tween the analysis of the original egg albumen and those of the protalbinic and lysalbinic acids. Indeed, the differences are so small as to be in most of the fractions well within the experimental error of the analysis. Protalbinic acid is significantly lower in ammonia N than is either the original egg albumin or the lysalbinic acid and both of the derived "acids" are higher in lysine content. It must be pointed out, however, that in all probability the figures in a Van Slyke analysis of materials prepared in this manner are misleading for if the guanidine group of arginine in the original egg albumen is attacked by the alkali, ornithine would result and this would be calculated as lysine in Van Slyke's method. To our mind the apparent increase in lysine in the protalbinic and lysalbinic acids is due to the presence of ornithine. This hypothesis, however, has not as yet been tested experimentally.

Summary.

Lysalbinic and protalbinic acids have been prepared from egg albumen by Paal's method and the nitrogen distribution of these preparations has been determined by Van Slyke's method and compared with a similar analysis of the original egg albumen. This comparison leads to the following conclusions:

(1) The nitrogen distribution in protalbinic and lysalbinic acids is not markedly different from that of the original egg albumen.

(2) Both protalbinic and lysalbinic acids show a somewhat greater apparent lysine content than does the original egg albumen.

(3) We believe that this apparently greater lysine content is due to the presence of ornithine, derived from arginine by the action of the alkali, inasmuch as ornithine if present would appear in the lysine fraction in Van Slyke's method.

(4) The analyses here recorded furnish no evidence as to whether or not the protalbinic and lysalbinic acids are true chemical compounds or as to whether or not their chemical structure is more simple than is that of egg albumen. It is extremely improbable, however, that either preparation has as low a molecular weight as 800.

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THE EFFECT OF PROLONGED ACID HYDROLYSIS UPON THE NITROGEN DISTRIBUTION OF FIBRIN WITH ESPECIAL REFERENCE TO THE AMMONIA FRACTION.[1]

BY ROSS AIKEN GORTNER AND GEORGE E. HOLM.

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Introduction.

In an article devoted to the study of "Different Forms of Nitrogen in

[1] Published with the approval of the Director as Paper No. 87, Journal Series, Minn. Agr. Exp. Station.

Proteins" Osborne, Leavenworth and Brautlecht[1] conclude that "ammonia originates from an amide union in the protein molecule" and that "none of the amino acids which are known products of protein hydrolysis yield any ammonia by long boiling with strong hydrochloric acid and cannot be considered as contributing any part of the ammonia formed from proteins by acid hydrolysis."

Henriques and Gjaldbaek[2] found that when proteins are hydrolyzed with acid both amino nitrogen and ammonia increase up to a certain point at which the amino nitrogen attains a maximum and that further hydrolysis causes a transformation of amino nitrogen into ammonia indicating a deamination of some amino acid or acids. They showed that deamination occurs when cystine is boiled with hydrochloric acid. Van Slyke has since shown that "cystine is partially destroyed during hydrolysis and gradually altered into a substance or substances which are not precipitated by phosphotungstic acid."

Henriques and Gjaldbaek conclude that the most certain method for hydrolyzing proteins so as to obtain the maximum amount of amino nitrogen and the least possible ammonia formation due to deamination is to conduct the hydrolysis with 3 N hydrochloric acid in an autoclave at 150° for one and one-half hours.

Van Slyke[3] hydrolyzed different proteins at 100° and atmospheric pressure for different periods of time and also at 150° and 160° in an autoclave for 1.5 and 3 hours. He found that at 100° and atmospheric pressure twenty-fours was necessary for the production of maximum amino nitrogen. Prolonged hydrolysis beyond this point caused an increase in ammonia. At 150° hydrolysis beyond 1.5 hours caused an increase in the ammonia nitrogen in five of the six proteins studied, casein alone showing no such increase. The increases for an additional 1.5 hour hydrolysis ranged from 4.5% of the original ammonia nitrogen for wheat gliadin through 10.8% for oxyhaemoglobin, 13.7% for wheat gluten, 31% for edestin to 60% of the original ammonia nitrogen for egg albumen. That deamination does occur with casein is shown by an increase of 23% in the ammonia nitrogen found for the hydrolysate at 160° and 3 hours over that at 150° for a similar period of time. These increases in ammonia nitrogen occurred at the expense of the amino nitrogen. However, Van Slyke concludes "that any of the other natural amino acids (than cystine) are deaminized to an appreciable extent unless heated under pressure

[1] T. B. Osborne, C. S. Leavenworth, and C. A. Brautlecht, "The Different Forms of Nitrogen in Proteins," *Am. J. Physiol.*, 23, 180–200 (1908).

[2] V. Henriques and J. K. Gjaldbaek, "Über quantitative Bestimmung der im Proteine oder in dessen Abbauprodukten vorhanden Peptidbindungen," *Zeitschr. physiol. Chem.*, 67, 8–27 (1910).

[3] Van Slyke, D. D., "The Conditions for Complete Hydrolysis of Proteins," *J. Biol. Chem.*, 12, 295–299 (1912).

appears doubtful" and agrees with Osborne, Leavenworth and Brautlecht that the "ammonia" arises chiefly from the acid amide group of the asparagine and glutamine in the protein molecule.

Gortner[1] recently published figures which show that ammonia nitrogen is increased by prolonged hydrolysis at atmospheric pressure. Thus he finds an increase of 19.5% in the ammonia nitrogen of casein hydrolyzed for 120 hours over that hydrolyzed for 26 hours, an increase of 35.4% in the ammonia fraction of fibrin hydrolyzed 115 hours over that hydrolyzed for 25 hours and an increase of 15.0% for egg albumen hydrolyzed 96 hours over the figure obtained for a 48-hour hydrolysate.

All evidence, therefore, seems to indicate that the ammonia nitrogen of protein hydrolysis is not a fixed quantity but varies with the strength of acid employed, the length of hydrolysis and, we believe, with the vigor of boiling. Our problem, therefore, is not to show that variations in the amount of the ammonia fraction do occur when the time of hydrolysis is extended but to gather some information as to whether or not the deamination which takes place under such conditions affects only one or two amino acids or whether it is a general phenomena affecting all of the amino acids more or less equally, the completeness of the reaction being limited only by the length of the hydrolysis.

Experimental.

During the progress of our work we have noted that both (*a*) length of time of hydrolysis, and (*b*) vigor of hydrolysis, have a marked effect upon the ammonia fraction and also upon the amount of "humin." In order to gain some insight into the nature of the changes produced by prolonged hydrolysis, the following experiments were made:

Three grams of fibrin, finely ground in a Seck mill, were boiled vigorously with 75 cc. of 20% hydrochloric acid for different periods of time ranging from 1 hour to 72 hours. In order to keep an absolute check upon the time of hydrolysis the acid was brought to boiling before being added to the fibrin and the hydrolysate immediately placed upon the hot sand bath. At the expiration of the specified time the hydrolysate was cooled in running water, and the "insoluble humin" filtered off and determined separately by the Kjeldahl method. The ammonia was determined according to Van Slyke's directions and the calcium residue filtered off and Kjeldahled, the nitrogen being recorded as "soluble humin." The filtrate from the soluble humin was slightly acidified and made up to 500 cc., 10 cc. of which was used for amino nitrogen determinations in Van Slyke's apparatus. To make all figures of amino nitrogen absolutely comparable, the determination in each case was continued exactly six

[1] R. A. Gortner, "The Origin of the Humin Formed by the Acid Hydrolysis of Proteins. II. Hydrolysis in the Presence of Carbohydrates and of Aldehydes," *J. Biol. Chem.*, **26**, 177–204 (1916).

minutes. On account of the slight variation in length of hydrolysis, the amino nitrogen was determined only upon selected filtrates. The figures obtained are shown in Table I.

TABLE I.—SHOWING COMPARATIVE ANALYSES OF 3-G. SAMPLES OF FIBRIN HYDROLYZED FOR DIFFERENT PERIODS OF TIME (1 HOUR TO 72 HOURS).

Length of hydrolysis. Hrs.	Mg. insol. humin N.	Mg. ammonia N.	Mg. soluble humin N.	Mg. amino N.
1	0.72	34.50	7.10	305.5
2	1.40	34.45	7.28	....
3	1.80	34.70	8.20	338.0
4	1.75	34.70	8.30	355.0
5	2.66	35.70	8.45	....
6	2.82	36.40	8.00	347.5
7	3.20	36.25	8.00	....
8	3.70	36.60	8.00	....
9	3.25	36.80	9.00	....
10	4.00	37.77	....	343.5
12	4.05	37.55	8.60	....
14	4.15	38.25	8.30	332.5
16	5.25	38.50	....	....
20	6.02	40.80	8.00	....
24	6.25	40.65	....	....
36	7.00	47.25	10.25	328.0
72	7.55	51.00	10.00	316.0

The figures for ammonia nitrogen agree with the conclusions of Henriques and Gjaldbaek and with those of Gortner. In order to visualize the results the curves for ammonia nitrogen and amino nitrogen are plotted in Fig. 1. There is at first a steady increase in amino nitrogen but during this time very little change in ammonia. *After 4 hours' hydrolysis deamination begins and there is an increase in ammonia, this increase becoming greater with length of hydrolysis.* Four hours' hydrolysis may or may not represent the point of complete hydrolysis. *It may only be the point where deamination is less rapid than is the formation of free amino groups from the peptide linkings.* If such be the case the amino nitrogen is no indication of the completion of hydrolysis after this point has been reached.

The following figures show the increase in milligrams of ammonia nitrogen and also in percentage increase for different periods of time:

Period.	Mg. N increase.	Percentage increase.
1–12 hrs.	3.05	8.84
12–24 "	3.10	8.98
24–36 "	6.60	19.13
36–72 "	3.75	10.87

With hydrolysis prolonged beyond 36 hours, there seems to be a tendency for the ammonia to increase more slowly due probably to the greater difficulty of deaminizing some acids than others. The series of hydrolyses

shows that there is a deamination of acids with an increase in ammonia according to the length of time of hydrolysis and that it cannot be attributed to any one acid, for if such were the case there should be no increase after this one acid present had been completely deaminized and apparently deamination continues indefinitely becoming less and less with each successive interval of time.

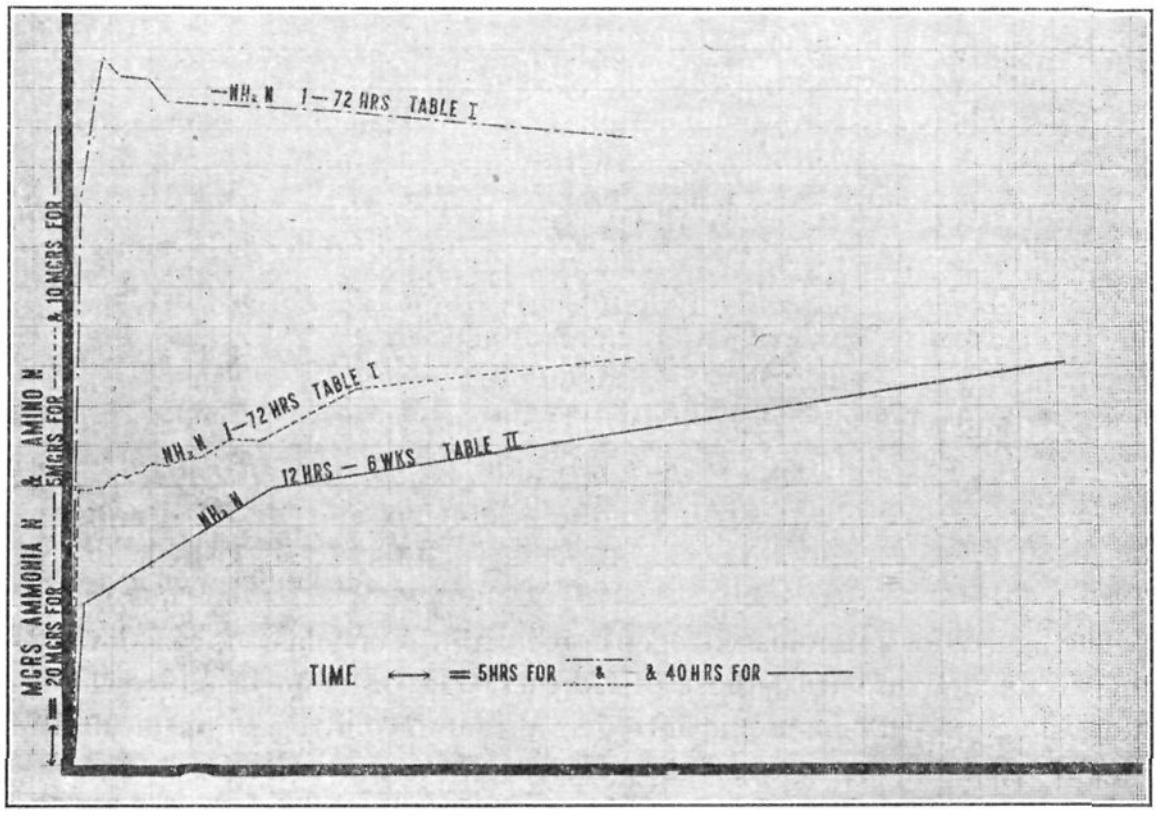

Fig. 1.—Showing Changes in Ammonia and Amino Nitrogen with Length of Hydrolysis.

In order to ascertain how long deamination would continue, 10 g. of fibrin were hydrolyzed for 24 hrs. with 20% hydrochloric acid and ammonia determined in the usual manner. The residue was filtered, acidified, concentrated and made up to its former volume with 20% hydrochloric acid and again boiled for 14 days. At the end of this time ammonia was again determined, the residue filtered, acidified, concentrated and made up to volume as before. The final hydrolysis was then continued for 8 weeks and ammonia determined.

The results were as follows:

1st fraction	(24 hrs. hydrolysis)	157.00 mg. N.
2d "	(24 hrs.–14 days ")	115.00 " "
3rd "	(2 wks.–8 wks. ")	79.00 " "

The ammonia obtained in the first fraction undoubtedly contains all of the amide nitrogen found in 10 g. of fibrin and in addition some ammonia formed by deamination. The deamination for 10 g. according to Table I and Fig. 1 would equal about 20.50 mg. or 13.05% of the amount ob-

tained. The second and third fractions represent deamination entirely and show that deamination continues with prolonged hydrolysis but becomes increasingly difficult.

That the nitrogen splits off as ammonium chloride is shown by the following experiment: The second fraction representing 115 mg. nitrogen was evaporated to dryness yielding 0.4105 g. hydrochloride. 0.25 g. of this salt was dissolved in 50 cc. of water and duplicate 10 cc. portions were Kjeldahled. From the remaining 30 cc. the platinum salt was prepared yielding the usual yellow prisms of ammonium chloroplatinate.

	Found.	Theoretical for NH_4Cl.
Per cent. nitrogen in salt.....	26.00%	26.16%
Pt. content of chloroplatinate.....	43.20%	43.91%

In order to gain some information as to whether the bases or the monamino acids were the more susceptible to deamination, Van Slyke analyses[1] were made upon 3 g. of fibrin[2] hydrolyzed with 20% hydrochloric acid for varying lengths of time. The results are shown in Tables II and III.

TABLE II.—SHOWING NITROGEN DISTRIBUTION IN MILLIGRAMS OF NITROGEN IN 3 G. OF FIBRIN HYDROLYZED FOR VARYING PERIODS OF TIME.

	12 hrs. hydrolysis.			48 hrs.[4] hydrolysis.	201 hrs. hydrolysis.			6 wks.[5] hydrolysis.
	I.	II.	Av.		I.	II.	Av.	
Mg. ammonia N.....	40.50	40.25	40.37	47.30	68.25	70.40	69.32	100.30
Mg. insol. humin N.....	5.70	5.80	5.75	7.25	9.70	11.00	10.35	11.85
Mg. sol. humin N.....	7.00	7.25	7.12	7.20	6.63	6.65	6.64	5.70
Mg. phosphotungstic humin N.....	4.50	3.50	4.00	1.45	4.10	3.40	3.75	5.60
Mg. total N in bases.....	147.25	146.00	146.62	138.00	134.25	133.00	133.82	124.12
Mg. amino N in bases.....	83.10	84.66	83.88	85.37	79.42	79.40	79.41	78.00
Mg. total N in filt. from bases.....	252.40	258.00	255.00	259.12	236.40	236.00	236.20	204.00
Mg. amino N in filt. from bases.....	251.20	253.82	252.51	256.20	229.82	227.66	228.74	198.55
Mg. N recovered[3].....	457.35	460.80	459.06	460.32	459.32	460.45	460.08	451.57
Distribution of N in bases:								
Arginine N.....	.....	.....	.....	50.00	.....	.....	56.00[7]	46.00
Histidine N.....	.....	.....	.....	22.69	.....	.....	18.61	17.43
Lysine + cystine N[6]....	.....	.....	.....	65.31	.....	.....	59.21	60.69

[1] D. D. Van Slyke, "The Analysis of Proteins by Determination of the Chemical Groups Characteristic of the Different Amino Acids," *J. Biol. Chem.*, 10, 15–55 (1911).

[2] This sample of fibrin was obtained from a German supply house, while that used in the preceding experiments was of American origin.

[3] The total nitrogen in each 3-g. sample used was 0.4561 g.

[4] A single analysis.

[5] One of the hydrolysates was lost before the 6 weeks' hydrolysis had been completed, hence the single analysis for this period.

[6] Cystine not determined on these hydrolysates. Gortner (*loc. cit.*) found 2.4 mg. cystine N in the 48-hour hydrolysate of 3 g. of fibrin.

[7] Probably too high a value.

TABLE III.—SHOWING THE AVERAGE NITROGEN DISTRIBUTION IN PERCENTAGES OF THE TOTAL NITROGEN OF FIBRIN HYDROLYZED FOR VARYING PERIODS OF TIME.

	12 hrs. hydrolysis.	48 hrs. hydrolysis.	201 hrs. hydrolysis.	6 wks. hydrolysis.	Loss (—) or gain (+) between 12 hrs. and 6 wks. hydrolysis.
Ammonia N	8.85	10.37	15.19	21.99	+13.14
Insoluble humin N	1.26	1.59	2.27	2.60	+ 1.34
Soluble humin N	1.56	1.58	1.46	1.25	— 0.31
Phosphotungstic humin N	0.87	0.32	0.82	1.23	+ 0.36
Total basic N	32.14	30.25	29.33	27.21	— 4.93
Amino N of bases	18.38	18.71	17.40	17.10	— 1.28
Total N in filt. from bases	55.90	56.80	51.73	44.72	—11.18
Amino N in filt. from bases	55.35	56.16	50.14	43.52	—11.83
Total N regained	100.58	100.91	100.70	99.00	....
Distribution of N in bases:					
Arginine N	....	10.96	12.28	10.08	....
Histidine N	....	4.97	4.08	3.82	....
Lysine + cystine N	....	14.32	12.98	13.30	....

Table II shows a marked decrease in both the total nitrogen and in the amino nitrogen of the bases and the filtrate from the bases due to the prolonged hydrolysis, while the ammonia fraction shows a steady increase. The ammonia curve is plotted in Fig. 1. The increase in ammonia nitrogen and the decrease of nitrogen in the bases and filtrate from the bases between 12 hrs. and 6 wks. hydrolysis are as follows:

Increase in ammonia N	59.93 mg.
Decrease in total N in filtrates	51.00 "
Decrease in amino N in filtrates	53.96 "
Decrease in total N in bases	22.50 "
Decrease in amino N in bases	5.88 "

In the case of the filtrate from the bases the decrease in total nitrogen is approximately equal to the decrease in amino nitrogen showing that the loss in total nitrogen is nearly all deamination.

The figures for the bases show that the decrease here is not primarily due to deamination. That the principal reaction is not due to the breaking off of the α-amino group from a heterocyclic diamino acid and the formation of a compound which would not be precipitated by the phosphotungstic acid seems to be proven by the fact that the non-amino nitrogen of the filtrate from the bases increases only slightly (3.03 mg.) by prolonged hydrolysis. There is however a loss of 16.62 mg. of non-amino N from the bases. A part of this must be due to the breaking up of tryptophane. We have already shown[1] that some of the nitrogen of indole boiled with HCl appears in the ammonia fraction, although of course this might be due to unchanged indole. Almost conclusive proof

[1] R. A. Gortner and G. E. Holm, "On the Origin of the Humin Formed by the Acid Hydrolysis of Proteins. III. Hydrolysis in the Presence of Aldehydes. II. Hydrolysis in the Presence of Formaldehyde," THIS JOURNAL, **39**, 2477 (1917).

that tryptophane is the principal amino acid involved is obtained when we compare the loss of non-amino nitrogen for the entire period of 6 weeks with the loss between 12 hours' and 48 hours' hydrolysis. Of a total loss of 16.62 mg. of non-amino N, 9.11 mg. or 54% is lost between 12 hours and 48 hours. We have already shown[1] that tryptophane is responsible for the formation of humin.

The further loss of non-amino nitrogen from the bases is apparently distributed about equally among arginine, histidine and lysine, as may be seen by a reference to Tables II and III. It is difficult to conceive of the imidazole ring of histidine being broken down, yet the percentage of histidine falls slightly when hydrolysis is prolonged. It is probable that the α-amino group is slowly broken off and the imidazole residue passes into the filtrate from the bases. Possibly the guanadine group of arginine is somewhat affected for Beilstein[1] states that guanidine is broken down into ammonia and CO_2 by boiling with concentrated acids. However, as the tables show, this reaction if it takes place at all, takes place very slowly. It is doubtful whether lysine suffers appreciable deamination under prolonged hydrolysis. The decrease of 5.88 mg. in amino nitrogen in the bases can easily be accounted for by complete decomposition of the cystine which escaped the 12-hour hydrolysis and which was precipitated by phosphotungstic acid.

We must therefore conclude that histidine, arginine and lysine are not readily deaminized by prolonged boiling with acids and that fairly accurate values may be obtained for these amino acids even though the hydrolysis of the protein were extended for as long a period as six weeks. On a 12-hour hydrolysis, however, some other amino acid (tryptophane) is, in part, precipitated with the bases which would be calculated as histidine. For example, assume an arginine content of 50 mg. in the 12-hour hydrolysate. This would give a histidine content of 42.86 mg. or practically twice the amount which is probably present.[2]

The figures for the filtrates from the bases strongly indicate that the ammonia fraction of protein hydrolysis is derived from at least two sources, *i. e.*, from acid amides and from the deamination of amino acids. Among the amino acids it is principally the mono amino acids which are attacked.

When a protein is hydrolyzed for only a few hours the amount of deamination can be disregarded and the ammonia figures may be taken as representing fairly accurately the nitrogen of acid amides. However,

[1] Beilstein, F., article on "Guanidine" in *Handb. Organ. Chem.*, 3d Ed. Bd. 1., pp. 1161–2. Hamburg and Leipzig (1893).

[2] Histidine N will not fall from 42.86 mg. to 22.69 mg. in the period of 12–48 hrs. and then remain practically unchanged for 6 weeks' further hydrolysis. This shows that some tryptophane has been added to the histidine N of the 12-hour hydrolysis.

such is not the case for a 24- or 48-hour hydrolysis, where the error may be much greater. The ammonia nitrogen of a fibrin 24-hour hydrolysate probably contains nearly 20% of "deamination" nitrogen, while the ammonia nitrogen is increased practically 50% by a 72-hour hydrolysis.

Just which amino acids undergo deamination the more readily, it is impossible to determine without additional experiments. It is certain however that amino acids other than cystine are attacked.

Aside from the changes due to deamination the only other change of significance is in the humin figures, especially in those for insoluble humin. The change from 12 hours to 48 hours may be chemical although we are inclined to doubt it. The further changes are almost certainly due to physical causes, *i. e.*, carbonization. We have already shown[1] that insoluble humin is formed from tryptophane plus some other substance presumably an aldehyde, and we have shown that there is not sufficient of the second component in the fibrin molecule to combine with all of the tryptophane which is present. This being the case, it is difficult to see how an additional formation of *true humin* can result from a longer hydrolysis. In those hydrolysates which ran for 201 hours and for 6 weeks a part of the black "humin" adhered so tenaceously to the sides of the hydrolyzing flask that it could not be removed by washing and the insoluble humin N was therefore determined by Kjeldahling the insoluble humin in the original hydrolyzing flask. We are certain that a very considerable part, if not all, of the increase in this fraction after 12 hours is due to carbonization but at present there is no means of proving or of disproving this hypothesis.

Summary.

Fibrin was hydrolyzed with 20% hydrochloric acid for varying periods of time ranging from 1 hour to 6 weeks and various analyses made upon the resulting hydrolysates. These analyses lead to the following conclusions:

1. The figures for ammonia nitrogen in an acid hydrolysate are not necessarily a true measure of the amide nitrogen in the protein molecule but they also include some ammonia derived from the deamination of certain of the amino acids.

2. The extent of this deamination depends upon the length of hydrolysis.

3. The mono-amino acids are much more easily deaminized than are the histone bases.

4. Cystine is not the only amino acid which undergoes deamination when boiled with hydrochloric acid.

5. The figures for arginine, histidine and lysine in a Van Slyke analysis are not appreciably altered by a hydrolysis extending over six weeks, providing that all tryptophane has been so altered that it does not precip-

[1] *Loc. cit.*

itate on the addition of phosphotungstic acid. If it does precipitate it will be calculated as histidine.

6. Increases in the insoluble humin N due to prolonged hydrolysis we regard as due to carbonization.

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SOME NITROGENOUS AUXOAMYLASES.

BY ELBERT W. ROCKWOOD.

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For some time it has been known that starch-splitting enzymes were made more active by some component of serum. Thus Chittenden and Ely[1] found that the hydrolysis of glycogen and starch by saliva was increased by peptones. Chittenden and Cummins[2] found that the action of malt diastase was stimulated by peptones and albumins.

Langley and Eves[3] ascribed this to the binding of sodium carbonate by peptone and found that other proteins acted similarly, myosin, alkali and acid metaproteins, egg albumin and blood serum. Wohlgemuth[4] extracted from serum a heat-stable activating principle and thought it might be a lipoid. Pozerski[5] obtained similar results with salivary and pancreatic amylases by the action of intestinal juice and press-juice from macerated glands and from serum. He believed that salts of serum were the activating agents, though possibly proteins or their decomposition products.

In the following experiment the effect of various nitrogen compounds have been tested as regards their ability to increase the hydrolytic activity of the amylases of the saliva on boiled starch. Those which do increase the activity of the starch-splitting enzyme are here called auxoamylases.

The substrate was 10 g. of cornstarch, moistened, boiled 15 minutes and diluted to a liter. The saliva was diluted with water and filtered before adding to the starch solution. The saliva percentages refer to the volume of saliva in the diluted solution which was added to the starch.

The digestions were carried out in flasks suspended in a water bath heated to 38° by an electric heater. Only those digested at the same time and with the same saliva and starch are compared. Ordinarily, 200 cc. were used and in all cases 5 cc. of toluene were added. 25 cc. samples were removed at intervals by a pipet and, after the addition of an excess of Fehling's solution, were heated for an hour on a steam bath or boiled

[1] Chittenden and Ely, *Am. Chem. J.*, 4, 107 (1882).

[2] Chittenden and Cummins, *Trans. Conn. Acad.*, 7 (1885).

[3] Langley and Eves, *J. Physiol.*, 4, 18 (1883).

[4] Wohlgemuth, *Biochem. Z.*, 33, 303 (1911).

[5] Pozerski, *Thesis*, Inst. Pasteur, Paris, 1902; *Compt. rend. soc. biol.*, **55**, 429.