bromine capacity under the conditions adopted for the bromination of the final solutions obtained from the protein digests (Section III). Bromination of weighed amounts of the solids themselves did not yield concordant results.

Solutions of indol and skatol in absolute alcohol were brominated: the bromine absorption of a volume of alcohol corresponding to that used in the titrations of indol and skatol was estimated and deducted from the value for the bromine absorption of the solutions: again no concordant results could be obtained.

In another series of experiments the indol and skatol were dissolved in a small volume of absolute alcohol (25 cc.) and the solution was diluted with water to 100 cc. Aliquot parts of these solutions were treated with bromine and a correction was introduced for the bromine absorption of the alcohol. It is with diffidence that the results are published: the author does not consider that much reliance can be placed in them, they merely serve to show the order of the bromine absorption of these substances.

The following results were obtained:

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[14] Homer, *loc. cit.*, 117.

TABLE II.

The Absorption of Bromine by Aqueous-Alcoholic Solutions of Indol and Skatol.

Weight of substance in solution.	Weight of bromine added.	Weight of residual bromine.	Weight of bromine used by volume of solvent taken.	Weight of bromine absorbed by 1 gm. of substance.	Weight of bromine absorbed by 1 gm. molecular weight of substance.
Mean results.	(Nascent)				
gm.	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*
Indol					
0.0364	0.4683	0.2651	0.0745	3.54	414
0.0402	0.4620	0.2965	0.0745	3.44	402
Skatol					
0.03045	0.4683	0.3128	0.0745	2.66	348

The bromine absorption of indolacetic and indolpropionic acid in aqueous solution was also measured and the results have been embodied in Table III.

TABLE III.

The Absorption of Bromine by Indolacetic and Indolpropionic Acid in Aqueous Solution.

Weight of substance in volume of solution taken.	Weight of bromine added.	Weight of residual bromine.	Bromine absorbed by 1 gm. of substance.	Bromine absorbed by 1 gm. molecular weight of substance.
Mean results. Time of reaction 30 min.				
gm.				
Indolacetic acid	*gm.*	*gm.*	*gm.*	*gm.*
0.0402	0.4856	0.3483	3.40	595
0.0400	0.3793	0.2402	3.43	600
Indolpropionic acid				
0.0400	0.4856	0.3408	3.62*	674
0.0401	0.4683	0.3358	3.30	623
0.0227	0.2295	0.1583	3.13	591

* The high value in this set of determinations was probably due to the fact that a small amount of sodium carbonate solution had been added to dissolve the acid.

Section III. The Baryta Hydrolysis of Casein and the Estimation of the Tryptophane Liberated Therein.

a. The Preparation from the Hydrolysis Mixture of the Solution for Estimation with Bromine.

100 grams of casein were treated with 350 grams of barium hydrate in 2.5 liters of water, and the mixture was heated on a water bath for periods varying from twenty to one hundred and twenty hours. The baryta was removed by slight excess of sulphuric acid and the liquid filtered. The filtrate was made 5 per cent acid with sulphuric acid, treated with an excess of mercury sulphate reagent and allowed to stand for forty-eight hours. The mercury sulphate precipitate may contain tyrosine, cystine, various incompletely digested protein substances, and other indol derivatives as well as tryptophane.

The tryptophane was separated from the other substances present in the mercury sulphate precipitate and likely to absorb bromine by the following process: The precipitate, after having been freed from tyrosine by repeated washing with 5 per cent sulphuric acid until the washings no longer gave Millon's test, was suspended in 2 per cent sulphuric acid and decomposed with sulphuretted hydrogen. The filtrate from the mercury sulphide was freed from sulphuretted hydrogen and while still acid (approximately 2 per cent) was treated with a solution of phosphotungstic acid with avoidance of excess. After standing, the precipitate of polypeptides, etc., if any, was filtered off; the excess of phosphotungstic acid was removed by means of baryta, and the latter by means of sulphuric acid. The liquid was again tested with Millon's reagent, and if there were any indications of tyrosine the precipitation with the mercury sulphate reagent was repeated. In the experiments under consideration it was found unnecessary to reprecipitate.

Cystine, although precipitable by the mercury reagent, can not interfere because it is destroyed by the alkaline hydrolysis.

In view of these observations (Section II) with regard to the precipitation of indol derivatives by the reagent it was necessary to make tests for the presence of indol derivatives other than tryptophane in the solutions for bromination.

The following four color tests are in vogue for the detection of indol derivatives in solution:

1. *The glyoxylic reaction* originally employed as a specific test for tryptophane, is given by all indol derivatives although the actual shade of color produced varies considerably.[10]

2. *The purple color reaction with bromine water* is specific to tryptophane and, under certain conditions, for its additive compounds. The other indol derivatives absorb bromine but do not give any characteristic color reaction with this reagent.

3. *Sodium nitrite and hydrochloric acid* give color reactions with all indol derivatives. Indol, skatol, indolaldehyde, indolcarboxylic acid, indolacetic, indolaceturic acids treated with these reagents give color reactions which are characterized by well defined absorption bands, whereas indolpropionic acid gives no characteristic color under the same conditions.[15]

4. Indol derivatives treated with Ehrlich's *p-dimethylaminobenzaldehyde* according to the directions given by Herzfeld yield characteristic color reactions. Indol gives a characteristic red color, and tryptophane, as shown by Herzfeld, gives a beautiful blue color. However in the present research it was ascertained that a similar blue color is given by indolacetic, indolaceturic, and indolpropionic acids and by skatol: indolaldehyde and indolcarboxylic acid give a greenish blue color.

The original protein digests and also the solutions finally obtained after the decomposition of the mercury sulphate precipitate were therefore tested for the presence of indol, skatol, indolaldehyde, indolcarboxylic and indolacetic acids, and for tryptophane by the color tests given above. Indolpropionic acid can not be detected in presence of tryptophane except by extraction of the solution with ether, in which tryptophane is insoluble, removal of the solvent, and conversion of the residue, if any, into the nitroso-derivative.

If indol, skatol, etc., are present in a digest, presumably they must have been formed from tryptophane during the process of hydrolysis, and they will be precipitated by the mercury reagent together with tryptophane. Owing to the slight solubility of indol, skatol, indolaldehyde, and indolcarboxylic acid there can

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[15] Homer, *loc. cit.*

be, at the most, but traces of these substances in the final solutions for estimation with bromine: as regards indolacetic and indolpropionic acids their absorption of bromine is so close to that of tryptophane (page 377) that for the small amounts present they will not appreciably affect the estimation: that is to say, they will influence the bromine absorption to the same extent as though they had been present as the parent substance, tryptophane.

As a matter of experience in fourteen hydrolyses with baryta there were three only of the final solutions which gave any indication of the presence of indol derivatives other than tryptophane. Of these, two indicated traces of indol, and one traces of indolacetic acid. Moreover, that there could not have been any appreciable conversion of tryptophane into derivatives not appearing in the final solutions is shown by the results obtained from the hydrolyses of the same sample of casein for varying periods of time (page 381) and from the results of the hydrolyses of pure tryptophane.

b. Estimation of the Tryptophane Content of the Solutions Obtained after the Decomposition of the Mercury Sulphate Precipitates, etc.

An aliquot part (50 cc.) of the solution for estimation was treated with excess of nascent or molecular bromine of known strength and the unabsorbed bromine estimated by means of potassium iodide and standard thiosulphate solution. The bromination was carried out in well stoppered bottles fitted with ground glass stoppers or with tightly fitting waxed corks. The bromine absorption of the solutions was compared with that of a freshly prepared solution of pure recrystallized tryptophane treated with the brominating solutions under comparable conditions of time and temperature; from these observations the tryptophane content of the hydrolysis mixtures could be calculated.

It was found that concordant results could be obtained if the conditions as indicated above (page 373) were kept constant during each series of experiments.

Samples of commercial casein obtained from three different firms were hydrolyzed with baryta and the hydrolysis mixture was treated in the manner described above to ascertain (α) the tryptophane content of the protein by the method under investigation and to see how it compared with the values given by other observers; (β) the length of time of hydrolysis for a maximum yield of tryptophane; and (γ) to what extent further hydrolysis would lead to a diminution of tryptophane in the final solution.

The results of the determinations have been embodied in Table IV and in each case the mean results of a series of observations have been given.

TABLE IV.

The Tryptophane Content of Commercial Casein as Estimated by the Method Described in This Paper.

Sample.	Weight of casein taken.	Time of hydrolysis.	Volume of final solution.	Bromine absorbed by final solution.		Bromine absorbed by 1 gm. tryptophane under the same conditions.	Tryptophane content of 100 gm. casein.
				(a) Nascent.	(b) Molecular.		
	gm.	*hrs.*	*cc.*	*gm.*	*gm.*	*gm.*	*gm.*
A	200	40	500	10.45		3.30	1.59
B (1)	100	20	1000	3.24		3.29	0.99
					3.64	3.44	1.06
(2)	100	40	1000	3.41		3.29	1.04
					3.85	3.44	1.1
(3)	100	60	1000	3.41		3.29	1.04
					3.75	3.44	1.09
(4)	100	80	1000	3.41		3.29	1.04
					3.88	3.44	1.13
C (1)	100	120	1000	4.07		3.30	1.24
(2)	100	120	1000	4.07		3.30	1.24

An examination of the results given in Table IV shows that except for Sample A the percentages of tryptophane indicated are all lower than those given by Hopkins and Cole but higher than those given by Fasal and by Herzfeld. Hopkins and Cole obtained 1.5 grams of tryptophane from 100 grams of Hammarsten's pure casein; but to the best of my knowledge their average yield from commercial casein was not much more than 1 per cent.

From the determinations made with Sample B it appears that the length of time suitable for a complete separation of tryptophane from casein is about forty hours.

That there could not have been any appreciable loss of tryptophane due to the formation of indol derivatives or other compounds not present in the final solution is evidenced first by the results obtained from the hydrolysis of the same sample (Sample B) for varying periods of time and secondly, by the fact that duplicate hydrolyses of Sample C conducted for the same periods of time and treated separately have given concordant results.

The solutions for estimation with bromine were all tested for the presence of indol derivatives (possibly precipitated by the reagent): where detected they were present as traces only.

The advantages in favor of the use of the above described method for the estimation of the tryptophane content of proteins are:

1. The ease with which the hydrolysis can be conducted.
2. The fact that the tryptophane does not tend to disappear during the hydrolysis.
3. The values obtained for casein are nearer those obtained by Hopkins and Cole, who actually isolated and weighed the tryptophane, than those given by investigators using colorimetric methods. In any case the values obtained in this research, in view of the possible formation of decomposition products from tryptophane and their subsequent loss in the processes employed, are doubtless too low rather than too high.
4. The method is proving of the utmost service in a series of experiments in which the action of tissue enzymes on tryptophane is being studied.

From the consideration of the question of the possible destruction of tryptophane by prolongation of the time of digestion of the protein with baryta I was led to a consideration of the action of alkalis and acids on pure tryptophane.

Section IV. The Action of Acids and Alkalis on Pure Tryptophane.

a. The Action of Acids on Tryptophane.

It is a matter of common experience that tryptophane can not be estimated in any appreciable quantity from the products formed during an acid hydrolysis of tryptophane.

The following experiments demonstrate this clearly:

1. 100 grams of casein were heated with 150 cc. of a 25 per cent solution of sulphuric acid for periods of 1, 2, 4, 8, 12, and 20 hours. Baryta was used to remove the acid and after filtration of the barium sulphate the filtrate was made 5 per cent acid with sulphuric acid and treated with mercury sulphate reagent. After washing the precipitate free from tyrosine it was found that in each case there was little more than a trace of precipitate left on the filters, at any rate not sufficient for estimation.

Thus at no period of the digestion was tryptophane present in any appreciable quantity.

In order to ascertain whether the poor yield of tryptophane in an acid hydrolysis is due to the direct action of acid on the substance as soon as it is liberated from the protein molecule the following experiments were made with pure tryptophane.

2. 25 grams of pure tryptophane dissolved in 150 cc. of a 25 per cent solution of sulphuric acid were heated on a sand bath for forty-two hours. The acid was removed by baryta and after filtration of the barium sulphate, removal of the last trace of baryta or of acid, the liquid was evaporated to crystallizing point and 12.5 grams of tryptophane were recovered. The recovery of so much tryptophane showed that the decomposing action of the acid had been slight.

In two other experiments 5.5 and 10 grams of tryptophane, to each of which had been added 1 gram of ferric sulphate, were heated on sand baths with 100 cc. of a 25 per cent solution of sulphuric acid for twenty-one and twenty-four hours respectively. The reacting liquids became deeply pigmented and in neither case was it possible to recover any tryptophane nor was any indol derivative isolated from the gummy residues although they both gave a marked glyoxylic color reaction.

3. Small weighed amounts of pure tryptophane were treated (a) with 25 per cent sulphuric acid alone, (b) with 25 per cent sulphuric acid in presence of a small amount of ferric sulphate, and (c) with the acid in presence of small amounts of copper sulphate. At the end of the time allowed for the reaction the liquids were made slightly alkaline with baryta, filtered, the precipitates well washed, and the filtrate after the removal of the baryta was made up to a certain bulk (Solution A).

An aliquot part of Solution A was made 5 per cent acid and after filtration from barium sulphate was precipitated with mercury sulphate reagent. The mercury sulphate precipitate was treated in the usual way (page 378) and the resulting neutral solution made up to a known volume (Solution B) aliquot parts of which were treated with nascent bromine.

The other portion of Solution A was made slightly alkaline with baryta and steam distilled. Both distillate and residue were tested colorimetrically for the presence of indol derivatives; the tests were negative.

In some cases the bromine absorption of both Solutions A and B was estimated with a view to ascertaining whether after the hydrolysis of tryptophane with acid there were present in the digest substances which absorbed bromine but which were not precipitated by the mercury sulphate reagent.

An examination of Table V shows that the bromine capacity of Solution A when estimated directly is considerably higher than can be accounted for by the tryptophane precipitated as the mercury sulphate compound and estimated in Solution B. It is thus obvious that there are formed during an acid hydrolysis of tryptophane substances which absorb bromine but which are not precipitated by the mercury sulphate reagent.

It is also evident that acid alone has a slow action on pure tryptophane whereas in the presence of an oxygen carrier there is considerable decomposition of the substance: for the same period of time the effect of a salt of iron is somewhat more marked than that of a salt of copper. The rate of decomposition, however, does not provide a simple explanation of the disappearance of tryptophane in acid digests.

Some observers consider that during an acid hydrolysis of tryptophane an internal anhydride is formed, but from the evidence adduced in this paper (Section II) it is clear that so long as the indol ring remains intact, it should be precipitated by mercury sulphate reagent and should absorb bromine.

The results recorded above demonstrate the presence of substances in the hydrolysis mixture which absorb bromine but which are not precipitated by the mercury sulphate reagent. It may be that the tryptophane newly split off from the protein molecule is very susceptible to chemical reaction, and it is con-

TABLE V.

The Action of 25 per cent Sulphuric Acid on Pure Tryptophane.

Kind of metallic salt added.	Weight of tryptophane taken.	Weight of metallic salt added.	Time of hydrolysis.	Bromine absorption of *Solution A* measured *directly*.	Bromine absorption of the *tryptophane* content of Solution A (calculated from the absorption of bromine by Solution B).	Bromine absorbed by 1 gm. of tryptophane under the same conditions.	Tryptophane content of Solution A.	Loss of tryptophane during the hydrolysis.
	gm.	*gm.*	*hrs.*	*gm.*	*gm.*	*gm.*	*gm.*	*per cent*
(a) —	2	—	20	6.38	6.58	3.30	1.99	0.5
(b) $Fe_2(SO_4)_3$	1.5	0.15	17	4.70	3.78	3.30	1.14	23
"	2	0.2	40	4.84	3.84	3.30	1.16	42
"	1	1	40		0.74	3.00	0.25	75
(c) $CuSO_4$	1	1	40		1.05	3.00	0.35	65

Solution A was the hydrolysis mixture after the removal of acid and metallic salt.

Solution B was prepared from the decomposition of the mercury sulphate precipitate thrown down from an aliquot part of Solution A and represents the tryptophane content of Solution A.

ceivable that during an acid hydrolysis of protein some product of hydrolysis acts as an oxygen carrier, or it may be that the other amino-acids serve as catalysers and induce the destruction of the tryptophane as quickly as it is split off from the complex molecule of the protein.

b. The Action of Baryta on Pure Tryptophane.

Before the completion of this research Herzfeld[8] had published the results of his observations on the action of alkalis on proteins and on tryptophane itself. He hydrolyzed small quantities of protein and of tryptophane itself with different alkaline solutions. After distilling the hydrolysis mixtures, he estimated the indol in the distillate. His results showed that there is slight production of indol from the action of water, more from the action of 0.5 per cent sodium carbonate solution, and a considerable amount from that of a 9 per cent solution of caustic soda in presence of copper sulphate. He states that the indol in the distillates from the baryta hydrolysis is even less than from the hydrolysis with 0.5 per cent sodium carbonate.

My experiments on the action of baryta on tryptophane and on tryptophane in presence of ferric salts recorded in this paper had been done before the appearance of Herzfeld's paper. Since then I have also tried the action of baryta on tryptophane in presence of copper sulphate. The results of my experiments confirm the observation of Herzfeld, although he estimated the indol production, whereas I estimated the tryptophane content of the hydrolysis mixtures. It will be seen that after forty hours' hydrolysis there has been practically no loss of tryptophane, and even in the presence of salts of iron and copper the loss was not very marked.

As in previous cases the hydrolysis mixtures after being freed from baryta and from copper or iron were made up to a known volume (Solution A). An aliquot part of Solution A was precipitated with mercury sulphate reagent and the remainder was used for colorimetric tests.

The mercury sulphate precipitate was decomposed in the usual way and the filtrate made up to a known volume, Solution B. Part of Solution B was estimated for its tryptophane content by means of solutions of nascent bromine: the remainder was used for colorimetric tests.

Of the final solutions, that obtained from the hydrolysis with baryta in presence of copper was the only one which showed any indication of indol, but even so the intensity of the color reaction revealed the presence of traces only.

In other experiments the hydrolysis mixtures were not used for precipitation with the mercury reagent but were made slightly alkaline with baryta and distilled; the distillates were extracted with xylol and tested with *p*-dimethylaminobenzaldehyde; there was a distinct indol coloration but the intensity of color given by the xylol extract of the distillate obtained from one-half the total bulk of the hydrolysis mixture was not as great as that given by 0.005 gram of pure indol dissolved in xylol and treated with the reagent in the same way. It was also noticed that if the baryta were completely removed from the hydrolysis mixture and the latter then made alkaline with sodium carbonate and distilled, the intensity of the color reaction of the xylol extract of the distillate was much greater than in the previous case. This observation is also in confirmation of Herzfeld's statements.

It is obvious that at the temperature at which the hydrolysis of tryptophane with baryta was conducted, there was little production of indol. Colorimetric tests failed to reveal the presence of other indol derivatives in the hydrolysis mixtures or in the final solutions for estimation with bromine.

TABLE VI.

The Action of Baryta on Tryptophane.

Kind of metallic salt added.	Weight of tryptophane taken.	Weight of metallic salt added.	Time of hydrolysis.	Bromine absorption of Solution A calculated from that of Solution B.	Bromine absorption of 1 gm. tryptophane under the same conditions.	Tryptophane content of hydrolysi mixture.	Loss of tryptophane during hydrolysis.
	gm.	*gm.*	*hrs.*	*gm.*	*gm.*	*gm.*	*per cent*
—	1	—	20	3.48	3.30	1.06	—
—	0.5	—	40	1.50	3.00	0.50	—
$Fe_2(SO_4)_3$	1	1	40	2.53	3.00	0.84	16
$CuSO_4$	0.5	1	40	1.42	3.00	0.41	18

Solution A was the hydrolysis mixture after the removal of baryta and metallic salt.

Solution B was prepared from the decomposition of the mercury sulphate precipitate thrown down from an aliquot part of Solution A and represents the tryptophane content of Solution A.

From the results indicated in Table VI it is obvious that there is practically no loss of tryptophane during the hydrolysis with baryta even for forty hours. If the hydrolysis was conducted in the presence of a relatively large amount of metallic salts there was less than 20 per cent loss during the forty hours' hydrolysis.

SUMMARY.

The baryta hydrolysis of proteins, the precipitation of the tryptophane from the hydrolysis mixtures with mercury sulphate reagent, and the subsequent bromination of the tryptophane in solution after the decomposition of the mercury sulphate precipitate is advocated as a method for the estimation of the tryptophane content of proteins for the following reasons:

1. The ease with which the hydrolysis can be conducted.
2. The separation of the tryptophane from the other substances precipitated by the reagent is simplified by the fact that cystine is destroyed during an alkaline hydrolysis.
3. Prolongation of the time of hydrolysis does not lead to a destruction of the tryptophane split off from the protein complex.
4. The results obtained by this method for the tryptophane content of casein more nearly approximate those of Hopkins and Cole who actually isolated the tryptophane in a crystalline form than do those of other observers who have based their results on colorimetric methods.
5. The precipitation of tryptophane in solution and the estimation of the tryptophane content of the filtrate from the decomposition of the mercury compound by means of its bromine absorption are proving of great service and reliability in experiments now under progress in which the action of various tissue enzymes on tryptophane is being measured.

A criticism of the method, one shared by the methods of Fasal and Herzfeld and others, can be made, *viz.*, that the reaction used in the final determinations is not specific to tryptophane but is shared by other members of the indol group. In this case the bromine absorption of other indol derivatives present in the solutions, presumably formed from tryptophane, would not affect the final results to any appreciable extent. However, as a matter of experience, it has been found that the final solutions do not contain more than traces of indol derivatives and, further, that the results obtained indicate that there is no appreciable formation of substances from tryptophane which do not appear in the final solutions for estimation with bromine.

From these considerations we see that, although the value obtained for the tryptophane content of a protein may be somewhat lower than the true value, yet it can be taken as the lowest value assignable to the protein under consideration. Such values, though only approximate, may be of the utmost service in experiments on nutrition and in the preparation of synthetic diets.

Finally a separate series of experiments has shown the greater resistance of tryptophane to the action of baryta than to the action of acids with or without the presence of oxygen carriers.

THE RELATION BETWEEN THE ADMINISTRATION OF TRYPTOPHANE TO DOGS AND THE ELIMINATION OF KYNURENIC ACID IN THEIR URINE.

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It is known from the work of Willcock and Hopkins[1] and from that of Osborne and Mendel[2] that tryptophane is an essential constituent of diet for the maintenance of the life of an animal and it is generally accepted that there is some close connection between tryptophane and blood pigment although up to the present this conjecture has not received the support of experimental evidence.

The only direct indication of the fate of this substance in the animal body has been furnished by Ellinger[3] who showed that in dogs and in rabbits the excretion of kynurenic acid was increased by the administration of tryptophane by the mouth or by subcutaneous injection: in the human being there was no excretion of kynurenic acid after the administration of tryptophane by the mouth.

The formation of kynurenic acid from tryptophane presents an interesting problem, as it involves the conversion of an indol into a quinoline compound. Kynurenic acid has hitherto been regarded as γ-hydroxy-β-carboxytryptophane,[4] and both Ellinger and Dakin have suggested possible ways of formation of the acid from tryptophane. Ellinger regards indol glyoxylic acid as an intermediate stage in the conversion whereas Dakin considers the formation of indolpyruvic acid as a prelude to the opening of the pyrrol ring thus:

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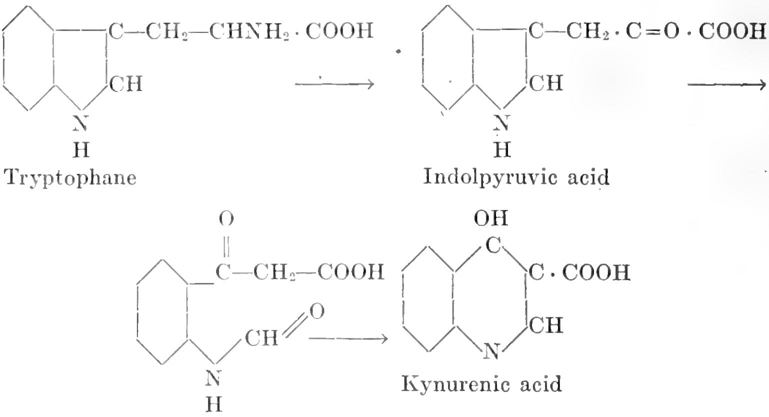
[1] Willcock, E. G., and Hopkins, F. G., *Jour. Physiol.*, 1906–07, xxxv, 88.

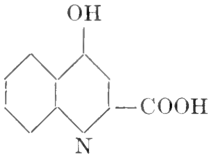
[2] Osborne, T. B., and Mendel, L. B., *Jour. Biol. Chem.*, 1914, xvii, 325.

[3] Ellinger, A., *Ztschr. f. physiol. Chem.*, 1904–05, xliii, 325.

[4] Camps, R., *ibid.*, 1901, xxxiii, 390.



But in view of the fact that kynurenic acid has been shown to be γ-hydroxy-α-carboxy quinoline[5]



these suggestions are no longer tenable. However, for the present the author is not prepared to offer any suggestion as to the method of formation of this compound from tryptophane.

Before proceeding to certain metabolism experiments, undertaken conjointly with Dr. Hopkins of Cambridge, England, in which it was desired to obtain some evidence of the way in which tryptophane becomes converted into kynurenic acid in the metabolic processes of the dog, it seemed advisable to ascertain whether the kynurenic acid eliminated represented a complete picture of the metabolism of tryptophane in the body or whether a certain amount of the tryptophane administered became "side-tracked" into kynurenic acid while the remainder was utilized in furnishing either the energy or the particular compounds necessary for the maintenance of the life of the individual.

With this object in view varying doses of tryptophane were administered to dogs of different sizes and ages and the output of

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[5] Homer, A., *Jour. Biol. Chem.*, 1914, xvii, 509.

kynurenic acid in the urine was estimated by Capaldi's method[6] as follows:

> The urine was treated with half its bulk of a 10 per cent solution of barium chloride to which had been added 5 per cent concentrated ammonia. After standing for half an hour the liquid was filtered and the filtrate evaporated to half the bulk of the original volume of urine taken for the estimation. The residual liquid was decomposed with a 4 per cent solution of hydrochloric acid and allowed to stand for a period of sixteen to twenty-four hours. The crystalline precipitate was filtered, washed with dilute hydrochloric acid, dissolved in ammonia, and the excess of ammonia removed by evaporation on a water bath. The solution of kynurenic acid in ammonia was then treated with a 4 per cent solution of hydrochloric acid and allowed to stand for six hours. The crystalline precipitate was filtered through a Gooch crucible, washed, dried at 110 C., and weighed.

In one series of determinations (Table I a) in which a dog was being fed on lean meat and in which, during the preliminary period the dog was excreting traces only of kynurenic acid, it was noticed that, in the period after the removal of the tryptophane from the diet, there was apparently an appreciable amount of kynurenic acid being excreted. An examination of this supposed kynurenic acid showed that the substance was practically pure uric acid.

It was evident that the determination of kynurenic acid in the urine by Capaldi's method could not be relied upon in presence of uric acid. In order to get a true idea of the amount of kynurenic acid in the urine it was necessary therefore, either to modify Capaldi's method so as to eliminate the possible contamination of the precipitate with uric acid, or to make separate estimations of the uric acid content of the Capaldi precipitates, or to choose a diet for the dogs on which they would excrete an amount of uric acid insufficient to interfere with the estimations.

In one series of experiments (Table I b) in which a dog on a meat diet was given constant doses of tryptophane, the kynurenic acid (admixed with uric acid) was estimated by Capaldi's method (pptes. A) and the uric acid content of the precipitate was estimated as follows:

> The kynurenic-uric acid precipitates (pptes. A) were dissolved in ammonia and the excess of ammonia was evaporated off on the water bath.

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[6] Capaldi, A., *Ztschr. f. physiol. Chem.*, 1897, xxiii, 92.

The solution was made slightly alkaline with ammonia and saturated with Kahlbaum's pure recrystallized ammonium chloride. The precipitated ammonium urate was decomposed and ultimately weighed on a Gooch crucible as uric acid.

At the same time determinations of the total amount of uric acid present in the urine were made by Hopkins' method in order to ascertain whether there was any simple relationship between the amount of uric acid present in the urine and that carried down with the Capaldi precipitates: there was none.

In view of the interference of uric acid in the estimation of kynurenic acid by Capaldi's method and the labor involved in the subsequent estimations of the uric acid content of the mixed precipitates of kynurenic and uric acids, it seemed worth while to determine whether uric acid was excreted to any appreciable extent in the urine of dogs on a diet of a lower purine value.

Of all the observers who have studied the question of the origin of kynurenic acid in the metabolic processes of the dog, amongst whom may be mentioned Liebig, Schmidt, Schmeiderberg, Schultzen, Hauser, Voit and Reiderens, and Solomin, the last mentioned alone seems to have recognized that uric acid was excreted by dogs in any appreciable amount.

Solomin[7] made determinations of the relative amounts of kynurenic acid and uric acid excreted by dogs on a diet of 400 gm. of horse flesh, 250 cc. of milk, and 250 gm. of bread a day. He determined the total output of uric acid by the Salkowski-Ludwig method and the kynurenic acid by Capaldi's method. For a dog of about 9 kg. weight the average output of uric acid was 0.11 gm. and of kynurenic acid 0.30 gm. a day.

It will be seen (Tables I a and I b) that the dogs on a diet of 500 grams of meat per day were excreting somewhat larger amounts of uric acid than were excreted by the dogs in Solomin's experiments. Further, it was found (Tables IV a, b, and c) that traces only of uric acid were excreted by dogs on a diet of bread and milk, of bread and milk and a small amount of meat (25 to 50 grams), of dog biscuits alone, and on a starvation diet.

If a dog can be induced to take any of these diets with regularity then Capaldi's method can be used for the estimation of the kynurenic acid content of the urine. The results thus obtained will give a true representation of the acid present and will not be too high on account of its contamination with uric acid.

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[7] Solomin, P., *ibid.*, 1897, xxiii, 501.

Since the presence of uric acid interferes with the estimation of kynurenic acid it was necessary to ascertain whether the estimation of uric acid by (a) Hopkins' and (b) by Folin's methods could be relied on in presence of kynurenic acid.

(a) Mixtures of uric and kynurenic acids were taken. The mixture of acids was dissolved in the least possible quantity of sodium carbonate solution and the uric acid estimated by Hopkins' method.

Mixture of acids.		Uric acid precipitated as ammonium urate and estimated as uric acid by	
Weight of uric acid taken.	Weight of kynurenic acid taken.	1. Titration with $KMnO_4$ solution.	2. Weighing on a Gooch crucible.
gm.	*gm.*	*gm.*	*gm.*
0.1209	0.1232	0.1202	
0.2458	0.1506	0.2432	
0.3406	0.1256		0.3320
0.1980	0.1720		0.1920

There was no indication of the contamination of the final precipitates of uric acid with kynurenic acid.

(b) Mixtures of uric and kynurenic acids were taken and the uric acid was estimated by Folin's colorimetric method.

Three separate solutions of weighed amounts of pure uric and kynurenic acids were made by dissolving the mixed acids in a solution of lithium carbonate and making the volume up to 100 cc. Aliquot parts of these solutions (2 cc.) were treated with Folin's phosphotungstic reagent and estimated colorimetrically against a standard solution of pure uric acid treated in the same way.

Mixture of acids.		Weight of uric acid in total volume of solution estimated colorimetrically by Folin's method.
Weight of uric acid taken.	Weight of kynurenic acid taken.	
gm.	*gm.*	*gm.*
0.100	0.100	0.100
0.100	0.100	0.998
0.100	0.100	0.101

From these results it is obvious that the presence of kynurenic acid does not interfere with the estimation of uric acid by Hopkins' and by Folin's methods.

In the accompanying tables are given the results of the estimations of the amount of kynurenic acid eliminated in the urine of dogs after the administration of tryptophane. Tables I a, b, and c deal with the administration of repeated similar doses of tryptophane to dogs and puppies; Tables II a, b, and c with the administration of increasing doses of tryptophane to dogs and to puppies and a consideration of the output of total nitrogen during the tryptophane feeding; Table III, with the administration of large doses of tryptophane; Tables IV a, b, and c with the excretion of uric acids by dogs on various diets.

Before attempting to draw any conclusion as to the significance of the data given in these tables with regard to the possibility of the elimination of kynurenic acid being of the nature of a side issue, and not representing the main line of breakdown of tryptophane in the animal body, it was necessary to ascertain whether kynurenic acid, once formed, could be further dealt with by the body: in other words, does the kynurenic acid isolated from the urine represent the total amount produced from the tryptophane absorbed?

Hauser[8] administering kynurenic acid to dogs by the mouth and by subcutaneous injection was able to recover in one case, 36 per cent and in another case, 56 per cent of the tryptophane given. He found that after the administration of the substance to man none could be recovered from the urine. On the other hand Solomin[7] found that there was as much as 90 per cent of the kynurenic acid administered by injection excreted in the unchanged form by dogs and 60 per cent by rabbits. The amount of the substance recovered after administration by the mouth was much less for both animals. In the case of man none or only a very small percentage of the substance could be recovered after administration by the mouth.

It is possible that the samples of kynurenic acid used by Solomin, isolated by Capaldi's method from the urine of dogs kept on a high meat diet for some time, were contaminated with uric acid. The administration of a "mixed" product would lead to results showing a higher loss than had actually taken place. In Hauser's experiments with dogs a considerable loss was shown; here again the investigator may not have been dealing with a pure product.

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[8] Hauser, A., *Arch. f. exper. Path. u. Pharmakol,* 1895, xxxvi, 1.

With Hauser's results indicating a possible utilization of at least 50 per cent of the kynurenic acid administered, it was necessary to ascertain whether, with pure kynurenic acid, the results would confirm those of Solomin or those of Hauser.

One of my dogs who had been on varying amounts of tryptophane was put on a diet on which he was excreting traces only of uric acid and no kynurenic acid. Doses of chemically pure kynurenic acid prepared according to the directions given in a previous paper (Homer)[5] were administered to the dog. The kynurenic acid content of the urine excreted during the thirty-six hours following the administration of the substance was estimated by Capaldi's method.

Weight of kynurenic acid administered.	Weight of kynurenic acid isolated from the urine by Capaldi's method.
gm.	*gm.*
0.690 (subcutaneously)	0.630
0.330 (by mouth)	0.266

These results support the view that the body of the dog does not utilize the kynurenic acid to any appreciable extent; whence it follows that, in the consideration of the experimental results recorded in the accompanying tables, the output of kynurenic acid can be taken as a true representation of the total amount of kynurenic acid formed by the body from the tryptophane administered to the animal.

A study of the accompanying tables shows that:

1. Kynurenic acid is not utilized by the body of the dog but is eliminated in the urine in an unchanged condition.

2. There is no simple relation between the amount of tryptophane administered and the kynurenic acid eliminated in the urine.

If the tryptophane were completely changed into kynurenic acid the proportion should be: 1 gram of tryptophane: 0.95 gram of kynurenic acid.

The greatest amount of kynurenic acid excreted by any of the dogs was only 38 per cent of the theoretical amount possible; the average was about 10 per cent and in some cases it was as low as or even lower than 5 per cent of the theoretical amount.

3. In each animal the excretion of kynurenic acid reached a maximum beyond which further increase in the dose of tryptophane was without effect.

The amount of tryptophane excreted by any one dog may depend upon the relative size of its liver. Thus a dog of 23½ pounds' weight excreted as much as 1.8 grams of kynurenic acid; a dog of 13½ pounds' weight excreted 0.64 gram of the acid; puppies of 6 pounds 14 ounces', and 4 pounds 5½ ounces' weight respectively excreted 0.3 and 0.13 gram of the acid on the same weights of tryptophane, etc.

4. The young animals have a greater capacity for the utilization of tryptophane than the fully grown dog has (Tables I c and II b).

5. There is no marked increase in the output of nitrogen during the tryptophane feeding period. The administration of the substance has therefore no marked effect on the general metabolic processes of the body (Table II c).

6. Even after flooding the digestive organs with a large dose of tryptophane, the corresponding amount of kynurenic acid was not eliminated; neither could any unchanged tryptophane be detected in the urine, nor could any other indol derivatives be detected in the urine by colorimetric methods (Table III).

SUMMARY.

The results of the investigation seem to indicate that:

1. Kynurenic acid apparently is not readily capable of further utilization in the body of the dog as all which is administered is excreted in the urine. It is therefore justifiable to assume that all the tryptophane converted into kynurenic acid in the body is excreted in the urine as such.

2. The production of kynurenic acid from tryptophane does not represent either the only or the main line of breakdown of tryptophane in the animal body; this is specially true of the young animal.

The evidence adduced in this paper seems to indicate that a certain amount of the tryptophane administered becomes "side-tracked" into kynurenic acid while the remainder is utilized in some direction not yet elucidated, but which may probably have

some intimate connection with the furnishing of the particular substance or substances necessary for the maintenance of life of the individual.

The work described in this paper was undertaken originally at the suggestion of Dr. Hopkins, Cambridge, England. The author wishes to express her appreciation of the help he has given and the interest he has shown during the progress of the investigation.

TABLE I a.

The Repeated Administration of Tryptophane.
Dog A. (Weight 13½ lbs.)

Date.	Diet.	Volume of urine.	Kynurenic acid in urine estimated by Capaldi's method.
		cc.	gm.
Mar. 31–Apr. 1	250 gm. lean meat	190	
Apr. 1– 2	" " " "	170	Traces.
2– 3	500 " " "	Not measured	
3– 4	500 gm. meat, 3 gm. tryptophane	400	0.648
4– 5	" " " " " "	475	0.512
5– 6	" " " " " "	253	0.578
6– 7	" " " " " "	300	0.640
7– 8	" " " " " "	339	0.625
8– 9	" " " " " "	250	0.540
9–10	" " " " " "	350	0.518
10–11	" " " " " "	400	0.542
11–12	" " " " " "	310	0.535
12–13	500 gm. lean meat	350	0.1403
13–14	" " " "	440	0.0877
14–15	" " " "	500	0.2025
15–16	" " " "	Not estimated	
16–17	" " " "	370	0.1072
17–18	" " " "	290	0.0920
18–19	" " " "	390	Spoilt.
19–20	" " " "	360	0.0982

It was shown afterwards that the precipitates weighed as kynurenic acid between the dates April 12–20 were practically pure uric acid.

TABLE Ib.

The Repeated Administration of Tryptophane to a Dog on a Meat Diet. Dog B. (Weight 23½ lbs.) Estimation in the Urine (1) of the Kynurenic Acid by Capaldi's Method. (2) Estimation of the Uric Acid Contaminating the Capaldi Precipitates and Hence the True Value for the Kynurenic Acid Output. (3) Estimation of the Uric Acid Content of the Urine by Hopkins' Method.

Date.	Diet.	Total volume of urine.	(1) Weight of *kynurenic acid* by Capaldi's method. *Pptes. A.* =kynurenic — uric acid.		(2) Kynurenic acid after deducting uric acid contaminating Pptes A.		(3) Total amount of *uric acid* in the urine estimated by Hopkins' method on 100 cc. of the urine.	
			(a) per cc.	(b) Total.	(a) per cc.	(b) Total.	(a) per cc.	(b) Total.
		cc.	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*
May								
21–22	500 gm. meat	288	0.000344	0.099	0.000277	0.080	0.000472	0.136
22–23	" "	380	0.000934	0.355	0.000724	0.275	0.000482	0.183
23–24	" "	340	0.00168	0.502	0.00130	0.441	0.000483	0.164
24 25	" "	544	0.00106	0.578	0.000901	0.491	0.000404	0.220
25–26	" "	464	0.00094	0.436	0.000728	0.338	0.000342	0.168
26–27	500 gm. meat and 5 gm. tryptophane	282	0.00218	0.707	0.00226	0.636	0.00056	0.155
27–28	" " " " "	349	0.00261	0.901	0.00300	0.832	0.000535	0.187
28–29	" " " " "	558	0.00349	1.946	0.00307	1.812	0.000571	0.319
29–30	" " " " "	322	0.00498	1.603	0.00487	1.573	0.000639	0.206
30–31	" " " " "	818	0.00221	1.804	0.00209	1.712	0.000309	0.253
June								
1–2	500 gm. meat	384	0.000810	0.247	Spoilt		0.000560	0.215
2–3	" "	366	0.000752	0.275	0.000511	0.187	0.000441	0.161
3–4	" "	316	0.000538	0.170	0.000332	0.105	0.000371	0.117
4–5	" "	461	0.000455	0.210	0.000276	0.127	0.000324	0.149
5–6	" "	338	0.000462	0.150	0.000248	0.0838	0.000426	0.143
			Mixed precipitates A (Capaldi)		*True value for kynurenic acid.*			

TABLE I c.

The Administration of Tryptophane to Puppies from the Same Litter, Aged 2 Months. Dogs C and D.

Date.	Diet.	Dog C. Weight 4 lbs. 5½ oz.		Dog D. Weight 6 lbs 14 oz.	
		Volume of urine.	Kynurenic acid excreted.	Volume of urine.	Kynurenic acid excreted.
July		*cc.*	*gm.*	*cc.*	*gm*
22–23	Bread and milk		Traces		Traces.
23–24	“ “ “		“		“
24–25	“ “ “		“		“
25–26	Bread and milk and 3 gm. tryptophane	290	0.0885	204	0.170
26–27	“ “ “ “ “ “	136	0.158	132	0.255
27–28	“ “ “ “ “ “	152	0.148	160	0.283
28–29	“ “ “ “ “ “	170	0.135	220	0.316
29–30	“ “ “ “ “ “	166	0.131	202	0.292
30–31	“ “ “ “ “ “	144	0.125	150	0.253
July 31–Aug. 1	“ “ “ “ “ “		Traces		Traces.
Aug. 1–2	“ “ “ “ “ “		Nil		Nil.

It was ascertained that on a diet of bread and milk there was no excretion of uric acid.

TABLE II a.

The Administration of Increasing Doses of Tryptophane to a Dog. Dog B. (Weight 23½ lbs.)

Date.	Diet.	Volume of urine.	Uric acid excreted.	Kynurenic acid excreted.
		cc.		*gm.*
Nov.				
18–19	Bread and milk and 50 gm. meat		Traces	Traces.
19–20	“ “ “ “ “ “ “		“	“
20–21	“ “ “ “ “ “ “	295	“	“
21–22	The same and 1 gm. tryptophane	305	“	0.0939
22–23	“ “ “ “ “ “	None		
23–24	The same and 2 gm. tryptophane	560	“	0.156
24–25	“ “ “ “ “ “	345	“	0.354
25–26	“ “ “ 4 “ “	None		
26–27	“ “ “ 6 “ “	180	“	0.224
27–28	“ “ “ “ “ “	500	“	0.679
28–29	Dog died			

A post mortem examination of the dog showed considerable hemolysis of the liver and kidney.

TABLE II b.

The Administration of Increasing Doses of Tryptophane to a Puppy Aged 5 Months. Dog C. (Weight 16 lbs.)

Date.	Diet.	Volume of urine.	Uric acid excreted.	Kynurenic acid excreted.
		cc.		*gm.*
Oct.				
27–28	Bread 150 gm., milk 250 cc., meat 25 gm.		Nil	Traces.
28–29	“ “ “ “ “ “ “ “ “		“	“
29–30	“ “ “ “ “ “ “ “ “		“	“
30–31	“ “ “ “ “ “ “ “ “		“	“
Oct.31–Nov. 1	The same and 1 gm. tryptophane	380	“	Spoilt.
1– 2	“ “ “ 2 “ “	255	“	0.1326
2– 3	“ “ “ 4 “ “	406	“	0.0854
3– 4	“ “ “ 6 “ “	134	“	0.0670
4– 5	“ “ “ 8 “ “	260	“	0.0359
5 - 6	Bread, milk, and meat	390	“	0.0156

TABLE II c.

Administration of Increasing Doses of Tryptophane. Estimation of the Total Output of Kynurenic Acid and of Nitrogen during the 24 Hour Periods. Dog F. (Weight 15 lbs.)

Date.	Diet.	Volume of urine.	Kynurenic acid excreted.	Total nitrogen (Kjeldahl).	Uric acid excreted.
		cc.	*gm.*	*gm.*	
Feb. 27–Mar. 4	150 gm. dog biscuits and 25 gm. meat daily		Not estimated		Nil.
Mar.					
4– 5	150 gm. biscuits and 25 gm. meat	132	0.0134	4.03	"
5– 6	" " " " "	128	0.0110	3.56	"
6– 7	" " " " "	218	0.100	5.21	"
7– 8	The same and 1 gm. tryptophane	188	0.0438	4.03	"
8– 9	" " " 2 " "	149	0.1880	4.40	"
9–10	" " " 3 " "	183	0.1994	4.17	"
10–11	" " " 4 " "	256	0.1736	4.05	"
11–12	" " " 5 " "	143	0.4332	4.16	"
12–13	" " " 6 " "	221	0.3038	5.20	"
13–14	" " " 7 " "	169	0.4120	4.46	"
14–15	150 gm. dog biscuits and 25 gm. meat	183	0.0048	4.58	"
15–16	" " " " " "	147	0.0078	5.06	"
16–17	" " " " " "	103	0.0060	2.38	"
17–18	" " " " " "	174	0.0074	4.71	"

There was no free tryptophane in the urine and no indication of the elimination of indol pigments.

1 gm. of kynurenic acid contains 0.074 gm. of nitrogen.
1 " " tryptophane " 0.134 " " "

TABLE III.

The Administration of Large Doses of Tryptophane to a Dog. Dog B. (Weight 23½ lbs.)

Date.	Diet.	Volume of urine.	Kynurenic acid excreted.
		cc.	*gm.*
Oct.			
13–14	Bread 250 gm., milk 250 cc., meat 50 gm.	540	Traces.
14–15	" " " " " " " " "	342	"
15–16	The same and 10 gm. tryptophane (Only about ⅓ of food eaten.)	66	0.212
16–17	The same and 10 gm. tryptophane	685	1.256

A further dose of 10 gm. of tryptophane was given with the dog's food on Oct. 17 but by this time the dog refused to touch any more food in the cage whether with or without the addition of tryptophane.

Tryptophane has a very bitter taste and it was difficult to administer as much as 10 gm. at a time and successfully mask its bitterness.

TABLE IV a.

The Excretion of Uric Acid by a Dog on a Meat Diet. (Compare with Results for Dog B, Table I b.) Dog H.

Date.	Diet.	Volume of Urine.	Total output of uric acid for the 24 hours estimated by	
			Folin's method on 5 cc. urine.	Hopkins' method on 100 cc. urine.
		cc.	*gm.*	*gm.*
Apr.				
1– 2	Biscuits	150	Traces	
2– 3	"	235	"	
3– 4	1 lb. lean meat	167	0.0729	
4– 5	" " " "	264	0.131	
5– 6	" " " "	160	0.0929	
6– 7	" " " "	150	0.0622	
7– 8	" " " "	175	0.155	0.160
8– 9	" " " "	324	0.239	0.250
9–10	" " " "	232	0.130	
10–11	Biscuits	52	0.0150	
11–12	"	160	Traces	

TABLE IV b.

The Excretion of Uric Acid by Dogs on Diets with Low Purine Value.

Date.	Diet.	Uric acid excreted.	Kynurenic acid excreted.
Dog A on diet of bread and milk for some days previous.			
June			
13–14	Bread and milk	Traces	0.0148
14–15	“ “ “	“	0.0083
15–16	“ “ “	“	Traces.
Dog B on diet of bread and milk and small amount of meat for some days previous.			
Nov.			
18–19	Bread, milk, and 50 gm. meat	Traces	Traces.
19–20	“ “ “ “ “ “	“	“
20–21	“ “ “ “ “ “	“	“
Dog E on diet of dog biscuits for some days previous.			
Dec.			
16–17	150 gm. dog biscuits	Traces	Traces.
17–18	“ “ “ “	“	“
18–19	“ “ “ “	“	“
Dog F on diet of dog biscuits and small amount of meat.			
Mar.			
4–5	150 gm. biscuits and 25 gm. meat	Traces	0.0134
5–6	“ “ “ “ “ “ “	“	0.0110
6–7	“ “ “ “ “ “ “	“	0.0110

TABLE IV c.

The Excretion of Uric Acid by a Dog Kept Without Food from Feb. 24 to Mar. 4. Dog G.

Date.	Volume of urine.	Total excretion of uric acid (estimated by Folin's method on 5 cc. of urine).
	cc.	*gm.*
Feb.		
24–25	410	0.013
25–26	516	0.019
26–27	344	0.030
27–28	710	0.032
Feb. 28–Mar. 1	260	0.031
Mar.		
1–2	452	0.023
2–3	398	None.
3–4	276	None.

THE DEXTROSE AND DIASTASE CONTENT OF THE BLOOD AS AFFECTED BY ETHER ANESTHESIA OF ANIMALS FED ON DIFFERENT DIETS.

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In 1904 Hawk[1] reported glycosuria following anesthesia of dogs. Seelig[2] confirmed his results. In 1914 Ross and Hawk[3] published work in which it was shown that ether anesthesia of dogs fed on a mixed diet was not followed by glycosuria.

Many suggestions have been advanced to explain this glycosuria. Shaffer[4] thinks it is probably due to asphyxia during the anesthesia. The work of Araki, Grube, and others[5] indicates that post anesthetic glycosuria may be due to a reduction of the body temperature during the anesthesia. But the fact that the glycosuria is just as pronounced in animals if the body temperature has been kept normal by artificial heat during the anesthetic period argues against this explanation. It is well known that asphyxia may cause glycosuria. Still this can not be an important factor since it has been shown that[3] there is no detectable difference in the glycosurias caused by the administration of ether well supplied with air and anesthesia when a very limited amount of air is mixed with the ether.

Post anesthetic glycosuria then is considered by some to be due to excitement; by others to the specific action of the ether; and by others to both of these factors. If both are involved the relative importance of each requires investigation.

In any glycosuria, either one of two changes may be immediately operative: (1) a change in the kidney itself, or (2) changes in the blood, the most important of which is a rise in the concentration of the sugar content. That post anesthetic glycosuria is

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[1] Hawk, P. B., *Am. Jour. Physiol.*, 1903-04, x, p. xxxvii.

[2] Seelig, A., *Arch. f. exper. Path. u. Pharmakol.*, 1905, lii, 481; 1906, liv, 206.

[3] Ross, E. L., and Hawk, P. B., *Arch. Int. Med.*, 1914, xiv, 779.

[4] Shaffer, P. A., *Jour. Biol. Chem.*, 1914, xix, 297.

[5] See review of literature in reference 3.

extremely transient argues against changes in the kidney, such as increased permeability. Therefore changes in the amount of sugar in the blood appear to be the most important immediate cause of sugar in the urine. Consequently it was thought that a study of the effect of anesthesia on the dextrose and diastase contents of the blood of dogs which had been fed on mixed and meat diets might throw some light on the mechanism of ether glycosuria.

EXPERIMENTAL WORK.

Healthy dogs were chosen for the work. Each animal was fed on a constant pure lean meat diet for from five to eight days and then anesthetized and the necessary samples of blood for analysis were taken. For an equal period the same dogs were then fed on a mixed diet which consisted of about three-quarters of the amount of meat of the preceding week, but to which were added 100 to 200 gm. of soda crackers per day. They were again anesthetized and samples taken for analysis as before. In several instances the animals died during the first anesthesia and consequently could not be used for the second. We have noticed that accidents under ether happen more frequently when the dogs are fed on a meat diet than on a mixed diet, an observation deserving notice in medical practice. The animals were anesthetized in the early forenoon in each case, and had been last fed the noon preceding.

The method of giving the ether provided an abundant admixture of air. This was accomplished by blowing air through a bottle of ether from which a tube led to a mask for the nose. No attempt was made to warm or to moisten the vapors passed to the animal.

Blood samples were taken from the jugular vein, just before the ether was administered and again after the animal had been tied on the board. Ether was then given and other samples of blood were taken after 15, 60, and 120 minutes of anesthesia. Another sample was taken thirty minutes after the ether was discontinued. Each time when blood was taken about 8 cc. were drawn in a clean syringe and quickly placed in a centrifuge tube. Then 8 cc. more were drawn into a syringe which contained a very small amount of powdered potassium oxalate. The first sample was centrifuged and the serum used for a determination of the diastase; the second sample was used for determination of the dextrose.

The method of determination of the diastase content of the serum was that of von Hess.[6] The Benedict colorimetric method[7] was used for the dextrose. All determinations of dextrose were made in duplicate and the average value was taken.

The dogs were weighed each day just before feeding. Tables I and II show the average weights in pounds, for the first two and the last two days

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[6] McGuigan, H., and von Hess, C. L., *Am. Jour. Physiol.*, 1914–15, xxxvi, 359.

[7] Lewis, R. C., and Benedict, S. R., *Jour. Biol. Chem.*, 1915, xx, 61.

of each period of feeding. The following tables give the results of the work done.

TABLE I.

Body Weights in Kilos While on a Mixed Diet.

No. of dog.	Average of first two days.	Average of last two days.	Gain or loss.
1	11.97	12.33	+0.36
2	9.49	9.45	−0.04
3	7.15	7.29	+0.14
5	10.84	10.22	−0.62
6	13.95	13.81	−0.14
8	9.90	9.90	0.00
9	7.38	7.56	+0.18
10	9.09	9.22	+0.13
11	10.57	10.80	+0.23
12	12.69	12.60	−0.09
Average			+0.01

TABLE II.

Body Weights in Kilos While on a Meat Diet.

No. of dog.	Average of first two days.	Average of last two days.	Gain or loss.
1	12.60	11.61	−0.99
2	9.76	9.54	−0.22
4	9.67	9.45	−0.22
5	9.54	9.45	−0.09
6	13.59	13.23	−0.36
7	12.37	11.56	−0.81
8	10.21	9.67	−0.54
13	13.95	12.96	−0.99
14	14.94	13.23	−1.71
15	11.25	10.35	−0.90
Average			−0.68

DISCUSSION.

There seem to be differences of opinion regarding the influence of anesthetics on the dextrose content of the blood. Macleod[8] claims that the changes are very slight and can be disregarded.

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[8] Macleod, J. J. R., Diabetes: Its Pathological Physiology, New York, 1913, 187.

Shaffer[4] reports considerable increase of blood sugar due to anesthesia. He shows increases of 100 to 300 per cent. The results given in Tables III, IV, and V agree with those of Shaffer. The animals on a mixed diet showed a sugar content from 0.058 to 0.085 per cent or an average of 0.075 per cent before ether. During the first fifteen minutes of surgical anesthesia the blood sugar content rose to from 0.077 to 0.156 per cent or an average of 0.117 per cent. The increase amounted to 56 per cent of the original content.

TABLE III.

Per cent of Dextrose in Blood of Dogs on a Mixed Diet.

No. of dog.	Before ether.	During anesthesia.			Half hour after ether was discontinued.
		During 1st 15 min.	End of 1 hr.	End of 2 hrs.	
1	0.082	0.114	0.093	0.080	0.088
2	0.076	0.109	0.172	0.179	0.109
3	0.058	0.114	0.147	0.138	0.224
5	0.085	0.128	0.106	0.138	0.170
6	0.076	0.110	0.123	0.157	0.109
8	0.079	0.119	0.106	0.167	0.134
9	0.055	0.077	0.138	0.130	0.122
10	0.085	0.109	0.118	0.116	0.126
11	0.082	0.156	0.164	.....	.....
12	0.072	0.137	0.139	0.128	0.169

TABLE IV.

Per cent of Dextrose in Blood of Dogs on a Lean Meat Diet.

No. of dog.	Before ether.	During anesthesia.			Half hour after ether was discontinued.
		During 1st 15 min.	End of 1 hr.	End of 2 hrs.	
1	0.153	0.200*	0.214*	0.221*	0.205*
2	0.081	0.163	0.163	0.175	0.203
4	0.127	0.160*	0.274*	0.300*	0.357*
5	0.091	0.109	0.165	0.179	0.185
6	0.048	0.103	0.114	0.196	0.185
7	0.082	0.062*	0.127	.....	.....
8	0.059	0.117	0.114	0.118	0.124
13	0.057	0.085	0.091	0.099	0.090
14	0.061	0.099	0.067*	0.092	.....
15	0.064	0.093	0.169	.....	.....

TABLE V.

Average Dextrose Content of Blood of Dogs on Different Diets.

	Before ether.	During anesthesia.			Half hour after ether was discontinued.
		During 1st 15 min.	End of 1 hr.	End of 2 hrs.	
Mixed diet. Results expressed in per cent	0.075	0.117	0.130	0.137	0.139
Meat diet. Results expressed in per cent	0.082	0.119	0.150	0.172	0.193
Meat diet. Results expressed in per cent with (*) results rejected from average	0.068	0.110	0.135	0.143	0.157
Mixed diet. Results expressed in relation to normal	1.0	1.56	1.73	1.83	1.85
Meat diet. Results expressed in relation to normal	1.0	1.45	1.83	2.10	2.34
Meat diet. Results expressed in relation to normal with (*) results rejected	1.0	1.62	1.99	2.10	2.31

After feeding with meat the sugar content of the normal blood varied from 0.048 to 0.153 per cent or an average of 0.082 per cent. After the animals had been under ether for about fifteen minutes the blood sugar content rose to from 0.062 to 0.200 per cent with an average of 0.119 per cent. The average increase in this case was 45 per cent. It may be claimed that the starred values in the tables are out of harmony with the majority. If we leave these out, before ether the values range from 0.048 to 0.091 per cent or an average of 0.068 per cent and after ether their range is from 0.085 to 0.163 per cent or an average of 0.110 per cent. The increase is then 62 per cent which is still larger than when all the figures were included.

It is clear that in our twenty cases there was, because of the anesthesia, a marked increase in the dextrose content of the blood. The changed diet seems to have shown no marked change in the normal blood or in the hyperglycemia of the first fifteen minutes of anesthesia. There does seem, however, to be some change in the general condition of the animal that makes it less resistant to the ether, and this becomes more pronounced the longer the period of anesthesia.

Without going into the individual values of the dextrose content of the blood in the periods following the first, averages only will be considered. The dogs on a mixed diet increased the blood sugar content from 0.117 per cent to 0.130 per cent in the first hour, from 0.130 to 0.137 per cent the second hour, and in the thirty minutes immediately following the removal of the ether from 0.137 to 0.139 per cent.

The animals on a meat diet during the first hour of ether, increased the blood sugar from 0.119 to 0.150 per cent, in the second hour from 0.150 to 0.172 per cent, and during the thirty minutes immediately after removal of the ether from 0.172 to 0.193 per cent. If the starred values are omitted from the averages the values for the meat-fed dogs are 0.110, 0.135, 0.143, and 0.157 per cent respectively.

If the per cent of dextrose be expressed in relation to the normal, valued as 1, the values for the successive periods for the dogs fed on a mixed diet are 1.0, 1.56, 1.73, 1.83, and 1.85. For the animals when fed on a meat diet the values are 1.0, 1.45, 1.83, 2.10, and 2.34, or omitting the starred values in the last, 1.0, 1.62, 1.99, 2.10, and 2.31.

From a consideration of the increase of the sugar following the first fifteen minutes of anesthesia several interesting facts are clear. First the increase is general and consistent, and still continues after cessation of the ether. Second, the increases on the meat diet were markedly more than on a mixed diet. The increase on the meat diet during the first hour was approximately equal to the increase on the mixed diet during the two and one-half hours.

The excitement factor in producing hyperglycemia, suggested by Shaffer, should grow less or disappear in two hours of anesthesia, and the longer the animal remain under the ether the more should the amount of blood sugar decrease. The opposite, however, occurs. The regular and gradual increase in the hyperglycemia argues strongly against preliminary excitement being an important factor in producing an increase in the blood sugar.[9]

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[9] Morita, S. (*Arch. f. exper. Path. u. Pharmakol.*, 1915, lxxviii, 188), comes to the same conclusion from work on decerebrate animals.

Often this hyperglycemia is attributed to asphyxia. The fact that both groups of animals show a positive increase in the blood sugar in the thirty minutes following the removal of the ether does not support this contention. Furthermore the fact[3] that an abundant supply of air with ether is just as productive of hyperglycemia as a minimal allowance also argues against it. However if Verworn's[10] theory, which holds that anesthesia is essentially an asphyxiation, can be sustained, the validity of our conclusions is questionable. In that case we must assume that after the removal of the ether the nervous system must return to normal much more quickly than the muscular or glandular tissues, an assumption we are not prepared to accept.

According to Carlson and Luckhardt[11] serum diastases are very slightly, if at all, decreased by ether anesthesia. Moeckel and Rost[12] conclude that cold increases the diastases. Allen[13] states that the nutritive condition of the animal has no influence on the diastases. Carlson and Luckhardt fed dogs on constant diets for four weeks; two on meat alone and two with bread and milk. They conclude that there is no relation in the diastatic content of the blood and the amount of carbohydrate in the diet.

Tables VI, VII, and VIII give the values of the serum diastases in minutes required for the diastases in 1 cc. of serum to hydrolyze the starch in 10 cc. of 1 per cent solution of soluble starch to such an extent that no blue color is produced by the addition of 1 cc. of 0.002 per cent iodine solution to one drop of the serum-starch mixture.

The values given in Table VI indicate a very wide individual variation of from 27 to 67. In this series the concentrations of the blood diastases of six dogs were not altered by the ether, one was increased, and three were decreased. These variations due to ether were small and insignificant in every case, as is shown by the table of averages, Table VIII.

The influence of ether on the blood diastase of meat-fed dogs is indicated in Table VII. The individual variation here is from 20 to 50. The diastatic activity after ether was unchanged in

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[10] Verworn, M., *Bull. Johns Hopkins Hosp.*, 1912, xxiii, 97.

[11] Carlson, A. J., and Luckhardt, A. B., *Am. Jour. Physiol.*, 1908-09, xxiii, 148.

[12] Moeckel, K., and Rost, F., *Ztschr. f. physiol. Chem.*, 1910, lxvii, 433.

[13] Allen, F. M., Studies Concerning Glycosuria and Diabetes, Boston. 1913, 113.

TABLE VI.

Diastase Content of Blood of Dogs on Mixed Diet.

No. of dog.	Before anesthesia.	During anesthesia.			Half hour after ether was discontinued.
		During 1st 15 min.	End of 1 hr.	End of 2 hrs.	
1........	35	35	35	35	35
2........	53	49	49	49	49
3........	60	60	67	67	67
5........	..	38	38	38	..
6........	39	39	39	39	39
8........	27	27	30	30	30
9........	43	43	43	43	43
10........	41	41	41	41	41
12........	37	37	40	..	..
13........	31	31	31	31	31

TABLE VII.

Diastase Content of Blood of Dogs on Lean Meat Diet.

No. of dog.	Before anesthesia.	During anesthesia.			Half hour after ether was discontinued.
		During 1st 15 min.	End of 1 hr.	End of 2 hrs.	
1........	24	24	30	30	30
2........	40	40	40	40	40
4........	40	40	40	40	40
5........	44	44	50	50	50
6........	20	20	20	20	20
7........	30	30	30	..	..
8........	31	31	34	34	..
13........	29	35	35	35	35
14........	34	34	34	34	..
15........	36	36	36	..	..

TABLE VIII.

Average Diastase Content of Blood of Dogs on Different Diets.

	Before anesthesia.	During anesthesia.			Half hour after ether was discontinued.
		During 1st 15 min.	End of 1 hr.	End of 2 hrs.	
Mixed diet..............	41	40	41	41	42
Meat diet...............	33	33	35	35	36

six cases or slightly decreased in four, the decrease being 33 to 35. This tendency toward a decrease is greater on a meat than on a mixed diet.

On studying the general trend of the values in Tables VI and VII it is apparent that there is a distinct range in each table. The averages from both are given in Table VIII, which shows a clear difference in the diastatic activity as a result of differences in diet. The meat diet has increased the blood diastases.

At this point it may be well to make a superficial study of the condition of dogs under the two diets as indicated from the tables of body weights given in Tables I and II. In the first table it is shown that one dog lost an appreciable amount and the others either gained or lost very little. The average for the ten dogs was 0.01 kilo per period. Table II shows that six out of ten dogs fed on a meat diet lost weight appreciably. The average loss of weight was 0.68 kilo per period. Judging from the variations in the body weights of the dogs, it is probable that the meat-fed animals were no more laden with glycogen than those on the mixed diet.

Macleod[14] asks: "How, then, are we to harmonize these apparently contradictory observations—*viz.*, that the glycogen rapidly disappears without there being any increase (indeed, in the case of the sugar-fed dogs, a decrease) in the percentage of reducing substance in the blood? We can do so only by assuming that the glycogen may leave the liver, ... in some other form than sugar—possibly as some dextrin, which is either precipitated along with the proteins or is unable to reduce cupric salts."

Our results indicate that there is an increase in the blood dextrose produced by ether anesthesia and that the increase is greater when the dogs have been fed on a meat diet. We also find that ether anesthesia has no effect on the serum diastase but that the absence of carbohydrate from the diet increases it.

It seems to us therefore that Dr. Macleod's problem quoted above may be more simply solved than he has suggested. Suppose that ether in itself is an activator or sets free an activator, which makes the glycogen of the liver more susceptible to attack by the diastase of the blood serum. Then ether anesthesia would

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[14] Macleod, *loc. cit.*, 190.

cause a disappearance of the glycogen from the liver and an increase of sugar in the blood. This accords with the facts. Then too if the blood diastases were increased ether would cause a proportionally greater hyperglycemia. This is also in accord with our data. Taking the diastase values before ether in Table VIII, there is found an increase of 24 per cent when the animals were changed to a meat diet. On referring to Table V, a hyperglycemia of 83 per cent is indicated in two hours, due to the ether, on a mixed diet, and a hyperglycemia of 110 per cent on a meat diet—or a difference of 32 per cent (of 83) due to the absence of carbohydrates from the diet. Therefore the increase in the sugar seems to follow the increase in the diastase; for when we increase the diastase 1.24 times we increase the extra sugar 1.32 times or approximately to the same degree. While this may be a coincidence, it seems more like a causative relation which requires consideration. It suggests the probability that in ether anesthesia the glycogen of the liver so changes that it is more readily acted on by the diastases which are common to both blood and liver.

SUMMARY AND CONCLUSIONS.

Ten dogs were fed on a mixed diet for a period of about a week. At the end of that time they were anesthetized for two hours. The dextrose and diastase contents of the blood were determined at intervals before and during the anesthesia. The animals were then fed on a meat diet for a similar period, and the sugar and dextrose determinations made as before, during and after ether anesthesia, in such a way that the concentration of each could be directly compared. The results indicate the following:

1. Ether anesthesia causes a hyperglycemia.
2. Ether causes a greater hyperglycemia in dogs which have been fed on a pure meat diet than on a diet including carbohydrate.
3. Ether itself, and not asphyxia or excitement, is responsible for the greater part of the blood sugar change.
4. Ether anesthesia does not increase the diastatic power of the blood serum.
5. The diastase concentration of the blood is on the average higher in animals which have been fed on a meat diet than on a mixed diet.

PEPTONE HYPOGLYCEMIA.

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Hypoglycemia has not been so frequently recorded nor has it been considered so important as hyperglycemia. The reason is very apparent. Diabetes is so common and so fatal a disease that all phases of it have attracted attention. When, therefore, it was found that the blood sugar is usually increased and that when it reaches a certain concentration it passes into the urine, the cause of the disease was thought to be closely associated with the blood. On the other hand we know of no disease where hypoglycemia has been considered so important as the hyperglycemia of diabetes. If increase in the blood sugar is at all important the measures which will cause a decrease must also be of importance. The methods which reduce the blood sugar are worthy of attention for the following reasons: First, they may illuminate the process of normal sugar metabolism; and second, they may reveal something of value in the treatment of perverted sugar metabolism. While such possibilities exist it must be confessed that all of the known methods of blood sugar reduction are brutal and of much more immediate injury to the animal than the most rapidly progressing hyperglycemia.[1]

Reduction of the sugar content of the blood is a rather difficult matter. The conditions that may cause a reduction of the normal sugar concentration are extreme exhaustion,[2] adrenal insufficiency,[3] Addison's disease,[4]

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[1] The treatment of diabetes recently advocated by Allen, F. M. (*Boston Med. and Surg. Jour.*, 1915, clxxii, 693–730), apparently does not deserve this condemnation.

[2] Weiland, W., *Deutsch. Arch. f. klin. Med.*, 1907–08, xcii, 223.

[3] Mayer, A., *Compt. rend. Soc. de biol.*, 1908, lxiv, 219.

[4] Porges, O., *Ztschr. f. klin. Med.*, 1910, lxix, 341; 1910, lxx, 243.

thyroid insufficiency,[5] poisoning by phosphorus[6] and hydrazine,[7] high section of the cord,[8] anaphylactic shock, intercurrent affections,[9] moribund states,[10] and the injection of foreign proteins. The striking physiological changes that result from the application of most of the methods are: low blood pressure, splanchnic dilation, depleted vitality, and, in larger doses, a moribund state. In none of them is there the remotest suggestion of anything that operates for the welfare of the organism. Various artificial hyperglycemias and glycosurias have been reported to be lessened by the administration of certain glandular extracts or salt solutions. Stenström[11] found that pituitrin lessened adrenalin hyperglycemia. Dresel[12] reports that extracts of the pituitary, thyroid, ovary, or pancreas when injected with epinephrin reduce the height of the hyperglycemia. Miculicich[13] found that hirudin inhibits and Glaessner and Pick[14] found that pancreatic juice and Witte's peptone lessen epinephrin glycosuria. Bock and Hoffman[15] showed that salt glycosuria is lessened by the further action of the injected saline. It is now known that calcium chloride will lessen salt glycosuria. Underhill[16] found that the glycosuria produced by morphine, pyridin, etc., is reduced by the free administration of oxygen. Other cases of the kind are reported by Allen.[17] It should be stated, however, that the whole subject is unsatisfactory and contradictory to such an extent that many substances are recorded by some to produce hyperglycemia or glycosuria, while according to others the same drug causes hypoglycemia. Phosphorus, salt solutions, and peptones are among such drugs. Also in most moribund states the blood sugar increases immediately before death.

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[5] Cushing, H., The Pituitary Body and Its Disorders, Philadelphia, 1910, 132 and 262.

[6] Frank, E., and Isaac, S., *Arch. f. exper. Path. u. Pharmakol.*, 1911, lxiv, 274.

[7] Underhill, F. P., *Jour. Biol. Chem.*, 1911–12, x, 159.

[8] Falta, W., quoted by Cammidge, P. J., Glycosuria and Allied Conditions, London, 1913, 144. Chauveau, A., and Kaufmann, M., *Compt. rend. Acad. d. sc.*, 1893, cxvi, 298, 551, and 613. Bernard, C., Leçons sur la physiologie et la pathologie du système nerveux, Paris, 1858, i, 466 and 482.

[9] Cammidge, *loc. cit.*, 180.

[10] Macleod, J. J. R., Diabetes: Its Pathological Physiology, New York, 1913, 58. Also Bock, C., and Hoffman, F. A., *Jahresb. f. Thierchem.*, 1874, iv, 440.

[11] Stenström, T., *Biochem. Ztschr.*, 1913–14, lviii, 472.

[12] Dresel, K., *Ztschr. f. exper. Path. u. Therap.*, 1914, xvi, 365.

[13] Miculicich, M., *Arch. f. exper. Path. u. Pharmakol.*, 1912, lxix, 128.

[14] Glaessner, K., and Pick, E. P., *Ztschr. f. exper. Path. u. Therap.*, 1909, vi, 313.

[15] Bock and Hoffman, Reichert and Du Bois-Reymond's *Arch. f. Anat.*, 1871, 550; *Jahresb. f. Thierchem.*, 1874, iv, 435.

[16] Underhill, F. P., *Jour. Biol. Chem.*, 1905–06, i, 113.

[17] Allen, Studies Concerning Glycosuria and Diabetes, Boston, 1913, 855ff.

In making blood transfusions from one normal animal to another we have noticed that there is a general tendency for the blood sugar of the recipient to fall. The reduction may be small in amount but at the same time it is suggestive. We have since found that this is a common result of the intravenous injection of proteins and especially of peptones. It is of this later action that we wish to report. Henderson and Underhill[18] have reported glycosuria following the injection of peptone. We can not confirm their results, and look upon the glycosuria they describe not as a peptone but as either an ether glycosuria or as an exception to the regular peptone action. Similar exceptions may be seen in those cases in which peptone hastens rather than retards the coagulation of the blood.[19] The whole subject of the action of sera, organ extracts, and toxins on the sugar content of the blood is in an unsatisfactory state, and the action perhaps varies with conditions. Our own work is concerned only with the action of peptone and it shows that the usual result of the intravenous injection of peptone, especially if it causes splanchnic dilation, is a fall in the level of the blood sugar.

In the present work we used Witte's peptone, and think that it is more active in causing a lowering of the blood sugar when it is dissolved in cold water and not boiled after solution. We obtained similar results with silk peptone and with gelatin; and in several cases of anaphylactic shock, which resembles peptone action, we found a hypoglycemia; not in all cases, however.[20]

METHODS.

To determine the action of peptone on the blood sugar our work may be divided into three parts:

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[18] Henderson, Y., and Underhill, F. P., *Am. Jour. Physiol.*, 1911, xxviii, 280.

[19] Stewart, G. N., A Manual of Physiology, Philadelphia, 5th edition, 1906, 47.

[20] Woodyatt's case of human transfusion may be cited to show the harmful influence of the intravenous injection of proteins. It also agrees with the work of Henderson and Underhill. At least part of the harmful result in this case may be attributed to the anesthetic (Woodyatt, R. T., and Raulston, B. O., *Jour. Biol. Chem.*, 1914, xvii, p. 1).

First: Experiments in which the dogs were first etherized and a sample of blood was taken for sugar analysis after which the peptone was immediately injected and the ether removed. In some cases blood samples were taken before etherization. The later samples were taken without the use of ether. If in all cases samples of blood had been taken before etherization the data would have been more complete. However, we know the general action of ether on the blood sugar, and the additional data that might have been gained would in no way affect the general conclusions. The results of this work, tabulated in Table I, show clearly that under these conditions peptones at least do not increase the sugar content of the blood. There is always an increase in the blood sugar under ether; if after the short period of etherization, which was never more than fifteen minutes in this work, the ether be removed, we must expect a rather sudden return of the sugar to the original level. If, however, peptone causes a great hyperglycemia, this drop should not take place, or at least the hyperglycemic state should soon be manifest. Clearly, such is not the case. Any rise in the sugar concentration under these conditions seems to be due to the ether.

Second: In this group of experiments, as recorded in Table II, no ether or other anesthetic was used. The normal sugar was determined immediately before the injection of the peptone and at intervals for several hours afterward. In about two hours after the injection the sugar content of the blood reaches a minimum. The usual result is a fall of the sugar to about one-half or one-third of the original. Between the injection of the peptone and the hypoglycemic state there may be an increase in the sugar concentration of the blood, but as a rule this is transient and insignificant, and due, we think, to asphyxial conditions. So far as we have found it has never led to glycosuria. In one extreme case the blood sugar rose to 0.45 per cent, but the rise could not be attributed to the peptone action, as the animal was moribund and the symptoms indicated asphyxia. A few other cases showed considerable rise from the same reasons. In those cases where any marked rise occurred the animal died in a short time. Such cases we consider moribund, and from them no legitimate conclusion can be drawn.

Third: Those cases in which the animal was kept under the influence of ether throughout the experiment. The results are summarized in Table III. This indicates that it is the ether and not the peptone that raises the sugar concentration. When the ether is removed the sugar falls; when it is again administered the sugar content rises. This last rise takes place notwithstanding that the animals after this amount of peptone are so depressed that their resistance is low and consequently they exhibit but little excitement. In all cases, ether is decidedly antagonistic to the action of peptone.

Method of Sugar Determination.—Between 5 and 10 gm. of blood were drawn from the femoral or jugular vein with a syringe. When it was thought that the concentration was low, slightly more blood was taken. On the average about 8 gm. were used. It was added directly to a weighed solution of sodium sulphate and acetic acid (crystalline sulphate 20 per cent and acetic acid 1 to 1.5 per cent) and again weighed. The precipitation was completed by heat, and the coagulum was filtered and washed with some of the sulphate acetic acid solution. About the same amount of solution was used in each case. Consequently the final volume was the same. The permanganate method of titration of the reduced copper from Fehling's solution was used and the permanganate solution was prepared so that 1 cc. represented 1 mg. of dextrose. The solution was standardized by adding a known solution of dextrose to the same volume of the acid-sulphate as was used to precipitate and to wash the blood proteins. This old method we believe has as yet no superior.

It is well known that if peptone be present in a solution of sugar which is to be determined by a Fehling solution, it will hold the reduced copper in solution. It is also known that it is very difficult to precipitate peptone and we do not think that this method could be relied on for this purpose. The solvent effect of even small amounts of peptone on cuprous oxide is enormous. For this reason it may be thought that the reduction of the sugar reported may be more apparent than real. We think the factor of peptone interference has been eliminated for the following reasons: Substances injected into the blood soon leave it except in the merest traces. We also tested the filtrate after the proteins were precipitated, and could never detect peptone by the ninhydrin and biuret tests, the limit of which is delicate enough to detect peptone present in sufficient concentration to interfere with sugar determination. Finally, the strongest argument against such an assumption is the fact that the tests show a larger amount of sugar one hour after the injection of the peptone than some hours later.

DISCUSSION.

Regarding the mechanism of the action of peptone or an explanation of its action we have only suggestions to offer. As pointed out above, the most striking common physiological change is splanchnic dilation. This, together with the well known action of albumoses and peptones on the liver, leads us to think that the fundamental cause is a change in the circulation of the liver. The whole action of the peptone when administered in the way stated is a condensed fatigue effect. The question of the glycogen in the liver is important. We determined this in three cases only. In these we found it to be 0.45, 1.91, and 3.18 per cent. The blood sugar in the last case was lower than in any of the others. From these facts it is evident that depletion of the liver glycogen is not essential to the hypoglycemia, although the average of the glycogen content seems to be diminished.[21]

CONCLUSION.

The intravenous injection of albumose and peptone in most cases causes a pronounced hypoglycemia. This action seems to be due to circulatory changes in the liver.

TABLE I.

Animals Given Ether Only during the Injection of Peptone—15 to 20 Minutes.

Date.	Weight of dog.	Weight of peptone.	Volume of water.	Blood sugar at beginning.	Blood sugar.		Blood sugar.	
						After		After
1914	*kg.*	*gm.*	*cc.*	*per cent*	*per cent*	*hrs.*	*per cent*	*hrs.*
Dec. 14	12.1	5	50	0.068	0.047	3	0.038	5
May 28	5.9	5	100	0.160	0.095	3		
June 19	16.6	10	100	0.054 before ether	0.197	15 min.	0.033	24
" 29	13.3	5	100	0.152	0.075	2		
July 15	10.3	6		0.061	0.048	24		
		Morphine	and ether					
May 25		5	100	0.104	0.049	15		
" 26		5	100	0.098	0.076	20		
June 18	16.0	10	100	0.072	0.077	2	Animal died during night.	
" 22	9.0	5	100	0.070	0.037	20		
" 24	10.5	5	30	0.088	0.199	1	0.024	3
" 26	10.5	6	50	0.070	0.098	1	0.030	3

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[21] Bock and Hoffman (reference 10) have shown the marked influence of disturbed liver circulation on the sugar concentration of the blood. Their animals, however, were moribund.

TABLE II.

No Anesthetic at Any Stage of the Experiment.

Date.	Weight of dog.	Weight of peptone.	Volume of water.	Blood sugar at beginning.	Blood sugar minimum.	Time after peptone to attain minimum blood sugar.	Maximum sugar.	Time of maximum.	
1915	*kg.*	*gm.*	*cc.*	*per cent*	*per cent*	*hrs.*	*per cent*	*hrs.*	*min.*
Jan. 23	11.9	3.5	15	0.104	0.027	3	0.140	2	0
“ 25	11.8	3.5	?	0.082	0.047	3			
“ 25	13.5	1.8	8	0.065	0.046	18	0.068		20
“ 25	13.9	1.9	10	0.066	0.030	4			
“ 30	10.2	2.0	15	0.060	0.040	3¼	0.040	3	15
Feb. 1	10.5	10.0	7	0.068	0.055	2½	0.068		
“ 3	10.2	2.0	10	0.094	0.063	2½	0.063	2	30
“ 4	10.2	5.0	12	0.060	0.047	5½	0.103	1	25
	Same dog								
“ 4	7.7	1.75	10	0.049	0.034	2½	0.051	1	
“ 5	7.7	3.0	14	0.047	0.015	2	0.044	4	
	Same dog								
“ 6	9.0	3.0	10	0.050	0.022	5	0.040	1	30
“ 13	9.0	5.0	17	0.088	0.240	2	0.450	2	30
	Same as Feb. 6								Dies from asphyxia.
“ 23	4.0	8.0	40	0.072	0.050	2	0.050	4	
Mar. 3	10.0	3.0	15	0.055	0.018	2½	0.096	1	30
“ 11	10.2	3.0	?	0.040	0.015	2½	0.092		35
Feb. 12	10.2	3.5	11	0.038	0.032	2			
Same dog as Feb. 3.									
1914									
Dec. 10	10.6	3.0	?	0.085	0.062	3			
“ 12	18.5	5.0	100	0.080	0.041	5			

TABLE III.

Ether throughout Experiment.

Date.	Weight of dog.	Weight of peptone.	Volume of water.	Sugar before ether.	Sugar immediately after ether.	Maximum sugar after peptone.	Time of maximum after peptone.	Minimum sugar.	Time of minimum after removal of ether.
	kg.	*gm.*	*cc.*	*per cent*	*per cent*	*per cent*	*hrs.*	*per cent*	*min.*
Feb. 9......	9.2	6	20		0.056			0.030	30
“ 10......	6.5	2	10	0.052	0.090	0.152	4	0.064	30
“ 11......	9.0	6	30	0.073	0.140	0.165	4	0.067	1 hr.
Jan. 3......	12.0	6	50		0.095	0.160	4½	0.055*	2 hrs.
May 7......	12.0	6	75	0.060	0.110	0.149	1	0.085	30

* In the middle of the experiment the ether was removed and the sugar fell to the low point given. Etherization again raised the sugar to the figure mentioned.

ON THE KYRINE FRACTION OBTAINED ON PARTIAL HYDROLYSIS OF PROTEINS.

SECOND COMMUNICATION.

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The significance of the theoretical assumptions based on Siegfried's original conception of the composition of kyrines has been discussed in an earlier paper.[1] According to Siegfried the kyrines are fragments of the protein molecule—polypeptides, resembling in every respect the natural protamines, and as such carry evidence in favor of the assumption that the molecule of every protein is built around a protamine nucleus. Levene and Birchard succeeded in fractionating the kyrine fraction of gelatin into two peptides, one containing in its molecule only arginine and the other only lysine. One of the two was obtained in a state of satisfactory purity. The work was since then extended to the study of the kyrine fraction of casein. The object of the work was twofold. First, it was realized that the kyrines obtained from other sources than gelatin might actually represent the large fragments of the structure assumed by Siegfried. On the other hand, it was possible that most kyrines were composed of simple basic peptides. In that case, a comparative study of their structure could be expected to furnish some information as to the distinctive structure of the molecule of individual proteins.

The procedure employed for fractionating the gelatin kyrine was applied in a general way also for fractionation of the substance obtained from casein. Only one additional step was introduced. In the earlier work the kyrine was separated into one part forming a silver complex insoluble in water, while the other part was solu-

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[1] Levene, P. A., and Birchard, F. J., *Jour. Biol. Chem.*, 1912–13, xiii, 277.

ble. In the present work each of the two fractions was further fractionated into two parts, the one forming a phosphotungstate soluble in hot water, the other a phosphotungstate which was insoluble. The details of the procedure remained as in the previous work.

From one of the fractions it was possible to obtain a sulphate which had all the macroscopic appearance and character of a crystalline substance. It was no longer hygroscopic as were Siegfried's kyrines, or the amorphous peptides. It was composed of long, glistening needles. Under the microscope they presented in part perfect prismatic needles, in part tapering formations resembling Lehmann's liquid crystals (Fig. 1). By means of fractional crystallization this substance could not be altered in its elementary composition nor in its ratio of $\frac{\text{amino N}}{\text{total N}}$.

The ratio prior to hydrolysis was 50 per cent, and after hydrolysis, 75 per cent.

Furthermore, it was found that all the basic nitrogen was present in the form of amino nitrogen, hence the peptide contained in its molecule only one basic substance: lysine. This assumption was substantiated by the fact that the lysine could be isolated in the form of its picrate in a quantity required by the theoretical assumption. Hence there remains no doubt that the substance was a tripeptide containing lysine, and two amino-acids, of which one did not contain a primary NH_2 group and therefore might have been either proline or oxyproline. The elementary analysis of the sulphate had a complete agreement with the assumption that the tripeptide was composed of lysine, oxyproline, and valine. The theory for this assumption requires $C = 42.20$, $H = 7.04$, $S = 7.03$, and the figures found by analysis were $C = 42.29$, $H = 6.97$, $S = 6.60$.

Unfortunately we were not equally successful in the isolation and in the identification of the amino-acids as we were in that of lysine. Valine was obtained once in a rather small quantity; oxyproline has not been identified up to the present. This perhaps is due to the fact that in its pure form the substance was available for hydrolysis in every experiment in a rather small quantity. Furthermore, the possibility is not excluded that the sulphate consisted of two, or of several, lysine peptides.

However, the principal fact was established, namely, that the casein kyrine can also be fractionated into simple peptides each containing in its molecule only one basic substance.

Fig. 1.

EXPERIMENTAL.

500 grams of casein were hydrolyzed with 5.5 liters of 12.5 per cent hydrochloric acid for five weeks at 40°C. At the end of that time the solution was filtered, diluted with water to twice its volume, and to the solution phosphotungstic acid was added as long as a precipitate formed. After standing in the refrigerator over night, the phosphotungstic precipitate was filtered off and washed repeatedly with cold 5 per cent sulphuric acid until the wash water was free from hydrochloric acid. The precipitate was

then dissolved in 5 liters of 10 per cent ammonia, the solution brought to a boil, and the phosphotungstic acid removed by means of finely powdered barium hydrate. The excess of barium hydrate was removed with carbon dioxide and the filtrate concentrated to a small volume.

This material was further fractionated by means of silver nitrate and barium hydrate.

The solution was first slightly acidified with dilute nitric acid and then silver nitrate was added in slight excess. To the solution barium hydrate was then added until no more precipitate was formed. Both filtrate and precipitate were acidified with sulphuric acid and decomposed with hydrogen sulphide, and, after filtering, neutralized with barium hydrate and concentrated. The peptides were precipitated with phosphotungstic acid and the precipitates freed from nitric acid by repeatedly washing with cold 5 per cent sulphuric acid.

The phosphotungstic acid precipitates were then extracted four times with large volumes of boiling hot water. The phosphotungstic acid was removed from the aqueous solution by lead acetate and the excess of lead by hydrogen sulphide. The phosphotungstic precipitates were dissolved in ammonia and freed from phosphotungstic acid in the way previously described. Thus four fractions were obtained, namely:

Silver precipitate............	1. Phosphotungstate soluble in hot water. 2. Phosphotungstate extracted with hot water.
Filtrate from silver nitrate treatment..................	3. Phosphotungstate soluble in hot water. 4. Phosphotungstate extracted with hot water.

Fraction 4, obtained after the treatment with silver nitrate and barium hydrate and extraction of its phosphotungstic precipitate with hot water, had the ratio of amino nitrogen to total nitrogen of about 1 to 3, which ratio did not change after repeatedly fractionating it by means of silver nitrate and barium hydrate.

The final solution was concentrated to a small volume, acidified with sulphuric acid, and precipitated in about 6 liters of 99.8 per cent alcohol. The precipitate of the sulphate was filtered off, redissolved in a small amount of water, and again precipitated in alcohol. It was found that on repeating the process the ratio of amino nitrogen to total nitrogen had increased after each precipitation until it reached the value of 0.50, after which two precipitations in alcohol did not give any marked change in the nitrogen ratio.

Amino Nitrogen Ratio in the Purified Sulphate.

The ratio of amino nitrogen to total nitrogen was determined according to the method of Van Slyke.

0.6000 gm. of sulphate was dissolved in 20 cc. of water.

Total N : 5 cc. of solution neutralized 10.6 cc. $\frac{N}{10}$ H_2SO_4. N = 14.84 mg.

Amino N : 5 cc. of solution gave 13.20 cc. gas at 23°, 768 mm. N = 7.47 mg.

$\frac{\text{Amino N}}{\text{Total N}}$ .... 50.5 per cent.

Analysis of the Substance.

I. 0.1098 gm. of substance gave 0.1664 gm. CO_2 and 0.0668 gm. H_2O.
0.0668 gm. of substance gave 0.0310 gm. $BaSO_4$.
II. 0.0929 gm. of substance gave 0.1450 gm. CO_2 and 0.0604 gm. H_2O.

	Calculated for $C_{16}H_{32}N_4SO_9$:	Found: I	II
C	42.20	42.29	42.58
H	7.04	6.97	7.33
S	7.03	6.60	

Hydrolysis: Nitrogen Distribution.

2 grams of the sulphate were hydrolyzed for twenty-four hours with 50 cc. of 20 per cent hydrochloric acid, the excess of hydrochloric acid was then distilled off *in vacuo*, and the solution made up to 100 cc.

Total N : 5 cc. of solution neutralized 6.95 cc. $\frac{N}{10}$ H_2SO_4. N = 9.73 mg.

Amino N : 5 cc. of solution gave 13.10 cc. gas at 22°, 763 mm. N = 7.40 mg.

$\frac{\text{Amino N}}{\text{Total N}}$ .... 76 per cent.

From this it is evident that after hydrolysis among the components there was present a substance containing nitrogen in a different form from a primary amino group. To that class belong proline, oxyproline, tryptophane, arginine, and histidine. Further hydrolysis has shown that all the basic nitrogen was composed of amino nitrogen, and hence was made up of lysine.

Hydrolysis Aiming to Show the Quantity of Lysine in the Peptide.

Another 20 grams of hydrolyzed sulphate were, after removal of the hydrochloric acid and sulphuric acid as before, made up to 500 cc., and 75 cc. of concentrated sulphuric acid and 1 liter of 10 per cent phosphotungstic acid solution added. After standing in the refrigerator the phosphotungstic precipitate was filtered off and the phosphotungstic acid removed in the usual way by means of barium hydrate.

Of the 100 cc. solution 2 cc. were taken, the ammonia was boiled off with calcium oxide, and the solution made up to 10 cc.

Total N : 5 cc. solution neutralized 3.10 cc. $\frac{N}{10}$ H_2SO_4. N = 4.34 mg.
Amino N : 2 cc. solution gave 3.10 cc. gas at 25°, 765 mm. N = 1.73 mg.

$\frac{\text{Amino N}}{\text{Total N}}$ .... 99.9 per cent.

Total 100 cc. contain 0.4320 gm. of amino N, corresponding to 2.27 gm. of lysine.

The solution was concentrated to 50 cc. and 3 grams of picric acid were added. On boiling, a precipitate was formed which was filtered off, washed with ether, and dried. Yield: 5.6 grams, corresponding to 2.3 grams of lysine.

0.1500 gm. of the picrate gave 20.20 cc. N gas in the Van Slyke apparatus, at 22°, 765 mm.

	Calculated for $C_6H_{14}N_2O_2.C_6H_2(NO_2)_3OH$:	Found:
N	7.80	7.65

Hence it is made clear that the substance contained only one base, and that this was lysine.

Analysis of the Mono-Amino-Acids.

10 grams of the sulphate were hydrolyzed by boiling for twenty-four hours with 200 cc. of 20 per cent hydrochloric acid; the sul-

phuric acid was removed with barium hydrate and the hydrochloric acid distilled off under diminished pressure. The remaining hydrochloric acid was removed by means of silver sulphate. The silver was removed finally with hydrogen sulphide and the sulphuric acid quantitatively with barium hydrate.

It was attempted to separate the amino-acids by converting them into the copper salts. This process was not successful. Hence the lysine was removed by means of phosphotungstic acid. The filtrate was freed from the excess phosphotungstic acid by means of barium hydrate. This again was removed quantitatively by means of sulphuric acid. The solution was concentrated to a very small volume. Absolute alcohol was then added to the solution. On standing, a precipitate formed which was filtered over a small Buchner funnel, washed with alcohol, and recrystallized from a minimal quantity of water. The yield was 0.200 gram.

Amino N : 0.020 gm. of substance gave 4.00 cc. gas at 21°, 768 mm. N = 2.28 mg.

The substance contained 6.37 per cent ash.

0.0970 gm. of substance gave 0.1841 gm. CO_2 and 0.0792 gm. H_2O.

	Calculated for $C_5H_{11}NO_2$:	Found:
C	51.30	51.68
H	9.40	9.11
Amino N	11.95	12.17

The substance had the following rotation:

$$[\alpha]_D^{20^\circ} = \frac{+0.70^\circ \times 2.8885}{0.5 \times 1.055 \times 0.1500} = +25.54^\circ$$

Attempt to Isolate α-Proline or Oxyproline.

The product of hydrolysis of 10 grams of the sulphate was precipitated with phosphotungstic acid and the filtrate freed from phosphotungstic acid with barium hydrate in the usual way. In the barium-free solution the amino-acids were converted into their copper salts. From the mixture α-proline could not be isolated. Hence the product was freed from copper, the solution was made up to a volume of 80 cc. to which 3 grams of barium hydrate were added, and heated in a sealed tube at 140-150° for five hours. The attempt to isolate the copper salt of the inactive α-proline was not successful.

In another experiment with 10 grams of the sulphate an attempt was made to isolate oxyproline. The product was treated with phosphotungstic acid, and the amino-acid fraction was converted into the copper salts. The process which had been successfully employed in this laboratory on a previous occasion was followed in this experiment.[2] The copper salts were fractionated by means of dilute alcohol (80 per cent) but no pure oxyproline could be isolated.

[2] Levene, P. A., and Beatty, W. A., *Ztschr. f. physiol. Chem.*, 1906, xlix, 256.

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SPECTROGRAPHIC STUDY OF AMINO-ACIDS AND POLYPEPTIDES.[1]

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The object of this investigation was twofold: (1) to gather spectrographic data with a view to using the same in the study of the constitution of proteins and their derivatives; and in particular (2) to throw light on the peptide linking; to ascertain for instance, whether the keto-enol tautomerism suggested by Dakin,[2] in explanation of the interesting and valuable phenomenon of the "racemization" of proteins, can be shown by spectrographic methods.

In regard to the first object, the results show that, with the exception of the aromatic derivatives phenylalanine and tyrosine, the amino-acids do not show any special absorption, it being general in the extreme ultraviolet. The same holds true with their alkaline or acid salts, *i.e.*, when they are dissolved in sodium hydroxide or hydrochloric acid. The results are in harmony with those of Hartley, Baly, Stewart, Hantzsch, Ley, Henri, and others who have shown that aliphatic acids,[3] alcohols,[3] esters,[4] amines,[5] and amides[5] produce no specific absorption, or in other words contain no chromophoric group.

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[1] Read before the Biological Section, American Chemical Society, Seattle, Wash., August, 1915.

[2] Dakin, H. D., *Jour. Biol. Chem.*, 1912–13. xiii, 357. Dakin, H. D., and Dudley, H. W., *ibid.*, 1913, xv, 263.

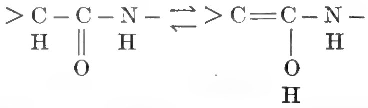
[3] Bielecki, J., and Henri, H., *Ber. d. deutsch. chem. Gesellsch.*, 1912, xlv, 2819.

[4] Bielecki and Henri, *ibid.*, 1913, xlvi, 1304.

[5] Bielecki and Henri, *Compt. rend. Soc. de biol.*, 1913, clvi, 1860.

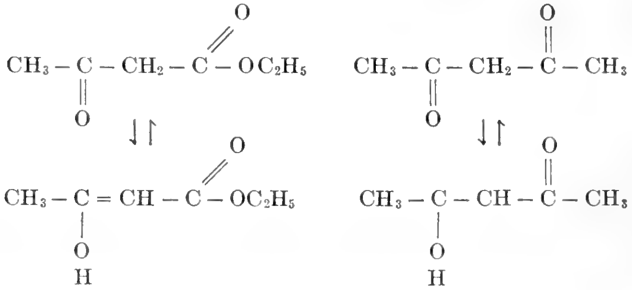
Qualitatively speaking, the absorption in the ultraviolet is of very little value, if any, for the aliphatic amino-acids, while for tyrosine and phenylalanine, the absorption is very marked, and may be useful in demonstrating the presence of such groups in protein derivatives, as for example, peptides. Quantitative results, which we were unable to make, owing to unavoidable delay in importing a sector photometer, will probably show greater usefulness.

On the other hand the equilibrium suggested by Dakin, according to previous work should give, if true, a specific absorption. Dakin's ideas are expressed by the reaction:



$$>\underset{H}{C}-\underset{O}{\underset{\|}{C}}-\underset{H}{N}- \rightleftharpoons >C{=}\underset{H}{\underset{O}{\underset{|}{C}}}-\underset{H}{N}-$$

which assumes that one of the hydrogen atoms of a carbon atom is labile. A similar tautomerism was studied by Baly and Desch[6] who had very good reasons for believing that the special absorption of ethyl acetoacetate and acetylacetone was due to a dynamic isomerism, between their keto and enol forms:



The explanation of this special absorption has since been somewhat modified, it being fairly well shown that it is not the oscillation of the hydrogen atom that causes the specific absorption, but the salts of these two forms, or the forms themselves.[7] These facts, together with the fact that acetone and similar substances having double bonds are chromophoric, would lead us to expect

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[6] Baly, E. C. C., and Desch, C. H., *Tr. Chem. Soc.*, 1904, lxxxv, 1029.

[7] Bielecki and Henri, *Compt. rend. Soc. de biol.*, 1913, clvi, 1322.

specific absorption in Dakin's "racemization" tautomerism. This expectation is increased by the fact that an excess of sodium hydroxide acts catalytically in Dakin's phenomenon as well as in the absorption of ethyl acetoacetate.

So far, however, we have found no absorption band which would support the tautomerism suggested by Dakin. It is possible that in other solvents and under more favorable conditions such specific absorption may be found, but it is not probable.

Owing to the importance of the matter, we should like to call attention to the following points, which, even though they have nothing to do with absorption, tend to show that Dakin's explanation of the facts is incorrect:

1. Dakin and Dudley state that their "racemized" protein is indigestible, *i.e.*, not digested by pepsin, trypsin, and erepsin; which they take as evidence of complete racemization. This seems very hard to understand, as it has been shown that *trypsin*[8] *digests many* and *erepsin*[9] *all racemic peptides*. The indigestibility seems to indicate more than racemization; it indicates a profound change in the protein molecule, either in the configuration other than optical, or in an extended adsorption of alkali. But if the racemization of their substance is complete, then there can be no question of an equilibrium and the equation becomes: active form → enol form → racemic form. This possibility would mean that there is a difference in chemical action between optically active and inactive substances, which would be the first instance on record. It is possible that Dakin and Dudley meant that the "racemized" protein[10] was more or less the enol form and that on hydrolysis equal quantities of optical antipodes were produced as a result of the law of chances. This would account for its indigestibility and other properties, but as stated above no evidence of such enol formation has yet been found.

2. Dakin and Dudley found that all amino-acids obtained from racemized proteins are not inactive, and explain this by assuming that those that are active are the ends of peptide chains, *i.e.*,

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[8] Fischer, E., and Abderhalden, E., *Ztschr. f. physiol. Chem.*, 1905, xlvi, 52.

[9] Abderhalden, E., London, E. S., and Voegtlin, C., *ibid.*, 1907, liii, 334.

[10] Their statement reads that *racemization*, and not enol formation, is complete.

their carboxyl groups are free in the original protein. This explanation seems purely hypothetical, as recent work shows that native proteins like gelatin and casein have very few, if any, free carboxyl groups. This heterodox statement is based on three sets of facts: (1) Cupric hydroxide reagent,[11] which is a very sensitive test for conjugated and unconjugated α- and β-amino-acids, does not show any free carboxyl groups in proteins. This does not prevent γ-amino-acids from being free and escaping detection with this reagent, but this is only possible in proteins when one of the carboxyl groups of glutamic acid[12] (γ position to the NH_2) would be free. This possibility would, however, only allow for one carboxyl group being free and not the one which, according to Dakin's hypothesis, would allow glutamic acid to escape enol tautomerism. (2) D. D. Van Slyke's reagent for free NH_2 groups showed in the proteins,[13] gelatin and casein, that only enough reaction is obtained to account for one NH_2 group, which seems to be that of the second NH_2 group of the diamino-acid, lysine. Van Slyke concludes that practically all amino groups are in peptide linkings. This agrees with the results of the copper method, that most of the carboxyl groups if not all, are conjugated. (3) The very careful work of L. L. Van Slyke[14] and collaborators with caseinates indicates that the number of carboxyl groups cannot be very large, probably not more than one or two. It is difficult to be certain, as salt formation of proteins with bases, without demonstrating the neutralization by its heat or otherwise, is no proof that carboxyl groups or replaceable hydrogen atoms are involved.[15] It is possible that amides, which are not detected by the copper method, or by Van Slyke's reagent, might escape enol tautomerism, and thus account for some of the active amino-acids, or it is possible that the alkali used by Dakin and Dudley increased the number of carboxyl groups through hydrolysis, but the finding of active amino-acids in "racemized" protein cannot be taken as a test for free carboxyl groups in the original protein.

[11] Kober, P. A., and Sugiura, K., *Jour. Am. Chem. Soc.*, 1913, xxxv, 1580.

[12] Kober and Sugiura, *loc. cit.*, 1573.

[13] Van Slyke, D. D., and Birchard, F. J., *Jour. Biol. Chem.*, 1913–14, xvi, 539.

[14] Van Slyke, L. L., and Bosworth, A. W., *ibid.*, 1913, xiv, 203, 207, 211, 227. Van Slyke, L. L., and Winter, O. B., *ibid.*, 1914, xvii, 287.

[15] Kober and Sugiura, *Am. Chem. Jour.*, 1912, xlviii, 384, 410.

The statement of Dakin and Dudley, that the racemization of amino-acids at a high temperature with alkali has nothing in common with the racemization of proteins at a lower temperature, seems not yet warranted, since the difference between the two processes may only be one of speed, due to a difference in temperature and other accelerating conditions. Dakin and Dudley required eighteen to twenty days for partial racemization of their substance at 40°C., whereas at 180°C. with barium hydroxide, 7 grams of valine[16] were racemized completely within twenty hours. Aspartic acid[17] was racemized completely with hydrochloric acid in several hours.

Our interest in the peptide linking, besides that in connection with the general constitution of proteins, was in reference to the salt or complex formation of the substances with cupric hydroxide. Dakin himself realized the effect of such an equilibrium on the constitution of the salts, stating that it would throw "light on the relative position of some groups in protein and peptide complexes." Dakin[18] supposed that the enol form would (as it is quite natural to suppose) make salts by the replacement of the labile hydrogen atom. The fact that dipeptides act as dibasic acids, *i.e.*, combine with one molecule of cupric hydroxide, in forming complexes, is in harmony with Dakin's hypothesis, but tri- and tetrapeptides also act as dipeptides, *i.e.*, combine with only one molecule of cupric hydroxide. Therefore if the enol form is responsible for the "dibasicity" of dipeptides, then tri- and tetrapeptides would have only one enol group, since they are also "dibasic." This would not harmonize at all with the facts obtained by Dakin and Dudley, that the majority of amino-acids in "racemized" proteins are inactive. On the basis of an extended study[19] of the copper complexes and the biuret reaction we find, however, that evidence of enol forms having a rôle in the formation of complexes is wholly lacking.

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[16] Ehrlich, F., and Wendel, A., *Biochem. Ztschr.*, 1908, viii, 399.

[17] Micheal, A., and Wing, J. F., *Ber. d. deutsch. chem. Gesellsch.*, 1884, xvii, pt. ii, 2984.

[18] Private communication.

[19] Kober and Sugiura, *Am. Chem. Jour.*, 1912, xlviii, 383. Kober, P. A., and Haw, A. B., *Proc. of Organic Sect., Am. Chem. Soc.*, Seattle, Wash., Aug., 1915.

SUMMARY.

1. The absorption of aliphatic amino-acids, in acid or alkaline solution, is only general in the extreme ultraviolet.

2. The aromatic amino-acids show absorption bands, which may be useful in detecting their presence in peptide chains.

3. The presence of an excess of alkali with these substances seems to increase the amount of absorption and tends to shift it towards the red end of the spectrum.

4. The spectrum of di- and tripeptides in acid or alkaline solution shows no special absorption, it being similar to that of the free amino-acids.

5. The lack of special absorption of the peptides in alkaline solution, and other points which were discussed, show that the keto-enol tautomerism in peptide linkings, as suggested by Dakin and Dudley in explanation of the "racemization" of proteins, is doubtful and unsupported.

EXPERIMENTAL.

Technique.

Instrument.—The Hilger[20] spectrograph, size *c*, was used in connection with a sparking outfit,[21] which consisted of a ¼ kilowatt rotary converter, transformer (giving about 15,000 volts), and condenser. This gave a strong spark between the electrodes, when they were one-fourth inch apart.

In order to eliminate fluctuations of the light, we had arranged in front of the slit a set of quartz prisms[22] so made that the light from the source was divided into two identical paths, one, however, going through the cell and the other going to the instrument, without the cell, as a control. When no absorbing medium was used, these two paths of light produced a photograph of the two spectra (to about 2000 Å. u.) in close juxtaposition, of equal intensity, so that any absorption in one of them was easily noticed. The correct position of the electrodes was determined and maintained as follows: A piece of paper was securely fastened under-

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[20] Made by Adam Hilger, Ltd., London.

[21] Made by Clapp-Eastham Co., Cambridge, Mass.

[22] Made especially for us by Adam Hilger, Ltd.

neath the source of light, on the table supporting the electrodes. Horizontal movements of the electrodes were then made and recorded, and photographs taken. The variations of heights were similarly recorded, by means of a plumb-bob held by a fixed metallic rack, fastened securely to the spectrograph. After the correct point was found, *i.e.*, the one giving the spectra from each of the two paths identically, the plumb-bob was fixed so that its end, when it was suspended, always gave the exact point for the source of light. The position of the cells (Baly's) so that they were axially centered was determined by a similar process. Noted positions were photographed until the correct one was found.

The electrodes were prepared according to Harry Jones[23] and the wave length of the lines used as standard was determined by comparison with a photograph of a cadmium spark taken on the same plate. The glass wave length scale was made after the instrument had been set up, from a photograph of an iron spark. The photographic plates were those of Wratten and Wainwright, ("Panchromatic A") 10 x 4 inches, which allowed fifteen exposures or double spectra on one plate. The exposures were each one and one-half minutes.

Solutions.—Stock solutions were made by dissolving 0.0500 gram of substance, either as a free base or as a salt, in water and making up to 100 cc., which gave a 0.0500 per cent solution. Alkaline solutions were made by taking 2.5 cc. of 2 N sodium hydroxide and making up to 50 cc. with stock solution. This gave a 0.0475 per cent solution of the substance in a $\frac{N}{10}$ solution of alkali. The acid solutions were similarly made $\frac{N}{10}$ with 2.5 cc. of 2 N hydrochloric acid.

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[23] Jones, H. C., *Carnegie Institution of Washington, Publication No. 110,* 1909, 9.

Amino-Acids.

Substance.	Wave lengths, at which edge of absorption is visible on photograph plate to naked eye, for different thicknesses of liquid.					
	1 mm.	5 mm.	10 mm.	20 mm.	40 mm.	
Glycine	245 μμ	248 μμ	252 μμ	259 μμ	269 μμ	
Glycine in 0.1 NaOH	226	250	257	269	280	
Glycine in 0.1 HCl	226	242	249	256	266	
Alanine	215	228	232	237	242	
Alanine in 0.1 NaOH	223	230	237	248	257	
Alanine in 0.1 HCl	234	243	244	245	254	
Amino-butyric acid	225	225	230	235	242	
Amino-butyric acid in 0.1 NaOH	230	233	237	246	256	
Amino-butyric acid in 0.1 HCl	232	242	248	249		
Valine	224	226	230	235	242	
Leucine	219	225	233	237	238	
Leucine in 0.1 NaOH	220	231	238	242	250	
Leucine in 0.1 HCl	221	226	230	235	244	
Aspartic acid	220	223	226	235	244	
Aspartic acid in 0.1 NaOH	220	229	235	245	255	
Aspartic acid in 0.1 HCl	220	227	231	234	246	
Asparagine in 0.1 NaOH	220	230	238	245	255	
Asparagine in 0.1 HCl	219	226	233	237	245	
Glutamic acid	224	227	231	233	238	
Glutamic acid in 0.1 NaOH	232	234	236	240	249	
Glutamic acid in 0.1 HCl	219	226	231	239	248	
Cystine in 0.1 NaOH	233	260	277	294	300	
Cystine in 0.1 HCl	231	252	270	289	300	
Histidine di-HCl	225	235	239	242	244	Absorption edges sharp.
Histidine di-HCl in 0.1 NaOH	230	239	244	250	256	
Histidine di-HCl in 0.1 HCl	230	236	241	244	245	
Arginine di-HNO_3	232	243	252	258	265	
Arginine di-HNO_3 in 0.1 NaOH	239	240	254	262	277	
Arginine di-HNO_3 in 0.1 HCl	242	254	260	270	280	
Phenylalanine	230	235 263	235 267	234 269	232 272	
Phenylalanine in 0.1 NaOH	232	239 268	238 270	238 272	238 276	

Amino-Acids—Concluded.

Substance.	Wave lengths, at which edge of absorption is visible on photograph plate to naked eye, for different thicknesses of liquid. $\mu\mu$				
	1 mm.	5 mm.	10 mm.	20 mm.	40 mm.
Phenylalanine in 0.1 HCl	227	236 269	236 270	236 271	236 274
Tyrosine	248	248 288	248 292	248 297	300
Tyrosine in 0.1 NaOH	269 310	268 317	320	324	328
Tyrosine in 0.1 HCl	248 287	248 291	248 295	248 299	300
Isoserine	224	227	233	242	258
Isoserine in 0.1 NaOH	223	235	247	255	290
Isoserine in 0.1 HCl	220	229	238	248	260

Dipeptides.

Alanyl-glycine	234	242	245	249	253
Alanyl-glycine in 0.1 NaOH (at once)	228	237	248	258	275
Alanyl-glycine in 0.1 NaOH (after 10 hrs.)	257	275	287	304	340
Alanyl-glycine in 0.1 HCl	224	234	242	250	255
d-Leucyl-*l*-leucine	256	258	261	265	274
d-Leucyl-*l*-leucine in 0.1 HCl	253	254	255	256	258
d-Leucyl-*l*-leucine in 0.1 NaOH (at once)	248	250	255	270	284
d-Leucyl-*l*-leucine in 0.1 NaOH (after 20 hrs.)	278	289	320	345	360
l-Alanyl-*d*-alanine	236	244	249	256	272
l-Alanyl-*d*-alanine in 0.1 HCl	235	244	250	258	274
l-Alanyl-*d*-alanine in 0.1 NaOH	246	253	264	272	285

Tripeptides.

Leucyl-alanyl-glycine	242	247	257	266	280
Leucyl-alanyl-glycine in 0.1 HCl	247	254	265	276	290
Leucyl-alanyl-glycine in 0.1 NaOH	245	260	272	298	323

STUDIES ON THE PHYSIOLOGICAL ACTION OF SOME PROTEIN DERIVATIVES.

I. ARE PROTEOSES PREPARED FROM ZEIN AND GLIADIN PHYSIOLOGICALLY ACTIVE?[1]

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(From the Sheffield Laboratory of Physiological Chemistry, Yale University, New Haven.)

(Received for publication, July 27, 1915.)

Two of the more important recent developments concerning the physiology of proteins have been the discovery of the phenomenon of anaphylaxis and the demonstration of the relation of the amino-acid content of the different proteins to their ability to meet the nitrogen requirements of the animal body. These properties have become so prominent in the minds of students of physiology that a number of long known reactions have been interpreted in terms of one or the other.

Under the stimulation of these new viewpoints the subject of "peptone" intoxication is receiving renewed attention. Anaphylactic shock and the physiological action of "peptone" are two varieties of protein intoxication. The exact relation of these reactions has been extensively studied by many investigators and many points of resemblance have been noted. Others have been interested in the amino-acid make-up of the protein in relation to the toxicity of derived cleavage products. Although several investigations on this subject exist, there is no unanimity of opinion and almost every study along this line points to a different amino-acid as the cause of the "peptone" effect. For the final solution of the problem renewed study from various viewpoints is essential. The first paper of this investigation is concerned with one phase of the question.

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[1] The data in this paper are taken from the dissertation presented by Byron M. Hendrix for the degree of Doctor of Philosophy, Yale University, 1915.

Nearly all of the early work (for literature see Underhill, '03) on the physiological action of proteoses was carried through with proteoses prepared from Witte's "peptone" or by using untreated Witte's "peptone." It is stated that this substance is made by digesting fibrin with pepsin and hydrochloric acid. The suggestion was made, especially in Hofmeister's laboratory, that the toxic body exists preformed in the animal tissues, and is added unchanged to the proteoses either by fibrin or the enzyme. Chittenden, Mendel, and McDermott ('98) investigated this hypothesis by employing proteoses which were made by using papain, a plant enzyme, as the proteolytic agent. They found the proteoses thus obtained were just as toxic as those made from Witte's "peptone." Chittenden, Mendel, and Henderson ('98) went a step further by using proteoses which had been prepared by employing acid or superheated water as the hydrolytic agent. These also were found to be toxic.

Pick and Spiro ('01) have held the view that the physiological action of the proteoses is not a property of pure proteoses, but is caused by an adhering toxic substance which they called "peptozyme." They believed that it is present in the animal tissues as a "peptozymogen." In support of their conclusions they claimed that casein and edestin do not yield poisonous proteoses under any circumstances, and that the products of tryptic or autolytic digestion, or of the hydrolysis by alkali are never toxic. They were able to remove a portion of the physiologically active substance from Witte's "peptone" by boiling with 50 per cent alcohol. Their digestion mixtures lost the poisonous properties when boiled with 50 per cent alcohol which had been made alkaline with sodium hydroxide. They regard the "peptozyme" as soluble in alcohol, but believe that it is destroyed by boiling with alkaline alcohol.

Underhill ('03) pointed out that Pick and Spiro had failed to separate the proteoses in their experiments and did not know whether the material which they injected contained a noticeable amount of proteoses. He also called attention to the hydrolytic action of sodium hydroxide on proteoses, stating that the loss of toxic action of their mixtures when treated with alkaline alcohol was undoubtedly caused by a destruction of the proteoses. In his own work Underhill was able to obtain toxic proteoses by digesting crystallized vegetable proteins with acid, superheated water, or vegetable enzymes, demonstrating beyond a doubt that poisonous proteoses are formed from pure protein. He injected the unchanged proteins and showed that they contained no adhering toxic impurity.

Popielski ('08, '09, '13) has continued to work with the alcoholic extracts of the commercial "peptone" preparations and of the separated proteoses. He regards the toxic portion of proteoses as a distinct compound to which he applies the name "vasodilatin." The chief chemical characteristics of this "compound" are its protein nature and its solubility in alcohol. There is little doubt that toxicity is exhibited by alcoholic extracts of proteose preparations, but inasmuch as certain of the proteoses are soluble in alcohol, this method of distinguishing the nature of the toxic substance is ineffective.

That an alcohol-soluble toxic substance can be prepared was conclusively proven by Vaughan ('13) and his coworkers. His method of preparation consists in boiling protein with 2 per cent sodium hydroxide dissolved in absolute alcohol. This treatment dissolves a portion of the protein. The soluble portion is very toxic, a few mg. being sufficient to kill a guinea pig. The "crude soluble poison" of Vaughan has many of the properties of proteoses, but is only partially precipitated by saturating the aqueous solution with ammonium sulphate. Both the precipitate and filtrate have a marked physiological action when administered intravenously.

The protamines were found by Thompson ('00) to have a physiological action similar to the proteoses. The protamines occur in combination with nucleic acids to form certain nucleo-proteins. They differ from other proteins in the high content of diamino-acids, relatively low molecular weight, and strongly basic character. Of the distinguishing chemical characteristics of the protamines, the only one which would seem sufficient to account for their toxic action, when introduced into the blood, is the large amount of diamino-acids in the molecule; but this peculiarity can not be the only one concerned in the physiological action, because the protamines lose their toxicity when hydrolyzed by acid.

Abderhalden ('12) has prepared a fourteen-amino-acid polypeptide consisting entirely of leucine and glycocoll which, he says, causes intoxication and death in guinea pigs which have received previous injections of the same substance. If the theory of anaphylaxis of Biedl and Kraus is accepted, this would indicate that the toxic material is even a simpler polypeptide than the one used by Abderhalden; for, according to this theory, the intoxication results from a substance formed from the injected material by parenteral digestion. It seems impossible for the "peptozyme" of Pick and Spiro to exist in the tissues if the toxic body is such a simple polypeptide as the work of Abderhalden suggests. His investigation also discredits the hypothesis that the diamino-acids are of importance in proteose intoxication.

The question of the specific amino-acid or group, responsible for the proteose action, has received much attention by recent workers, and the results are somewhat conflicting. Barger and Dale ('10–'11) and Dale and Laidlaw ('10–'11) have suggested β-iminazolylethylamine, a derivative of histidine, as a possible factor in this phenomenon. They were able to isolate it from extract of intestinal contents where it undoubtedly originated by protein putrefaction. β-Iminazolylethylamine causes a very distinct fall in arterial pressure, but has no effect on the coagulation of the blood. Heyde ('11–'12) claimed that methyl guanidine and guanidine when injected into the circulation produce all of the effects characteristic of proteoses, immunity to subsequent injections of proteose being a prominent feature. He believed that his experiments showed guanidine and methyl guanidine to be responsible for proteose intoxication. It is easy to see that these compounds may arise from arginine, but the cleavage of this amino-acid does not ordinarily occur during the course of proteolysis produced by enzymes or acids. If this were true the digestion products of protamines would be exceedingly toxic on account of the large amount of arginine in them.

The most recent work along this line is that of Knaffl-Lenz ('13). He claims that the typical proteose action is produced only when tryptophane is present in the molecule of the injected proteose. His conclusions were based on experiments with the digestion products of zein and gliadin. He found the digestion products of zein entirely inert when administered intravenously, but his gliadin product had a slight action, and other digestion mixtures were still more toxic. His experiments were not made with isolated proteoses, but with evaporated digestion mixtures. For this reason the results of his investigation are open to criticism as no data are given showing the actual amount of proteoses injected. Other investigators have always found the proteoses from zein to be toxic. Szumowski ('02) prepared a "soluble zein" by digestion with strong alkali, which gave a typical though diminished proteose action; Underhill ('03) found the zeoses toxic; and Wells and Osborne ('11) have shown that zein gives a typical anaphylactic reaction.

EXPERIMENTAL.

Are Zeoses and Gliadoses Physiologically Active?

Since the results of Knaffl-Lenz ('13) differ so greatly from those of previous workers, and the methods employed are open to so much criticism because of his failure to inject definitely isolated substances, the physiological action of zeoses has again been subjected to investigation.

Technique.

Dogs were used as experimental animals. They were made to fast for twenty-four hours before the experiment, and narcosis was produced by a subcutaneous injection of 10 milligrams of morphine sulphate and 1 of atropine sulphate per kilo of body weight. During the operation anesthesia was produced, whenever necessary, by means of the usual A.C.E. mixture. The blood pressure was recorded from the left carotid artery by a mercury manometer, the fluid in the connecting tube being a saturated solution of magnesium sulphate.

The proteoses which were dissolved in from 30 to 50 cc. (depending on the size of the dose) of physiological salt solution, to which a little sodium carbonate had been added, were injected into the right jugular vein. The injection never took longer than thirty seconds. Blood samples (2–3 cc.) from the right femoral artery were caught in small cylinders, a clean dry cannula being used for each sample. Blood was considered clotted when the containing cylinder could be inverted without the loss of a drop.

Preparations.

A portion of the zein used in these experiments was very kindly furnished by Dr. T. B. Osborne of the Connecticut Experiment Station, and the remainder was made in this laboratory by extracting corn gluten with boiling 85 per cent alcohol. The extract was filtered, poured into a large volume of cold salt solution, and allowed to stand over night. The precipitated zein was strained off and dried on a plate over a radiator. Both portions of zein were free from tryptophane as shown by the Hopkins-Cole test.

The zeoses were prepared by digesting zein with 2 per cent sulphuric acid in an autoclave for two hours. The liquid was then filtered off, a second quantity of the acid added, and the zein again digested for two hours. This process was repeated until most of the zein had gone into solution. The filtrates were finally united and neutralized with ammonia. The small amount of acid protein was removed by filtration, and the filtrate evaporated to a convenient volume. While still hot the solution was saturated with ammonium sulphate. The precipitated zeoses were dialyzed against running water, in the presence of toluene and chloroform, until free from sulphates. A small amount of heteroproteose was removed by filtration and the filtrate evaporated to dryness on a water bath. The residue, consisting of mixed proteoses, was a brownish material which could easily be reduced to a fine powder.

The gliadin used in preparing the gliadoses was obtained from flour, by first washing out most of the starch, drying, and then extracting with 75 per cent alcohol. The alcoholic extract was filtered and then poured into a large volume of salt solution from which the gliadin separated in doughy masses which adhered to the stirring rod and the sides of the vessel. The salt solution was poured off and the gliadin was dried on plates over a radiator.

In the preparation of gliadoses the method used in preparing zeoses was followed.

Experiments with Zeoses.

The following experiments show distinctly that zeoses have a typical though reduced physiological action. That immunity is produced by zeoses was shown by the resistance of the animal to subsequent injections of Witte's "peptone."

Zeoses Prepared by Acid Digestion of Zein.

Experiment 5.—May 16, 1914. Bitch, 8.2 kilos.

Injected 4.1 gm. zeose (0.5 gm. per kilo). Animal remained quiet during and after injection.

Normal clotting time of blood—8 minutes.

Clotting time of blood after injection.

Sample collected after	Clotting time.
3 minutes	3 hrs. 30 minutes
5 "	3 " 30 "
20 "	30 "
30 "	30 "
1 hr.	9 "

(After 1¼ hrs. 4.1 gm. Witte's "peptone"—0.5 gm. per kilo.)

5 minutes	47 minutes
10 "	12 "

Blood pressure in mm. mercury.

Before injection	128
1 minute after injection	38
12 minutes " "	92
16 " " "	118

(Witte's "peptone" injected.)

1 minute after injection	54
3 minutes " "	38
11 " " "	60

Zeoses Prepared by Acid Digestion of Zein.

Experiment 6.—May 26, 1914. Dog, 6 kilos.

Injected 1.8 gm. zeose (0.3 gm. per kilo). Animal remained quiet during and after injection.

Normal clotting time of blood—8 minutes.

Clotting time of blood after injection.

Sample collected after	Clotting time.
1 minute	12 minutes
3 minutes	7 "
10 "	11 "
20 "	9 "

Blood pressure in mm. mercury.

Before injection	130
30 seconds after injection	120
1 minute " "	90
5 minutes " "	110
9 " " "	130

Zeoses Prepared by Acid Digestion of Zein.

Experiment 40.—March 15, 1915. Dog, 8 kilos.

Injected 4 gm. zeose (0.5 gm. per kilo). Animal remained quiet during and after injection.

Normal clotting time of blood—11 minutes.

Clotting time of blood after injection.

Sample collected after	Clotting time.
2 minutes	39 minutes
6 "	43 "
14 "	25 "
23 "	6 "

Blood pressure in mm. mercury.

Before injection	150
1 minute after injection	90
3 minutes " "	120
7 " " "	150

The results of the experiment, in which only 0.3 gram per kilo of zeoses was given, resemble those of Knaffl-Lenz ('13) ("Versuche II, III"). This indicated that his digestion mixture contained, at most, a small amount of zeoses; for he used as much as 1.5 grams per kilo in one of his experiments. The only conclusion which is tenable is that Knaffl-Lenz failed to inject sufficient zeoses.

It is unquestionable that zeoses produce effects less marked than other typical proteoses. The reason for this is not known. Whether the diamino-acid content of a protein has anything to do with the toxicity of the proteoses derived from it has not been investigated. It is interesting to note, however, that the diamino-acid content of zein is less than that of any other true protein which has been studied. The toxic proteoses of Witte's "peptone" are relatively rich in these substances. Gelatin, on the other hand, contains a relatively large amount of diamino-acids and yields proteoses which are entirely without action. It contains very little histidine which may be of importance in this connection. Vaughan found that his "crude soluble poison," which is perhaps the most toxic cleavage product of protein yet prepared, contains only 1 to 2 per cent of diamino nitrogen.

Some preparations of racemized zein which have not been purified have a typical though less marked physiological action than similar material prepared from other proteins (see second paper, Experiment 8). This fact supports the contention that toxic cleavage products can be prepared from zein.

Knaffl-Lenz compared the action of his zein preparation with that of a similar one from gliadin, and found evidence supporting his statement concerning the toxic properties of tryptophane groups. Two experiments are given here to show the action of purified gliadoses.

Experiments with Gliadoses.

Gliadoses Prepared by Acid Digestion of Gliadin.

Experiment 23.—December 1, 1914. Bitch, 10.75 kilos.

Injected 5.37 gm. gliadose (0.5 gm. per kilo). Animal remained quiet during and after injection.

Normal clotting time of blood—7 minutes.

Clotting time of blood after injection.

Sample collected after	Clotting time.
2 minutes	*
5 "	*
30 "	*
1 hr.	*
2 hrs.	*
2½ "	**
4 "	15 minutes

Blood pressure in mm. mercury.

Before injection	130
1 minute after injection	64
3 minutes " "	42
6 " " "	58
8 " " "	94
10 " " "	134

* Not clotted 24 hrs. later.

** Found clotted 24 hrs. later.

Gliadoses Prepared by Acid Digestion of Gliadin.

Experiment 26.—December 8, 1914. Dog, 5.75 kilos.

Injected 1.75 gm. gliadose (0.3 gm. per kilo). Animal remained quiet during and after injection.

Normal clotting time of blood—8 minutes.

Clotting time of blood after injection.

Sample collected after	Clotting time.
2 minutes	*
8 "	*
20 "	*
40 "	*
1 hr.	**
1½ hrs.	5 hrs.
3 "	35 minutes

	Blood pressure in mm. mercury.
Before injection	98
1 minute after injection	34
3 minutes “ “	32
8 “ “ “	80
3 hrs. “ “	80

* Not clotted 24 hrs. later.

** Found clotted 24 hrs. later.

From Experiments 23 and 26 it appears that gliadoses have a very pronounced influence on the coagulability of the blood and a somewhat less marked action on the arterial pressure. When these results are compared with those of Knaffl-Lenz, it will be seen again that the purified proteoses have a stronger action than the digestion mixture which he employed; though there is less difference in regard to blood pressure than in the time required for the blood to clot. Very few records of the coagulation time are given by Knaffl-Lenz. In his "Versuch VI" he shows a typical fall in blood pressure after injecting his gliadin digestion mixture, but offers no data on clotting of the blood. He asserts that a large dose was given and that it acted like a Witte's "peptone" injection. This supports the idea that he might have obtained the typical proteose effect had he used an amount of his digestion mixture equivalent, in proteose content, to the ordinary dose of the purified product.

The gliadoses appear to have a somewhat less pronounced action on the blood pressure than such proteoses as those prepared from Witte's "peptone," but the effect on the coagulability of the blood appears to be just as marked.

CONCLUSIONS.

1. The intravenous injection of zeoses in relatively large doses (0.5 gram per kilo) causes a fall in arterial pressure and inhibits the coagulation of the blood.
2. Smaller doses are without marked effect.
3. Gliadoses have a very strong inhibiting action on the coagulation of the blood and a somewhat less marked effect on the arterial pressure.

4. The statement of Knaffl-Lenz that the presence of tryptophane in proteoses is responsible for their physiological action could not be confirmed.

5. The suggestion is made that the failure of Knaffl-Lenz to obtain characteristic effects with his preparations is probably due to the relatively small amount of proteoses contained in the digestion mixtures employed.

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STUDIES ON THE PHYSIOLOGICAL ACTION OF SOME PROTEIN DERIVATIVES.

II. THE RELATION OF RACEMIZATION TO THE PHYSIOLOGICAL ACTION OF PROTEINS AND PROTEOSES.[1]

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(Received for publication, July 27, 1915.)

The power of living organisms to distinguish between stereoisomeric compounds is a matter of common knowledge, but until recently the nature of racemized proteins had not been subjected to biological investigation. Inasmuch as these substances have been shown to differ from the native proteins in at least two fundamental biological properties it was deemed important to study the physiological action of racemized proteins and their cleavage products when injected into the circulation.

The racemization of proteins by alkali was accomplished by Kossel and Weiss ('09), but physiological studies were first made by Dakin and Dudley ('13). They prepared racemized casein by digesting this protein with half normal sodium hydroxide for three weeks at 37°C. The optical rotation of the solution changed from $-5.°$ to $-3.°$ and remained constant at the lower value. It was shown that racemized casein and caseoses are not affected by the proteolytic enzymes *in vitro* and can be completely recovered from the feces of a dog to which they have been fed.

Ten Broeck ('14) found that racemized egg albumin does not sensitize guinea pigs or rabbits to injections either of racemized egg albumin, or of the naturally occurring material, and animals sensitized to egg albumin do not show the symptoms of anaphylactic shock when an injection of racemized egg albumin is administered. He believes that these results support the enzyme theory of anaphylaxis.

Whether the change has been sufficient to destroy the power of the protein to yield a toxic cleavage product is not considered by

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[1] The data in this paper are taken from the dissertation presented by Byron M. Hendrix for the degree of Doctor of Philosophy, Yale University, 1915.

Dakin and Dudley ('13), or by Ten Broeck ('14). The following experiments were performed in hope of clearing up this point. Racemized casein, zein, and egg albumin were used, together with the proteoses formed from each of these during the process of racemization. Proteoses were also employed which were made from racemized casein by digestion with acid.

Technique.

These experiments were conducted under conditions similar to those of the preceding paper. The racemized proteins are rather insoluble; therefore, more sodium carbonate was used in dissolving them than was employed for the proteoses.

Preparations.

The racemization was carried out according to the methods of Dakin and Dudley and the same procedure was employed for all the proteins. A portion of the casein was the commercial Hammarsten product, and a second portion was prepared in the laboratory according to the method of Hammarsten. The egg albumin was the commercial material which had been purified by precipitation with ammonium sulphate after removal of the globulin by half saturation with the same salt. The purified albumin was separated from the adhering ammonium sulphate by dialysis. The zein used in these experiments was a portion of the material which was employed in preparing the zeoses described in the preceding paper.

The general description of the preparation of the racemized proteins follows. 500 gm. of protein were dissolved in 5 liters of half normal sodium hydroxide, and the solution was allowed to stand in a warm room for twenty-eight days when the polariscopic reading, in the case of casein, had changed from −5.° to −2.5°. The solution was then filtered, and carefully neutralized with acetic acid. The racemized protein separated out of the slightly acid solution in a gummy mass. The precipitate was removed from the acid solution, washed as thoroughly as possible, and dried on a plate over a radiator. The resulting material could be easily reduced to a fine brownish powder.

The filtrate from the racemized protein was saturated with ammonium sulphate and heated on a water bath for about thirty minutes. The precipitated proteoses were filtered and dialyzed against running water until free from sulphate. The dissolved proteoses were then filtered and evaporated to dryness on the water bath. Caseoses were also prepared by digesting racemized casein with acid.

Experiments with Crude Racemized Protein.[2]

Racemized Casein (Unpurified).

Experiment 7.—May 27, 1914. Dog, 14 kilos.

Injected 7 gm. crude racemized casein (0.5 gm. per kilo). Animal remained quiet during and after injection.

Normal clotting time of blood—7 minutes.

Clotting time of blood after injection.

Sample collected after	Clotting time.
2 minutes	7 hrs. 45 minutes
5 "	7 " 40 "
20 "	2 " 35 "
30 "	1 hr. 45 "
1 hr.	10 "

Blood pressure in mm. mercury.

Before injection	154
1 minute after injection	90
3 minutes " "	126
6 " " "	154

The dog was not immune to subsequent injections of Witte's "peptone."

Racemized Egg Albumin (Unpurified).

Experiment 9.—June 4, 1914. Dog, 11.5 kilos.

Injected 5.75 gm. crude racemized egg albumin (0.5 gm. per kilo). Animal remained quiet during and after injection.

Normal clotting time of blood—10 minutes.

Clotting time of blood after injection.

Sample collected after	Clotting time.
2 minutes	*
9 "	*
29 "	*
1 hr.	**
3 hrs.	**

Blood pressure in mm. mercury.

Before injection	104
30 seconds after injection	50
4½ minutes " "	74
10 " " "	104

* Not clotted 24 hrs. later.

** Found clotted 24 hrs. later.

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[2] These experiments are submitted as examples of the type of reaction produced. In order to save space a large number of duplicate experiments is omitted.

Racemized Zein (Unpurified).

Experiment 8.—June 1, 1914. Bitch, 5.5 kilos.

Injected 2.75 gm. crude racemized zein (0.5 gm. per kilo). Animal remained quiet during and after injection.

Normal clotting time of blood—3 minutes.

Clotting time of blood after injection.

Sample collected after	Clotting time.
3 minutes	1 hr. 35 minutes
5 "	1 " 30 "
10 "	1 " 30 "
30 "	49 "
1 hr.	19 "

Blood pressure in mm. mercury.

Before injection	120
1 minute after injection	60
2 minutes " "	100
5 " " "	120

Racemized Zein (Unpurified).

Experiment 39.—March 29, 1915. Dog, 11 kilos.

Injected 5.5 gm. of crude racemized zein (0.5 gm. per kilo). Animal remained quiet during and after injection.

Normal clotting time of blood—12 minutes.

Clotting time of blood after injection.

Sample collected after	Clotting time.
2 minutes	7 minutes
6 "	14 "
9 "	13 "

Blood pressure in mm. mercury.

Before injection	140
1 minute after injection	160

No further change in pressure.

These experiments indicate that racemized casein and racemized egg albumin are quite toxic. The action on the coagulation of the blood is more marked than on the arterial pressure. The racemized egg albumin had a more marked action than the racemized casein. This latter substance failed to produce immunity to Witte's "peptone."

The evidence of the physiological action of racemized zein is conflicting. Some of the experiments show that it has a slight poisonous action, while others indicate that it is without effect.

In any case the action is slight, perhaps depending on the sensitivity of the animal used in the experiment.

That proteins which are so little changed as racemized casein and egg albumin should have such a marked action, as shown by the preceding experiments, suggests that their action may be caused by some adhering proteose which was carried down with the protein in the process of precipitation. Purification of these products was carried out by dissolving in sodium carbonate and then reprecipitating them by making the solution acid with acetic acid. The precipitate was filtered and the process of dissolving and reprecipitating repeated four times.

That this process deprived the racemized proteins of their toxic properties is shown by the following experiments.

Racemized Casein (Purified).

Experiment 14.—October 24, 1914. Bitch, 16 kilos.

Injected 8 gm. of purified racemized casein (0.5 gm. per kilo). Animal remained quiet during and after injection.

Normal clotting time of blood—4 minutes.

Clotting time of blood after injection.

Sample collected after	Clotting time.
2 minutes	9 minutes
14 "	12 "
20 "	5 "
29 "	7 "

Blood pressure in mm. mercury.

Before injection	102
1 minute after injection	94
5 minutes " "	124

No change after 5 minutes.

Racemized Egg Albumin (Purified).

Experiment 15.—October 27, 1914. Dog, 12.5 kilos.

Injected 6.25 gm. of purified racemized egg albumin (0.5 gm. per kilo). Animal remained quiet during and after injection.

Normal clotting time of blood—4 minutes.

Clotting time of blood after injection.

Sample collected after	Clotting time.
2 minutes	12 minutes
5 "	14 "
10 "	10 "
16 "	10 "
34 "	8 "

Blood pressure in mm. mercury.

Before injection	84
1 minute after injection	36
6 minutes “ “	70
9 “ “ “	100

Racemized Zein (Purified).

Experiment 11.—October 9, 1914. Dog, 8 kilos.

Injected 4 gm. purified racemized zein (0.5 gm. per kilo). Slight respiratory disturbances during injection.

Normal clotting time of blood—7 minutes.

Clotting time of blood after injection.

Sample collected after	Clotting time.
2 minutes	17 minutes
5 “	15 “
11 “	15 “
16 “	10 “

Blood pressure in mm. mercury.

Before injection	135
1 minute after injection	56
4 minutes “ “	90
6 “ “ “	112

The toxicity of racemized casein and racemized egg albumin was greatly reduced if not entirely removed by the purification. No experiments with the purified racemized zein gave any more than the slightest action, showing that whatever poisonous properties it had possessed in the crude state had been removed by washing. The loss of the toxic action may have been due, either to the removal of some poisonous impurity, or to a change in the racemized proteins brought about by the action of the alkali and acid which were used in dissolving and reprecipitating.

In order to settle this point the filtrate from washing the racemized egg albumin was investigated. To this solution just enough sulphuric acid was added to replace the acetic acid, the solution was heated for several hours at a constant volume, and finally evaporated to dryness. The residue was extracted with 95 per cent alcohol in order to dissolve any alcohol-soluble toxic body contained in the mixture. The alcoholic extract was small. About 1.5 grams were used in the following experiment.

Alcohol-Soluble Washings from Racemized Egg Albumin.

Experiment 27.—December 17, 1914. Bitch, 4.6 kilos.

Injected approximately 1.5 gm. of alcohol-soluble washings from racemized egg albumin (0.3 gm. per kilo). Animal remained quiet during and after injection.

Normal clotting time of blood—5 minutes.

Clotting time of blood after injection.

Sample collected after	Clotting time.
2 minutes	7 hrs. 30 minutes
5 "	*
10 "	7 hrs. 30 minutes
30 "	4 "
1 hr.	1 hr.
1½ hrs.	40 minutes
2 "	35 "

Blood pressure in mm. mercury.

Before injection	114
1 minute after injection	98
2 minutes " "	30
9 " " "	35
30 " " "	62
40 " " "	75

*Found clotted 24 hrs. later.

This experiment shows that the racemized protein loses its poisonous properties when washed, not because the nature of the racemized protein is changed during the process of purification, but because the toxic portion is removed.

In order to get more evidence in this direction a portion of crude racemized casein was extracted with cold 95 per cent alcohol. This extract was evaporated to dryness, dissolved in physiological salt solution, and injected. The extracted racemized casein was also tested in the usual way.

Alcoholic Extract of Crude Racemized Casein.

Experiment 36.—March 2, 1915. Dog, 12.25 kilos.

Injected 4 gm. of alcohol-soluble portion of crude racemized casein (0.3 gm. per kilo). Animal remained quiet during and after injection.

Normal clotting time of blood—9 minutes.

Clotting time of blood after injection.

Sample collected after	Clotting time.
1 minute	*
5 minutes	*
15 “	*
29 “	*
50 “	*
1 hr. 20 minutes.	*
3 hrs.	*

Blood pressure in mm. mercury.

Before injection	150
1 minute after injection	70
3 minutes “ “	50
3 hrs. “ “	54

* Not clotted 24 hrs. later.

Crude Racemized Casein Washed with Alcohol.

Experiment 37.—March 5, 1915. Bitch, 13.5 kilos.

Injected 6.75 gm. alcohol-washed crude racemized casein (0.5 per kilo). Animal remained quiet during and after injection.

Normal clotting time of blood—12 minutes.

Clotting time of blood after injection.

Sample collected after	Clotting time.
2 minutes	1 hr. 8 minutes
5 “	2 hrs. 20 “
15 “	2 “ 5 “
30 “	1 hr. 15 “
1 hr. 25 minutes	30 minutes
1 “ 50 “	15 “

Blood pressure in mm. mercury.

Before injection	100
$\frac{1}{2}$ minute after injection	34
2 minutes “ “	24
10 “ “ “	34
1 hr. 10 minutes after injection	40*
2 hrs. 10 “ “ “	90

* The blood clotted in the cannula about an hour after the injection. Subsequent records of pressure are inaccurate.

While complete separation of the toxic substance was not accomplished by this washing, the portion soluble in alcohol has a much more marked physiological action than either the crude racemized casein (see Experiment 7), or the racemized casein which had been extracted with alcohol (see Experiment 37).

Since racemized proteins are not available for nutrition and do not give the anaphylactic reaction, it might be supposed that the proteoses formed by their digestion with acid would have a physiological action different from that of other proteoses. The following experiment demonstrates that caseoses prepared by acid digestion of racemized casein have the usual physiological action.

Caseoses Prepared by the Acid Digestion of Racemized Casein.

Experiment 2.—April 22, 1914. Dog, 12 kilos.

Injected 6 gm. of caseoses prepared by acid digestion of racemized casein (0.5 gm. per kilo). Animal gave a few cries after injection.

Normal clotting time of blood—7 minutes.

Clotting time of blood after injection.

Sample collected after	Clotting time.
3 minutes	*
8 "	*
18 "	*
38 "	*
1 hr. 8 minutes	*
1 " 54 "	*
3 hrs. 35 "	*

Blood pressure in mm. mercury.

Before injection	122
1 minute after injection	38
31 minutes " "	46
50 " " "	52
3 hrs. 30 minutes after injection	36

* Not clotted 24 hrs. later.

The preceding experiment shows clearly that proteoses prepared by acid hydrolysis of racemized proteins are just as toxic as those prepared from native proteins. Although the caseoses used in this experiment were obtained from unpurified racemized casein, their marked poisonous properties can hardly be ascribed to the impurities of the racemized casein.

The only physiological study of the properties of the proteoses formed during the racemization of protein is that of Dakin and Dudley, in which it was observed that these substances are resistant to the action of proteolytic enzymes. The following experiments were performed to determine whether such proteoses differ in toxic properties from the usual type.

Racemized Caseoses.

Experiment 33.—February 12, 1915. Dog, 13 kilos.

Injected 5 gm. racemized caseoses (0.4 gm. per kilo). Animal remained quiet during and after injection.

Normal clotting time of blood—6 minutes.

Clotting time of blood after injection.

Sample collected after	Clotting time.
2 minutes	*
5 “	*
47 “	*
1 hr. 30 minutes	**
1 “ 40 “	20 minutes
2 hrs. 39 “	12 “

Blood pressure in mm. mercury.

Before injection	96
1 minute after injection	26
3 minutes “ “	12
14 “ “ “	32
21 “ “ “	44
31 “ “ “	56
56 “ “ “	80

* Not clotted 24 hrs. later.

** Found clotted 24 hrs. later. Immunity to a subsequent injection of Witte's peptone was produced.

Racemized Egg Albumoses.

Experiment 32.—February 9, 1914. Bitch, 18.75 kilos.

Injected 9.37 gm. racemized albumoses (0.5 gm. per kilo). Animal remained quiet during and after injection.

Normal clotting time of blood—8 minutes.

Clotting time of blood after injection.

Sample collected after	Clotting time.
2 minutes	*
5 “	*
11 “	*
48 “	*
1 hr. 16 minutes	*
2 hrs. 16 “	*

Blood pressure in mm. mercury.

Before injection	140
1 minute after injection	30
4 minutes “ “	25
33 “ “ “	30
43 “ “ “	50
1 hr. 16 minutes	90
2 hrs. 16 “	110

* Not clotted 24 hrs. later.

Racemized Zeoses.

Experiment 22.—November 22, 1914. Dog, 3.5 kilos.

Injected 1.45 gm. racemized zeoses (0.5 gm. per kilo). Animal remained quiet during and after injection.

Normal clotting time of blood—10 minutes.

Clotting time of blood after injection.

Sample collected after	Clotting time.
2 minutes	18 minutes
5 "	15 "
11 "	10 "
16 "	11 "
21 "	10 "

Blood pressure in mm. mercury.

Before injection	60
15 seconds after injection	50
30 " " "	80
1 minute " "	60

These observations demonstrate that racemized caseoses and racemized albumoses in equivalent doses are nearly if not quite as toxic as other proteoses. With racemized zeoses the case is quite different, for out of several experiments performed not one showed any evidence of physiological action.

CONCLUSIONS.

1. Crude racemized proteins produce toxic symptoms when introduced into the circulation. The evidence in case of racemized zein is not decisive, inasmuch as some preparations exhibit typical effects, whereas with others no influence can be demonstrated.
2. Purified racemized proteins show no poisonous action.
3. The washings from crude racemized proteins contain a toxic substance.
4. A portion of the active substance can be removed from racemized proteins by extraction with alcohol. Efforts to remove the poisonous material completely by this means were unsuccessful.
5. Proteoses prepared by acid digestion of racemized proteins are probably as toxic as those prepared from the native proteins.
6. Of the racemized proteoses the caseoses and albumoses are quite toxic but the zeoses seem to be inert.

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STUDIES ON THE PHYSIOLOGICAL ACTION OF SOME PROTEIN DERIVATIVES.

III. THE PHYSIOLOGICAL ACTION OF VAUGHAN'S "CRUDE SOLUBLE POISON."[1]

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(Received for publication, July 27, 1915.)

Vaughan ('13) and his coworkers have shown that a very toxic body can be prepared by digesting any true protein with an alcoholic solution of sodium hydroxide. Evidences of toxicity of this substance of Vaughan's were yielded by subcutaneous injections into guinea pigs. The symptoms observed under these circumstances consist of very marked respiratory disturbances, struggles, followed by coma, and finally death. The picture is one of asphyxiation. The reaction is apparently identical with anaphylactic shock and proteose intoxication in these animals.

As far as we are aware the only experiments designed to determine the physiological action of Vaughan's "crude soluble poison" after injection into the blood of the dog were those of Edmunds ('13) who failed to notice any influence on the coagulation of the blood. These results are so different from those obtained in anaphylactic shock and proteose intoxication in this animal that a repetition of his experiments was thought to be imperative.

Preparation.

60 grams of Hammarsten's casein were boiled on a water bath with a liter of absolute alcohol in which 20 grams of dry sodium hydroxide had been dissolved, a reflux condenser being used to prevent evaporation. The digestion was continued for

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[1] The data in this paper are taken from the dissertation presented by Byron M. Hendrix for the degree of Doctor of Philosophy, Yale University, 1915.

two hours, and then the undissolved portion was subjected to a second digestion in the same manner. The united filtrates were neutralized with concentrated hydrochloric acid, care being taken to avoid an excess of the acid. The sodium chloride resulting was removed by filtration and the alcoholic solution evaporated to dryness over an incandescent electric light bulb. The brown residue was dissolved in a small amount of absolute alcohol, filtered, and again evaporated to dryness. A similar preparation was made from zein also.

Vaughan's "Crude Soluble Poison" (from Casein.)

Experiment 24.—Dec. 3, 1914. Bitch, 7 kilos.

Injected 3.5 gm. Vaughan's "crude soluble poison" (0.5 gm. per kilo). Animal remained quiet during and after injection.

Normal clotting time of blood—9 minutes.

Clotting time of blood after injection.

Sample collected after	Clotting time.
2 minutes	*
7 "	*

Blood pressure in mm. mercury.

Before injection	82
1 minute after injection	14
2 minutes " "	2
3 " " "	0

*Not clotted 24 hrs. later. Animal died in less than 4 minutes after injection.

Vaughan's "Crude Soluble Poison" (from Casein).

Experiment 25.—Dec. 5, 1914. Bitch, 15 kilos.

Injected 4.5 gm. Vaughan's "crude soluble poison" (0.3 gm. per kilo). Animal remained quiet during and after injection.

Normal clotting time of blood—7 minutes.

Clotting time of blood after injection.

Sample collected after	Clotting time.
2 minutes	*
5 "	*
7 "	*

Blood pressure in mm. mercury.

Before injection	100
1 minute after injection	28
3 minutes " "	16
7 " " "	4

*Not clotted 24 hrs. later. Animal died within 8 minutes after injection.

It is distinctly shown by Experiments 24 and 25 that Vaughan's "crude soluble poison" from casein has a more marked physiological action than any proteose employed in our experience. Death rapidly followed the injection, but it was possible to collect samples of blood which remained liquid for more than twenty-four hours.

With the product prepared from zein the effects while still comparable with those yielded by typical proteoses are not so pronounced as the reactions induced by the substance formed from casein (See Experiment 44).

Vaughan's "Crude Soluble Poison" (from Zein).

Experiment 44.—Dog, 14.0 kilos.

Injected 7 gm. (0.5 gm. per kilo) Vaughan's "crude soluble poison." Animal remained quiet during and after injection.

Normal clotting time of blood—7 minutes.

Clotting time of blood after injection.

Sample collected after	Clotting time.
2 minutes	*
5 "	*
12 "	*
21 "	*
40 "	*
55 "	*
1 hr. 30 min.	*

Blood pressure in mm. mercury.

Before injection	140
1 minute before injection	60
10 minutes " "	120
20 " " "	130
25 " " "	140

* Not clotted 24 hrs. later.

These observations differ from the findings of Edmunds who failed to observe any effect upon the clotting of the blood. The fact that our doses were somewhat larger than those of Edmunds may account for the difference in results.

It is well established that the intravenous injection of proteoses into rabbits fails to call forth the symptoms induced in the dog and cat except when very large doses are employed. We have taken advantage of this fact in order to determine whether the toxicity of Vaughan's "crude soluble poison" can be entirely accounted for by its proteose content or whether the poisonous properties must be assigned to some additional factor.

Experiment 45.—A rabbit was given through the marginal ear vein in about 2 minutes an injection of 0.6 gm. (0.2 gm. per kilo) of Vaughan's "crude soluble poison" prepared from casein. The animal struggled greatly and expired in less than 2 minutes after the completion of the injection.

Experiment 46.—To a pregnant rabbit was administered intravenously as above 0.075 gm. per kilo of the same product as that in Experiment 45. During and for some minutes after injection the animal cried and struggled but survived. The ear into which the injection was made became black and necrotic.

These experiments show conclusively that the effects brought about by Vaughan's product must be ascribed to some substance or substances other than the proteoses contained therein since the dosage employed here is far too small to produce any symptoms in the rabbit even though the entire amount of material injected consisted solely of proteoses.

We have attempted to differentiate the proteoses from the other toxic substances present in Vaughan's product by the destruction of the former, that is, by hydrolysis with acid beyond the biuret stage. Vaughan's product was boiled under a reflux condenser with a 3 per cent solution of hydrochloric acid until biuret-free. The acid solution was neutralized with sodium carbonate and injected into the circulation of the dog. As may be seen from the protocol of Experiment 47 no influence upon blood coagulation was noted. A distinct but transitory fall of pressure resulted which was also given by a second injection. This fall of pressure may be ascribed to the high sodium chloride content since the same strength of sodium chloride solution will bring about an entirely comparable fall in pressure. It is therefore obvious that the prolonged boiling of Vaughan's "crude soluble poison" destroys not only the proteoses but also the other unknown toxic principles.

Vaughan's "Crude Soluble Poison," Hydrolyzed with Acid to the Abiuret Stage.

Experiment 47.—June 14, 1915. Bitch, 5.5 kilos.

Injected 2.75 gm. (0.5 gm. per kilo) of Vaughan's product after digestion. Animal remained quiet during and after injection.

Normal clotting time of blood—7 minutes.

Clotting time of blood after injection.

Sample collected after	Clotting time.
2 minutes	2 minutes
5 "	2 "
8 "	2 "
(25 minutes after the first injection a second similar dose was given.)	
2 minutes	2 minutes
3 "	2 "
(5 minutes after the second injection 2 gm. of sodium chloride dissolved in 50 cc. of water were injected intravenously.)	
1 minute	2 minutes
3 minutes	2 "
5 "	2 "

Blood pressure in mm. mercury.

Before injection	160
½ minute after first injection	90
1 " " " "	80
2 minutes " " "	140
3 " " " "	165
Before second injection	140
½ minute after second injection	110
1 " " " "	90
2 minutes " " "	170
Before third injection (NaCl)	180
1 minute after third injection	120
2 minutes " " "	90

CONCLUSIONS.

1. Vaughan's preparation is much more toxic than Witte's "peptone."

2. The statement of Edmunds that Vaughan's "crude soluble poison" has no action on the coagulation of the blood is not confirmed. On the contrary, a very marked effect was noted.

3. In its action on blood pressure and on blood clotting, Vaughan's "crude soluble poison" strongly resembles the proteoses.

4. Vaughan's preparation differs from the proteoses in that it produces marked symptoms or even death in the rabbit in relatively small doses.

5. Boiling with dilute hydrochloric acid to the abiuret stage destroys the toxicity of Vaughan's product.

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DOES FAT FORMATION OCCUR IN THE PERFUSED KIDNEY?

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(Received for publication, July 27, 1915.)

The recent communications of Gross and Vorpahl[1] have opened for renewed discussion the long debated question of the formation of fat from protein, a controversy around which an enormous literature[2] has grown up without furnishing a decisive answer. In particular there has been involved the subject of fatty degeneration, in Virchow's sense, versus fatty infiltration. At the present moment it would seem that the consensus of opinion tends to discredit the probability of a direct formation of fat from protein, and the assertions of Gross and Vorpahl are therefore of particular interest. These investigators claim that during a long continued perfusion with Ringer's solution an increase of fat takes place in the cortex of the kidney as indicated both by microscopical and chemical methods. In these experiments only the cortex of the kidney was analyzed for fat. The fat content of this portion of the perfused kidney is not only relatively but may be absolutely greater than in the normal or unperfused organ, and the authors call attention to the fact that mere washing out of soluble substances by perfusion will not account for the augmented fat content. They conclude that the kidney cells are able to form fat from fatty acids derived from the protein of the kidney.

In view of the theoretical as well as the practical importance of the subject it seemed desirable that the experiments of Gross

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[1] Gross, O., and Vorpahl, F., *Arch. f. exper. Path. u. Pharmakol.*, 1914, lxxvi, 336; 1914, lxxvii, 317.

[2] For a discussion of the literature upon the subject see Wells, H. G., Chemical Pathology, Chicago, 1914.

and Vorpahl should be repeated. This has been done, the technique and methods employed being those advocated by the above mentioned investigators. The perfusion was carried on for a period of twenty-four hours, fat being estimated by the procedure of Kumagawa and Suto.[3] In our investigation the fat content was determined in the cortex alone and also was extended to a consideration of that contained in the *entire* kidney. The results obtained are detailed in Tables I and II.

It may be seen from the data that the results of Gross and Vorpahl are corroborated in almost every instance whether the fat is determined in the cortex only or in the whole organ. On the other hand if it is assumed that the two kidneys of a rabbit weigh equal amounts, an assumption which is approximately correct, the reference of the fat content of the perfused kidney to the dry weight of the unperfused organ would be of interest. With the figures obtained from the cortex of the kidney only, this procedure is open to criticism and much weight can not be placed upon the data. Applied to the entire kidney, however, a greater degree of accuracy may be expected. Under these circumstances it may be seen that both relatively and absolutely four experiments of the nine fail to corroborate the findings of Gross and Vorpahl, in other words the *percentile increase is an apparent rather than a real augmentation.* Even when the results are positive the figures obtained by this method of calculation show only small differences whether judged from the viewpoint of relative or absolute values.

Although our figures apparently support the views of Gross and Vorpahl one can see the possibility that an entirely different interpretation may be read into them—involving the question of difficulty of technique which applies especially to fat determinations in the cortex. After a kidney has been perfused for twenty-four hours it is edematous and very soft and the separation of the cortex from the medulla is accomplished with very great difficulty. The pelvis always contains appreciable amounts of fat, small quantities of which undoubtedly act as a source of contamination for the portion selected for analysis. Only small quantities of material are available in the rabbit's kidney; hence

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[3] Kumagawa, M., and Suto, K., *Biochem. Ztschr.*, 1908, viii, 212.

TABLE I.

Fat Content of the Cortices of Kidneys Perfused with Ringer's Solution Compared with That of the Normal Organ.

Experiment.	Normal kidney.					Perfused kidney.					Percentage of fat in perfused kidney compared with normal kidney.
	Weight.		Solids.	Fat.		Weight.		Solids.	Fat.		
	Moist.	Dry.		Weight.	Percentage.	Moist.	Dry.		Weight.	Percentage.	
	gm.	*gm*	*per cent*	*gm.*		*gm.*	*gm.*	*per cent*	*gm.*		
11	3.549	0.820	23.12	0.100	12.23	4.407	0.510	11.59	0.096	18.92	11.78
12	3.866	0.805	20.84	0.117	14.55	3.826	0.484	12.66	0.115	23.99	14.17
14	4.177	0.988	23.89	0.105	10.66	5.052	0.640	12.65	0.145	22.67	14.79
15	4.327	1.065	24.63	0.127	11.93	4.437	0.631	14.22	0.168	26.76	15.85
*15 A	4.117	0.879	21.38	0.108	12.34	4.681	0.686	14.65	0.120	19.06	13.73

* 15 A was not perfused but was merely suspended in Ringer's solution for 24 hrs.

TABLE II.

Fat Content of the Entire Kidney Perfused with Ringer's Solution Compared with That of the Normal Organ.

Experiment.	Normal kidney.					Perfused kidney.					Percentage of fat in perfused kidney compared with normal kidney.
	Weight.		Solids.	Fat.		Weight.		Solids.	Fat.		
	Moist.	Dry.		Weight.	Percentage.	Moist.	Dry.		Weight.	Percentage.	
	gm.	*gm.*	*per cent*	*gm.*		*gm.*	*gm.*	*per cent*	*gm.*		
1	7.290	1.528	20.95	0.163	10.72	7.410	0.725	9.78	0.156	21.57	10.23
2	6.126	1.251	20.43	0.144	11.55	6.995	1.182	16.89	0.217	18.42	17.38
3	5.494	1.157	21.06	0.181	15.64	6.319	0.926	14.66	0.189	20.41	16.34
4	6.009	1.302	21.68	0.194	14.95	10.767	1.159	10.76	0.150	12.96	11.53
6	6.766	1.496	22.11	0.137	9.18	9.974	1.203	12.06	0.174	14.50	11.60
7	7.078	1.369	19.34	0.157	11.53	8.382	0.982	11.74	0.151	15.40	11.05
8	3.819	0.964	25.24	0.120	12.52	5.198	0.689	13.26	0.101	14.72	10.52
9	6.576	1.374	20.89	0.171	12.45	8.894	1.120	12.59	0.177	15.82	12.91
10	5.730	1.182	20.64	0.148	12.54	5.069	0.649	12.79	0.155	23.86	13.18

even traces of fat adherence would be of significance. The determination of the fat content in the cortices of several kidneys as was done by Gross and Vorpahl in some of their experiments does not militate against our contention; for the same source of error is possible since each cortex must be separately isolated. A similar source of error involving the fat of the pelvis is also possible when the entire kidney is considered. We believe then that the possibility of the unavoidable inclusion of small quantities of kidney fat affords a reasonable explanation for the data obtained by Gross and Vorpahl and ourselves. It must also be admitted that a part of the apparent increase in fat content is caused by the washing out of substances from the kidney as may be seen in certain instances, especially with the entire kidney. This may be seen from an inspection of the solids present after perfusion.

Gross and Vorpahl ascribe to the kidney cells the capacity to form fat from fatty acids derived from protein, and the inference one gains from their papers is that the kidney cells under the nutritive influence of warm Ringer's solution remain alive and active for many hours, since their experiments were carried over periods of from twenty-four to forty-eight hours. From unpublished work upon kidney perfusion with Ringer's solution, even in the presence of oxygen, we are convinced that the renal cells lose all activity within a period of two to three hours. In our own investigation, although all possible precautions were taken, the kidney at the completion of a day's perfusion invariably was possessed of a more or less putrescent odor. We are also skeptical as to whether *perfusion* with Ringer's solution plays any rôle in the establishment of data like those obtained by Gross and Vorpahl and by ourselves, for the results in Table I, Experiment 15 A, were obtained from a kidney which was simply suspended in warm Ringer's solution for twenty-four hours. Under these conditions cellular activity must have ceased very rapidly. The figures show an apparent gain in fat content which we believe may readily be accounted for by the hypothesis advanced above.

SUMMARY.

If warm Ringer's solution is perfused through the kidney of the rabbit for a period of twenty-four hours there may be a percentile or an absolute increase in the amount of fat contained in the cortex of the kidney or of the entire organ. These results confirm those of Gross and Vorpahl.

Unlike Gross and Vorpahl, who cite these findings as evidence for the formation of fat from protein, we believe that the data may be interpreted merely as an expression of the mechanical difficulty of preventing contamination with fat of that portion of material to be analyzed.

Doubt is also expressed concerning the influence of the perfusion with Ringer's solution, for a kidney simply suspended in this medium may yield results entirely comparable with those obtained after perfusion.

THE CHARACTER OF THE WATER-SOLUBLE NITROGEN OF SOME COMMON FEEDINGSTUFFS.[1]

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(Received for publication, July 26, 1915.)

Since Stutzer[2] in 1880 proposed his copper hydroxide method for the estimation of the "amide" nitrogen in feeding materials there has been so much progress in protein chemistry that one is surprised to find that more modern methods have not been applied to our common feedingstuffs for the estimation of free acid-amide, amino-acid, and ammonia nitrogen, thereby giving us a more perfect picture of the nitrogen distribution in such materials.

It is certain from experimental evidence to be presented later that copper hydroxide does not make a complete precipitation of all protein and polypeptide structures and thereby give a clean quantitative separation of "amide" nitrogen from protein nitrogen. It is also evident that the so called "amide" nitrogen, or what is not precipitated by the copper hydrate, is a mixture of acid-amide nitrogen, amino-acid nitrogen, and ammonia nitrogen. There may still be other forms of nitrogen present in this filtrate, but since clean cut methods are now available for direct measurement in the feedingstuff of the forms of nitrogen mentioned, it is evident that data should be accumulated with these newer and more precise methods. We are certain that amino-acid nitrogen has direct nutritive value, while we are not so certain that ammonia nitrogen or acid-amide nitrogen have any value in normal protein storage. As fast as accurate methods become available we should discontinue the differentiation of

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[1] Published with the permission of the Director of the Agricultural Experiment Station.

[2] Stutzer, N., *Jour. Landw.*, 1880, xxviii, 103.

forms of nitrogen in our feeding materials by a method which makes no attempt at differentiation on the basis of nutritive value. Grindley and associates[3] have recently published preliminary data on the estimation of lysine, arginine, histidine, and cystine by Van Slyke's method, involving the crude feeding material in direct acid hydrolysis. Whether accurate determinations of any or all amino-acids can be secured when the hydrolyzing proteins are in contact with hydrolyzing carbohydrates, etc., must first be determined before these data can be accepted as final. The very fact that there is no approximate agreement between the results secured by Grindley on cottonseed meal and those of Nollau[4] on the same material indicates inherent difficulties in the quantitative estimation of amino-acids by direct hydrolysis of the feedingstuff. For example, Grindley reports 19.5 per cent of the total nitrogen in cottonseed meal as arginine nitrogen and 4.7 per cent as lysine nitrogen, while Nollau reports 12.7 per cent arginine nitrogen and 1.9 per cent lysine nitrogen. Is it possible that different samples of the same feed will vary so widely in their protein make-up? It appears more than probable that condensation of nitrogenous complexes with derivatives of hydrolyzing carbohydrates may arise, leading to complete failure in separating and detecting certain amino-acids or at least grossly vitiating their quantitative determination. For example, Nollau reports the complete absence of histidine in the cow-pea while as a matter of fact Osborne[5] isolated 3.08 per cent of histidine from vignin, the globulin of that seed.

Nollau further reports no proline, oxyproline, and tryptophane in corn meal. This again indicates gross inaccuracies in the method of analysis, because if corn meal contains no tryptophane animals confined to it as the sole source of the nutrients probably could not grow. The necessity of tryptophane in the diet for maintenance and growth has been emphasized by the work of Osborne and Mendel,[6] and Hart and McCollum[7] have shown that

[3] Grindley, H. S., Joseph, W. E., and Slater, M. E., *Jour. Am. Chem. Soc.*, 1915, xxxvii, 1778.

[4] Nollau, E. H., *Jour. Biol. Chem.*, 1915, xxi, 611.

[5] Osborne, T. B., and Heyl, F. W., *Am. Jour. Physiol.*, 1908, xxii, 362.

[6] Osborne, T. B., and Mendel, L. B., *Jour. Biol. Chem.*, 1914, xvii, 325.

[7] Hart, E. B., and McCollum, E. V., *ibid.*, 1914, xix, 373.

the growth of swine can take place on a corn meal, gluten feed, and salt-mixture diet. It is needless to say that the hopes expressed in certain quarters of formulating rations on their amino-acid content, secured by *direct* hydrolysis, will not even begin realization until at least safer methods for the quantitative estimation of these substances are available.

In our own work we have attempted nothing more than a dissection of the water-soluble nitrogen of some feeding materials for the purpose of securing a clearer picture of the composition of the so called "amide" nitrogen.

EXPERIMENTAL.[8]

Inaccuracy of Stutzer's Separation.—5 gm. of Witte's peptone were dissolved in water and treated with an excess of Stutzer's reagent; the precipitated material was filtered off, the excess of copper hydrate removed with hydrogen sulphide, and after expelling the hydrogen sulphide by boiling, the filtrate was diluted to 200 cc. The amino-acid nitrogen of the solution was determined directly by Van Slyke's nitrous acid method. A second portion of the filtrate from Stutzer's reagent was treated with sulphuric acid to 30 per cent concentration and hydrolyzed by boiling for five hours under a reflux condenser. The sulphuric acid was removed with barium hydrate, the excess of barium removed with carbon dioxide, and the amino-acids were determined as before. A third portion of the filtrate from Stutzer's reagent was hydrolyzed with sulphuric acid as above, but the sulphuric acid was neutralized with strong alkali and the amino-acids were directly determined. The results secured were as follows:

Amino-acid nitrogen from 0.1 gm. of Witte's peptone after treating with Stutzer's reagent, 2.01 mg. N.

Same as above after hydrolysis with sulphuric acid and the removal of the acid, 9.20 mg. N.

Same as above except that the sulphuric acid was neutralized instead of removed, 9.25 mg. N.

The data show a large increase in amino-acid nitrogen upon hydrolysis of the filtrate after treating with Stutzer's reagent, and indicate that complexes of the nature of peptide linkings were still present in the solution. This makes it clear that there is

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[8] After our work was completed and in manuscript an abstract appeared of similar work carried on by H. S. Grindley and E. C. Eckstein (*Science*, 1915, xlii, 70). We have not had the opportunity of knowing the details of the methods used and consequently can make no comparison of the results.

no distinct separation by Stutzer's reagent of free amino-acids from the more complex polypeptides.

Preparation of Samples.—In all cases the plant materials, when harvested green were dried at 37–40°C. and then allowed to stand a number of days to become air dried. The preparation of the water extract was made as follows: 25 grams of the air dried material were extracted with small portions of hot water until nearly 500 cc. of filtrate were obtained. The filtrations were made through linen cloth, which hastened the process, and from which the filtrate could be squeezed out quite completely. The filtrate was then acidified with acetic acid and boiled for a few minutes to precipitate the soluble proteins; the solution was cooled, diluted to 500 cc., and then filtered through paper. This gave a clear solution for further analysis.

Estimation of Ammonia Nitrogen.—Direct distillation with a weak alkali, such as magnesium oxide, has been recommended by a number of investigators, but Denis[9] confirmed the observation of Embden that magnesium oxide distillation at 100°C. liberates nitrogen in the form of ammonia from cystine.

We have made the further observation that practically a quantitative yield of the acid-amide nitrogen of asparagine can be obtained by magnesium oxide distillation at 100°C., while the work of Van Slyke and Hart[10] showed that none of the nitrogen from acetamide could be obtained by magnesium oxide distillation. This distinction in the behavior of the two acid-amides, and the probable presence of asparagine in plant tissues made it necessary to abandon the magnesium oxide method as an accurate procedure for the direct estimation of ammonia nitrogen. The results by direct distillation of asparagine and acetamide with magnesium oxide follow:

	Acid-amide N. *mg.*	N obtained. *mg.*
Asparagine	10.0	9.7
Acetamide	70.0	0.0

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[9] Denis, W., *Jour. Biol. Chem.*, 1910–11, viii, 429.

[10] Van Slyke, L. L., and Hart, E. B., *New York Agricultural Experiment Station Technical Bulletins*, No. 215, 1902.

We consequently adopted the Folin aeration method for determination of the ammonia nitrogen. This method gives none of the acid-amide nitrogen of asparagine or acetamide and Denis[11] has shown that cystine is stable under the conditions of this method. 25 cc. were used in the estimation.

Estimation of Acid-Amide Nitrogen.—Since magnesium oxide distillation at 100°C. may disturb cystine, and does not give the acid-amide nitrogen of all acid-amides, its use for direct estimation for this kind of nitrogen could not be made. We therefore adopted the method of acid hydrolysis and aeration. To 25 cc. of the solution hydrochloric acid was added to a concentration of 20 per cent and boiled under a reflux condenser for thirty minutes; the solution was cooled and maintained cool while being exactly neutralized with strong alkali; it was then made to 100 cc. The total ammonia nitrogen was then determined by the Folin method. The ammonia nitrogen previously determined by direct aeration was subtracted from this result, giving the acid-amide nitrogen.

Estimation of Amino-Acid Nitrogen.—The amino-acid nitrogen was determined directly upon the solution by Van Slyke's nitrous acid method.

It appeared possible that peptides might still be present in the water solution of the feed even after boiling with acetic acid and their exposed amino groups react with nitrous acid, thereby giving too high results for amino-acid nitrogen. This might also introduce an error into the acid-amide nitrogen determination. To determine the extent of this error a portion of the acidulated water extract was treated with tannic acid, filtered, and the amino-acid and acid-amide nitrogen were determined without the removal of tannic acid. In another portion the tannic acid was removed with barium hydrate and the excess of barium with carbon dioxide, after which the determination of the amino-acid and acid-amide nitrogen was repeated.

On the addition of the tannic acid there was found to be in each case but very slight precipitation, the largest occurring from the solution of the rape leaf, the data of which are given below.

Water extract boiled with acetic acid and filtered.

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[11] Denis, *loc. cit.*

Rape	Total N. *per cent*	Amino-acid N. *In per cent of*	Acid-amide N. *total N.*
(Air dried)	4.45 4.57	14.7	4.52
Extract treated with tannic acid and filtered		15.1	4.74
After the removal of the tannic acid		14.5	4.46

TABLE I.

Distribution of the Water-Soluble Nitrogen in Some Common Plant Materials.

Feed. (Air dried.)	Stage of growth.	Total N.	In per cent of total nitrogen.					
			Water-soluble N.	NH_3 N.	Acid-amide N.	Amino-acid N.	Rest N.	Peptide N.
		per cent						
Alfalfa	In blossom	3.67 3.74	27.9	0.87	1.74	17.7	7.6	
Red clover	In blossom	4.48 4.58	18.2	0.35	0.35	9.9	7.6	
Sweet clover	Before flowering	4.15 4.07	33.3	1.09	6.81	16.0	9.4	
Rape*	1 ft. high, young	4.45 4.57	36.0	2.30	4.52	14.7	14.5	
Rape	6 in. high, young	5.26 5.30	42.1	4.88	1.92	25.9	9.4	2.6
Corn	1 ft. high, young (green)	3.79 3.76	34.6	0.52	2.64	17.9	13.6	
Peas	In flower	3.26 3.26	36.0	0.48	2.12	23.4	10.0	
Oats (a)	Not flowered	2.90 2.72	36.8	0.54	2.26	20.7	13.3	5.5
Oats (b)	Milk stage	1.26 1.30	45.3	0.78	1.72	25.2	17.6	
Barley	Milk stage	1.36 1.33	24.4	0.58	1.80	16.5	5.5	
June grass	In flower	1.69 1.76	16.3	1.50	1.28	11.4	2.1	
Timothy	Headed green	1.48 1.48	25.9	0.26	1.34	17.1	7.2	
Sugar beet	Tuber only	0.87	49.5	Trace	None	18.3	31.2	
Rape	Fall growth	4.44	29.2	1.47	4.22	10.3	13.2	
Cabbage	Small head	3.28	34.3	1.72	8.3	13.0	11.3	
Corn stover	Mature	0.86	65.5	3.05	25.6	25.7	11.2	
Clover hay	Mature	2.53	9.3	None	None	4.2	5.1	
Alfalfa hay	Mature	2.28	23.5	None	7.3	10.0	6.2	

* Rape dried at 96°C.

The very small differences recorded above indicate that little if any complex protein material existed in the water solution after boiling with acetic acid. The fact, however, that not all of the nitrogen in the solution could be accounted for by the direct determination of the amino-acid, acid-amide, and ammonia nitrogen indicated the possibility of there still being present in the solution non-reacting amino nitrogen, which was not precipitated by tannic acid. To determine if all this was peptide nitrogen, the water extract of rape and oats was hydrolyzed with sulphuric acid for five hours and an amino-acid nitrogen determination made after neutralizing and diluting to a definite volume. The difference between the results so obtained and the direct amino-acid nitrogen determination of the original extract is recorded in the table as peptide nitrogen. It will be noticed that in the case of rape and oats peptide nitrogen constituted 30 to 40 per cent of the rest nitrogen but does not account for all of it. By rest nitrogen is meant the nitrogen constituting the difference between the total water-soluble nitrogen and the sum of the nitrogen fractions determined directly as ammonia, acid-amide, and amino-acid nitrogen.

The data secured by the above methods are recorded in Table I.

SUMMARY.

The "amide" nitrogen of feedingstuffs is largely composed of free amino-acids and peptide linkings. In most cases the nitrogen in these structures constitutes 50 to 70 per cent of the water-soluble nitrogen.

The acid-amide nitrogen is relatively small, seldom exceeding 20 per cent of the water-soluble nitrogen, and more often being below 10 per cent. Corn stover is an interesting exception, showing approximately 40 per cent of the water-soluble nitrogen in acid-amide form.

The ammonia nitrogen rarely exceeded 5 per cent of the total water-soluble nitrogen, and in some instances was wholly absent.

THE EFFECT OF PITUITARY SUBSTANCE ON THE EGG PRODUCTION OF THE DOMESTIC FOWL.

By LEWIS NEILSON CLARK.

(From Oldham Farm, Port Hope, Ontario.)

(Received for publication, July 31, 1915.)

Dr. Carey Pratt McCord[1] in his experiments with the pineal body, taken from growing mammals, has used chicks as subjects, with satisfactory results. While following his work on poultry with some experiments on the effect upon the offspring, of this substance when fed to the parents, I was led to the study of others of the organs of internal secretion. The relation between the pituitary gland and the genital system made this gland an interesting source of experiment to test its effect on the ovulation of the domestic fowl. When extracting the pineal body from the brains of calves and lambs, I also dissected out the pituitary gland, preparing it for use in the same way as the pineal. This will explain why the pituitary glands, used in the experimental work described in this paper, were all from the brains of *growing* mammals. The heads were supplied by the local butchers.

The pituitary substance was prepared as follows: The pituitary gland was dissected out and the posterior lobe and all adhering tissue discarded. The anterior lobe was weighed, ground to a paste with pestle and mortar, and to it was added three times its weight of milk sugar, and the whole thoroughly mixed. This paste was dried at room temperature, ground to a powder, and weighed, the loss in weight amounting to 13.5 per cent. In the experiments recorded below 69 mg.[2] of this powder, representing 20 mg. of fresh pituitary substance (anterior lobe) were administered to each hen, per day.

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[1] McCord, C. P., *Jour. Am. Med. Assn.*, 1914, lxiii, 234.

[2] Grains (avoirdupois) were used for measurements in this work on account of the convenient scales available. (0.33 grain = 20 mg.)

The first experiment attempted dealt with 35 Single Comb White Leghorn hens hatched in April, 1913, mated, in two pens, to two cockerels of the same breed, hatched in May, 1914. These two pens were housed in two colony-houses, on free range, and had been used as breeding-pens for this season's work before the dosing was commenced. I have, therefore, records of their egg production for several months previous, the laying being very steady and consistent. For the purpose of illustrating the results of this experiment, it will be necessary to give only the egg production for a period of fourteen days prior to first dosing. It will be noted in Table I that the production curve was declining, previous to dosing, this being only natural in view of the heavy and consistent laying of these hens since February. Both hens and cockerels were forcibly fed at night time with the powder, enclosed in gelatin capsules, the dose in each case being 69 mg. The first dose was given on the evening of May 20, and the last dose on the evening of May 28. Table I shows the daily egg production. A remarkable increase in production was noted on the fourth day after the administration of the first dose and continued some time after the last dose, but was apparently dropping back to normal when, owing to the exigencies of the management of a large poultry farm, it was necessary to break up these isolated pens. The hens were kept under close observation for a period of one month after the last dosing and their general health continued exceptionally good, there being not one case of sickness or death among any of the subjects, and the general appearance of the birds being far above the average for this season of the year.

I felt sure that the marked increase shown in the production of these hens, whose laying had been most consistent, and whose production curve was *on the decline* at the time of experiment, was due solely to a direct stimulating effect on the ovaries, produced by pituitary substance and to no other causes. A 100 per cent egg production is almost unknown in my experience, at this season of the year, especially with flocks of this size (seventeen and eighteen in a pen). The conditions for egg production were practically ideal, but the same conditions, and very much the same weather, had obtained for a month previous. In order to meet the natural query as to whether the weather or other conditions might not

TABLE I.

Preliminary period. Daily egg production.				Feeding period. 0.33 grain pituitary substance daily to each experimental hen. Daily egg production.				Post period. Daily egg production.			
Date.	Experimental pens. 35 hens.	Laying-houses (for comparison).		Date.	Experimental pens. 35 hens.	Laying-houses (for comparison).		Date.	Experimental pens. 35 hens.	Laying-houses (for comparison).	
		657 hens.	431 hens.			657 hens.	431 hens.			657 hens.	431 hens.
May				May				May			
7	22	301	184	21	15	310	197	29	35	310	180
8	21	300	179	22	15	285	178	30	29	270	166
9	20	265	153	23	18	291	175	31	25	285	169
10	21	322	168	24	27	311	191	June			
11	18	299	185	25	31	280	189	1	21	283	168
12	18	292	175	26	35	271	175	2	26	307	180
13	18	307	194	27	35	300	185	3	23	299	167
14	16	294	190	28	32	284	188	4	21	297	185
15	14	315	180					5	18	273	162
16	12	301	182					6	17	309	176
17	14	301	192					7	Pens broken up		
18	18	298	190								
19	23	295	200								
20	16	305	210								

First dose evening of May 20. Last dose evening of May 28.

Average daily egg production of Experimental Pens May 7 to May 14 (inclusive) 19.25 eggs = 55 per cent of possible.

Average production May 15 to May 23 (inclusive) (to 3 days after first dose) 16.11 eggs = 46 per cent of possible.

Average production May 24 to May 30 (inclusive) (from 3 days after first dose to 2 days after last dose) 32 eggs = 91.43 per cent of possible.

Average production May 31 to June 6 (inclusive) 21.57 eggs = 61.63 per cent of possible.

have influenced the production of the experimental fowl, the daily egg records of two large laying pens were studied and are given in Table I for comparison. Conditions in these two pens and in the experimental pens were very similar. Feeding conditions, weather, and the nature of the soil were identical, the only difference being in the fact that the experimental pens were in small isolated houses, on free range, whereas the large flocks were in long continuous laying-houses each with a fenced in run of three and one-half acres of land.

In order to test the effect, if any, on the hatchability of the eggs and the viability of the chicks, 100 eggs from the experimental fowl were collected previous to dosing—that is, from May 14 to May 20—and were set in an incubator on May 20, the eggs being from two hours to six days old. On the fifth day after the first dose, eggs were again collected from the same fowl, 100 eggs being gathered from May 26 to May 28, and were set in an incubator of the same make on May 29, the eggs being one to three days old. The results of these hatches are even more remarkable than the increased egg production, as is seen from Table II.

TABLE II.

Date set.	No. of eggs set.	Infertile.	Dead germs.	Dead in shell.	Hatched.	Per cent of possible.
May 20	100 (collected before dosing)	6	8	15 (7 shells pipped)	71	71
May 29	100 (collected after dosing)	0	0	4 (all pipped)	96	96

Prairie State Sand Tray Incubators of the same size were used. Conditions of temperature were closely regulated by thermostats. In both experiments the temperature ranged from 102–103.5°F., as required to give the best conditions for hatching. The chicks are being kept under observation to note sex and any peculiarities of growth.

Feeling it desirable to have a further experiment to more fully prove the effect of this substance, I determined to dose all the hens in one of the large laying-houses. The house so chosen contains 655 one year old Single Comb White Leghorn hens (no males), and the dose administered to them was 45 grams of the powder per day. Owing to the large scale on which the experiment was being conducted, it was only possible to obtain sufficient material for four daily doses. It was unfortunate that the dosing could not be continued over a longer period, but the results obtained from these few doses were so marked and striking as to leave no doubt as to the stimulating effect of pituitary substance (anterior lobe) on the egg production of the domestic fowl.

As will be noted in Table III, in the preliminary period, the production curve of these 655 hens was again, in this case, on the decline. In order to show that weather or other conditions did not apparently tend to increase production on these dates, I have again given (in Table III) the daily egg record of the flock of 431 hens. It will be noted in this case that the stimulating effect on the 655 hens lasted only about seven or eight days after the last dosing, but it will be seen that the total dose administered only provided, during the four days, the equivalent of 80 mg. of pituitary substance per hen, and there is no assurance that the material was equally divided among the hens. It being impracticable to forcibly feed so many hens, the following method was employed in this case: 45 grams of powder were divided into three equal parts, each part being thoroughly mixed into a pailful of dry mash. This mash was then moistened with sour skim milk and fed to the hens. The same quantity of this same moist mash had been fed to these hens every day for months past. It had been mixed in exactly the same way except for the addition of the pituitary substance, and feeding conditions and general care were identical during the experiment with conditions previous, as far as it is possible to make conditions of this kind identical. It was noted that on the second day after dosing, these hens required, and were given, a larger allowance of grain than previously, and the dry mash being always before them in open feed-hoppers, it is probable that they consumed a greater quantity of this material than under normal conditions. The increased appetite was undoubtedly occasioned by the increase in production, the writer having often noted the fact that a sudden increase in the appetite of a flock is usually a precursor of an increase in egg production.

TABLE III.

Preliminary period. Daily egg production.			Feeding period. Daily egg production. 0.33 grain pituitary substance daily to each experimental hen.			Post period. Daily egg production.		
Date.	Experimental pen. 655 hens.	Laying-house (for comparison). 431 hens.	Date.	Experimental pen. 655 hens.	Laying-house (for comparison). 431 hens.	Date.	Experimental pen. 655 hens.	Laying-house (for comparison). 431 hens.
June			June			June		
1	283	168	22	250	147	26	314	153
2	307	180	23	249	156	27	361	156
3	299	167	24	228	166	28	366	172
4	297	185	25	293	164	29	367	153
5	273	162				30	321	134
6	309	176				July		
7	298	145				1	352	143
8	302	176				2	304	142
9	298	160				3	291	143
10	275	171				4	252	130
11	263	168				5	247	147
12	265	159				6	267	138
13	241	163				7	260	140
14	223	149				8	269	114
15	236	161				9	247	129
16	208	171				10	225	140
17	224	180				11	202	141
18	231	165						
19	220	167						
20	246	155						
21	235	165						

First dose afternoon of June 21. Last dose afternoon of June 24.

Average daily egg production of Experimental Pen June 1 to June 8 (inclusive) 296 eggs = 45.09 per cent of possible.

Average production June 9 to June 17 (inclusive) 248.11 eggs = 37.88 per cent of possible.

Average production June 18 to June 24 (inclusive) (up to fourth day after first dose) 237 eggs = 36.18 per cent of possible.

Average production June 25 to July 1 (inclusive) (from fourth day after first dose to seventh day after last dose) 339.14 eggs =

I am publishing this paper as a preliminary report. Further experiments will follow to determine:

1. The post period during which the stimulating effect lasts.
2. The smallest dose producing the desired results.
3. The general health, growth curve, and age at maturity of chicks from dosed parents.
4. A comparison of the effect of pituitary substance taken from growing mammals and from adult mammals.

I have other experiments under way at the present time.

At the time these two experiments were completed I did not know that work of a similar nature had been done by Pearl and Surface.[3] These two observers administered the pituitary substance hypodermically to hens whose ovaries were in a state of complete rest (at the moulting season). They found no activation but their experiments were carried out under very different conditions, as regards the physiological condition of the subjects, the method of administering the substance, etc. It seems possible to me that the negative results they obtained as compared with my positive results might be explained by the fact that the pituitary substance I used was from growing mammals while it is probable that their material was from adults, as it was purchased from the Organotherapeutic Laboratory of Armour and Company. It is known from the work of McCord[4] that positive results are obtained from the use of pineal body taken from growing mammals, and negative results from this substance taken from adults, and the same may be the case with pituitary gland. Experiments are in progress to test this point.

SUMMARY.

These experiments show that:

1. Feeding of pituitary gland substance (anterior lobe) increased the egg production of hens whose production curve was on the decline: Case 1, with 35 hens in isolated pens; Case 2, with 655 hens.
2. The dosage was effective on the fourth day after the first dose and lasted for several days after the last dose.
3. The hatchability of eggs from dosed parents was increased.

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[3] Pearl, R., and Surface, F. M., *Jour. Biol. Chem.*, 1915, xxi, 95.

[4] McCord, *loc. cit.*

THE ASH OF CLAM MUSCLE IN RELATION TO ITS OSMOTIC PROPERTIES.

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(Received for publication, August 20, 1915.)

A knowledge of the ash of the tissues of marine animals is likely to throw light on a number of interesting physiological problems. It is generally supposed that the ash of animal tissues represents to a large extent their inorganic salt content, and that the salts in question dissolved in the fluids of the living tissue play an important part in giving to those fluids their characteristic osmotic pressure. But the physicochemical conditions in the tissues which have hitherto been examined are by no means simple—there is no doubt that a part of the ash comes from the organic constituents of the tissue, and there is much reason to believe that a considerable proportion of the water is so combined with the colloids that it cannot act as a solvent for the salts.[1]

The salt content of the blood and tissues of most vertebrates is much lower than that of the blood and lymph of the marine invertebrates.[2] It is a very interesting question, therefore, whether the salt content of the tissues of these latter is low like that of the vertebrate tissues or high enough to correspond with the high salt content of their surroundings. The answer to this question can hardly fail to throw light on the osmotic properties and relations of animal tissues in general.

The vitreous portion of the adductor muscle of *Venus mercenaria* has been analyzed for total ash, water, solids, sodium, potassium,

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[1] Overton, E., *Arch. f. d. ges. Physiol.*, 1902, xcii, 128–142. Meigs, E. B., and Ryan, L. A., *Jour. Biol. Chem.*, 1912, xi, 410–413. Meigs, E. B., *ibid.*, 1914, xvii, 91-93.

[2] Höber, R., Physikalische Chemie der Zelle und der Gewebe, Leipsic, 3rd edition, 1911, Chapt. 2.

calcium, magnesium, iron, phosphorus, sulphur, and chlorine. The methods of analysis were in general the same as those used in a previous investigation.[3] The total ash was determined by heating the dried tissue moderately in a platinum crucible until it was carbonized, pulverizing the mass of carbon, and extracting it with dilute hydrochloric acid. The extracted carbon was replaced in the crucible and completely ashed, the ash so obtained added to the acid extract, and the whole solution then evaporated. The residue was heated until it just began to glow, cooled, and weighed.

The sulphur was determined in one case by fusing the tissue with sodium peroxide; in another, by ashing it without the addition of any reagent. The two sets of results are given in the table.

For a description of the kind of muscle used the reader is referred to a previous article.[4]

The results of the investigation are given in the table and protocols at the end of this article.

GENERAL DISCUSSION.

Study of the figures given in the table reveals a number of interesting relations between the ash of the clam's muscle and that of the various kinds of vertebrate muscle which have hitherto been studied. The figures for the potassium, phosphorus, and total sulphur content of the clam's muscle are remarkably close to those which have been obtained for the smooth and striated muscle of the frog, the striated muscle of mammals, etc. The sodium and chlorine content of the clam's muscle are, as one would expect, higher than that for the muscle of fresh water and land vertebrates; but remarkably low when compared with the sodium chloride content of sea water and of lamellibranch blood.[5] Consideration of the figures for total ash and for the various constituents of the ash makes it clear that the salts contained in the muscle cannot be nearly sufficient to bring the osmotic pressure

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[3] Meigs and Ryan, *loc. cit.*, 401.

[4] Meigs, *loc. cit.*, 82 and 83.

[5] Fürth, O. v., Vergleichende chemische Physiologie der niederen Tiere, Jena, 1903, 73.

of the water of the muscle up to that of sea water. This adds to the evidence which has been given in an earlier article to show that a large proportion of the water of clam muscle is combined with the colloids in such a manner that it cannot act as a solvent for salts.

Some experiments have been carried out for the purpose of throwing light on the manner in which the elements of the ash are combined in the living tissue.

The sulphur has been determined in two portions of muscle which were fused with sodium peroxide, and in two other portions which were ashed at high temperature without the addition of alkali. The figures obtained are widely different from each other, as was to have been expected from a consideration of the sources of sulphur in the ash of such a tissue as muscle. It is generally accepted that the muscle proteins contain considerable quantities of sulphur in organic combination. This is held in the ash when the tissue is fused with alkali, but most of it escapes when the tissue is ashed without the addition of anything to hold it. Whether or not all of it would escape is doubtful, and some small portion of it may, therefore, be included under the figures given for "inorganic sulphur."

On the other hand, any sulphur combined as sulphate with sodium, potassium, magnesium, or calcium would remain in the ash whether or not alkali were added when the tissue was ignited, and the figures given for "inorganic sulphur" represent whatever sulphur may originally have been combined in this way plus an uncertain quantity of the organic sulphur. These figures represent, therefore, the maximum amount of sulphur which can be present in the living tissue as non-volatile sulphate, and show that this quantity must be very small.

Examination of the table shows that the sodium and chlorine of the ash are present in just about such proportions as would unite to form sodium chloride. That these elements exist as sodium chloride in the living tissue seems *a priori* highly probable, and is rendered still more so by the results of diffusion experiments. It has been found that all the chlorine will diffuse out of the living muscle into a surrounding 30 per cent saccharose solution in the course of about forty-five hours.[6] In more recent

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[6] Meigs, *loc. cit.*, 87.

experiments (Experiments 1 and 2 of this article) it has been found that under similar conditions the muscle loses about 86 per cent of its sodium. There is every reason to think, therefore, that at least 86 per cent of the sodium and chlorine found in the ash exists in the living muscle as sodium chloride.

Somewhat similar experiments have been carried out with respect to the potassium and phosphorus of the muscle's ash, and it has been found that while the tissue loses about 75 per cent of its potassium to a 30 per cent saccharose solution in forty-one hours, it loses under similar circumstances only about 28 per cent of its phosphorus (Experiments 1, 2, 3, and 4). It seems impossible, therefore, to suppose that more than about half of the tissue's potassium is combined with the phosphorus as inorganic phosphate. Most of the phosphorus which fails to diffuse out from the muscle to the sugar solution could be accounted for by supposing it to be combined with the calcium and magnesium of the ash as insoluble calcium and magnesium phosphate. But how the surplus potassium is combined is a problem which remains to be solved.

A consideration of the quantities of acids and bases in the ash of clam muscle, as shown by the figures given in the table, shows that the former are not present in sufficient quantity to yield a neutral solution when combined with the latter. No matter how the sodium, potassium, calcium, and magnesium of the ash were combined with the phosphorus, chlorine, and inorganic sulphur, the resulting solution would still be strongly alkaline. It is highly probable, therefore, that the tissue contains some additional acid, which does not appear in ash analyses as ordinarily made, and that this acid exists in the living tissue in combination with a part of the potassium. Whether the acid in question is carbonate or something else is another question that still remains to be answered.

It is a remarkable fact that the clam's muscle remains highly irritable after it has been for forty hours or more in 30 per cent saccharose solution. Experiment 2 shows that during this period the muscle may gain more than 60 per cent of its original weight, and in an earlier article it was shown that enough sugar diffuses into it to bring the concentration of saccharose in the water of the muscle up to 17.5 per cent; also, that during this period all the chlorine, so far as could be determined by chemical analysis, was lost.[7] Experiments 1, 2, 3, and 4 show that the muscle loses also to the sugar solution 86 per cent of its sodium, 75 per cent of its potassium, and 28 per cent of its phosphorus. There is left in the muscle, therefore, only about 0.03 per cent Na, 0.08 per cent K, 0.11 per cent P, and possibly most of the Ca, Mg, and S. And in spite of this remarkable loss of its own inorganic materials and the taking up of considerable quantities of water and sugar from the surroundings, the muscle remains as irritable as at the beginning of the experiment. Its behavior in this respect forms a noteworthy contrast to that of frog striated muscle, which very quickly loses its irritability in any potassium or non-electrolytic solution.

In conclusion, I wish to offer my most sincere thanks to Dr. John Marshall and the staff of the Hare Chemical Laboratory of the University of Pennsylvania, whose kind assistance and support greatly facilitated the work on which this article is based.

*Constituents of the Adductor Muscle (Vitreous Portion) of Venus mercenaria, Given as Percentages of the Fresh Tissue.**

No.	Water.	Solids.	Total ash.	K	Na	Fe	Ca	Mg	P	S† (Total.)	S‡ ("Inorganic.")	Cl§
I.......	73.65	26.35	1.44	0.335	0.247	0	0.042	0.052	0.156	0.242	0.033	0.350
II......	75.11	24.89	1.48	0.278	0.184	0	0.034	0.044	0.148	0.208	0.026	0.280
Average	74.44	25.56	1.46	0.311	0.205	0	0.038	0.048	0.153	0.225	0.030	0.322

* The figures given under No. I and No. II represent the widest differences found for the various constituents; the averages are made up from these and from the intermediate figures (see protocols).

† Total sulphur was determined in muscle fused with Na_2O_2.

‡ "Inorganic" sulphur was determined in muscle ashed at high temperature without the addition of alkali. The figures probably represent sulphur from inorganic sulphates plus an unknown quantity yielded by the muscle proteins, but they were thought to be interesting as showing the maximum amount of sulphur which can be attributed to inorganic sulphates.

§ The chlorine determinations are taken from an earlier article (Meigs, *loc. cit.*, 96, Experiment 8).

Protocols of the Diffusion Experiments.

In all experiments the vitreous portion of the muscle was used.

Experiment 1.—July 13, 1914. Portion of adductor muscle of *Venus* weighing fresh 15.175 gm., yielded 3.873 gm. dry substance, 0.0495 gm. or 0.326 per cent K, and 0.028 gm. or 0.184 per cent Na.

Experiment 2.—July 13, 1914. From the animals used in Experiment 1 15.053 gm. fresh muscle were taken and kept for 41 hours in 30 per cent saccharose solution at temperature between 11° and 12°. The sugar solu-

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[7] Meigs, *loc. cit.*, 95 and 96, Experiments 6, 7, 8, and 9.

tion was changed three times. At the end of the 41 hours the muscle was still quite irritable and weighed 24.370 gm. It yielded 6.502 gm. dry substance, 0.0121 gm. or 0.08 per cent K and 0.0044 gm. or 0.029 per cent Na.

Experiment 3.—May 25, 1915. Portion of adductor muscle of *Venus* weighing fresh 10.453 gm. kept for 22 hours in 30 per cent saccharose solution at temperature between 1° and 5° with preliminary one and one-half hour period at 23°. The sugar solution was changed twice. At the end of the 22 hours the muscle was still quite irritable. It yielded 0.0421 gm. $Mg_2P_2O_7$ or 0.112 per cent P.

Experiment 4.—May 25, 1915. Portion of adductor muscle of *Venus* weighing fresh 10.785 gm. kept for 46 hours in 30 per cent saccharose solution at temperature between 1° and 6° with preliminary one and one-half hour period at 23°. The sugar solution was changed four times. At the end of the 46 hours the muscle was still quite irritable. It yielded 0.0424 gm. $Mg_2P_2O_7$ or 0.110 per cent P.

Protocols of the Ash Analyses and Determinations of Water and Total Solids.

All the analyses were made on the vitreous portion of the adductor muscle of *Venus mercenaria.*

Potassium and Sodium.—23.254 gm. muscle yielded 0.295 gm. combined KCl and NaCl and 0.488 gm. K_2PtCl_6, equivalent to 0.149 gm. KCl or 0.0779 gm. K. 0.295-0.149 = 0.146 gm. NaCl equivalent to 0.0574 gm. Na. Muscle contained, therefore, 0.0779 gm. or 0.335 per cent K, and 0.0574 gm. or 0.247 per cent Na. In three other similar determinations the results were: K, 0.326, 0.278, and 0.306 per cent; Na, 0.184, 0.198, and 0.191 per cent.

Iron.—10.570 gm. muscle yielded no Fe. In another similar determination the result was the same.

Calcium.—10.570 gm. muscle yielded 0.0059 gm. CaO or 0.042 per cent Ca. In another similar experiment the yield was 0.034 per cent Ca.

Magnesium.—10.570 gm. muscle yielded 0.0198 gm. $Mg_2P_2O_7$ or 0.052 per cent Mg. In another similar experiment the yield was 0.044 per cent Mg.

Phosphorus.—11.140 gm. muscle yielded 0.059 gm. $Mg_2P_2O_7$ or 0.148 per cent P. In two other similar experiments the yields were respectively 0.155 and 0.156 per cent P.

Sulphur ("Inorganic").—13.764 gm. muscle ashed at high temperature without the addition of alkali yielded 0.036 gm. $BaSO_4$ or 0.033 per cent S. In another similar experiment the yield was 0.026 per cent S.

Sulphur (Total).—11.350 gm. muscle fused with Na_2O_2 yielded 0.200 gm. $BaSO_4$ or 0.242 per cent S. In another similar experiment the yield was 0.208 per cent S.

Total Ash.—10.570 gm. muscle yielded 0.152 gm. or 1.44 per cent total ash. In another similar determination the yield was 1.48 per cent ash.

Water and Solids.—14.902 gm. fresh muscle dried for 42 hours at temperature between 100° and 110° yielded 3.845 gm. or 25.80 per cent solids and 74.20 per cent water. In five other determinations, the results were: solids, 26.35, 25.87, 24.89, 24.95, and 25.52 per cent; water, 73.65, 74.13, 75.11, 75.05, and 74.48 per cent.

STUDIES IN CARBOHYDRATE METABOLISM.

X. THE INFLUENCE OF HYDRAZINE UPON THE RESPIRATORY QUOTIENT AND UPON HEAT PRODUCTION.

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(Received for publication, July 27, 1915.)

Previous communications[1] have demonstrated that carbohydrate metabolism in the dog is markedly influenced by the subcutaneous injection of hydrazine. It has been shown, for example, that subsequent to treatment with this substance blood sugar content[2] is noticeably diminished, the change being accompanied by a disappearance of glycogen from the liver[3] and muscles. Significant histological alterations[4] in the hepatic cells obtain simulating those of degeneration. With large doses of hydrazine death invariably ensues. On the other hand, with the dosage carefully regulated animals may, and usually do, recover from the influence of hydrazine, the liver likewise regaining a normal appearance.[5] The production of hypoglycemia is not accompanied by glycosuria nor can any evidence be obtained of an increased output of such substances as lactic acid, diacetic acid, or acetone.

An explanation has been sought which would account for the lowered blood sugar content and the disappearance of glycogen from the body. The problem has been attacked from various viewpoints but in each instance the result has failed to afford the de-

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[1] Underhill, F. P., *Jour. Biol. Chem.*, 1911–12, x, 159. Underhill, F. P., and Fine, M. S., *ibid.*, 1911–12, x, 271. Underhill, *ibid.*, 1914, xvii, 293, 295. Underhill, F. P., and Prince, A. L., *ibid.*, 1914, xvii, 299.

[2] Underhill, *ibid.*, 1911–12, x, 159.

[3] Underhill, *ibid.*, 1911–12, x, 159; 1914, xvii, 293.

[4] Wells, H. G., *Jour. Exper. Med.*, 1908, x, 457.

[5] Wells, *loc. cit.*

sired elucidation. Thus, it has been found[6] that dextrose solutions perfused through the hearts of hydrazinized rabbits are not utilized to a different degree than is true for the hearts of normal animals. The experiments affording this conclusion were carried through on the hypothesis that muscle tissue may play a significant rôle in the diminution of carbohydrate in the hydrazinized animal, perhaps by means of an accelerated enzyme action concerned in carbohydrate transformations. Again, with the same object in view experiments were made in which the hepatic glyoxalase[7] activity of dogs treated with hydrazine was compared with that of normal animals.

The present communication presents the results obtained from metabolism studies upon dogs at different periods subsequent to hydrazine treatment and indicates that the long sought explanation for the action of hydrazine is at hand.

Methods.

During hydrazine intoxication food is refused, or if eaten is promptly vomited; hence, in the present investigation experiments have been carried through only on fasting dogs, the influence of inanition alone also being determined. The basal metabolism having been determined by respiration experiments on one or two dogs, a single dose of hydrazine was administered subcutaneously in 2.5 per cent solution at the rate of about 50 mg. per kilo. In only one instance was the metabolism determined within the first twenty hours, since the animal in this period usually exhibits a high degree of irritability. From this time on, however, the period of excitation gives place to one of depression and the animal is then suitable for a respiration experiment.

The apparatus used was the respiration incubator recently described by one of us (M[8]). Alcohol checks performed during the course of this work proved it to be in perfect condition.

The urine was analyzed for nitrogen and the excretion of this element was used in the calculation of the heat production for the first two dogs, but not for the last two. Because of the small

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[6] Underhill and Prince, *loc. cit.*

[7] Underhill, F. P., and Hogan, A. G., *Jour. Biol. Chem.*, 1915, xx, 211.

[8] Murlin, J. R., *Am. Jour. Dis. Child.*, 1915, ix, 43.

amount of nitrogen excreted, no material difference is made by the omission.

From the data in the table it is obvious that after the administration of hydrazine to a fasting dog no change occurs in the rate of dextrose oxidation until about the thirty-sixth hour. From this period to the forty-sixth hour or longer the respiratory quotient is raised—evidence of increased combustion of carbohydrate material. Inasmuch as hypoglycemia obtains at approximately the same period, as has been determined previously and in unpublished work, it is reasonable to interpret the low blood sugar content as a result, in part at least, of an increased oxidation of dextrose. Coincident with an augmented rate of oxidation it is probable that the power to furnish dextrose from non-carbohydrate sources is temporarily inhibited. That this capacity is checked for the time being only is attested by the fact that the blood sugar level returns to the normal within four days and glycogen is then present in the liver and muscles.

When dextrose is injected subcutaneously into hydrazinized dogs in doses of 5 grams per kilo of body weight death almost invariably follows within a period of twelve hours. The data in the appended table show, as indicated by the respiratory quotient, that a portion at least of the administered dextrose is oxidized rapidly. Since hydrazinized animals apparently temporarily lose the power to store glycogen it is clear that the ability to store glycogen is not inseparable from the capacity to oxidize dextrose; that is, the one may be inhibited or retarded without impairing the other.

It may also be seen that hydrazine is without influence upon heat production aside from the changes induced (a) by the muscular activity in the stage of excitement and (b) by the muscular relaxation during the period of depression. On the other hand, the subcutaneous injection of dextrose into hydrazinized dogs appears to bring about an increased heat production; that is, it exerts a specific dynamic action.[9]

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[9] Compare Lusk, G., *Jour. Biol. Chem.*, 1912–13, xiii, 27.

CONCLUSIONS.

The subcutaneous injection of hydrazine into fasting dogs induced an increased rate of carbohydrate combustion as measured by the respiratory quotient.

This increased combustion of carbohydrate probably explains the diminished blood sugar content and the disappearance of glycogen from the liver and muscles observed in previous communications.

Subcutaneously introduced dextrose is oxidized more rapidly in dogs that have received hydrazine than in the normal fasting animal. Such injections of sugar to hydrazinized dogs also appear to exert a specific dynamic action.

Hydrazine exerts no specific influence upon heat production.

Respiratory Metabolism of Hydrazinized Dogs.

Dog 1. Fasting Ten Days.

Date.	Weight.	Hours after hydrazine.	Respiration period.	CO_2 liters per hour.	O_2 liters per hour.	R.Q.	N in urine per hour.	Heat produced.	Remarks.
Nov.	*kg.*							*cal.*	
10	5.05		10.44–11.44	1.631	2.309	0.706	0.076	10.73	
			11.44–12.44	1.656	2.321	0.713	0.076	10.79	
11	4.92		10.19–12.19	2.781	3.838	0.725	0.072	17.82	
			12.19– 1.19	1.500	2.141	0.701	0.072	9.95	10 cc. 2.5 per cent hydrazine at 3.00 p.m.
12	?	24	2.52– 3.52	1.373	1.904	0.721	0.037	8.88	Dog very sluggish.
		25	3.52– 4.52	1.371	2.037	0.673	0.037	9.50	
13	4.45	43	10.26–11.26	1.464	1.943	0.753	?	9.22	
		44	11.26–12.26	1.459	1.868	0.781	?	8.93	
		45	12.26– 1.26*	1.532	1.974	0.776	?	9.42	80 cc. 30 per cent glucose subcutaneously at 1.45.
			2.53– 3.53	1.681	2.113	0.796	0.012	10.10	Small amount of urine due to antidiuretic effect of glucose.
			3.53– 4.53	1.698	2.449	0.693**	0.012	11.45**	
14	Dog found dead in morning.								

Dog 2. Fasting Two Days.

Date.	Weight.	Hours after hydrazine.	Respiration period.	CO_2 liters per hour.	O_2 liters per hour.	R.Q.	N in urine per hour.	Heat produced.	Remarks.
18	8.25		2.43– 3.43	3.024	4.253	0.711	0.113	19.80	
			3.43– 4.43	3.467	4.808	0.721	0.113	22.40	18 cc. 2.5 per cent hydrazine at 5.00 p.m.
19	7.8	21½	2.30– 3.30	2.501	3.494	0.716	0.107	16.25	
		22½	3.30– 4.30	2.601	3.662	0.710	0.107	17.04	
20	7.32	Dog died at 11.00 a.m. while in respiration apparatus.							

Dog 3. Fasting Five Days.

Date.	Weight.	Hours after hydrazine.	Respiration period.	CO_2 liters per hour.	O_2 liters per hour.	R.Q.	N in urine per hour.	Heat produced.	Remarks.
Dec.									
2	4.00		10.15–11.15	1.291	1.842	0.701		8.63	
			11.15–12.15	1.235	1.726	0.715		8.10	
3			10.32–11.32	1.243	1.874	0.663			
			11.32–12.32	1.214	1.526	0.795			
					Average	0.723		8.00	8 cc. 2.5 per cent hydrazine at 1.00 p.m.
4		21½	10.29–11.29	1.298	1.587	0.818			
		22½	11.29–12.29	1.321	1.966	0.672			
					Average‡	0.737		8.39	10 gm. glucose in 10 per cent solution subcutaneously at 1.50 p.m.
		23½	12.29– 1.29	1.284	1.747	0.735			
		25½	2.22– 3.22	1.276	1.776	0.719		8.35	
		26½	3.22– 4.22	1.316	1.569	0.839		7.61	More sugar burning than would burn in fasting dog. See next experiment.
			4.22– 5.22	1.442	1.702	0.847		8.27	
5	Dog found dead in morning.								

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*Body temperature, 1.35, 37.8°; 5.10, 37.8°.

**Probably O_2 leak.

‡When the two periods do not agree, it is due to the fact that the dog moved at the end of a period while the oxygen was being admitted to the cage for the purpose of equalizing the volume (see Murlin, *loc. cit.*). Any deficiency of oxygen in one period, however, is compensated in the next if the dog is quiet.

Dog 4. Fasting Two Days.

Date.	Weight.	Hours after hydrazine.	Respiration period.	CO_2 liters per hour.	O_2 liters per hour.	R.Q.	N in urine per hour.	Heat produced.	Remarks.
Dec.	*kg.*							*cal.*	
10	5.7		1.44– 2.44	1.858	2.452	0.759		11.65	Dog received 10 gm. glucose subcutaneously at 12.50, 12 cc. 2.5 per cent hydrazine at 9.20 p.m.
			2.44– 3.44	1.739	2.269	0.766		10.80	
11	5.45	12½	9.55–10.55	1.931	2.630	0.734		12.41	Dog excited, hence higher heat production.
		13½	10.55–11.55	1.997	2.711	0.737		12.80	
12		36¼	9.38–10.38	1.654	2.192	0.754		10.40	
			10.38–11.38	1.659	2.176	0.762		10.34	20 gm. glucose subcutaneously at 11.55.
			3.25– 4.25	2.051	2.185	0.939		10.87	2.55 p.m., blood contains 0.201 per cent glucose.
			4.25– 5.25	2.091	2.366	0.884		11.61	
14	Dog still alive. Urine contains bile.								10 cc. 2.5 per cent hydrazine at 11.00 a.m.
15	10.30 a.m. 5 cc. of blood from carotid artery contains 0.12 per cent glucose. 10.40 20 gm. glucose given subcutaneously. 12.45 dog found dying: blood from carotid contains 0.18 per cent glucose.								

THE DETERMINATION OF THE BILE SALTS IN URINE BY MEANS OF THE SURFACE TENSION METHOD.

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CONTENTS.

I. INTRODUCTION.

Bile salts occur in the urine in certain diseases, especially jaundice, but the small quantities which occur are difficult to detect by chemical tests. Hay's method of testing by shaking flowers of sulphur upon the surface of the urine depends upon the unusual power which bile salts possess, even in small concentrations, of lowering the surface tension of a solution. When the surface tension of the urine is lowered the powdered sulphur sinks to the bottom, and the lower the surface tension the more rapidly this takes place. The method is very unsatisfactory, however, because of the large variation in the surface tension of normal urine and because the interpretation of the test is so largely a matter of the judgment of the operator. Some test for bile salts more accurate than Hay's, but still convenient, and if possible a quantitative method, would be very desirable.

The surface tension of a solution may be measured very accurately by the stalagmometric method. Donnan and Donnan[1] and Lyon-Caen[2] have measured the surface tension of normal and pathological urines and of solutions of different urinary constituents by this method. The present study was undertaken to determine the feasibility of estimating the amount of bile salts present in pathological urines from measurements of the surface tension taken with a portable Traube stalagmometer. The problem was undertaken under the direction of Dr. J. F. McClendon, who has given valuable assistance at many points.

II. THE METHOD OF MEASURING THE SURFACE TENSION.

The surface tension of solutions reported in this paper was measured by means of a portable Traube stalagmometer which drops distilled water at an average rate of 40.8 drops in about two minutes and a half at 22°C. Solutions were drawn up into the pipette with the assistance of a filter pump until the surface of the solution was above the upper mark on the stem of the instrument. The beaker of solution was then held with the surface of the solution in the beaker in contact with the lower end of the pipette. The solution was allowed to run out of the pipette until the upper meniscus of the liquid in the instrument reached the upper mark on the stem. The beaker was then quickly lowered, breaking the connection between the solution in the beaker and that in the pipette, and the number of drops flowing from the pipette was counted until the meniscus reached the lower mark on the stem of the stalagmometer. The procedure described enables one to estimate the fraction of the last drop very easily and at the same time to work rapidly.

The surface tension of a solution is computed in per cent of that of distilled water according to the formula:

$$\frac{\text{Number of drops of distilled water}}{\text{Number of drops of solution}} \times \text{specific gravity of the solution.}$$

The results obtained by these methods are in close agreement with those of Donnan and Donnan, who used more exact methods with a fixed pipette dropping about 201 drops of distilled water. As will be shown later, the determinations of Lyon-Caen were very high. This discrepancy appears to be due to the pipette which he used and describes as dropping 120 drops of distilled

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[1] Donnan, W. D., and F. G., The Surface Tension of Urine in Health and Disease, *Brit. Med. Jour.*, 1905, pt. ii, 1636.

[2] Lyon-Caen, L., Recherches expérimentales sur la tension superficielle des urines, *Jour. de physiol. et de path. gén.*, 1910, xii, 526.

water in two to three minutes. This is nearly three times the rate of dropping of the Traube stalagmometer which we used, and is too great for accurate determinations. With too rapid dropping the substance which lowers the surface tension does not have time to accumulate in the surface layer enough to produce the maximum lowering. Bottazzi[3] states that in no case may more than 20 drops a minute be permitted. In another respect also the pipette described by Lyon-Caen seems open to criticism; that is in the fact that it lacked a broad dropping surface. When a standardized Traube stalagmometer is used it is our belief that measurements of the surface tension of urine can be made with sufficient accuracy for clinical purposes.

III. THE EFFECT OF BILE SALTS UPON THE SURFACE TENSION.

Bile salts possess the unusual property of lowering the surface tension of a solution very markedly even when present in small concentrations. Table I shows a lowering of the surface tension of distilled water from 100 per cent to 68.2 per cent by the addition of 0.1 per cent sodium glycocholate.

TABLE I.

Showing the Surface Tension of Solutions of Sodium Glycocholate in Distilled Water.

Solution.	Drops.	S. T.
Distilled water	40.8	100.0
Na-Glyc. per cent in distilled water.		
0.1	59.8	68.2
0.05	57.1	71.4
0.025	55.0	74.1
0.0125	52.6	77.5
0.00625	49.5	82.4
0.003125	46.3	88.1
0.0015625	42.5	96.0

Upon successive dilutions of this solution the surface tension was gradually raised, but so small a concentration of the bile salt as 0.00156 per cent caused a lowering of 4 per cent.

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[3] Bottazzi, F., in Neuberg's, Der Harn sowie die übrigen Ausscheidungen und Körperflüssigkeiten von Mensch und Tier, Berlin, 1911, pt. ii, 1709.

When bile salts are added to normal urine their effect upon the surface tension is similar to that in distilled water. A specimen of normal urine diluted with distilled water to a specific gravity of 1.010 had a surface tension of 95.6 per cent. The reason for diluting with distilled water to this specific gravity will be explained later. When 0.1 gram of sodium glycocholate was added to 100 cc. of this diluted urine the surface tension was lowered to 62.7 per cent. Upon successive dilutions of this solution with the original urine (sp. gr. 1.010) the surface tension gradually increased. So small an amount of sodium glycocholate, however, as 0.00078125 gram in 100 cc., *i.e.*, less than one part in 100,000 caused a lowering of nearly 3 per cent.

TABLE II.

Showing the Surface Tension of Solutions of Sodium Glycocholate in Urine.

Solution.	Sp. gr.	Drops.	S. T.
Distilled water		40.8	100.0
Urine Sample I.			
Diluted	1.010	43.1	95.6
Urine + Na-Glyc. gm. per 100 cc.			
0.1		65.7	62.7
0.05		63.0	65.4
0.025		59.8	68.9
0.0125		56.0	73.6
0.00625		52.4	78.6
0.003125		49.0	84.1
0.0015625		46.0	89.6
0.00078125		44.4	92.8

Figure 1 shows that increasing amounts of sodium glycocholate lowered the surface tension of this sample of urine (Sample I) at first very rapidly and with further increases in concentration more slowly as the curve of surface tension approaches the horizontal at about 60 per cent.

Donnan and Donnan found that 0.0664 gram of sodium glycocholate in 100 cc. of urine lowered the surface tension from 91 per cent to 62.4 per cent. Lyon-Caen determined the surface tension of a 0.1 per cent solution of sodium glycocholate as 80.9 per cent. As explained above this high figure must be due to instrumental error.

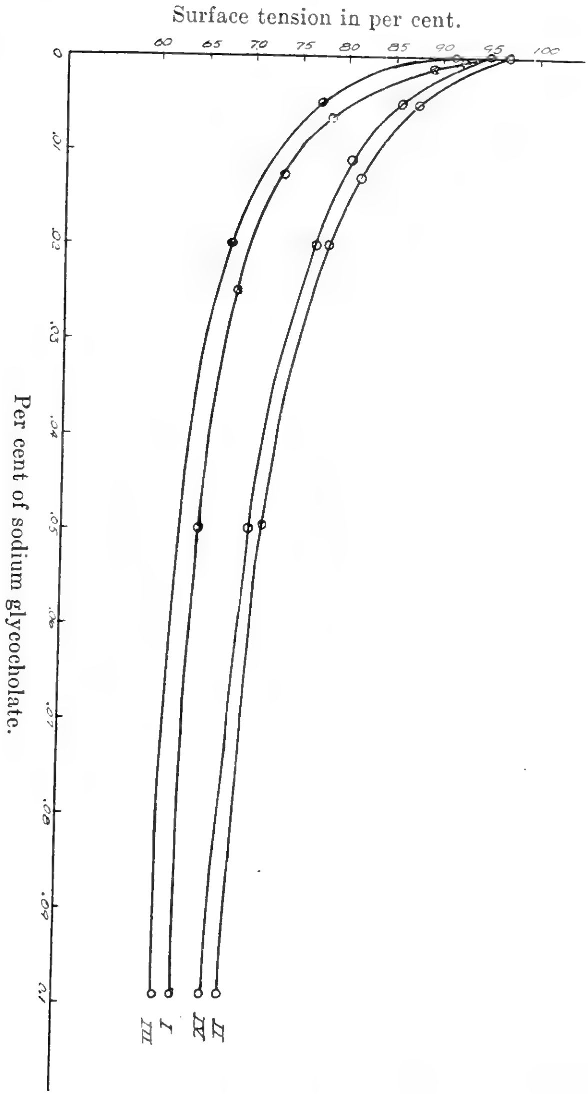

FIG. 1. Curves showing the surface tension of solutions of sodium glycocholate in four samples of normal urine diluted to a standard specific gravity of 1.010.

IV. VARIATION IN THE SURFACE TENSION OF NORMAL URINE.

The surface tension of normal urine varies considerably even in the same individual. Donnan and Donnan found a variation in the same individual between 82.4 per cent and 93.6 per cent. They found the morning urine sometimes characterized by high density and low surface tension due to the greater concentration. Unusual exercise caused a fall in surface tension and a rise in density. In general a high specific gravity is associated with a low surface tension. One cause of the variation in the surface tension of normal urine, therefore, is the varying dilution with water. If all samples of urine could be reduced to the average specific gravity, 1.020, this source of error could be eliminated. Since this is not practicable we have attempted to dilute all samples to a standard specific gravity of 1.010. When this was done the variation was found to be reduced. Table III shows the surface tension of the urine of the same individual taken at differ-

TABLE III.

Showing the Surface Tension of Samples of Urine from the Same Individual at Different Times, and Showing the Surface Tension of the Same Samples after Being Diluted to a Standard Sp. Gr. of 1.010.

Date.	Time.	As collected.			Diluted to sp. gr. 1.010.	
		Sp. gr.	Drops.	S. T.	Drops.	S. T.
(Distilled water)		1.000	40.8	100.0	40.8	100.0
Feb. 16	On rising	1.021	46.0	90.6	42.8	96.3
“ “	Noon	1.023	46.8	89.2	42.7	96.5
Feb. 19	On rising	1.022	48.3	86.1	43.4	94.9
“ “	Noon	1.019	43.2	96.2	41.6	99.1
“ “	6 p.m.	1.024	45.7	91.4	42.3	97.4
“ “	10 p.m.	1.028	46.8	89.6	42.1	97.9
Feb. 20	3 p.m.	1.025	45.7	91.5	42.1	97.9
Feb. 24	On rising	1.026	48.2	86.8	43.5	94.8
“ “	11 a.m.	1.011	44.4	92.9	43.8	94.1
“ “	1 p.m.	1.021	45.4	91.8	42.6	96.7
“ “	6 p.m.	1.0195	44.7	93.1	42.6	96.7
“ “	10 p.m.	1.024	46.2	90.4	42.6	96.7
Feb. 25	On rising	1.019	44.6	93.2	42.5	97.0
“ “	Noon	1.0215	44.0	94.7	42.0	98.1
Mar. 1	On rising	1.021	45.7	91.2	42.4	97.2
Mar. 9	24 hrs.	1.0225			43.1	95.6

ent times of the day, and the surface tension of the samples when diluted to the standard specific gravity of 1.010.

The surface tension of samples at the standard specific gravity was above 94 per cent while the surface tension of the samples as collected ran down to 86.1 per cent. A sample was found from another individual which had a surface tension of 83.9 per cent and a specific gravity of 1.028. After dilution to the standard specific gravity the tension became 91.9 per cent. It seems to be safe to conclude, therefore, that if the surface tension of normal urine may run as low as 80 per cent, then the surface tension of samples of normal urine diluted to a standard specific gravity of 1.010 will lie above 90 per cent. In other words, the range of variation of normal urine is practically cut in half by this procedure. The measurements of the effects of bile salts and other substances upon the surface tension of urine have usually been made in this study upon samples of urine diluted to the standard specific gravity of 1.010.

V. URINARY CONSTITUENTS NOT AFFECTING THE SURFACE TENSION.

If the bile salts in urine are to be measured quantitatively by the surface tension method, it must be certain that the surface tension of the sample is not materially lowered or raised by other constituents. None of the normal constituents of urine are known to have any considerable effect upon the surface tension. Inorganic salts increase the surface tension of distilled water, but only very slightly. Donnan and Donnan found that urea, sodium urate, dextrose, acetone, and albumin (egg white) had no effect upon the surface tension of urine, or only very little effect in concentrations that are relatively high for urine. Lyon-Caen added creatinine, thiocyanates, hippuric acid, and biliary pigments to this list as not affecting the surface tension of distilled water. We have confirmed these observations for urea, uric acid, hippuric acid, and creatinine in distilled water, and have found that creatine, oxalic acid, allantoin, inosite, and leucine are also without effect upon the surface tension of distilled water in the concentrations indicated in Table IV.

TABLE IV.

Showing the Surface Tension of Solutions of Various Urinary Constituents in Distilled Water.

Substance.	Concentration.	Sp. gr.	Drops.	S. T.
Distilled water		1.000	40.8	100.0
Urea	5.0 gm. per 100 cc.	1.013	41.4	99.8
"	2.5 " " " "	1.006	41.0	100.1
Uric acid	Saturated solution	1.002	40.7	100.2
" "	1 gm. in 2000 cc. + 50 cc. $\frac{N}{10}$ NaOH + solid NaOH till acid dissolved = 0.0487 gm. acid per 100 cc.	1.002	40.8	100.2
Hippuric acid	0.04 gm. per 100 cc.		40.8	100.0
Oxalic acid	0.004 " " " "		40.7	100.2
" "	0.002 " " " "		40.8	100.0
Creatinine	0.1 " " " "		41.0	99.5
Creatine	0.2 " " " "		40.9	99.8
"	0.1 " " " "		40.7	100.2
Allantoin	0.2 " " " "		40.9	99.8
"	0.1 " " " "		40.9	99.8
Inosite	1.0 " " " "		40.8	100.0
Distilled water	(Temperature 26°C.)		41.0	100.0
Leucine	1.0 gm. per 100 cc.		41.7	98.3

VI. THE EFFECT OF PEPTONES UPON THE SURFACE TENSION OF URINE.

The only other urinary constituents which we have found described as materially affecting the surface tension of urine are peptones. Lyon-Caen found that Witte's peptone lowered the surface tension of distilled water to 88 per cent in a 0.1 per cent solution. We have found the effect to be still greater than is indicated by this figure. A 0.1 per cent solution in distilled water was found to have a surface tension of 81.2 per cent and 0.1 gram added to 100 cc. of urine lowered the tension from 94 per cent to 80.6 per cent. Peptonuria, therefore, may cause appreciable error in attempting to measure the amount of bile salts present by the surface tension method.

TABLE V.

Showing the Surface Tension of Solutions of Witte's Peptone in Distilled Water and in Urine.

Solution.	Sp. gr.	Drops.	S. T.
Distilled water	1.000	41.0	100.0
Witte's peptone 0.1 gm. per 100 cc.		50.5	81.2
" " 0.05 " " " "		49.0	83.7
" " 0.02 " " " "		45.9	90.0
" " 0.005 " " " "		41.6	98.6
Distilled water	1.000	41.0	100.0
Urine. Diluted	1.010	44.0	94.0
Urine + Witte's peptone 0.1 gm. per 100 cc.		51.3	80.6
" + " " 0.05 " " " "		50.1	82.6
" + " " 0.02 " " " "		47.2	87.7
" + " " 0.005 " " " "		44.9	92.2

VII. THE EFFECT OF ACETONE BODIES UPON THE SURFACE TENSION OF URINE.

The acetone bodies, acetone, aceto-acetic acid, and β-oxybutyric acid, may occur in the urine in considerable amounts, especially in diabetes. Donnan and Donnan found that acetone added to urine had very little effect unless as much as 1 per cent by volume was added when the surface tension was depressed almost 3 per cent. We found that 2 per cent by volume of acetone in urine depressed the surface tension from 91.3 per cent to 87.2 per cent,

TABLE VI.

Showing the Surface Tension of Solutions of Acetone in Distilled Water and in Urine.

Solution.	Sp. gr.	Drops.	S. T.
Distilled water	1.000	40.9	100.0
Acetone 2.0 cc. in 100 cc.	0.9975	45.9	88.7
" 1.0 " " " "	0.998	43.9	92.9
" 0.5 " " " "	0.999	42.6	95.9
" 0.25 " " " "	1.000	41.9	97.6
Urine	1.020	45.7	91.3
" + 2.0 cc. acetone in 100 cc.	1.0155	47.6	87.2

although 2 per cent of acetone in distilled water depressed the tension from 100 per cent to 88.7 per cent.

β-Oxybutyric acid is much more important in its action on the surface tension than acetone. A 1 per cent solution by weight in distilled water was found to have a surface tension of 78.5 per cent, and 1 gram in 100 cc. of urine depressed the surface tension from 91.3 per cent to 75.3 per cent.

TABLE VII.

Showing the Surface Tension of Solutions of β-Oxybutyric Acid in Distilled Water and in Urine.

Solution.	Sp. gr.	Drops.	S. T.
Distilled water	1.000	40.9	100.0
Gm. per 100 cc.			
β-Oxybutyric acid 1.0		52.0	78.5
" " 0.75		51.0	80.0
" " 0.5		49.8	81.9
" " 0.25		47.8	85.4
" " 0.125		46.0	88.7
" " 0.0625		44.5	91.7
" " 0.03125		43.2	94.7
" " 0.007125		41.6	98.1
Distilled water	1.000	40.8	100.0
Urine	1.020	45.7	91.3
" + 1.0 gm. β-oxybutyric acid per 100 cc.	1.022	55.3	75.3
Distilled water	1.000	40.9	100.0
β-Oxybutyric acid 0.634 gm. per 100 cc. after being made neutral to methyl orange and alizarin with NaOH		47.4	86.1
β-Oxybutyric acid 0.645 gm. per 100 cc. after being made neutral to litmus with NaOH		47.6	85.7

When this acid was neutralized with sodium hydrate it was found to lose only about one-fourth of its effectiveness in lowering the surface tension of distilled water. When the 1 per cent β-oxybutyric acid solution in distilled water was neutralized to litmus with $\frac{N}{10}$ sodium hydrate the resulting solution, which was equivalent to a 0.645 per cent solution of β-oxybutyric acid, had a surface tension of 85.7 per cent; while a distilled water solution of this concentration of the unneutralized acid has a surface tension of 80.7 per cent as determined from a graph based upon Table VII.

In severe acetonuria the β-oxybutyric acid may be found in larger amounts than the acetone itself, and these observations indicate that such a condition would have a pronounced effect upon the surface tension of the urine. It would be important to determine also the effect of aceto-acetic acid upon the surface tension of urine for this substance would be found accompanying the oxybutyric acid, and it is also present in less severe acetonuria when the oxybutyric acid is not found. β-Oxybutyric acid, like the bile acids, is difficult to demonstrate in urine. Some simple test for it would be of great value in the study of glycosuria. Whether the surface tension method would be of value for this purpose is not certain. If aceto-acetic acid also lowers the tension very strongly, then these two bodies could not be distinguished by this means. It is evident, however, that conditions of acetonuria must be avoided in testing for bile salts by this method. According to von Noorden[4] acetonuria can be eliminated, except in diabetes, by feeding carbohydrates.

TABLE VIII.

Showing the Surface Tension of Solutions of Lactic Acid and of Benzoic Acid in Distilled Water.

Solution.	Sp. gr.	Drops.	S. T.
Distilled water	1.000	40.7	100.0
Lactic acid 1.0 gm. per 100 cc.		44.3	92.1
" " 0.59 " " " " after being made neutral to litmus with NaOH		41.3	98.8
Distilled water		40.8	100.0
Benzoic acid 0.2 gm. per 100 cc.		44.0	92.7
" " 0.1 " " " "		42.0	97.1
" " 0.05 " " " "		41.3	98.8

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[4] von Noorden, C., Clinical Treatises on the Pathology and Therapy of Disorders of Metabolism and Nutrition. Part VII. Diabetes Mellitus, New York, 1905.

VIII. BENZOIC ACID AND LACTIC ACID.

Lactic acid and benzoic acid have been found to have an effect upon the surface tension of distilled water, but a less effect than that of the peptones and the oxybutyric acid. These substances might become a minor source of error when present in considerable amounts.

IX. THE "RECIPROCAL ACTION" OF MINERAL SALTS AND BILE SALTS IN LOWERING THE SURFACE TENSION.

It has been shown by Traube[5] and by Billard and Dieulafé[6] that while very dilute solutions of bile have a surface tension very much below that of distilled water, the tension is lowered still further if a little mineral salt is added. This is true in spite of the fact that mineral salts themselves do not lower the surface tension of distilled water. Sodium salts were found more active in this respect than those of potassium, and among the different salts the chlorides were the most effective.

Lyon-Caen confirmed the results of Billard and Dieulafé. He found that a 0.1 per cent solution of sodium glycocholate in distilled water had a surface tension of 80.2 per cent of that of distilled water. The same concentration of glycocholate in 0.1 per cent sodium chloride solution had a surface tension of 76.4 per cent. With increasing concentrations of sodium chloride the surface tension became successively lower until it reached 69.3 per cent in an 0.8 per cent sodium chloride solution. This author made the further observation that increase of sodium chloride above the concentration of 0.8 per cent, up to 1.5 per cent at least, failed to cause greater lowering of the tension.

We have repeated these experiments with sodium chloride in 0.1 per cent and 0.003125 per cent solutions of sodium glycocholate in distilled water, and have verified the fact that a minimum surface tension is reached when the sodium chloride content reaches 0.8 per cent. The surface tension of a 0.1 per cent solution of glycocholate in distilled water was found to be 69.3 per cent. Increasing amounts of sodium chloride lowered this

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[5] Traube, J., *Jour. f. prakt. Chem.*, 1885. xxxi, 214.

[6] Billard and Dieulafé, *Compt. rend. Soc. de biol.*, 1902, liv, 405.

TABLE IX.

Showing the Surface Tension of Solutions of Sodium Glycocholate in Distilled Water and in Salt Solutions.

Solution.	Sp. gr.	Drops.	S. T
Distilled water	1.000	40.7	100.0
Na-Glyc. 0.1 per cent	1.001	58.4	69.8
“ “ “ + 0.1 gm. NaCl per 100 cc.	1.0025	64.6	63.2
“ “ “ + 0.2 “ “ “ “ “	1.0035	65.7	62.2
“ “ “ + 0.4 “ “ “ “ “	1.005	66.6	61.4
“ “ “ + 0.8 “ “ “ “ “	1.008	68.0	60.3
“ “ “ + 1.2 “ “ “ “ “	1.011	68.0	60.5
“ “ “ + 1.6 “ “ “ “ “	1.0135	68.4	60.3
Na-Glyc. 0.003125 per cent	1.000	44.8	90.8
“ “ “ + 0.2 gm. NaCl per 100 cc.	1.002	45.6	89.4
“ “ “ + 0.4 “ “ “ “ “	1.0045	45.7	89.4
“ “ “ + 0.6 “ “ “ “ “	1.006	46.0	88.9
“ “ “ + 0.8 “ “ “ “ “	1.008	46.4	88.4
“ “ “ + 1.2 “ “ “ “ “	1.011	46.5	88.4
“ “ “ + 1.6 “ “ “ “ “	1.014	46.6	88.5
Distilled water	1.000	40.9	100.0
Acetone 2.0 cc. per 100 cc. distilled water	0.997	46.0	88.6
“ “ “ “ “ “ 1.0 per cent NaCl solution	1.005	46.3	88.7

TABLE X.

Showing the Surface Tension of Solutions of Sodium Glycocholate in Distilled Water and in Sodium Sulphate Solutions.

Solution.	Sp. gr.	Drops.	S. T.
Distilled water	1.000	40.8	100.0
Na-Glyc. 0.1 per cent	1.001	61.4	66.5
“ “ “ + 0.2 gm. Na_2SO_4 per 100 cc.	1.0035	65.6	62.3
“ “ “ + 0.4 “ “ “ “ “	1.005	66.3	61.3
“ “ “ + 0.6 “ “ “ “ “	1.0065	67.3	61.0
“ “ “ + 0.8 “ “ “ “ “	1.008	67.7	60.7
“ “ “ + 1.0 “ “ “ “ “	1.009	68.1	60.4
“ “ “ + 1.2 “ “ “ “ “	1.0105	68.8	59.9
“ “ “ + 1.4 “ “ “ “ “	1.012	69.6	59.3
“ “ “ + 1.6 “ “ “ “ “	1.013	70.6	58.5
“ “ “ + 2.0 “ “ “ “ “	1.016	71.2	58.2
“ “ “ + 10.0 “ “ “ “ “	1.065	76.3	56.9

TABLE XI.

Showing the Effect upon Surface Tension of Urine of Adding Sodium Chloride.

Solution.	Sp. gr.	Drops.	S. T.
Distilled water	1.000	40.9	100.0
Urine Sample I.	1.010	43.4	94.4
" " " + 0.5 gm. NaCl per 100 cc.	1.013	44.0	94.1
Distilled water	1.000	40.8	100.0
Urine Sample II	1.025	46.6	89.7
" " " + 0.5 gm. NaCl per 100 cc.	1.0285	47.1	89.1
" " " + 1.0 " " " " "	1.0315	47.4	88.6
" " " + 1.5 " " " " "	1.0345	47.7	88.4
" " " + 2.0 " " " " "	1.0385	48.0	88.2
Distilled water	1.000	40.6	100.0
Urine Sample II. Diluted	1.010	42.1	97.4
" " " " + 0.5 gm. NaCl per 100 cc.	1.0135	42.5	96.8
" " " " + 1.0 gm. NaCl per 100 cc.	1.0175	42.6	96.9
" " " " + 2.0 gm. NaCl per 100 cc.	1.024	43.5	95.4
Distilled water	1.000	40.6	100.0
Urine Sample III. Diluted	1.010	42.3	98.7
" " " " + 0.01 gm. Na-Glyc. per 100 cc.		56.3	72.8
" " " " + 0.01 gm. Na-Glyc. per 100 cc. + 1.0 gm. NaCl per 100 cc.	1.0185	57.8	71.5
" " " " + 0.01 gm. Na-Glyc. per 100 cc. + 2.0 gm. NaCl per 100 cc.	1.025	59.0	70.5

figure until it reached 60.3 per cent for 0.8 per cent sodium chloride. Further increase of sodium chloride up to 1.6 per cent caused no further lowering. Similarly the surface tension of a 0.003125 per cent solution of sodium glycocholate in distilled water was lowered from 90.8 per cent to 88.4 per cent by adding 0.8 per cent sodium chloride. Sodium sulphate was found to have a weaker "reciprocal action," as Lyon-Caen also reports.

Sodium chloride is the chief inorganic constituent in urine, and the fact that its "reciprocal action" reaches its maximum in an 0.8 per cent solution is suggestive. Urine normally contains a little more than this amount of salt, and adding a little more would not be expected to alter the surface tension. On the other hand diluting the samples to a standard specific gravity of 1.010 would reduce the concentration of this salt. Would it be possible to eliminate the small error from this source by adding an excess of sodium chloride? Table XI shows the effect upon the surface tension of urine of adding increasing amounts of sodium chloride.

The surface tension of normal urine to which no bile salts had been added was lowered by adding the salt. This was true both before and after diluting to 1.010 specific gravity. And the lowering continued after the 0.8 per cent solution of NaCl was exceeded. To the third sample of urine 0.01 per cent sodium glycocholate was added which lowered the surface tension from 98.7 per cent to 72.8 per cent. Then an addition of 1.0 per cent NaCl lowered the tension 1.3 per cent more and a second addition of 1.0 per cent NaCl lowered the tension an additional 1.0 per cent. The rule, therefore, that a maximum lowering is reached in an 0.8 per cent solution does not hold true for urine, and it is not possible to obtain uniform readings by adding an excess of NaCl. The error from varying concentrations of NaCl does not appear to be large, however.

X. THE EFFECT OF ACIDITY UPON THE SURFACE TENSION.

Samples of normal urine which have been diluted to a standard specific gravity of 1.010 have a surface tension above 90 per cent. It might be expected that the initial variation in different samples would tend to disappear with the addition of increasing amounts of bile salts. Thus a 10 per cent lowering of the surface tension of urine is caused by the addition of 0.003 per cent sodium glycocholate, but when 0.01 per cent glycocholate is already present the addition of 0.003 per cent more of glycocholate causes a lowering of the tension of only 2 per cent, and when 0.1 per cent glycocholate is present a difference of 0.003 per cent more or less hardly causes an appreciable difference in the tension. For this reason the curves in Figure 1 based on observations upon four

TABLE XII.

Showing the Range of Surface Tension of Solutions of Sodium Glycocholate in Acid and in Alkaline Samples of Urine.

Solution.	Sp. gr.	Drops.	S. T.
Distilled water	1.000	40.8	100.0
Urine Sample II. (Reaction not noted.)	1.012	42.4	97.4
" " " Diluted	1.010	42.3	97.5
" " " " + 0.1 gm. Na-Glyc. per 100 cc.		60.7	67.9
" " " " + 0.05 gm. Na-Glyc. per 100 cc.		57.3	71.9
" " " " + 0.02 gm. Na-Glyc. per 100 cc.		53.0	77.7
" " " " + 0.005 gm. Na-Glyc. per 100 cc.		47.0	87.7
Distilled water	1.000	40.8	100.0
Urine Sample III. (Reaction acid.)	1.028	50.0	83.9
" " " Diluted	1.010	44.8	91.9
" " " " + 0.1 gm. Na-Glyc. per 100 cc. (cloudy)		67.7	60.8
" " " Diluted + 0.02 gm. Na-Glyc. per 100 cc. (clear)		60.6	68.0
" " " Diluted + 0.005 gm. Na-Glyc. per 100 cc.		53.0	77.7
Distilled water	1.000	40.7	100.0
Urine Sample IV. (Reaction alkaline.)	1.015	45.4	90.9
" " " Diluted	1.010	43.2	95.1
" " " " + 0.1 gm. Na-Glyc. per 100 cc.		62.5	65.8
" " " " + 0.05 gm. Na-Glyc. per 100 cc.		58.3	70.5
" " " " + 0.02 gm. Na-Glyc. per 100 cc.		53.2	77.3
" " " " + 0.01 gm. Na-Glyc. per 100 cc.		50.7	81.0
" " " " + 0.005 gm. Na-Glyc. per 100 cc.		47.8	86.0

different samples of urine (Tables I and XII) would be expected to approach each other toward their lower ends. As the figure indicates, a very different result was obtained. One sample of urine to which 0.1 per cent glycocholate was added had a surface tension as high as 67.9 per cent and another sample as low as 60.8 per cent. The original samples (sp. gr. 1.010) in these two cases showed a difference in surface tension of 6.6 per cent.

The following observations called attention to the effect of the acidity of the urine upon its surface tension, and this factor seems to account for the discrepancy. Some samples of urine do not readily dissolve 0.1 gram of glycocholate in 100 cc. A sample of this sort had a specific gravity of 1.028 and a surface tension of 83.9 per cent. When 0.1 gram of sodium glycocholate was added to 100 cc. of this urine a dense cloudy solution was obtained. The solution was then made clear by adding three drops of strong sodium hydrate, and it then had a surface tension of 64.3 per cent. When to a portion of this solution a drop of acid was added it became slightly cloudy and the tension dropped to 62.6 per cent. Another portion was diluted with the original urine to a 0.005 per cent glycocholate solution, when it had a tension of 75.1 per

TABLE XIII.

Showing the Effect upon the Surface Tension of Urine of Adding Acid and Alkali.

Solution.	Sp. gr.	Drops.	S. T.
Distilled water	1.000	40.7	100.0
Urine. Diluted	1.010	43.4	94.6
" " + 1 drop concentrated HCl per 100 cc.	1.011	44.4	92.6
" " + 2 drops " " " " "	1.011	46.0	89.4
" " + 3 " " " " " "	1.011	47.2	87.1
" " + 4 " " " " " "	1.011	48.4	85.0
" " + 5 " " " " " "	1.011	49.6	82.9
" " + 7 " " " " " "	1.011	51.3	80.2
" " + 10 " " " " " "	1.0115	50.6	81.3
" " + 10 " " " " " " (1½ hrs. later)	1.0115	50.4	81.8
Distilled water	1.000	40.9	100.0
Urine. Diluted. (Another portion of same sample as above.)	1.010	43.8	94.2
" " + 1 drop concentrated NaOH per 100 cc.	1.010	43.2	95.5
" " + 2 drops " " " " "	1.011	43.6	94.8
" " + 3 " " " " " "	1.011	44.1	93.7
" " + 5 " " " " " "	1.011	44.0	93.9

cent. After adding a drop of acid to this solution the tension dropped to 71.7 per cent. The remainder of the original sample of this acid urine was diluted to a specific gravity of 1.010, when the surface tension became 91.9 per cent. Addition of 0.1 gram of glycocholate to 100 cc. gave a partly cloudy solution having a tension of 60.8 per cent. This sample furnished the data for Sample III, Table XII and Figure 1. These observations indicate that a more acid reaction of icteric urine is accompanied by a lower surface tension.

The effect of acidity upon the surface tension of normal urine was then tested by adding increasing amounts of hydrochloric acid to one portion and increasing amounts of sodium hydrate to another portion of the same sample of urine. The results are given in Table XIII, and show that a maximum difference of 15.3 per cent in the surface tension of a non-icteric urine was produced by changing the acidity in this way.

The sample of urine which furnished the data for Curve III was known to be strongly acid. The reactions of Samples I and II had not been noted, but it was inferred that the latter was probably alkaline or weakly acid. To test this a fourth sample was obtained which was distinctly alkaline to litmus, and this sample (IV) gave a curve which approached that of Sample II.

We conclude, therefore, that the surface tension of both normal and icteric urines varies considerably with the reaction, being lower when the acidity is greater.

XI. DISCUSSION.

When peptonuria and acetonuria are not present and when samples of urine are diluted to a standard specific gravity of 1.010 the chief remaining error in attempting to estimate the amounts of bile salts present by the surface tension method seems to be due to the varying acidity of the samples. Upon the basis of the data given above a table may be constructed showing the amounts of sodium glycocholate in urine diluted to a specific gravity of 1.010 which should be present in urine of any observed surface tension. Such a table is given in Table XIV which is based upon Figure 1. It indicates, for example, that if the surface tension is 85 per cent a minimum of 0.001 gram of sodium glycocholate per 100 cc. of the diluted sample may be present if the sample is acid, or a maximum of 0.008 gram may be present if the sample is alkaline.

Table XIV is based entirely upon data obtained with sodium glycocholate. When bile is found in urine there is a mixture of glycocholate and taurocholate in unknown proportions, and, in many cases at least, biliary pigments are also found. Lyon-Caen reports finding that biliary pigments do not affect the surface tension. The taurocholate lowers the surface tension just a little less than the glycocholate does. Accordingly, the figures given in Table XIV are a little too low for a mixture of the two salts actually present in the urine. They represent the glycocholate equivalent of the mixture in its effect upon the surface tension.

TABLE XIV.

Showing the Amounts of Sodium Glycocholate Required to Give Any Observed Surface Tension in a Sample of Urine Diluted to a Standard Sp. Gr. of 1.010.

S. T. of sample diluted to 1.010 sp. gr.	Minimum of sodium glycocholate which may give observed S. T. (Sample is acid.)	Maximum of sodium glycocholate which may give observed S. T. (Sample is alkaline.)
Per cent of that of distilled water.	Diluted sample.	Diluted sample.
	gm. per 100 cc.	*gm. per 100 cc.*
95	0	0.001
90	0	0.004
85	0.001	0.008
80	0.003	0.015
75	0.007	0.03
70	0.015	0.065
65	0.033	0.1+
60	0.1+	0.1+

While the results reported in this paper may be disappointing in not finding the stalagmometric measurement of the surface tension a very accurate means of determining the amount of bile salts in urine it must be remembered that no other simple test is as satisfactory. The Hay test also depends upon the same phenomenon of surface tension, and it gives no satisfactory objective numerical result. The stalagmometer does give a numerical result which is not dependent upon the judgment of the operator. When the reaction of the sample is taken into account together with the percentage surface tension, this method of estimating the amount of bile in the urine may be found of clinical value.

A CRITICISM OF THE BANG AND LEWIS-BENEDICT METHODS FOR THE ESTIMATION OF BLOOD SUGAR, WITH SUGGESTIONS FOR A MODIFICATION OF THE LATTER METHOD.

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The importance of a method for the determination of blood sugar which is rapid and accurate, and which requires a minimum amount of blood, is indicated by the numerous so called micro-methods which have appeared recently. For a number of years the writer, in collaboration with Professor Macleod, has been seeking for such a method, but it is only within the past year that one which meets our requirements has been found. In the present paper an account of this and other less satisfactory methods recently employed in our laboratory will be given.

The micro-method proposed by Bang[1] in 1913 was thoroughly tried and found to be unsatisfactory. It must be admitted, however, that these trials were made according to the directions outlined in the original paper. Since Bang revised the method[2] we have not tried it in its entirety. With the original method the chief trouble was in removing the protein by means of acetic acid and potassium chloride. As our experiments did not make it necessary for us to use the small quantities of blood suggested by Bang, we attempted to modify the method by using 2 cc. of blood and precipitating the protein with dialyzed iron by the well known method of Rona and Michaelis. To apply Bang's titration, a portion of the clear filtrate properly diluted with potassium chloride solution was used, and the sugar estimated ac-

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[1] Bang, I., Der Blutzucker, Wiesbaden, 1913.

[2] Bang, Über die Mikromethode der Blutzuckerbestimmung, *Biochem. Ztschr.*, 1913, lvii, 300.

cording to Bang's directions for his micro-method. If the greatest care was taken to heat the flask so that the solution boiled in the time specified by Bang, and to see that no air was admitted in the flask while cooling, and that the titration was done in a pure atmosphere of CO_2, it was possible to recover added sugar in the blood within a 7 per cent error. The method, however, is slow and tedious, and requires a large number of duplicate examinations to be made on each sample in order that one may feel sure of accuracy.

The main difficulty we encountered was in regulating the time required for the solution to reach the boiling point. A variation of from ten to thirty seconds made an appreciable difference in the final result. Different flasks, although apparently alike, will vary in the time they must be heated by a flame to make their contents boil, and unless one gives his undivided attention to the heating of the flask, and also takes the same more or less arbitrary sign for the moment the solution begins to boil, variations result in the time of heating, and these variations affect the determination in a marked degree. This makes the method very slow, and renders it of little use in any research where a great number of bloods must be tested at one time.

With the hope of standardizing the heating conditions and of decreasing the time and attention required for the estimation, we tried a number of modifications of the method. At first we attempted to find a bath which when boiling would bring the solution to be titrated to the boiling point in one minute and ten seconds. A salt solution boiling at 110° will accomplish this, but the salt crystallizes and the water evaporates, and it is difficult to maintain the boiling point of the bath constant. We then tried placing the flasks in baths of glycerin, sand, or oil heated above 100° and regulated by a thermoregulator. In using these flasks the actual time the flasks were immersed in the bath was noted. No notice of the exact moment that the solution began to boil was taken, since it is presumable that this is a constant interval under these conditions. Russian White Oil was found to be the most satisfactory bath, since it may be heated to 140° without suffering any change. However, at this temperature, because of its low specific heat, solutions immersed in it will not boil in less than two minutes. By experiment it was determined that on leaving a flask in such a bath at 140° for five minutes, a reduction similar to that obtained by Bang's original method is obtained.

By placing the flasks in a large oil bath at intervals of a few minutes, it is possible to make a number of determinations in a comparatively short time. The greatest care must be taken to remove the flasks exactly at the end of the five minute period, and to close the rubber tubes guarding the neck at the same moment so as to prevent the entrance of air into the flask. The flasks are then placed in cold water and cooled rapidly, after which the vacuum, resulting from the condensation of the steam, is reduced by allowing CO_2 to enter. To accomplish the latter without allowing any oxygen to enter requires that, at the moment the clamp is released, the tube guarding the neck of the flask must be tightly attached to a gasometer from which CO_2 is flowing. The flask is then quickly removed and the titration is done in an atmosphere of CO_2. If there is the slightest evidence that air has obtained entrance into the flasks the result must be discarded since experience shows that the slightest trace of oxygen will oxidize some of the reduced copper of the solution. The chief drawback to Bang's method, besides its comparative complexity, is the extraordinary readiness with which the reduced copper becomes oxidized.

Since the method and time of heating are changed, the table prepared by Bang for his method is not of service, and a new one should be made. We were, however, able to confirm Bang's statement that the amount of reduction of the copper is, within certain limits, almost directly proportional to the amount of dextrose present in the solution. For example, he found that a factor could be used which represents the number of mg. of dextrose corresponding to 1 cc. of $\frac{N}{100}$ iodine solution. He found that this factor was almost constant between approximately 0.1 and 1.5 mg. of sugar. We found that it was best to determine this figure for each series of estimations by determining the iodine value of a sugar solution of known strength. From this number we could obtain the factor. In the case of the experiment shown in the table it is seen that each 0.1 mg. of sugar equaled about 0.42 cc. of iodine solution. The calculated value from the blank was 0.45.

Since we have a series of experiments, which will be reported in the near future, in which the above method was used, I will briefly describe the method in detail.

Two cc. of blood are added to 19 cc. of water. 10 cc. of dialyzed iron and 1 cc. of saturated sodium sulphate solution are now added and the solution is filtered. 2 cc. of the clear filtrate are taken for estimation by the modified Bang method. This is treated with 10 cc. of saturated solution of potassium chloride and 1 cc. of Bang copper solution, in a 25 cc. Jena flask, on the neck of which a rubber tube is drawn. The flask is placed in an oil bath heated to 140° C. and left there for exactly five minutes. At the end of this period the rubber tube is clamped off with a hemostat. When cool, CO_2 is introduced to fill the vacuum which has been made by the condensation of the steam, and the solution is titrated in an atmosphere of CO_2 with $\frac{N}{100}$ iodine solution, starch being used as an indicator. A solution made up with pure water in place of sugar solution will give a reduction equal to 0.2 cc. of iodine. For this reason 0.2 cc. is subtracted from all titration results.

The value of the iodine in terms of sugar is obtained before each series of titrations by the method above outlined.

The following results were obtained in an experiment in which known amounts of sugar were added to blood samples and the total sugar content of the blood was then determined. This experiment is typical of a series of eight similar ones which were done to determine the accuracy of the method.

The following table gives the average results of three titrations on the same sample.

Sample.	Sugar 0.395 per cent.	$\frac{N}{100}$ iodine, average 3 titrations.	Dextrose found.*	Dextrose calculated.	Actual difference.	Difference.
	cc.	*cc.*	*mg.*	*mg.*	*mg.*	*per cent*
I	0	1.10	0.242	0.242	0	
II	1	1.28	0.281	0.263	0.018	6.8
III	3	1.45	0.319	0.305	0.014	5.4
IV	5	1.74	0.382	0.347	0.035	10.0
V	7	1.96	0.421	0.389	0.032	8.2
VI	9	2.25	0.495	0.431	0.064	14.0

* In order to get the per cent of sugar in the blood, multiply the number of mg. dextrose found by 8, as the solution estimated represents one-eighth cc. of blood. Thus, Sample 1 contains 0.193 per cent dextrose.

Duplicate analyses of the same sample of blood are surprisingly close together, as the following figures indicate. These represent the actual mg. of dextrose found in one-eighth cc. of blood.

A.	B.	C.	D.
206	210	195	230
198	192	193	204
195	192	198	210

The error from these figures is seen to be about 6 per cent.

The extreme care necessary at each step, the delicacy of the reduced copper to the presence of oxygen, and the variations which slight changes in temperature cause, all contribute to render the above method unsatisfactory. It was with pleasure that we turned to the blood sugar method recently proposed by Lewis and Benedict,[3] which we have found to bear out all the claims which its authors make for it. We have used the method in our laboratory for the past six months and have found it to be very reliable.

The method in short consists in diluting 2 cc. of blood with 8 cc. of water and 15 cc. of concentrated picric acid. The blood proteins separate out, and after filtering, 8 cc. of the filtrate are treated with 2 cc. of concentrated picric acid and 1 cc. of 10 per cent sodium carbonate solution, and the mixture is evaporated over a free flame in a Jena test-tube to small volume or until a precipitate forms. A little water is added and the solution again brought to the boiling point; the contents of the tube are transferred to a 10 cc. measuring flask, and after being made up to volume, the solution is filtered and compared in a Duboscq colorimeter with a standard picramic acid solution, which is made to correspond to the color produced by 0.64 mg. of dextrose under the above conditions.

The method, while very well adapted to making analysis in a limited series of bloods, requires a great deal of patience and time for the evaporating of the 11 cc. of fluid down to the point required. without losing a little from the ebullitions or from shaking. From ten to fifteen minutes are required for this process if extreme accuracy is desired. If one tries to hurry by heating with a hotter flame and by more violent shaking, some of the fluid is likely to be lost, as can be shown by placing a piece of filter paper loosely

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[3] Lewis, R. C., and Benedict, S. R., A method for the estimation of sugar in small quantities of blood, *Jour. Biol. Chem.*, 1915, xx, 61.

over the top of the tube. A device which would permit a large number of tubes to be heated at once would make the method of far greater use for the routine examinations of a large number of bloods.

In seeking means by which to avoid the heating by hand over a free flame, many methods were tried, such as the use of oil baths at varying temperatures, sand baths, electric hot plates, etc., but without success. Either the solutions evaporated too slowly and became cloudy, or evaporations occurred so rapidly that the reaction was not completed, and readings that were too small were recorded for the sugar. The only method which gave uniform results was that recommended by the authors of the method. The foregoing observations indicate that the reaction requires a certain time at a certain temperature to complete itself. This suggested the possibility that it would take place if the boiling point of the liquid were raised. To accomplish this the tubes were placed in an autoclave and heated under a pressure of 2.5 kg. per sq. cm. for fifteen to thirty minute periods. Since the procedure does not entail any evaporation, it was found convenient to use 6 cc. of the filtrate in place of 8 cc. This amount is, under normal conditions, sufficient to give a fair reading in the colorimeter. After the autoclaving, the solutions were made up to 10 cc. in a measuring flask and compared with the standard. These solutions showed 75 per cent of the sugar contained in the tubes made up with 8 cc. of the blood filtrate, and in every case checked with estimations made in the original manner. The standard solution of picramic acid recommended by Benedict and Lewis may be diluted one-fourth, and the readings may then be made direct.

The use of the autoclave is of distinct advantage not alone because it allows a large number of estimations to be made at one time, but also because all danger of loss of fluid during the period of heating is removed, and the accuracy of the method is thus increased.

I have by this method prepared and estimated as many as forty samples of blood in the course of an afternoon. This entails eighty estimations, counting controls. This number is far in excess of that possible by the former method of heating over a free flame.

Another point which is of aid in obtaining perfectly relative readings of a large number of sugars is to compare all the estimations with the first of the series in place of comparing with the standard solution in each case. The color of the standard solution, since it contains no unchanged picric acid, is a little different from that obtained by the actual estimation of sugar and cannot be so easily matched with the reduced picric acid solution. By estimating the strength of the first one of the series against the picramic acid standard, which can be done within a very small error, and then using this one as the standard for the others, almost absolute comparative readings of the rest of the series are obtained.

Most accurate reading of the colorimeter can be obtained when the color intensity of the unknown is less than or nearly equal to that of the standard picramic acid solution. That this is mathematically true is seen by reference to the parabolic curve (Fig. 1). The ordinates represent the divisions on the colorimeter scale, and the figures on the abscissae the percentile strength of the unknown solution as determined by matching the unknown with the intensity of color obtained on immersing the colorimeter tube in the standard solution to 10 or 15 on the scale. It is seen that the divisions of the vernier progressively decrease in value in cases when the solutions are weaker than the standard, as is shown by the fact that the curve is here most steep, whereas with stronger solutions each division becomes progressively more and more important, the curve here rapidly becoming flat.

I have found curves of the above type, made to represent the relation of the unknown to the standard when the latter is set at 10 and at 15 in the colorimeter, of great convenience in rapidly determining the value of the solutions in terms of sugar. One simply reads the scale and vernier of the unknown when the standard is set at 10 or 15, and then determines on the curve the per cent of strength of the unknown in terms of the standard which corresponds to this reading. When many solutions are to be tested much calculation is thereby saved.

The exact details of the method as we have used it are as follows: 2 cc. of blood are added to 8 cc. of water in a test-tube, and 15 cc. of concentrated picric acid solution are then added after the blood is thoroughly laked. The mixture is then filtered.

6 cc. of the filtrate are then introduced into a test-tube, and 2 cc. of concentrated picric acid and 1 cc. of 10 per cent sodium carbonate solution added; the tube is then placed in an autoclave and heated for fifteen to thirty minutes at a pressure of 2.5 kg. per sq. cm. (20 pounds to the square inch is sufficient). The tube is then removed, cooled, and the solution made up to 10 cc. in a measuring flask. The solution is then compared with the standard in the colorimeter. The standard recommended by Lewis and Benedict may be diluted one-fourth or allowed for by calculation, since 6 cc. of filtrate are used in place of 8 cc.

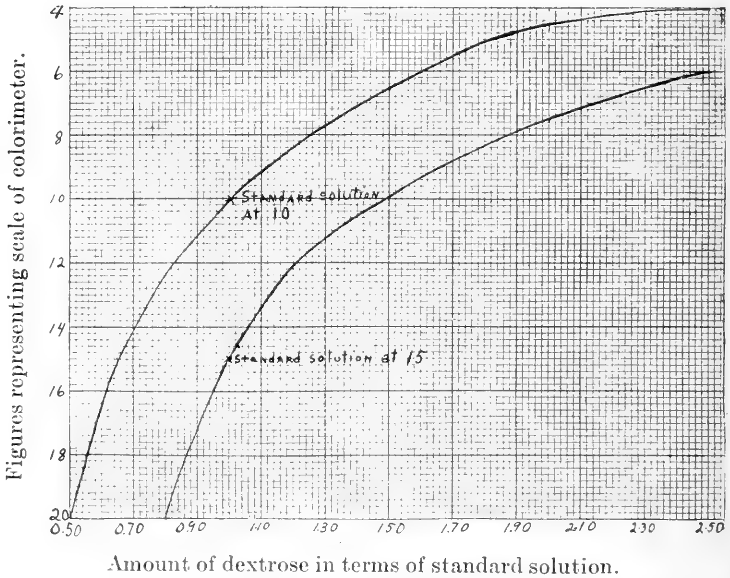

Fig. 1. Curves which show the amount of dextrose in terms of a standard solution as determined by the Lewis-Benedict blood sugar method.

We have been able to confirm the claims of the authors of the method for its accuracy, as the following figures will show. Measured quantities of a 0.1 per cent dextrose solution (accurately made up by weight, and, as tested with Bertrand's sugar method, shown to be approximately correct) were estimated both by the method as outlined by Lewis and Benedict and by the modified method of heating proposed above. The following data give the results.

Dextrose by weight.	Sugar estimated.	
	Heated in autoclave.	Heated by hand.
mg.	*mg.*	*mg.*
0.8	0.79	0.81
1.0	1.00	1.00
1.2	1.22	1.18
1.4	1.38	1.43
1.6	1.61	1.62
1.8	1.84	1.73
2.0	2.04	2.00

STUDIES ON REDUCTASE.

IV. THE INFLUENCE OF ALKALOIDAL AND OTHER NARCOTIC POISONS ON REDUCTASE.

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The poisonous influence of various substances towards enzymes has been largely investigated during the past few years. Certain substances are known which are so highly poisonous that, even in exceedingly small concentrations, they are capable of retarding or destroying the action of different enzymes. The poisonous action of a number of substances on the catalase of the blood has been investigated by Senter.[1] During recent years, Bredig and his pupils have shown that many of the enzymic poisons are also capable of inhibiting the action of certain inorganic catalysts. The similarity in effect of a number of different poisons on inorganic catalysts and enzymes is illustrated in Table I, in which is shown the concentration of the different poisons that is necessary to destroy entirely the catalytic action of colloidal platinum[2] and of catalase[3] on hydrogen peroxide.

Our previous work has shown[4] that the liver and other organs of the vertebrata contain a reducing enzyme reductase; and attempts to isolate it have indicated[5] that its activity is considerably retarded by contact with foreign substances. It seemed

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[1] Senter, G., *Ztschr. f. physikal. Chem.*, 1903, xliv, 257.

[2] Bredig, G., and Berneck, R. M. v., *ibid.*, 1899, xxxi, 258.

[3] Senter, *loc. cit.*

[4] Harris, D. F., and Creighton, H. J. M., *Proc. Roy. Soc., Series B*, 1912, lxxxv, 487; *Jour. Biol. Chem.*, 1915, xx, 179.

[5] Harris and Creighton, *Biochem. Jour.*, 1914, viii, 585.

TABLE I.

Poison.	Colloidal platinum.	Catalase.
H_2S	1: 300,000 molar	1: 1,000,000 molar.
HCN	1: 20,000,000 "	1: 1,000,000 "
$HgCl_2$	1: 2,000,000 "	1: 2,000,000 "
$Hg(CN)_2$	1: 200,000 "	1: 300 "
I in KI	1: 5,000,000 "	1: 50,000 "
$NH_2(OH)HCl$	1: 25,000 "	1: 80,000 "
$C_6H_5NH_2$	1: 5,000 "	1: 40,000 "
As_2O_3	1: 50 "	1: 2,000 "
HCl	1: 3,000 "	1: 100,000 "
NH_4Cl	1: 2,000 "	1: 1,000 "
HNO_3	No paralysis	1: 250,000 "
CO	Very poisonous	No paralysis.

desirable to extend these observations to include the possible action of organic poisons on reductase. Several well known, powerful, alkaloidal and narcotic poisons have been studied with a view of determining whether their toxicity towards reductase was in any way comparable with their toxicity towards living matter itself. It seemed of interest to know whether, if these substances were poisonous for reductase, their dosage in this connection was comparable with the dosage fatal to the entire animal. For this purpose the reductase contained in cat liver juice and brain juice was employed, being prepared in the manner previously described.[6]

The action of the foreign substances on reductase was measured by determining the difference between the time required for the reduction of oxyhemoglobin to the one-banded condition by pure liver or other juice, and by juice containing some of the foreign substance with which it had been in contact for a definite time. The progress of the reduction was followed by the spectroscopic method previously described.[7] In the experiments, 1 cc. of fresh liver juice and 1 cc. of an aqueous solution of the poison were allowed to remain in contact for ten minutes, at the end of which time 2 cc. of blood solution (prepared by diluting 10 cc. of cat's freshly defibrinated blood to 250 cc. with distilled water) were added and the mixture was introduced into narrow, conical, glass

[6] Harris and Creighton, *Jour. Biol. Chem.*, 1915, xx, 179.

[7] Harris and Creighton, *ibid.*, 1915, xx, 181.

observing tubes which were then placed in a thermostat at 40°. The spectrum of these mixtures was examined every half minute. The times required for the reduction at 40° by liver juice which contained foreign substances were compared with the time required for the reduction, at the same temperature, of a *normal* mixture containing 1 cc. of liver juice, 1 cc. of distilled water, and 2 cc. of the blood solution. The results of the action of a number of pure alkaloids are given in Table II. The times required for the reductions recorded in the following tables represent the mean of at least two experiments which seldom differed by more than a few per cent.

TABLE II.

4.5 Minutes Required for Reduction of Normal Mixture.

Poison.	Time in minutes required for the reduction of mixtures containing 1 cc. of *liver* juice, 2 cc. of blood solution, and 1 cc. of an aqueous solution of the poison having a normal concentration of					
	0.1	0.02	0.01	0.001	0.0005	0.0001
	min.	*min.*	*min.*	*min.*	*min.*	*min.*
Nicotine	8	11.5	11.5	10.0	—	13.5
Cocaine				4.5	—	—
Morphine				11.0	8.5	8.5
Strychnine				—	7.0	7.0
Aconitine				4.5	4.5	4.5
Atropine				8.0	10.0	8.5

On account of the extreme insolubility of these alkaloids in water, it was not possible to employ concentrations higher than 0.001 normal, except with nicotine. It was deemed advisable, therefore, to investigate the action of solutions of salts of these alkaloids on the activity of reductase. In addition to the action of salts of the foregoing alkaloids, the influence of hyoscine hydrobromide, over a large range of concentrations, has been determined. The results obtained are recorded in Tables III and IV.

Owing to the fact that so many of the alkaloidal poisons are narcotics, that is, act specifically on the central nervous system, it was thought advisable to carry out a parallel series of experiments, substituting brain juice for liver juice. The brain juice was obtained by grinding with sand in a mortar the cortex cerebri of a large cat with the addition of 20 cc. of 0.9 per cent sodium

TABLE III.

Poison: Hyoscine Hydrobromide.

4.5 Minutes Required for Reduction of Normal Mixture.

Concentration.	Time required for the reduction of mixtures containing 1 cc. of *liver* juice, 2 cc. of blood solution, and 1 cc. of an aqueous solution of the poison.
	min.
0.1 normal	5.5
0.05 "	6.5
0.02 "	7.5
0.01 "	6.5
0.001 "	4.0
0.00017 "	3.5
0.0001 "	3.5
0.00001 "	4.5

TABLE IV.

4.5 Minutes Required for Reduction of Normal Mixture.

Poison.	Time in minutes required for the reduction of mixtures containing 1 cc. of *liver* juice, 2 cc. of blood solution, and 1 cc. of an aqueous solution of the poison having a normal concentration of					
	0.1	0.02	0.01	0.001	0.0001	0.00001
	min.	*min.*	*min.*	*min.*	*min.*	*min.*
Hyoscine hydrobromide	5.5	7.5	6.5	4.0	3.5	4.5
Cocaine hydrochloride	5.5	8.0	8.5	10.0	4.5	—
Morphine sulphate	6.0	7.0	7.5	6.5	7.5	4.5
Atropine sulphate	8.0	6.0	6.5	—	—	—
Strychnine sulphate	Destroys blood	8.0	6.0	6.5	6.5	—
Quinine hydrochloride	—	11.0	9.0	8.0	9.0	6.0
Caffeine citrate	Destroys blood	25.0	24.5	12.5	10.0	9.0
Alcohol	10.0	—	10.0	—	9.0	—
Ether	—	5.0	—	5.0	—	—
Chloroform	—	10.0	—	9.0	8.0	7.0

chloride to the pulp so as to give a mixture which could be squeezed through cheese-cloth. In this way a remarkably homogeneous juice was obtained, of which 1 cc. plus 1 cc. of distilled water completely reduced 2 cc. of the blood solution in six and one-half minutes. The results of observations of the action of a number of different poisons on brain juice are recorded in Table V.

TABLE V.

6.5 Minutes Required for Reduction of Normal Mixture.

Poison.	Time in minutes required for the reduction of mixtures containing 1 cc. of *brain* juice, 2 cc. of blood solution, and 1 cc. of an aqueous solution of the poison having a normal concentration of			
	0.1	0.05	0.001	0.00001
	min.	*min.*	*min.*	*min.*
Hyoscine hydrobromide	9.5	—	—	9.5
Morphine sulphate	13.0	—	—	13.0
Aconitine	—	—	9.0	8.5
Caffeine citrate	Destroys blood		—	13.0
Alcohol	—	11.5	—	12.0
Ether	—	13.5	—	13.5
Chloroform	—	22.0	—	12.0

In a previous communication,[8] it has been shown that certain inorganic substances also exert a poisonous action on reductase.

TABLE VI.

10 Minutes Required for Reduction of Normal Mixture.

Poison.	Time in minutes required for the reduction of mixtures containing 1 cc. of *liver* juice, 2 cc. of blood solution, and 1 cc. of an aqueous solution of the poison having a molar concentration of	
	0.01	0.1
	min.	*min.*
As_2O_3	33	
KCN	30	34
$HgCl_2$	17	Destroys blood.
Na_3AsO_3	17	Destroys blood.
$AnCl_3$	15	16
OsO_4	13	19
$MnCl_2$	11	25
NH_4Br	11	13
HCHO	10	48
NH_4Cl	9	9

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[8] Harris and Creighton, *Biochem. Jour.*, 1914, viii, 585.

For the purpose of comparison the data obtained for a number of these poisons are given in Table VI.

Of the highly poisonous salts of the alkaloids hyoscine, cocaine, morphine, atropine, strychnine, only one, cocaine (0.001 normal), succeeded in doubling the time of reduction of oxyhemoglobin by hepatic reductase. The inference derivable from a survey of Tables II and IV is that the toxicity of the alkaloids examined is not directed preeminently against the reducing enzymic factor in the internal respiration of the tissues;[9] for in the end there is reduction, although after periods of varying length of retardation. These deadly alkaloidal poisons are not poisons of enzymes in the same sense that they are poisons of living protoplasm. In no case was the ultimate complete reduction of oxyhemoglobin to reduced hemoglobin prevented, even by the deadliest alkaloid or its salt. Our observations are in general agreement with the conclusions reached by Bywaters and Waller in 1910, after investigating the action of the powerful protoplasmic poison aconitine.[10] These observers found that the aconitine had no action on ptyalin as measured by the saccharification of starch. Using 0.01, 0.001, and 0.00001 normal solutions of the alkaloid, they could discover no inhibition of the enzyme even after twelve hours' contact. In Table V it will be noticed that when the reductase of cortex cerebri reduced oxyhemoglobin to the one-banded pigment in six and one-half minutes, 0.001 normal aconitine lengthened the time only to nine and 0.00001 normal to eight and one-half minutes. Now this is strictly not a toxic action at all; it is more accurately merely one of retardation. The same is true of hyoscine hydrobromide and morphine sulphate, the latter in each of two concentrations (0.1 and 0.00001 normal) just doubling the time of complete reduction. We therefore agree with Waller in distinguishing between poisons for protoplasm and poisons for enzymes. When we say, "alkaloids are deadly poisons," we mean for living protoplasm itself and not for its products, whether these products be secretion enzymes (like ptyalin, invertase, etc.) or endo-enzymes like reductase. The marked difference between the action of poisons and drugs on living matter and on enzymes may be further illustrated by the

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[9] Harris and Creighton, *Science Progress*, 1915, xxxvii, 50.

[10] Bywaters, H. W., and Waller, A. D., *Proc. Physiol. Soc.*, 1910, p. xlv.

following table[11] where the poisons are arranged in the relative order of their toxicity on isolated frog muscle, aconitine being taken as 1000:

TABLE VII.

Aconitine	1000.00
Quinine	100.00
Nicotine	33.00
Theobromine	18.00
Caffeine	12.00
Chloroform	6.00
Ether	0.72
Alcohol	0.06

Now this is very clearly by no means even approximately the state of matters as regards the action of these substances on the reductase of the liver. From Tables III and IV we see that if caffeine citrate is eliminated as the most toxic, then quinine hydrochloride, nicotine, chloroform, ether, and alcohol are all more or less in the same group as regards their retarding power on the activity of reductase. Table V shows the same thing in terms of the retardation of reductase from the cortex cerebri: thus for 0.00001 normal concentration, the figures for all poisons used are remarkably constant. Aconitine and hyoscine hydrobromide are actually the least poisonous (eight and one-half and nine and one-half minutes respectively), while morphine, alcohol, chloroform, and ether have practically the same amount of inhibiting effect on neural reductase. The powerful poisons aconitine and hyoscine hydrobromide are deadly for protoplasm, but comparatively innocuous for reductase.

When we turn from the alkaloidal poisons proper to consider such narcotics as alcohol, ether, and chloroform, we find that, as measured by the time they delay reduction by reductase from the central nervous system, they are actually more toxic than are the alkaloids. Thus, whereas the deadly hyoscine hydrobromide (0.1 normal) delayed reduction to nine and one-half minutes, six and one-half minutes being the normal, alcohol having one-half that concentration (0.05 normal) prolonged it to eleven and one-half minutes, ether (0.05 normal) delayed it to thirteen and one-half minutes, and chloroform of this strength prolonged the time

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[11] Waller, A. D., Physiology the Servant of Medicine, London, 1910, 93.

to twenty-two minutes. Thus if only the enzyme of internal respiration of the cortex cerebri be considered, alcohol, ether, and chloroform are more poisonous to that phase of metabolism than are such powerful poisons as hyoscine hydrobromide, morphine sulphate, and aconitine. Waller states[12] that as tested on muscle, one molecule of aconitine is one hundred times as toxic as a molecule of chloroform. No such difference obtains when these poisons are exhibited to neural reductase. The deadliest of narcotic alkaloids must, therefore, be directed against some constituent of the neural vital machinery other than its reductase.

Our observations contribute additional data as to the more poisonous character of chloroform as compared with ether, for chloroform was found markedly to inhibit the activity of reductase, while ether (0.02 normal) scarcely inhibited the action of liver reductase at all. Ether only increased the time of reduction by half a minute, while chloroform of the same strength increased it by five and one-half minutes or by a period eleven times as long. Again, whereas 0.1 normal alcohol delayed the time of reduction to ten minutes, chloroform five times as weak (0.02 normal) was able to prolong the reduction to the same time; judged by this, chloroform would be five times as poisonous as alcohol. Alcohol (0.01 normal) delayed reduction to ten minutes, while chloroform ten times as dilute (0.001 normal) delayed it to nine minutes or only one minute less. The greater toxicity of chloroform as compared with that of both alcohol and ether on liver reductase is very clearly brought out in these observations. The greater toxicity of chloroform compared with ether on the frog's isolated non-medullated nerve was conclusively shown by Waller[13] in 1897. Using electrical responses to single induction shocks, he demonstrated that whereas nerve could recover after treatment with the vapor of ether, it could not do so after being similarly treated with chloroform vapor. Chloroform, whether judged by its action on the protoplasm of the entire animal or by its action on the endo-enzyme reductase, is considerably more toxic than either ether or alcohol.

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[12] Waller, *loc. cit.*, 51.

[13] Waller, Lectures on Physiology, First Series, On Animal Electricity, London, 1897, 23.

It should be pointed out that the concentrations of the poisons employed in most of the experiments were of an order very much higher than would be attained by the maximum dose of these in the circulating blood. To take a definite case: The maximum single dose of hyoscine hydrobromide is 0.0006 gram. This distributed through 3 liters of blood would give 0.0000002 gram of poison per 1 cc. of blood. In the experiments described above, the mixtures contained 1 cc. of juice, 1 cc. of an aqueous solution of poison, and 2 cc. of diluted blood; *i.e.*, 1 cc. of poison to 4 cc. of fluid. In those experiments where 0.1 normal hyoscine hydrobromide was used, each cc. of the mixture contained 0.01095 gram of the poison; while where the most dilute hyoscine hydrobromide solution (0.00001 normal) was employed, each cc. of the mixture contained 0.000001095 gram of the alkaloid. This latter quantity is about five times as strong as the concentration in the blood of the maximum dose of hyoscine hydrobromide. Therefore, since 0.00001 normal hyoscine hydrobromide (Table IV) has no inhibitory action on hepatic reductase, it is to be presumed that this poison at its maximum dosage in the blood does not affect the inspiratory phase of tissue respiration; its "deadly" power must be directed towards some other aspect of the metabolic cycle.

The last feature of these poisons to which we wish to draw attention is one which has been remarked on by previous workers, for instance, Vernon who writes[14] of the "non-dependence of toxic effect upon concentration." Referring to Table IV, it is found that both 0.1 and 0.001 normal morphine sulphate delayed reduction for practically the same time, six and six and one-half minutes, respectively. Again, whereas 0.02 normal strychnine sulphate prolonged the time of reduction to eight minutes, 0.0001 normal concentration of the poison prolonged it to six and one-half minutes, or one and one-half minutes less for a solution two hundred times as dilute. The figures for chloroform in this connection are very interesting: A concentration of 0.02 normal prolonged the time of reduction to ten minutes, one of 0.001 normal to nine minutes, one of 0.0001 normal to eight minutes, while a solution as weak as 0.00001 normal actually delayed reduction

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[14] Vernon, H. M., *Jour. Physiol.*, 1909–10, xxxix, 157.

to as much as seven minutes when the normal mixture required four and one-half minutes. The figures for ether are still more striking, for exactly the same time, five minutes, was required for reduction in the presence of both 0.02 and 0.001 normal concentrations of the poison. Without doubt our observations contribute additional evidence in support of the belief that toxicity is to some extent independent of the concentration of the poison, as though a little dust in the cog-wheels of the vital machinery was as effective as coarser grit in compromising the perfection of the working of the constituent parts.

In certain cases it was actually found that a particular concentration of the poison inhibited reductase more powerfully than did a stronger solution. This is seen in the case of the salts of hyoscine, cocaine, morphine, and atropine. Thus 0.02 normal hyoscine hydrobromide delayed reduction for two minutes longer than a 0.1 normal solution, and 0.02 cocaine hydrochloride delayed reduction for two and one-half minutes longer than did a 0.1 normal solution of the poison. In the case of the salts of cocaine and morphine (Table IV) the 0.01 normal concentration delayed reduction for half a minute longer than did the 0.02 normal solution. Where the difference between two times is only half a minute, we do not naturally lay any stress on it, as thirty seconds may well represent the time error in judging of the end-point in the spectroscopic method; nevertheless the phenomenon of a higher dilution apparently inhibiting reduction more perfectly than a more concentrated solution has occurred so frequently that we have been forced to take notice of it. Possibly the increase in the ionization of the salts of the alkaloids may be a factor in determining this result. We would point out that this phenomenon was observed amongst the more virulent poisons only, and was not met with in quinine, caffeine, alcohol, ether, or chloroform. Indeed, caffeine citrate gave what might be called a typical set of readings, each decreasing concentration inhibiting reductase for a shorter time than the dilution immediately preceding it in the series.

In conclusion it should be pointed out that of the inorganic poisons previously studied (Table VI), the most deadly to the organism are also those which delay to the greatest extent the action of reductase, namely mercuric chloride, potassium cyanide, arsenious acid, and formaldehyde; as has already been pointed out, this similarity of action is not also true of the alkaloidal narcotics.

SUMMARY.

1. The influence of various poisonous substances (alkaloids including caffeine and alcohol, ether, and chloroform) in prolonging the time required by reductase derived from (a) liver (cat) and (b) cortex cerebri (cat) completely to reduce oxyhemoglobin has been studied by a method already described.

2. Substances which are known to be rapidly fatal to animals and which depress the vitality of isolated tissues (nerves, muscles, heart, etc.), that is, which are deadly protoplasmic poisons, are not poisons in the same sense for reductase from liver or from brain.

3. The relative order of toxicity (molecular toxicity) of a number of typical protoplasmic poisons is quite different from that of the same substances as tested by their powers of inhibiting reductase.

4. Substances which are virulent poisons to protoplasm (*e.g.*, aconitine and hyoscine hydrobromide) inhibit reductase no more effectively than substances very much less poisonous to protoplasm (*e.g.*, caffeine citrate and alcohol).

5. The poisonous deadly power possessed by the narcotic alkaloids would seem to be exerted against some physiological activity of the cellular metabolism other than the inspiratory phase of tissue respiration (action of reductase).

6. Our observations fully confirm the belief that chloroform is considerably more toxic than ether or alcohol. Chloroform, unlike certain substances usually regarded as more poisonous, inhibits reductase much more effectively than they.

7. We would point out the convenience of the method used in this and preceding investigations on reductase, as one whereby the action of poisons or drugs on one phase of metabolism can be studied in cases where the injection of poison into the intact animal could not yield the precise information desired.

The expenses of this research were met by a grant to one of us (D. F. H.) from the Royal Society which is hereby gratefully acknowledged.

QUANTITATIVE STUDIES ON THE IN VIVO ABSORPTION OF IODINE BY DOGS' THYROID GLANDS.

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It is common knowledge that iodine is taken up by the thyroid very rapidly when administered in any form and by any method. It is also known that the amounts taken up from a given intake vary with the size of the gland and the existing degree of hyperplasia. These are statements of fact to the best of our knowledge, but they are also vague generalizations and on that account neither satisfying nor convincing. It is for these reasons that criticisms and doubts have been expressed, and it is for the purpose of supplying definite figures that the following experiments, though somewhat old, are reported.

The plan of the experiments was as follows: With the usual aseptic precautions one lobe was removed, cleaned, and weighed. A weighed portion was kept for histological examination, and the remainder dried at 70° for the determination of its iodine content. 50 mg. of either potassium iodide or sodium iodide were given in 5 mg. doses by mouth for the following ten days; then, after an interval varying from five to eight days, the remaining lobe was removed, cleaned, drained of blood, and weighed. A weighed portion was kept for histological examination and the remainder dried for iodine determination.

Six experiments have the above mentioned constant features, and the principal data are tabulated on the following page.

As regards weight of the animals and size of the thyroid lobes, there is fairly wide range between minimum and maximum. So also as regards sex and age the data are sufficient to indicate that no important change is related to these factors.

The histological condition of the control lobes includes examples of our three arbitrary divisions of the several degrees of hyperplasia. It will be noted that with the exception of one (A-285), the second lobe was completely involuted to its colloid or resting stage. This exception happens to be the fifteen day or shortest interval between the administration of iodine and the removal of the lobe, as well as the largest thyroid. As the iodine content per gram is much above that at which hyperplasia disappears, it indicates that the interval of time allowed was a little short of that necessary to complete the change. The usual variations in the iodine content of the control lobes in relation to the degree of hyperplasia are present.

TABLE.

Experiment No.	Age and sex of dog.	Weight of dog.	Thyroid control lobe or iodized lobe.	Time interval between removal of control and iodized lobes.	Weight of lobe.	Histological condition.	Iodine per gm. of dried gland.	Total amount of KI or NaI administered. (10 days.)	Total iodine in lobe.	Ratio of thyroid weight to body weight.	Total iodine found in iodized lobe.
		kg.		*days*	*gm.*		*mg.*	*mg.*	*mg.*		*per cent*
A289	5 mos. ♀	5.0	Control	16	5.1	Marked hyperplasia	0.08	50.0 KI	0.10	1:980	16.0
			Iodized		6.1	Colloid	4.00		6.10		
A274	Young adult ♀	9.0	Control	18	2.5	Moderate hyperplasia	0.15	50.0 NaI	0.10	1:4500	5.6
			Iodized		2.0	Colloid	5.57		2.48		
A285	Young adult ♀	7.75	Control	15	9.5	Moderate hyperplasia	0.32	50.0 NaI	0.76	1:687	18.5
			Iodized		11.28	Colloid early	2.85		8.54		
A286	Middle aged ♂	8.7	Control	16	8.0	Moderate hyperplasia	0.14	50.0 KI	0.18	1:1641	10.2
			Iodized		5.3	Colloid	3.07		4.05		
A287	Middle aged ♀	10.0	Control	18	2.5	Early hyperplasia	0.52	50.0 KI	0.27	1:5000	7.0
			Iodized		2.0	Colloid	4.90		2.91		
A288	Middle aged ♂	14.6	Control	18	3.0	Early hyperplasia	0.53	50.0 NaI	0.35	1:3842	7.7
			Iodized		3.8	Colloid	3.85		3.61		

The variations in the size of the lobes are more marked than usual and show no constancy. In three the iodized lobes are larger and in the others the control lobes are larger. These variations illustrate one point often emphasized before, that, while iodine generally causes a reduction in the size of the thyroid of dogs, this is not necessarily the case—the necessary change is the involution.

In figuring the ratio of thyroid weight to body weight, obviously the iodized lobe is used, while the body weights are those taken after the first operation. The iodine contents per gram of dried gland, both for the controls and the iodized lobes, are given in one column. There is considerable variation in the amount of iodine stored per gram of thyroid, with a bare suggestion that the smaller the gland, the higher its iodine content. As to the biological significance of these variations, I have no suggestion. It is probably not a physical phenomenon.

The gain in iodine is in all cases very pronounced, and the figures obtained support the generalization that the thyroid has an extraordinary affinity for iodine up to the point of saturation, which is on the average between 5 and 6 mg. per gram of dried gland for dog thyroids. Passing to the total amounts of iodine recovered, it is obvious that the amount varies with the size of the gland. This also has long been known, and merely indicates that with the amount of iodine and the time of its administration definite, and with other organs, especially the kidney, competing, the surface area for absorption is the largest factor. This feature is brought out more clearly in the ratios of thyroid weight to body weight and in the percentages of the total intake of iodine recovered from the thyroid. In figuring these percentages, 50 mg. KI were figured as 38 mg. iodine, and 50 mg. NaI as 42 mg. iodine.

Another factor than the size of the gland is concerned. Unfortunately the series is too small to show it clearly, but it can be seen by comparing Experiment 289 with 285 and 286, that the more marked the hyperplasia or the lower the original iodine content, the greater the quantity stored. The percentage stored from a definite intake would therefore vary with the size of the gland and its degree of hyperplasia.

It should be added that the liver and spleen in each of these experiments were examined for iodine, but with uniformly negative results. This was to be expected, since the minimum interval between the last dose of iodine and the removal of the organs was five days.

It is recognized that the oral administration of such small quantities of iodine, even when given in very dilute solution, is more likely to cause losses than when introduced parenterally. With dogs, however, only care is necessary to prevent losses, and absorption of the soluble salts of iodine from the alimentary tract is probably complete.

CONCLUSIONS.

These experiments emphasize the extraordinary affinity of the thyroid tissue for iodine. When one considers that as high as 18.5 per cent of a given intake of iodine by mouth may be recovered from a thyroid whose ratio to the body weight is as 1:687, it stands alone at present among the specific affinities of tissues for inorganic substances. The results further emphasize the fact that maximum thyroid effects are induced by minimum amounts of iodine. The amount of a given intake absorbed depends, for the most part, on the size of gland and the existing degree of hyperplasia or the degree of saturation with iodine at the time of its administration.

THE NITROGEN EXCRETION OF THE CAT DURING A PURINE-FREE AND A PURINE-RICH DIET.

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In view of the fact that cats are frequently used in studies in biochemistry it seems opportune for a report on an examination of the distribution of nitrogen among the nitrogenous constituents of cat urine, together with the effect of a purine-free and a purine-rich diet upon the excretion of uric acid.

From the work of Mendel and Brown,[1] Kanger,[2] Wiechowski,[3] and Hunter and Givens,[4] it is well known that cats excrete but small amounts of uric acid, allantoin taking its place as the excretory product of the purines. That this is not due to a non-production of uric acid by the cat tissues, but is due to a more complete oxidation of the preformed purines, is shown by the high amounts of uric acid found in the glandular tissues of the cat and the non-existence of uric acid in normal cat urine. The work of the investigators just mentioned is chiefly qualitative.

All the analyses herein reported have been done according to the microchemical methods of Folin and his associates.[5] All were made in duplicate on fresh urine. The urines represent total twenty-four hour quantities obtained at approximately the same time each day through gentle massage of the bladder.

To determine if nitrogen equilibrium could be obtained when the animal was allowed to choose the quantity of food ingested,

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[1] Mendel, L. B., and Brown, E. W., *Am. Jour. Physiol.*, 1899–1900, iii, 261.

[2] Kanger, A., *Arch. f. d. ges. Physiol.*, 1903, c, 428.

[3] Wiechowski, W., *Beitr. z. chem. Phys. u. Path.*, 1908, xi, 109.

[4] Hunter, A., Givens, M. H., and Guion, C. M., *Jour. Biol. Chem.*, 1914, xviii, 393.

[5] Folin, O., and Farmer, C. J., *Jour. Biol. Chem.*, 1912, xi, 493. Folin, O., *ibid.*, 1912, xi, 507. Folin, O., and Macallum, A. B., *ibid.*, 1912, xi, 523.

a cat weighing 2.5 kilos was placed in a metabolism cage for six days and allowed to eat of a purine-free diet of crackers and milk freely twice a day.

The urines of the first three days were discarded. The figures in Tables Ia and Ib represent the final three day excretion. It is seen that nitrogen equilibrium was fairly well established during the experiment.

TABLE Ia.

Nitrogen Excretion of the Cat during a Purine-Free Diet.

Day.	Amount.	Total N.	Urea N.	NH_3 N.	Uric acid N.	Creatinine N.
	gm.	*gm.*	*gm.*	*gm.*	*mg.*	*gm.*
4........	128	1.31	1.12	0.06	0.21	0.05
5........	117	1.29	1.04	0.05	0.08	0.03
6........	144	1.27	1.01	0.10	0.17	0.04

TABLE Ib.

Nitrogen Excretion in Terms of Total Nitrogen.

Day.	Total N.	Urea N.	NH_3 N.	Uric acid N.	Creatinine N.
	gm.	*per cent*	*per cent*	*per cent*	*per cent*
4.........	1.31	85.5	4.6	0.01	3.9
5.........	1.29	80.6	3.9	0.01	2.5
6.........	1.27	79.5	7.9	0.01	3.3

The very low uric acid output and the low per cent of total nitrogen excreted as uric acid will prove that the uric acid of cat urine does not represent its nuclear metabolism. This question and a discussion of the distribution of the nitrogen in the determined constituents will be taken up later.

Mendel and Brown[1] report that the ingestion by the cat of food rich in nucleins results in an increase of uric acid excretion. In order to quanitatively confirm this and to determine if a cat could be brought to a nitrogen equilibrium during a high nitrogen purine-rich period of feeding, a male cat weighing 3.5 kilos was confined in the metabolism cage and given 200 grams of fresh beef spleen twice daily, with water freely. Tables IIa and IIb represent the analyses of the final three days of a six day period.

TABLE IIa.

Nitrogen Excretion of the Cat during a Purine-Rich Diet.

Day.	Amount.	Total N.	Urea N.	NH_3 N.	Uric acid N.	Creatinine N.
	gm.	*gm.*	*gm.*	*gm.*	*mg.*	*gm.*
4........	325	8.55	5.82	0.42	10.59	0.06
5........	308	9.33	8.35	0.41	9.76	0.06
6........	341	10.81	8.53	0.43	12.49	0.06

TABLE IIb.

Nitrogen Excretion in Terms of Total Nitrogen.

Day.	Total N.	Urea N.	NH_3 N.	Uric acid N.	Creatinine N.
	gm.	*per cent*	*per cent*	*per cent*	*per cent*
4.........	8.55	68.07	4.91	0.12	0.70
5.........	9.33	89.49	4.28	0.10	0.67
6.........	10.81	78.90	3.97	0.10	0.60

Here also there is a fair nitrogen equilibrium established. There is an increase in uric acid nitrogen of fifty times the amounts found during a purine-free diet with only ten times the per cent of total nitrogen excreted as uric acid. Moreover the per cent of total nitrogen excreted as uric acid is far below that excreted by man. Hence we must conclude that under any system of feeding the cat organism tends to destroy uric acid before excretion.

These two preliminary experiments demonstrate the ease of bringing a cat to nitrogen equilibrium during either a high or low protein diet. This possibility facilitates the study of the cat's nitrogenous metabolism in general and the uric acid excretion in particular, whether on a high or low nitrogen diet. In a comparison of the urines from the animals upon the different diets, certain differences in the distribution of the nitrogen are evident, which for confirmation require experiments of longer duration. However, it is possible from these results to compare the relative rates of metabolism of cat and man and to see whether or not we are justified in using the cat for experimental metabolic studies.

A male cat weighing 2.5 kilos was confined in the cage and allowed to eat 100 grams of beef spleen twice daily for six days.

For the next eleven days the diet was confined to crackers, eggs, and milk. This period was followed by the same system of feeding as in the beginning for ten days. Water was given freely. Tables IIIa and IIIb give the results of the analyses of the urines collected during the experiment. Accurate twenty-four hour quantities are represented and the analyses made as previously described.

TABLE IIIa.

Nitrogen Partition in Cat Urine.

Date.	Amount.	Total N.	Urea N.	NH_3 N.	Uric acid N.	Creatinine N.
1914	*gm.*	*gm.*	*gm.*	*gm.*	*mg.*	*gm.*
Feb.						
16	102	5.01	4.48	0.19	6.22	0.043
17	125	6.33	5.19	0.23	7.94	0.050
18	116	5.10	4.41	0.22	11.43	0.045
19	107	4.96	3.85	0.21	11.04	0.044
20	120	5.64	4.63	0.21	15.66	0.046
21	113	4.56	3.87	0.21	11.38	0.032
22	140	2.22	1.61	0.16	1.93	0.031
23	120	1.59	1.13	0.14	0.20	0.031
24	159	2.14	1.64	0.17	0.00	0.041
25	—	—	—	—	—	—
26	156	2.09	1.58	0.12	0.00	0.031
27	180	1.85	1.39	0.14	0.26	0.033
28	200	2.00	1.58	0.17	0.00	0.040
Mar.						
1	175	2.03	1.46	0.17	1.40	0.040
2	190	2.77	2.22	0.22	0.23	0.051
3	165	1.26	0.73	0.14	0.00	0.031
4	131	2.04	1.60	0.15	0.00	0.039
5	159	3.08	2.69	0.17	3.13	0.033
6	111	4.92	4.02	0.20	7.39	0.032
7	96	4.13	3.55	0.17	8.04	0.026
8	120	4.80	3.99	0.19	8.06	0.027
9	173	7.35	6.37	0.34	10.76	0.038
10	132	6.07	5.11	0.22	12.92	0.030
11	112	5.80	4.75	0.22	13.27	0.034
12	91	4.55	3.93	0.16	15.95	0.037
13	150	7.71	6.51	0.29	20.14	0.043

TABLE IIIb.

Nitrogen Partition in Cat Urine in Terms of Total Nitrogen.

Date.	Total N.	Urea N.	NH_3 N.	Uric acid N.	Creatinine N.
1914	*gm.*	*per cent*	*per cent*	*per cent*	*per cent*
Feb.					
16	5.01	89.42	3.79	0.10	0.86
17	6.33	81.99	3.63	0.10	0.79
18	5.10	86.47	4.31	0.22	0.70
19	4.96	77.62	4.23	0.22	0.89
20	5.64	82.09	3.72	0.28	0.82
21	4.56	84.87	4.61	0.25	0.70
22	2.22	72.52	7.20	0.09	1.40
23	1.59	71.07	8.81	0.01	1.96
24	2.14	76.64	7.94	0.00	1.92
25	—	—	—	—	—
26	2.09	75.60	5.75	0.00	1.49
27	1.85	75.13	7.57	0.01	1.78
28	2.00	79.00	8.50	0.00	2.00
Mar.					
1	2.03	73.60	8.37	0.01	1.97
2	2.77	80.13	7.94	0.01	1.84
3	1.26	57.93	11.10	0.00	2.46
4	2.04	62.30	7.35	0.00	1.91
5	3.08	87.34	5.52	0.10	1.07
6	4.92	81.71	4.07	0.15	0.65
7	4.13	85.95	4.12	0.19	0.63
8	4.80	83.12	3.96	0.17	0.56
9	7.35	86.67	4.63	0.15	0.52
10	6.07	84.18	3.63	0.21	0.49
11	5.80	81.88	3.62	0.23	0.59
12	4.55	86.37	3.52	0.35	0.59
13	7.71	84.44	3.76	0.26	0.56

In Table IIIa there are several points of interest in regard to the effect of high and low protein intake on the absolute amounts of nitrogen excreted in the various forms. As would be expected, the total and urea nitrogen are markedly lower during the purine-free low nitrogen period than during the purine-rich high nitrogen periods. The difference in amount of nitrogen excreted as ammonia is not as marked, yet shows a constant decrease during the low nitrogen period.

The nitrogen excreted as uric acid practically disappears during the purine-free diet. Correlating this with the fact that the glandular tissues of the cat, even when kept upon a purine-free diet previous to analysis, not only contain uric acid, but either liberate precombined uric acid or form more uric acid by oxidation of the purines therein contained, evidence is furnished that, although the normal metabolism of the cat leads to the production of uric acid, yet this compound is not excreted as such. On the other hand there is an excretion of uric acid during the spleen diet that apparently increases in quantity daily. This does not come from the preformed uric acid of the spleen as fed, for if a cat be fed uric acid during either a purine-free or a purine-rich diet no increase in urinary uric acid is observed. Hence it must come from the uric acid precursors in the fed spleen absorbed during assimilation. Now the cat kidney can excrete uric acid freely, for on intravenous injection of uric acid there is found an accumulation of uric acid in the kidneys and the tied off urinary bladder, but in no other place. Normally but minute traces of uric acid are found in cat blood. After spleen feeding an increase, slight it is true, but nevertheless an increase, in uric acid is found in cat blood. Hence as a tentative explanation of the gradual increase in uric acid excretion during the spleen periods it is suggested that through a flooding of the system with the uric acid precursors the uricolytic activity of the organism is unable to keep pace with the changes leading to the uric acid production.

The excretion of nitrogen as creatinine throughout the three periods is a constant quantity. When the weights of the animals used are compared with the creatinine excretion it is evident that here as well as with man we have in creatinine an index of a phase of endogenous metabolism that is unaffected by extreme variations in both kind and quantity of food. This similarity is further brought out in a consideration of the changes in the ratio of nitrogen excreted as urea, ammonia, and creatinine to total nitrogen with the change from high to low nitrogen intake and *vice versa.*

Considering Table IIIb it is seen that during the high protein period the per cent of total nitrogen excreted as urea is higher than during the low protein period. Coupling this with the fact that the absolute quantity of urea nitrogen is also lower during this period shows that qualitatively the cat metabolism leading to the formation of urea is governed by the same laws as the excretion of urea by man, as described by Folin.[6]

Although the excretion of nitrogen as ammonia is less during the low nitrogen intake yet this decrease is not large enough to prevent an increase in the per cent of total nitrogen excreted as ammonia, again a qualitative similarity with human metabolism.

With creatinine, where the output is constant, the per cent of total nitrogen excreted as creatinine naturally rises as the total nitrogen excretion falls.

The quantitative distribution of nitrogen in cat urine among urea, ammonia, and creatinine, indicates that the cat is well suited for experimental studies in nitrogenous metabolism with reference to human problems, since the excretion of these substances is apparently governed by the same laws in the cat as in man.

In the case of the excretion of uric acid there is no similarity. Under no conditions does the cat excrete its uric acid as does man. No significant part of its nitrogen is excreted as uric acid during the purine-free feeding. The exact cause of the increase in the nitrogen excreted as uric acid during the purine-rich periods is, without very grave doubts, due to an overflow into the blood of uric acid which is promptly excreted. This overflow is probably due to the flooding of the system with purines in such quantities that the uricolytic enzyme can not destroy the uric acid as fast as it is liberated from its combinations. The main point to be gathered from this is, that the cat is of no use for studies in uric acid metabolism as applied to man, and can only be used in studies in comparative metabolism of the purines.

Tables IVa and IVb give the averages of the three periods omitting the first day of each period. The changes are therein the more easily followed.

From a quantitative point of view it is obvious that kilo for kilo cat metabolism on either high or low nitrogen diet is ten times greater than human, as shown by these figures. Under these circumstances the use of the cat for studies in total metabolism is of service only from the point of view of comparative metabolism.

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[6] Folin, *Am. Jour. Physiol.*, 1905, xiii, 66.

TABLE IVa.

Showing the Averages of Table IIIa.

Period.	Amount.	Total N.	Urea N.	NH_3 N.	Uric acid N.	Creatinine N.
	gm.	*gm.*	*gm.*	*gm.*	*mg.*	*gm.*
Spleen..........	116	5.32	4.39	0.22	11.49	0.043
Milk, etc.......	164	1.97	1.49	0.16	0.23	0.038
Spleen..........	123	5.66	4.77	0.22	12.07	0.033

TABLE IVb.

Showing the Averages of Table IIIb.

Period.	Total N.	Urea N.	NH_3 N.	Uric acid N.	Creatinine N.
	gm.	*per cent*	*per cent*	*per cent*	*per cent*
Spleen..........	5.32	82.61	4.10	0.22	0.78
Milk, etc.......	1.97	72.38	8.15	0.02	1.92
Spleen..........	5.66	84.29	3.91	0.21	0.57

This, however, does not prevent the use of the cat as a subject for experimental work in nitrogenous metabolism because here we have practically a complete parallelism between cat and man.

CONCLUSION.

From the evidence presented it is concluded that, with the exception of uric acid, the excretion by the cat of nitrogenous urinary constituents is governed by the same laws as the nitrogen excretion of man. This makes the cat an animal that can well be used in studies on nitrogenous metabolism with possible application to problems of human metabolism.

From a quantitative standpoint the cat can be used in studies on total metabolism only when the work is on a comparative basis.

On a purine-free diet the per cent of total nitrogen excreted as uric acid is so small as to be of no significance when considering the nitrogen of the urine. On a purine-rich diet the increase probably denotes an overburdening of the uricolytic enzymes.[7]

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[7] These investigations were carried on at the Harvard Medical School as a part of the work required for the degree of Doctor of Philosophy.

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