

TX 560
.B5 W3
Copy 1

The University of Chicago

THE CHEMISTRY OF CHINESE PRESERVED
EGGS AND CHINESE EDIBLE
BIRDS' NESTS

A DISSERTATION
SUBMITTED TO THE FACULTY
OF THE OGDEN GRADUATE SCHOOL OF SCIENCE
IN CANDIDACY FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

DEPARTMENT OF CHEMISTRY

BY
CHI CHE WANG

Private Edition, Distributed By
THE UNIVERSITY OF CHICAGO LIBRARIES
CHICAGO, ILLINOIS

Reprinted from
THE JOURNAL OF BIOLOGICAL CHEMISTRY, Vol. XLIX, No. 2
December, 1921

The University of Chicago

THE CHEMISTRY OF CHINESE PRESERVED
EGGS AND CHINESE EDIBLE
BIRDS' NESTS

A DISSERTATION

SUBMITTED TO THE FACULTY
OF THE OGDEN GRADUATE SCHOOL OF SCIENCE
IN CANDIDACY FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

DEPARTMENT OF CHEMISTRY

BY

CHI CHE WANG

Private Edition, Distributed By
THE UNIVERSITY OF CHICAGO LIBRARIES
CHICAGO, ILLINOIS

Reprinted from
THE JOURNAL OF BIOLOGICAL CHEMISTRY, Vol. XLIX, No. 2
December, 1921

TX560
.B5W3

1110
University
MAR 16 1922



THE COMPOSITION OF CHINESE EDIBLE BIRDS' NESTS AND THE NATURE OF THEIR PROTEINS.*

By CHI CHE WANG.

(From the Nelson Morris Institute for Medical Research, Michael Reese Hospital, Chicago.)

(Received for publication, September 26, 1921.)

Appearance and Origin.

The edible birds' nests are gelatinous substances produced by certain swifts, the *Collacalia*, natives of Malaya (1) and Ceylon. The nests, constructed in caves on the seashore, are collected while they are still moist and made into various shapes. The lowest grade is sold in the form of coarse powder. The higher the grade, the whiter the color and fewer the feathers and twigs. Owing to their high price, their use is limited to a delicacy at the feasts of the wealthy and a food for convalescents and the aged.

The source from which the birds make the nests has been uncertain. Green (2) gives three suggestions: in the algæ found in caves where the swifts make their nests, fish spawn, or a secretion from the swifts themselves. The algæ theory is disproved by the lack of vegetable cells shown by microscopic examination of the nests. The secretion theory is believed by most of the natives and has the support of Home (3) and Bernstein (4). The latter author found in the birds two large salivary glands which secrete much viscous mucus. The observation given in this paper shows that the nests consist largely of a mucin-like substance and, therefore, is in accord with the latter hypothesis.

Review of Literature.

The literature on the subject is limited. Descriptive statements concerning chiefly the occurrence and appearance of the nest may be found in Encyclopedias, China year books, and some semiscientific articles written during the early part of the 19th century. Green (2) and Krukenberg (5) are the first to give a report of a scientific study of the nest. Their work

* The work reported in this article was conducted at the Nutrition Laboratory, Department of Home Economics, University of Chicago. It forms part of the thesis submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the University of Chicago.

covers the solubilities, the response to protein tests, and some observations on hydrolysis products. Their results prove that the birds' nest contains both the carbohydrate and the protein radicle, belonging to the class of mucin-like substances, the glycoproteins.

Scope of the Present Work.

The present work covers the study of the general properties, the chemical composition, the artificial digestion, the carbohydrate radicle, and the biological value of the proteins. Comparison is made with other work on mucin, especially Lothrop's, Müller's, and Levene's.

The material used for this work was supplied by the Hoo Loong Edible Birds' Nest Store, Chicago, which imported it directly from China. It was of the highest grade, having somewhat the appearance of agar-agar, but it was extremely crisp and had tiny feathers interwoven with the mucilaginous material. For quantitative analysis, the material was ground and sifted. On sifting, most of the feathers cling together and may be removed, but some go through the sieve so that it is difficult to obtain a pure sample.

General Properties.

A sample boiled in distilled water for 3 hours and left there for several days, swells like a piece of sponge, but shows no tendency to dissolve. The filtrate responds to neither protein nor carbohydrate tests. 5 per cent sodium hydroxide dissolves it on standing 2 hours in the cold. The colorless solution responds to Millon's, the biuret, xanthoproteic, and Hopkins-Cole tests. The last reaction shows only faintly. It also has a slight reducing power with Fehling's reagent. A dilute acid, such as 3 per cent hydrochloric acid, dissolves the birds' nests only on heating. The solution acquires a purplish brown color, gives both protein and carbohydrate tests, and has a strong reducing action.

So far the properties agree with those reported by Green (2) in every respect except that he found the nest insoluble in dilute sodium hydroxide in the cold. They also agree with the commonly recognized properties of mucin.

Chemical Analysis.

Samples between 1.5 to 2 gm. were taken for the determination of moisture and ash. Neumann's method given in Mathews (6) was used for the estimation of phosphorus, and Denis's method (7) for that of sulfur. An attempt was made to determine the ether-soluble substance, but the results were too small to be of significance, only 0.3 per cent. The estimation of total nitrogen by the Kjeldahl-Gunning method was made on samples treated in three different ways: (1) original birds' nest, (2) ground birds' nest with feathers partially removed, and (3) a sample hydro-

TABLE I.
Chemical Composition of Chinese Edible Birds' Nests.

No.	Moisture.	Ash.			Phosphorus.	Sulfur.	Total nitrogen.		
		Water-soluble.	Water-insoluble.	Total.			Original birds' nests.	Ground, and feathers partially removed.	Hydrolyzed 13½ hrs. in 20 per cent HCl.
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
1	11.88	0.75	1.74	2.49	0.035	1.10	8.81	9.16	10.57
2	11.41	0.74	1.80	2.54	0.034	1.10	8.75	9.14	10.24
3	11.52	0.74	1.78	2.52					10.33
4									10.31
Average....	11.60	0.74	1.77	2.51	0.035	1.10	8.78	9.15	10.29

lyzed for 13½ hours in 20 per cent hydrochloric acid. Results are given in Table I.

The total ash, 2.51, is almost seven times as high as that of submaxillary mucin, 0.37 per cent, reported by Lothrop (8). This high percentage of ash shows that the birds' nest is not a pure mucin, but more probably dried saliva. Of the total ash 29.48 per cent is insoluble in water, but none insoluble in acid. Hence no sandy material is present.

The sulfur content, 1.10 per cent, is in agreement with the figures given by Müller (9) for salivary mucin, 1.40 per cent, but it is higher than that reported by Lothrop (8), 0.55 per cent. The

discrepancy may be due to the different methods used or in case of birds' nests, the presence of feathers. The phosphorus, 0.035 per cent, is too small to be of any significance.

The different values for nitrogen found in birds' nests treated in three different ways may be explained by the variation in the feathers present. The original sample containing the most feathers, had the lowest figure, 8.78 per cent, while that hydrolyzed with the least feathers, the highest or 10.29 per cent. The ground and sifted sample gave 9.15 per cent. Some feathers were removed during grinding and sifting, but more of them separated out by clinging to the walls of the vessel on hydrolysis. They could then be easily removed. The percentage of nitrogen of the hydrolyzed material, 10.29, agrees with the value given by Müller (9) for salivary mucin, 10.70 for total nitrogen, but it is lower than that reported by Lothrop (8), 12.49 per cent.

Artificial Digestion.

Artificial digestion experiments carried out in comparison with hard boiled egg white showed that the birds' nests were digested by both pepsin hydrochloric acid and trypsin though not so quickly as the egg. The speed of digestion was determined by Sørensen's titration (6). Comparison was made of the increase during 24 hours in the volume of 0.1 N sodium hydroxide for titrating 25 cc. of the peptic digest. Results were expressed in cc. per gm. of nitrogen in the material acted upon. For the birds' nests in a typical experiment this value was 9.60 cc. and for the egg white 15.47 cc. Similarly, the increase of 0.1 N hydrochloric acid to titrate the tryptic digest was 19.02 cc. for the birds' nests and 38.75 cc. for the egg white.

The percentage of carbohydrate in the hydrolyzed birds' nests could not be found with accuracy. Efforts using Benedict's (10) Method 2 failed to give concordant results. The material was prepared in the following manner: 1 gm. of the ground and sifted nests was dissolved in a small amount of concentrated hydrochloric acid by standing over night. It was then diluted with distilled water to make a 5 per cent acid solution and boiled with a reflux condenser for 1½ hours. The hydrolyzed mixture was treated with phosphotungstic acid, filtered, and the filtrate was made up to a definite volume.

Concordant results were not obtained. Variations were therefore made in the strength of acid from 3 per cent to concentrated and the length of hydrolysis from $\frac{1}{2}$ to $8\frac{1}{2}$ hours. In some cases Levene's (11) method of introducing a little stannous chloride into the hydrolyzing mixture was followed. It was soon discovered that the reducing power of the birds' nest was gradually diminished by heating in an acid solution. A difference of 6.37

TABLE II.

Relation between Quantity of Reducing Sugar and Time of Hydrolysis.

Time.	Reducing sugar.
<i>hrs.</i>	<i>per cent</i>
$\frac{1}{2}$	17.36
1	15.99
1	16.03
2	13.63
3	13.11
$8\frac{1}{2}$	10.99

per cent of carbohydrate calculated as glucose between the samples hydrolyzed $\frac{1}{2}$ and $8\frac{1}{2}$ hours is shown in Table II. In this experiment 20 per cent hydrochloric acid was used, the highest figure, in the table, 17.36 per cent, is much lower than that reported by Müller (9) for the carbohydrate in salivary mucin. His is 37 per cent, estimated by making phenylosazone from the hydrolyzed mixture.

Distribution of Nitrogen.

For the distribution of nitrogen Van Slyke's (12) method was closely followed, except that the bases were precipitated from a volume of 250 cc. instead of 200 cc. and the correction for solubility of the basic phosphotungstates made accordingly. Approximately 2 gm. of the ground and sifted birds' nest were taken for each of the four series of experiments. Several months elapsed between each one of the series.

By a study of Table III it will be seen that it was possible to obtain a fairly complete result on the distribution of nitrogen, the results for the different nitrogen fractions in the three finished series totalling 99.84, 100.61, and 100.54 per cent, respectively.

Series II and III are in close agreement with each other and Series IV slightly different, probably due to the fact that the material used in Series IV was purchased at a different time from the others. In Series II and III with the exception of cystine nitrogen, humin nitrogen, and the non-amino nitrogen of mono-amino-acids, the differences between duplicate series are all

TABLE III.
Distribution of Nitrogen in the Edible Birds' Nests.

Series.....	I		II		III		IV		Average of II and III.	
	Birds' nests.	Total nitrogen.	Birds' nests.	Total nitrogen.	Birds' nests.	Total nitrogen.	Birds' nests.	Total nitrogen.	Birds' nests.	Total nitrogen.
Nitrogen.	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent
Amide.....	1.07	10.15	1.03	10.06	1.05	10.10	1.09	10.54	1.04	10.08
Humin.....	0.71	6.75	0.65	6.38	0.72	6.98	0.70	6.77	0.69	6.68
Arginine.....	1.45	13.69	1.43	13.96	1.44	13.93	1.25	12.12	1.44	13.95
Cystine.....	0.37	3.51	0.29	2.79	0.41	4.00	0.49	4.77	0.35	3.39
Histidine....			0.60	5.90	0.67	6.53	0.67	6.47	0.64	6.22
Lysine.....			0.26	2.55	0.25	2.37	0.19	1.84	0.26	2.46
Amino nitrogen of mono-amino-acids.			5.08	49.61	5.24	50.76	4.99	48.41	5.16	50.19
Non-amino nitrogen of monoamino-acids.....			0.87	8.54	0.61	5.90	1.00	9.62	0.74	7.22
Total nitrogen recovered...			10.21	99.84	10.40	100.61	10.38	100.54	10.31	100.23
Total nitrogen determined.	10.57		10.24		10.33		10.31		10.29	

within the maximum experimental error allowed by Van Slyke (12) for pure proteins. The presence of considerable fine feathers with their high sulfur content and the resulting difficulty in obtaining a pure sample may be the cause of the variation in the cystine nitrogen. The differences in the humin nitrogen are probably due to the carbohydrate in the nest proteins.

The humin nitrogen is shown much higher than that of any of the pure proteins. The highest Van Slyke (12) has reported is 3.6 per cent for ox hemoglobin, a difference of 3.08 per cent from that of the nest. This high humin nitrogen may be explained as the result of the presence of a carbohydrate radicle in the nest proteins. Gortner and Blish (13), Gortner (14), and Hart and Sure (15) reported that the presence of dextrose or any other carbohydrate caused an increase of humin nitrogen during the hydrolysis of zein, fibrin, and casein.

The influence of the presence of a carbohydrate radicle on the distribution of nitrogen is also shown by the comparatively low lysine nitrogen and the high histidine nitrogen of the nest proteins. Thus, in case of pure casein reported by Hart and Sure (15) the value given for lysine nitrogen is 9.41 per cent and that for histidine nitrogen is 5.95 per cent. On the addition of dextrose, sucrose, and starch, respectively, during the hydrolysis of casein the corresponding values for lysine nitrogen are 7.01, 6.38, and 5.54 per cent, and those for histidine nitrogen are 7.31, 7.65, and 7.30 per cent. In every case, therefore, there is a decrease in the lysine nitrogen and an increase in the histidine nitrogen. Although the nature of the carbohydrate radicle in the nest has not yet been completely determined, it seems to possess in common with other carbohydrates the power of causing a redistribution of amino-acids on hydrolysis of protein. The presence of feathers in the material is undoubtedly the cause of the high value, 3.39 per cent for the cystine nitrogen in the nest. The highest value Van Slyke gave for cystine nitrogen in pure proteins is 1.25 per cent in gliadin.

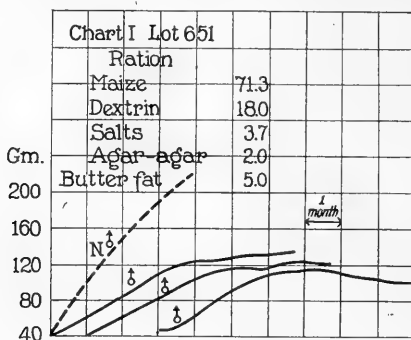
Biological Value of the Birds' Nests Proteins.

Feeding experiments were conducted on rats at Dr. McCollum's laboratory,¹ the School of Hygiene and Public Health, Johns Hopkins University, and the birds' nest was used to supplement a ration adequate in all respects but the character of the protein. Two unsatisfactory proteins, maize kernel and rolled oats, were chosen because they are of different character (16). Although

¹ For this part of the work I am indebted to Dr. McCollum and Miss Simmonds who kindly continued the experiments that I had started, and thus enabled me to secure the results.

when each is fed as the sole source of protein, they have approximately the same biological values for the support of growth, they are not at all similarly constituted. Oat proteins are supplemented well by the amino-acid mixture which comes from the digestion of gelatin, whereas the proteins of the maize kernel are not so supplemented in a degree which can be demonstrated by growth experiments with young animals.

The complexes which form the limiting factor in those two proteins are different, and, therefore, if the birds' nest protein has a high nutritive value it should at least supplement one of them. Since its addition failed to supplement either protein, it seems very probable that the birds' nest protein is of an inferior quality. It is of course possible that when taken together



with certain other foods it might have value, but its value is problematical in any case, and it is certain that it is far from being a complete food protein.

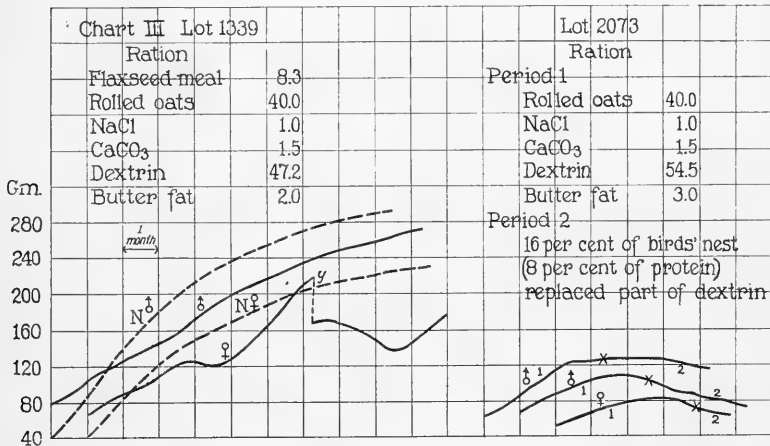
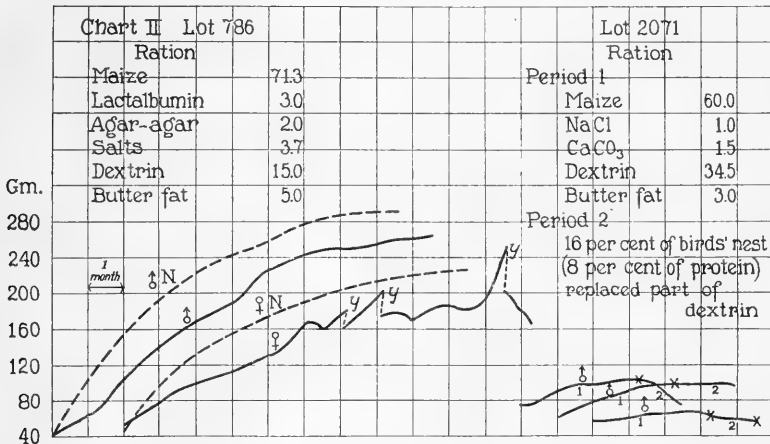
Lot 651 (Chart I) represents the growth curves for a group of rats which were restricted to a diet containing about 7 per cent of corn protein. The diet was otherwise fairly satisfactorily constituted. It failed to induce growth at a rate corresponding to about half the normal rate, and the animals became stunted when they were about half the normal adult size.

This diet is greatly improved by the addition of even so small an amount of purified protein as 3 per cent of lactalbumin. This is shown in the growth curves of Lot 786 (Chart II).

Lot 2071 (Chart II) shows the very slow rate of growth of a group of rats which were restricted to a diet comparable in all respects to those just mentioned except that the corn protein was fed at the plane of 6 per cent of the food mixture. This ration, when supplemented with a protein

having an appreciable biological value, would become capable of inducing growth, as illustrated in the case of Lot 786.

When a group of young rats were fed this diet for a period of 13 weeks and had been able to increase in weight but very little, and had finally completely failed to grow, 16 per cent of the Chinese edible birds' nest,



equivalent to 6 per cent of N \times 6.25, was added in the place of a portion of the carbohydrate of the diet. If the birds' nests protein had any appreciable nutritive value, it should have enabled the young animals to respond with growth. A wide experience has demonstrated that such response will be observed when the protein added is of such a nature as to

furnish certain essential amino-acids which form the limiting factor in the proteins of the corn (maize). In this experiment there was no growth following this addition of birds' nests. The only conclusion which can be drawn is that the birds' nests do not supplement the protein and the corn kernel.

Lot 2073 (Chart III) shows that young rats cannot grow much when the diet contains only the amount of protein which will be furnished by a content of rolled oats equivalent to 40 per cent of the food mixture. This amounts to approximately 6 per cent of protein in the diet. Lot 1339 (Chart III) shows that the same food mixture, supplemented with the proteins of flaxseed oil meal sufficient to furnish an additional 3 per cent of proteins, is sufficient for the maintenance of a rate of growth somewhat more rapid than half the rate at which the young rat is capable of growing. Animals on this diet have reached approximately the full adult size, and young have been produced.

The fact that Lot 2073 in Period 2 failed to respond with growth when 16 per cent of the edible birds' nest was added to the diet, leaves no room for doubt that this substance, although a protein of a peculiar character, does not supplement the proteins of the oat kernel in any appreciable degree.

SUMMARY.

1. The Chinese edible birds' nest has the properties of a protein as well as those of a carbohydrate. It belongs, therefore, to the class of glycoprotein.

2. Its percentage composition resembles that of salivary mucin. Its ash is high, but there is no sandy material present. It contains 10.29 per cent nitrogen and at least 17.36 per cent carbohydrate.

3. Artificial digestion experiments indicated that the birds' nest was digested by both pepsin hydrochloric acid and trypsin at a slower speed than boiled egg.

4. The distribution of nitrogen showed a higher value for both humin nitrogen and cystine nitrogen than for pure proteins. The former is probably due to the carbohydrate radicle in the nest while the latter is due to the presence of fine feathers. Other fractions were similar to those of pure proteins.

5. Feeding experiments indicate that the nest protein is probably of an inferior quality. It failed to supplement a ration adequate in all respects, except that the source of protein was derived from either maize kernel or rolled oats. Although both of them were unsatisfactory proteins they were different in character.

BIBLIOGRAPHY.

1. Ball, J. D., *Things Chinese*, 3rd edition, 1900.
2. Green, J. R., *J. Physiol.*, 1885, vi, 40.
3. Home, E., *Phil. Tr. Roy. Soc. London*, 1817, 337, quoted in Green, J. R., *J. Physiol.*, 1885, vi, 40.
4. Bernstein, *J. Ornithol.*, 1859, 111, quoted in Green, J. R., *J. Physiol.*, 1885, vi, 40.
5. Krukenberg, C. F. W., *Z. Biol.*, 1886, xxii, 261.
6. Mathews, A. P., *Physiological chemistry*, New York, 2nd edition, 1916.
7. Denis, W., *J. Biol. Chem.*, 1910-11, viii, 401.
8. Lothrop, A. P., *J. Allied Dental Soc.*, 1912, vii, 410.
9. Müller, F., *Z. Biol.*, 1901, xlii, 468.
10. Benedict, S. R., *J. Am. Med. Assn.*, 1911, lvii, 1193.
11. Levene, P. A., *J. Biol. Chem.*, 1916, xxvi, 143.
12. Van Slyke, D. D., *J. Biol. Chem.*, 1911-12, x, 15.
13. Gortner, R. A., and Blish, M. J., *J. Am. Chem. Soc.*, 1915, xxxvii, 1630.
14. Gortner, R. A., *J. Biol. Chem.*, 1916, xxvi, 177.
15. Hart, E. B., and Sure, B., *J. Biol. Chem.*, 1916, xxviii, 241.
16. McCollum, E. V., Simmonds, N., and Pitz, W., *J. Biol. Chem.*, 1916-17, xxviii, 483.



THE ISOLATION AND THE NATURE OF THE AMINO SUGAR OF CHINESE EDIBLE BIRDS' NESTS.*

BY CHI CHE WANG.

(From the Nelson Morris Institute for Medical Research, Michael Reese
Hospital, Chicago.)

(Received for publication, September 26, 1921.)

Literature on Carbohydrate Radicle of Glycoproteins.

Although many attempts have been made by different investigators to isolate the carbohydrate group from various glycoproteins, only two amino sugars have so far been isolated: chitosamine (glucosamine) and chondrosamine. Chitosamine (1) was first isolated from lobster shells and later from other substances. In some cases the hydrochloride of chitosamine was obtained in crystalline form while in others the base was estimated either from the reducing power of the hydrolysis solution or from the phenylsazone prepared from it. Bywaters (2) estimated the percentage of chitosamine in the white of eggs by the former method, and Osborne, Jones, and Leavenworth (3) by the latter. According to the latter authors the reduction method for the estimation of chitosamine is of but little value. Ross (4) succeeded in isolating the glycosamine hydrochloride in crystalline form from *Boletus edulis*. Her method is similar to the standard one given by Fischer (5) using concentrated hydrochloric acid to hydrolyze the substance. She demonstrated the fact that the glucosamine could not be isolated by hydrolyzing the substance with sulfuric acid. Ostwald (6) isolated a small amount of chitosamine from ovomucoid by dialyzing the hydrolysis solution with distilled water and evaporating the dialysate. Müller (7) made an investigation on the carbohydrate radicle of mucin from the sputum of tuberculosis patients and from the submaxillary saliva of dogs, and after having encountered many failures he succeeded in isolating a crystalline compound he called glucosamine hydrochloride. The properties of this chitosamine hydrochloride are somewhat different from those summarized by Levene and La Forge (8) for the ordinary chitosamine hydrochloride, in that Müller's is soluble in 95 per cent alcohol, while the latter is almost insoluble in 80 per cent

* The work reported in this article was conducted at the Nutrition Laboratory, Department of Home Economics, University of Chicago. It forms part of the thesis submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the University of Chicago.

alcohol; and that his is destroyed by standing in hydrochloric acid solution and therefore the work has to be carried through rapidly, while the latter is ordinarily prepared by hydrolyzing the substance with concentrated hydrochloric acid for many hours and then evaporating it on a water bath.

Levene (8, 9, 10) has made a most extensive study of the carbohydrate radicle of mucin from pigs' stomach, funis mucin, humor vitreous mucoid, cornea mucoid, and mucoids from cartilage and tendon. From the mucins he isolated the chitosamine while from the mucoids the chondrosamine. The difference between the two will be discussed later.

Isolation of the Carbohydrate.

In attempting to isolate the carbohydrate of the nest, the methods of the previous investigators were first tried, but met with only very moderate success or entire failure. However, since the experiments showed something of the properties of the carbohydrate in contrast with the properties of the previously known amino sugars, they are briefly described here.

Fischer's (5) method of preparing chitosamine by hydrolyzing the substance with concentrated hydrochloric acid and then evaporating it on a water bath was first tried. This method met with entire failure, as the reducing power of the birds' nest which had been so strong in a dilute hydrochloric acid solution hydrolyzed for a short time, was completely destroyed.

Levene's (11) method was next followed, isolating chitosamine from mucins by preparing the conjugated sulfuric acid first and then hydrolyzing the product with 20 per cent hydrochloric acid and a little stannous chloride. In following this method occasionally a few crystals of various forms, among which "Kreissektor" described by Müller (7) was also present, were obtained; but in no case was the yield sufficient for any analytical study.

The third method used was Müller's (7) for preparing chitosamine from salivary mucin. About 15 gm. of the nest were hydrolyzed with 150 cc. of 4 per cent hydrochloric acid for 3 hours on a water bath. The hydrolysis mixture was treated at once with dialyzed iron which threw down a heavy brownish black precipitate. The dark brown filtrate had a strong reducing power, but after evaporating in vacuum desiccators only a very few oblong crystals were formed which were not enough for recrystallization.

During the many failures some of the characteristic properties of the carbohydrate of the birds' nest were observed. It is unstable toward alkali and acid plus heat. It is soluble in 95 per cent ethyl and methyl alcohol. It seems to be partially absorbed or destroyed by protein precipitants. It is, therefore, essential to hydrolyze the material with dilute hydrochloric acid only long enough to split off the carbohydrate radicle but not to destroy it. The following method of preparation was then applied and 10 gm. of beautiful white crystals were obtained after three crystallizations from alcohol and ether.

About 300 gm. of the birds' nest were heated with 2,100 cc. of 3 per cent hydrochloric acid for 5 hours until the material had completely gone into solution, but no black precipitate was produced. (On long heating a black precipitate results.) The hydrolysis solution was evaporated to dryness over sulfuric acid and under solid sodium hydroxide in vacuum desiccators at room temperature. The thick black residue was extracted with 95 per cent alcohol and the alcoholic solution was separated by centrifuge. The extraction was continued until the alcoholic solution gave a little or no reducing power. It required from fifteen to twenty extractions, at least. The alcoholic solution was evaporated to a very thick dark brown syrup under diminished pressure (30 mm.). In another case where a better yield was obtained it stood for several months and evaporated gradually at room temperature. The syrup was then taken up with a large quantity (about 500 cc.) of methyl alcohol. A fairly large quantity of brownish crystals thus resulted. They were filtered off and redissolved in a little boiling water. The solution was filtered again and the brownish filtrate was treated with about eight times its volume of absolute alcohol and then with ether until no more precipitate formed. The crystallization was repeated until the crystals became pure white and seemed to be uniform on microscopic appearance; rods grouping themselves in the forms of an elaborate fern leaf. The yield was about 10 gm.

As the properties of this product seemed to be more or less different from those of any of the known amino sugars, it was thought possible that the product might be a mixture instead of being a single compound. Another preparation was, therefore, made according to the method given above except that in dissolving the brown crystals from methyl alcohol less water was used and instead of using both absolute alcohol and ether to bring about the precipitation, only the former was used. This required much more alcohol, at least 1,500 cc. of absolute alcohol. The alcoholic solution which was only cloudy at first, yielded a large

crop of about 5 gm. of beautiful precipitate on standing over night. It was filtered by suction and after being recrystallized twice, was dried over sulfuric acid. There are various forms of crystals in this fraction, among which the "Kreissektor" form of Müller predominates.

The alcoholic filtrate was treated with ether until the solution became cloudy. On standing over night another crop of about 5 gm. of pure white crystals was obtained. It was filtered and dried as before. The crystals of this fraction are large fluffy flakes.

Although the largest yield of the purified amino sugar was only about 3 per cent, the original protein contained at least 17.36 per cent of reducing substance as shown in the previous report. The small yield might be due to either the unstability of the amino sugar or the presence of some reducing substance other than amino sugar in the protein. Judging from the properties of the sugar, the former hypothesis is probably correct.

Properties of the Three Sets of Crystals.

The three sets of crystals are all very soluble in water and 80 per cent alcohol, fairly soluble in 95 per cent and in methyl alcohol, slightly soluble in absolute alcohol, and insoluble in ether, chloroform, and acetone. They all taste sweet and give a strong test with both Molisch's and Fehling's reagents. They fail to respond to any of the protein reactions. When boiled with strong sodium hydroxide, they give off ammonia. None of them melted when they were placed side by side in melting point tubes and heated to 250°C. in a glycerol bath. However, the alcohol-ether crystals started to turn dark at 142°C., the alcohol fraction at 170°C., and the ether fraction at 150°C. At 250°C. they all became almost black in color. On analysis they give the following percentage composition:

I. Analytical data of the alcohol-ether crystals.

A. Determination of carbon and hydrogen.

1. 0.1858 gm. substance: 0.2254 gm. carbon dioxide and 0.1121 gm. water.
2. 0.1631 gm. substance: 0.1978 gm. carbon dioxide and 0.0980 gm. water.

B. Determination of nitrogen by combustion.

- 0.1860 gm. substance: 10.1 cc. nitrogen at 20°C. and 758 mm. pressure.
- 0.1801 gm. substance: 10.1 cc. nitrogen at 20.5°C. and 756 mm. pressure.

C. Determination of chlorine.

- 0.1541 gm. substance required for titration of hydrochloric acid 13.92 cc. 0.05 N silver nitrate.
- 0.1597 gm. substance required for titration of hydrochloric acid 14.35 cc. 0.05 N silver nitrate.
- 0.1594 gm. substance required for titration of hydrochloric acid 14.41 cc. 0.05 N silver nitrate.

$C_8 H_{13} O_5 NCl$. Calculated. H 6.50, C 33.41, N 6.51, Cl 16.45.

Found. H 6.69, C 33.09, N 6.28, Cl 15.99.

D. Determination of the optical activity.

- 0.3335 gm. substance in 9.947 gm. water rotated in a 1 dm. tube with D-light $+2.58^\circ$ 7 minutes after the solution was made, and $+2.38^\circ$ 28 hours later.

Initial.	Equilibrium.
$[\alpha]_D^{20} = +79.5^\circ$	$[\alpha]_D^{20} = +73.4^\circ$

- 0.2821 gm. substance in 10.816 gm. water rotated in a 1 dm. tube with D-light $+1.81^\circ$ 10 minutes after the solution was made, and $+1.72^\circ$ 20 hours later.

Initial.	Equilibrium.
$[\alpha]_D^{20} = +71.2^\circ$	$[\alpha]_D^{20} = +67.7^\circ$

Average of (1) and (2).

Initial.	Equilibrium.
$[\alpha]_D^{20} = +75.4^\circ$	$[\alpha]_D^{20} = +70.6^\circ$

II. Analytical data of the alcohol fraction.

A. Determination of nitrogen by Kjeldahl-Gunning method.

- 0.3140 gm. substance required for titration of ammonia 14.56 cc. 0.1 N sulfuric acid.
- 0.2947 gm. substance required for titration of ammonia 13.42 cc. 0.1 N sulfuric acid.
- 0.1199 gm. substance required for titration of ammonia 5.54 cc. 0.1 N sulfuric acid.

$C_8 H_{13} O_5 NCl$. Calculated. N 6.51.

Found. N 6.46.

B. Determination of optical activity.

- 0.3056 gm. substance in 9.9028 gm. water rotated in a 1 dm. tube with D-light $+2.703^\circ$ 10 minutes after the solution was made, and $+2.153^\circ$ 22 hours later.

Initial.	Equilibrium.
$[\alpha]_D^{10} = +87.6^\circ$	$[\alpha]_D^{10} = +69.7^\circ$

- 0.2997 gm. substance in 10.0797 gm. water rotated in a 1 dm. tube with D-light $+2.56^\circ$ 15 minutes after the solution was made, and $+2.12^\circ$ 20 hours later.

Initial.	Equilibrium.
$[\alpha]_D^{10} = +86.1^\circ$	$[\alpha]_D^{10} = +71.3^\circ$

Average of (1) and (2).

Initial.	Equilibrium.
$[\alpha]_D^{10} = +86.9^\circ$	$[\alpha]_D^{10} = +70.5^\circ$

III. Analytical data of the ether fraction.

A. Determination of nitrogen by Kjeldahl-Gunning method.

1. 0.2152 gm. substance required for titration of ammonia
9.68 cc. 0.1 N sulfuric acid.
2. 0.2016 gm. substance required for titration of ammonia
9.02 cc. 0.1 N sulfuric acid.
 $C_6 H_{13} O_5 NCl$. Calculated. N 6.51.
Found. N 6.29.

B. Determination of optical activity.

1. 0.2329 gm. substance in 9.8419 gm. water rotated in a 1 dm. tube with D-light $+1.596^\circ$ 10 minutes after the solution was made, and $+1.675^\circ$ 22 hours later.

Initial.	Equilibrium.
$[\alpha]_D^{10} = +67.5^\circ$	$[\alpha]_D^{10} = +70.8^\circ$
2. 0.3512 gm. substance in 10.2822 gm. water rotated in a 1 dm. tube with D-light $+2.24^\circ$ 10 minutes after the solution was made, and $+2.425^\circ$ 20 hours later.

Initial.	Equilibrium.
$[\alpha]_D^{10} = +65.6^\circ$	$[\alpha]_D^{10} = +71.0^\circ$

Average of (1) and (2).

Initial.	Equilibrium
$[\alpha]_D^{10} = +66.6^\circ$	$[\alpha]_D^{10} = +70.9^\circ$

The phenylosazone was prepared from the alcohol-ether crystals, Garard and Sherman's (12) method was followed. About 1 gm. of the substance was placed in a small beaker with 10.80 gm. of freshly distilled phenylhydrazine, 12.60 gm. of glacial acetic acid, and 6.85 gm. of pure sodium acetate. The volume was made up with distilled water to 100 cc. and the mixture heated on a water bath for 3 hours. Unlike the osazone from glucose which crystallizes out during the course of heating under the same conditions, the osazone from this substance remained entirely in solution until it was cooled over night. This is similar to a preparation from glucosamine. It was so soluble in the 60 per cent alcohol used by Garard and Sherman that to recrystallize their glucosazone that solvent could not be used and Levene's method of recrystallization from water and pyridine was followed. After two recrystallizations it gave a melting point of 214° with rapid heating. On standing over sulfuric acid the color darkens and the melting point lowers markedly— 170° in one case during a week's standing. Samples of glucosazone made in exactly the same way for comparison both from glucose and glucosamine gave the usual melting point of 208° , and did not change on standing over sulfuric acid. Three tubes, one containing the

unknown osazone, the second glucosazone, and the third a mixture of the two, were heated in one bath. The unknown always melted at a temperature about 6° above the others, while the mixture was either about the same or a little below the glucosazone.

An attempt was made to study the optical activity of the osazone. As it did not dissolve completely in the usual amount of Neuberg's (13) alcohol pyridine mixture even after having stood for an hour with occasional shaking, the amount of solvent was doubled and a clear brown solution was secured after half an hour's standing. The color of the solution was, however, so deep that it was impossible to see through a 1 dm. tube. The solution was, therefore, diluted to one-fourth, the strength recommended by Neuberg and by Levene and it was found to rotate slightly to the left.

DISCUSSION.

An examination of the properties and the analytical data of the three sets shows that they are all hexosamine hydrochlorides and that they differ from each other only in their optical rotation and the temperature at which they begin to turn dark on heating. The latter difference might be explained on the basis of their purity. The one which is probably the purest, the alcohol fraction, turns dark at the highest temperature, 170°C., while the alcohol-ether crystals which begin to turn dark at 142°C., might be the least pure of the three. The degree of purity of the three sets of crystals is also shown by the slight difference in their nitrogen content. The alcohol fraction has a value of 6.46 per cent, the ether fraction 6.29 per cent, and the alcohol-ether crystals 6.28 per cent. The calculated value for the percentage of nitrogen of hexosamine is 6.51.

The difference in the optical rotation is, however, most interesting. The average of the two determinations of optical rotation of the alcohol-ether crystals is $[\alpha]_D^{20} = +75.4^\circ$ to $+70.6^\circ$ while those of the alcohol fraction and the ether fraction are $[\alpha]_D^{10} = +86.9^\circ$ to $+70.5^\circ$ and $[\alpha]_D^{10} = +66.6^\circ$ to $+70.9^\circ$, respectively. It is, therefore, clear that the three sets of crystals differ only in their initial rotations. The alcohol-ether crystals and the alcohol fraction have a descending rotation, while the ether fraction has an ascending one. Furthermore,

the value of the initial rotation of the alcohol-ether crystals lies between those of the other two fractions and is almost equal to the mean of the other two, which is $+ 76.8^\circ$. The duplicate determinations were made at least 6 months apart. These facts seem to indicate that the alcohol fraction is probably one of the hexosamine hydrochlorides of the α form; the ether fraction the β form, and the alcohol-ether crystals a mixture of the two. Thus Levene (14) reported that the optical rotation of chondrosamine hydrochloride of the α form was $[\alpha]_D^{20} = + 129.5^\circ$ to $+ 93.8^\circ$ and that of the β form was $[\alpha]_D^{20} = + 62.7^\circ$ to $+ 91.1^\circ$. No report on the properties of the α and β forms of other hexosamine hydrochlorides could be found in the literature.

Unfortunately, there is not enough material for more experimental work at present and the question as to whether the above hypothesis is correct has to be left unanswered.

What one of the possible hexosamines this is, has not been determined. A study of Tables I and II indicates that the properties of the unknown sugar are not in close agreement with either of the two known hexosamine hydrochlorides; the chitosamine hydrochloride or the chondrosamine hydrochloride. From Müller's (7) description of the "glucosamine" which he obtained from sputum mucin it seems probable that his substance was the same as the alcohol-ether crystals. The highest melting point which he gave from his osazone was only 205° , but the discrepancy with our 214° might be explained by the greater purity of the latter.

The unknown hexosamine hydrochloride differs from chondrosamine in its melting point and its optical activity, but resembles it in its solubility in alcohol and in giving an unstable osazone.

The most striking point of resemblance between chitosamine hydrochloride and the hydrochloride of the unknown sugar lies in their optical activity and their stability toward concentrated hydrochloric acid, solubility in alcohol, and in the stability and the character of their osazone. Thus, chitosamine hydrochloride is insoluble in 80 per cent alcohol, while the hydrochloride of the unknown sugar is very soluble in 80 per cent alcohol, and when pure, soluble in 95 per cent alcohol, methyl alcohol, as well as absolute alcohol. Chitosamine hydrochloride may be prepared by boiling with concentrated hydrochloric acid, while this sugar is

TABLE I.
Comparison of the Properties of Hexosamine Hydrochlorides.

Investigator.	Chondrosamine.		Chitosamine (glucosamine).			Unknown sugar.		
	Levene.	Levene.	Müller.	Ordinary.	Author.			
					Alcohol-ether crystals.	Alcohol fraction.	Ether fraction.	
Solubility in alcohols.								
80 per cent.....	Soluble.			Insoluble.	Very soluble.	Very soluble.	Very soluble.	
95 per cent.....			Soluble.	Insoluble.	Soluble.	Soluble.	Soluble.	
Absolute.....					Slightly soluble.	Slightly soluble.	Slightly soluble.	
Methyl.....	Soluble.	Insoluble.			Soluble.	Soluble.	Soluble.	
Stability in concentrated HCl.....			Unstable.	Stable.	Unstable.	Unstable.	Unstable.	
Melting point.....	182°C.	No.	No.	No.	No.	No.	No.	
Temperature at which sugar turns dark..					142°C.	170°C.	150°C.	

TABLE II.
Comparison of Optical Rotation of Hexosamine Hydrochloride.

Chondrosamine.		Chitosamine (glucosamine).			Unknown sugar.	
Origin.	Levene.	Origin.	Levene.	Ordinary.	Precipitation.	Author.
	[α] _b		[α] _b	[α] _b		[α] _b
	α form.	Funis mucin.	+84.8° to +71.9°	+100° to +73°	Alcohol-ether.	+75.4° to +70.6°
Tendon.	+129° to +93°	Stomach mucin.	+90.1° to +70.6°		Alcohol.	+86.9° to +70.5°
	β form.	Vitreous humor.	+96.8° to +72.2°		Ether.	+66.6° to +70.9°
Synthetic.	+60° to +91°	Cornea mucoid.	+95.4° to +71.9°			
Aorta mucoid.	+65.6° to +91.2°	Serum mucoid.	+89.5° to +71.4°			
Sclera mucoid.	+56.8° to +95.7°					

completely destroyed. Glucosazone melts at 208° and the osazone from this sugar at 214° . The former is more soluble in Neuberg's alcohol-pyridine mixture. It is stable on standing over sulfuric acid. The unknown osazone is unstable, darkening in color and lowering its melting point.

Which amino-hexose this sugar is is therefore still undetermined.

Levene (10) has recently made a grouping of his glycoproteins and their derivatives which throws light on the question of the birds' nest glycoproteins from tendons, cartilage, aorta, and sclera mucoids. He has isolated the conjugated sulfuric acid which he calls chondroitin sulfuric acid and which gives chondrosamine on hydrolysis, and from gastric mucosa, vitreous humor, funis mucin, cornea mucoids, and serum mucoids he has isolated mucoitin sulfuric acid from which chitosamine (glucosamine) is derived. Chondroitin sulfuric acid is highly resistant to hydrolytic agents, and mucoitin sulfuric acid is very easily hydrolyzed, and even amino sugar is often destroyed completely by the hydrolytic agent. It is plain from the work described in this paper that birds' nest glycoprotein belongs to the second group.

He also found that different members of the second group behave differently, especially in the properties of their conjugated sulfuric acid so that he distinguishes two subdivisions A and B. The precursor of the unknown sugar of birds' nest is more like subgroup B than A. Levene's subgroup A gives a fairly insoluble barium salt of the conjugated sulfuric acid and the subgroup B (the description of which was published after the work in this paper was done) a distinctly soluble one. The efforts of the writer to isolate a conjugated sulfuric acid from the birds' nest by the Levene early method met with almost complete failure, the substance behaving in the manner later described by Levene for subgroup B. It is, therefore, certain that the precursor of the unknown sugar belongs to the subgroup B of the second group of glycoproteins described by Levene.

SUMMARY.

1. A method for the solution of the amino sugar from Chinese edible birds' nest is described.
2. Three sets of pure white crystals having the percentage composition of a hexosamine, were obtained. They are similar

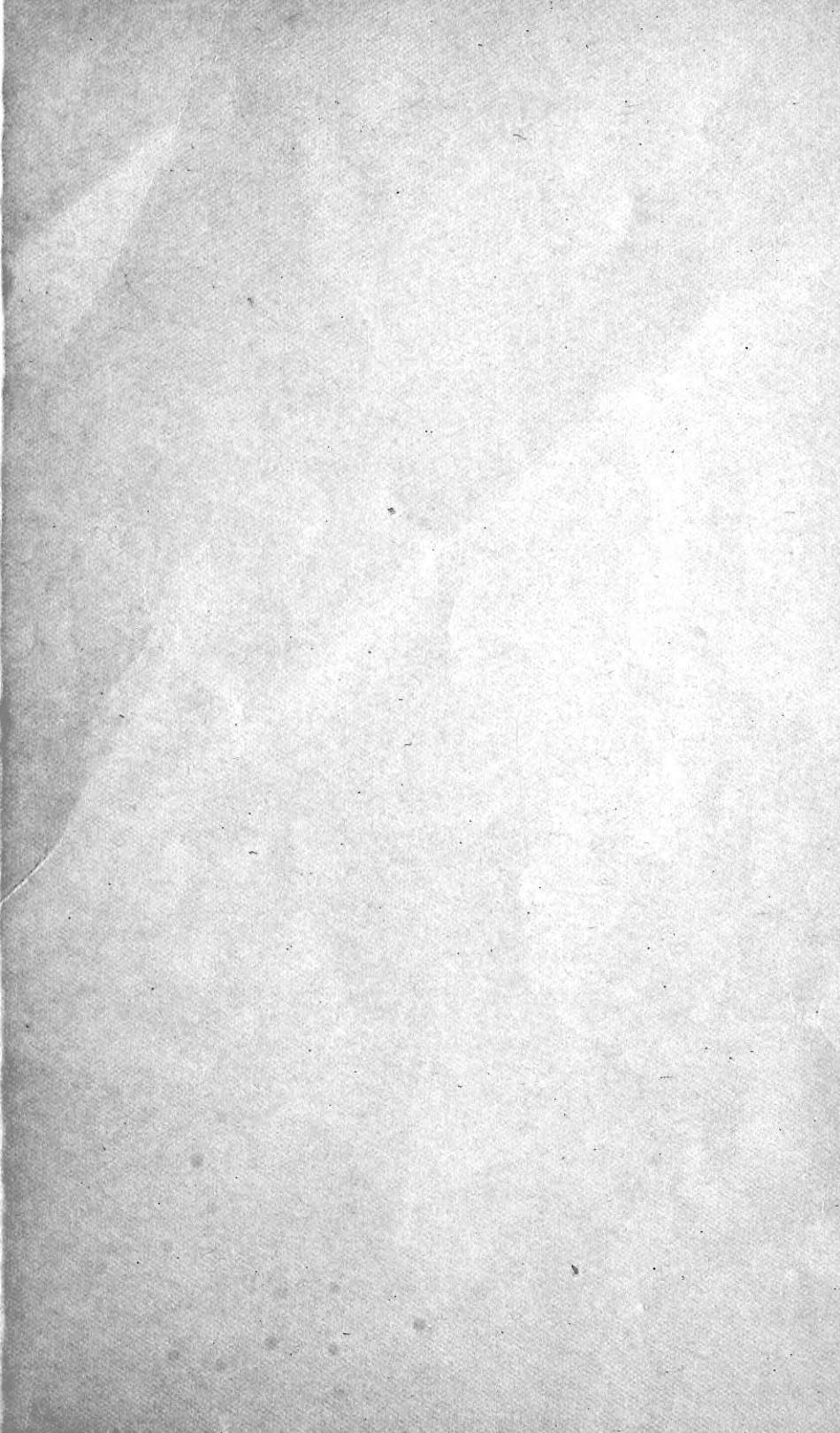
in all their properties except optical rotation which for two of them decreases on standing over night and of the third increases. The probable conclusion is that one of the two sets showing a decreasing of the optical rotation on standing is the α form of a hexosamine, the one which has a rise of the rotation the β form, and the third set a mixture of the two.

3. Levene's recent description of the different groups of glycoproteins and their derivatives shows that there is a striking resemblance between the properties of the precursor of this unknown sugar and those of his subgroup B of the second group.

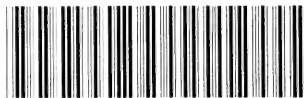
In conclusion I wish to express my obligation to Dr. Katharine Blunt, in whose laboratory the work reported in the last two papers was carried out, for her helpful suggestions and constant interest in the investigation.

BIBLIOGRAPHY.

1. Abderhalden, E., *Handbuch der biochemischen Arbeitsmethoden*, Berlin, 1912, vi, 76.
2. Bywaters, H. W., *J. Physiol.*, 1913, xlvi, p. xxxv.
3. Osborne, T. B., Jones, D. B., and Leavenworth, C. S., *Am. J. Physiol.*, 1909, xxiv, 252.
4. Ross, W., *Biochem. J.*, 1915, ix, 313.
5. Fischer, E., *Anleitung zur Darstellung organischer Präparate*, Braunschweig, 8th edition, 1908.
6. Ostwald, A., *Z. physiol. Chem.*, 1910, lxxviii, 173.
7. Müller, F., *Z. Biol.*, 1901, xlii, 468.
8. Levene, P. A., and La Forge, F. B., *J. Biol. Chem.*, 1914, xviii, 123.
9. Levene, P. A., and López-Suárez, J., *J. Biol. Chem.*, 1916, xxv, 511; xxvi, 373. Levene, P. A., and La Forge, F. B., *J. Biol. Chem.*, 1913, xv, 69, 155; 1914, xviii, 238.
10. Levene, P. A., and López-Suárez, J., *J. Biol. Chem.*, 1918, xxxvi, 105.
11. Levene, P. A., and López-Suárez, J., *J. Biol. Chem.*, 1916, xxvi, 373; xxv, 514.
12. Garard, I. D., and Sherman, H. C., *J. Am. Chem. Soc.*, 1918, xl, 955.
13. Neuberg, C., *Ber. chem. Ges.*, 1898, xxxii, 3384. Neuberg, C., and Schewket, O., *Biochem. Z.*, 1912, xlv, 481.
14. Levene, P. A., *J. Biol. Chem.*, 1917, xxxi, 609.



LIBRARY OF CONGRESS



0 014 338 893 1 ●