THE STAGES OF THE PEPTIC HYDROLYSIS OF EGG ALBUMIN.

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Numerous investigators have studied the stages in peptic digestion. The conclusions at which they have arrived may be grouped under two main heads.

On the one hand, Kühne (1) and Neumeister (2) concluded that the products of digestion arise by a serial degradation from the protein through acid albumin to proteose to peptone. On the other hand, Lawrow (3), Klug (4), Pick (5), Zunz (6), and Goldschmidt (7) found that the products of digestion appear more or less simultaneously.

The most comprehensive study of the stages of peptic digestion was made by Zunz. His general conclusion was that metaprotein, proteoses (primary and secondary), peptones, and simpler products, arise simultaneously and by inference directly from the protein molecule. The metaprotein and a large part of the primary proteose, he found to be converted into peptones. He also described large amounts of substances (61 per cent of the total nitrogen) simpler than peptones which appeared in the early hours of digestion and later decreased.

Klug concluded that the acid metaprotein formation in the early stages of digestion was due to the action of pepsin, while Maly and Boos (8) believed that it might be due to the action of acid alone.

A reexamination of the problem seemed desirable. The methods used were chiefly those described by Wasteneys and Borsook (9) for the fractional analysis of incomplete protein hydrolysates.

Metaprotein (acid albumin) was estimated by determination of the nitrogen content of the precipitate obtained on careful adjustment of the reaction to pH 6.0.

The proteose was precipitated by saturation of the solution with

sodium sulfate, at 33°C., and the peptone by precipitation with tannic acid under specially controlled conditions. The tannic acid filtrate was precipitated with alcohol and zinc sulfate. The nitrogen content of this precipitate was taken as subpeptone nitrogen, and the remainder as residual nitrogen, contained, it is assumed, in the simplest constituents of the hydrolysate.

No attempt was made to subdivide proteoses into primary and secondary classes except in one phase of the investigation which will be discussed later.

The results obtained are in accordance with those of Zunz and Pick that most of the products found in a peptic hydrolysate arise directly from the protein molecule. They differ in regard to the significance of acid metaprotein, and also in regard to the amount of, and variations in, the fraction simpler than peptone.

It was found that acid metaprotein is hydrolyzed more slowly than the native albumin from which it is derived; that the peptic hydrolysis of albumin can be effected at acidities less than pH 4.0, where no formation of metaprotein can be demonstrated; and that the hydrogen ion concentration for the minimum hydrolysis of undenatured albumin is distinctly different from that of acid metaprotein. For the former it is in the neighborhood of pH 6.0; for the latter at pH 4.0. From these results it was concluded that acid metaprotein formation, while a product of the action on protein of acid alone, as is well known, is not a necessary stage in the peptic hydrolysis of albumin, and its influence, if any, on hydrolysis, is to retard it.

Two-thirds of the initial amount of protein is hydrolyzed in 4 hours. The reaction then becomes progressively slower, so that in 12 hours 10 per cent, and in 7 days 6 per cent of the protein remains still unhydrolyzed. This rate of hydrolysis, however, holds only under the conditions of this experiment, but the rapid initial decomposition is characteristic of peptic hydrolysis under any conditions.

The results indicate that the peptic hydrolysis of albumin progresses in two stages. The first stage, occurring in the first 12 hours, consists of the hydrolysis of practically 100 per cent of the protein, with the formation of products of which 85 per cent may be spoken of as primary, *i.e.* undergo no further hydrolysis. The products at

this time consist of 55 per cent proteose, 17 per cent peptone, 12 per cent subpeptone, and 5 per cent residual nitrogen. A second stage, occurring later and progressing much more slowly, results in the hydrolysis of 15 per cent of the primary products into simpler fragments, which may be designated as secondary. The secondary hydrolysis occurs in both the proteose and subproteose fractions.

No methods are available for following subsequent changes in the subproteose fraction. In any case they affect the main picture of peptic hydrolysis very little, as only a very small fraction, about 6 per cent at most, of the total N is involved.

EXPERIMENTAL.

Metaprotein Formation and Its Relation to Hydrolysis.

Egg albumin (Merck) and pepsin (Merck) have been employed throughout. The metaprotein was prepared by allowing a solution of albumin at pH 1.6 to stand at room temperature for several days, thymol being employed as antiseptic.

The rate of formation of metaprotein is shown in Table I. After several weeks it attains a value of about 80 per cent of the total N, leaving approximately 15 per cent of the nitrogen still in the form of albumin soluble at neutrality.

A solution of metaprotein, on the other hand, prepared by solution at pH 1.6 of the precipitate obtained at pH 6.0 remains unchanged after standing for days at room temperature.

To compare the relative rates of hydrolysis by pepsin of metaprotein, and of albumin which has not been denatured by acid, two simultaneous hydrolyses were followed. The first hydrolysis was of an albumin solution which had been standing at room temperature at pH 1.6 for several days. The second was of an albumin solution of equal nitrogen content, to which the enzyme was added simultaneously with an amount of acid necessary to bring the pH to 1.6. This experiment was performed with 2.0, 1.5, 1.0, 0.5, and 0.25 per cent stock pepsin solutions, lasting 45 minutes. Considering the lowest enzyme concentration as 1 unit the others were, respectively, 10, 8, 6, 4, and 2 units. The results are recorded in Fig. 1.

It is clear from Fig. 1 that in mixtures of undenatured albumin and acid metaprotein, the velocity of peptic hydrolysis is greater where the concentration of acid metaprotein is less. Fig. 1 also shows the surprising result, that, at least in these experiments, strict proportionality between velocity of hydrolysis and concentration of pepsin obtains in those solutions where the conversion of undenatured protein to metaprotein has attained approximate equilibrium.

The slower rate of hydrolysis of acid metaprotein, whether the solution consists almost entirely of metaprotein, as in the previous experiment, or of a mixture of

metaprotein and undenatured albumin, is shown in an experiment the result of which is indicated in Fig. 2. In this experiment the relative rates of hydrolysis were compared in three solutions of identical nitrogen and enzyme content, one containing undenatured albumin, a second consisting of equal parts of undenatured albumin and acid metaprotein, and a third containing only metaprotein. As the curves show, peptic hydrolysis is most rapid in the undenatured albumin solution, and least rapid in the metaprotein solution, while the relative rate of hydrolysis of the mixture is intermediate.

Since acid metaprotein was hydrolyzed more slowly than undenatured albumin, it seemed probable that the formation of metaprotein indeed was not, as Maly, and Boos (8) maintained, a necessary stage in peptic hydrolysis. To test this, experiments were performed to ascertain the possibility of obtaining peptic hydrolysis of albumin at hydrogen ion concentrations where no metaprotein is formed.

TABLE I.

Rate of Formation of Acid Metaprotein from Egg Albumin at pH 1.6 and at 20°C.

Time	Metaprotein
	per cent total N
1 min.	10.6
5 "	17.0
20 "	18.9
30 "	21.3
1 hr.	26.5
2 "	33.3
6 "	39.0
1 day	54.6
3 "	56.9
5 "	68.6

It was necessary first to define the limit of acidity beyond which metaprotein formation does not occur. Portions of a neutral solution of 3.2 per cent albumin were adjusted to various acidities with hydrochloric acid. These were set away in a water bath at 37.7°C. for 45 minutes. They were then assayed for metaprotein by precipitation at pH 6.0. The results are given in Table II. They show that no metaprotein formation occurs at acidities less than pH 4.0.

As a result of this experiment a number of hydrolyses were carried out at acidities slightly less than pH 4.0 with several concentrations of pepsin. Typical results are given in Table III.

These experiments show that it is possible to effect considerable amounts of hydrolysis without the formation of any metaprotein. They also show that when hydrolysis is measured by following the

changes occurring in the protein substrate, it is possible to detect the progress of hydrolysis at acidities less than pH 4.0. Michaelis (10) found that peptic hydrolysis of albumin ceases at pH 4.0.

The reason for the difference between our findings and those of Michaelis lies in the probability that the latter worker was dealing

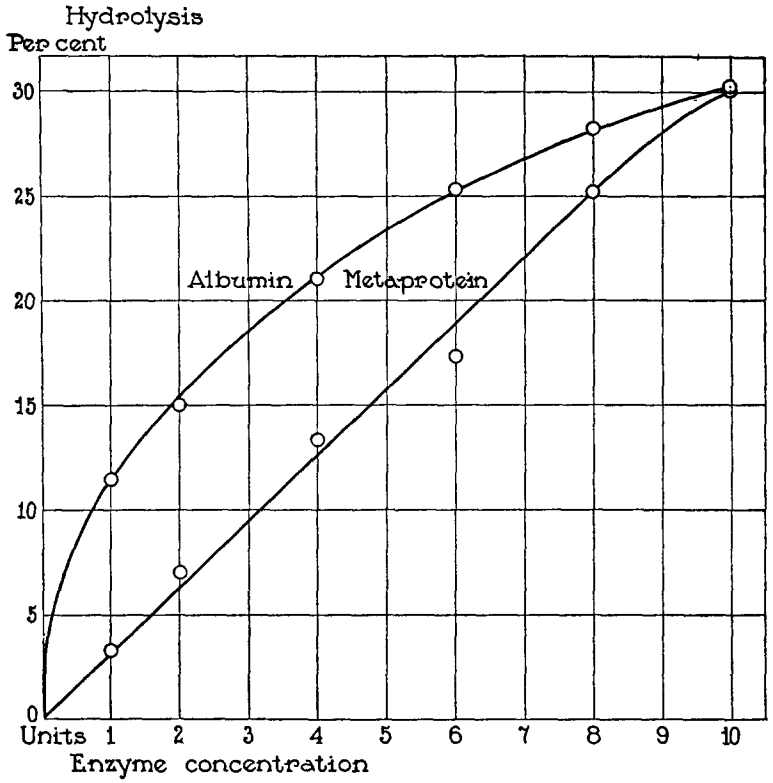

FIG. 1. Relative rates of hydrolysis of albumin and metaprotein.

with a solution consisting largely of metaprotein. The hydrogen ion concentration for the threshold of minimum hydrolysis of acid metaprotein is at pH 4.0; for egg albumin, which has not been denatured, it is in the neighborhood of pH 6.0. The optimum pH is approximately the same for both, near pH 1.6. This is shown in Fig. 3. In this experiment 3.2 per cent solutions of albumin and of acid

metaprotein were hydrolyzed for 1 hour at 37.7°C. with 0.2 per cent pepsin at the acidities indicated.

In the peptic hydrolysis of coagulated egg albumin observations were made, which, in contradistinction to the findings with uncoagulated albumin, do not conform to the view that acid metaprotein is not a necessary stage in the peptic hydrolysis of albumin. A 3.2

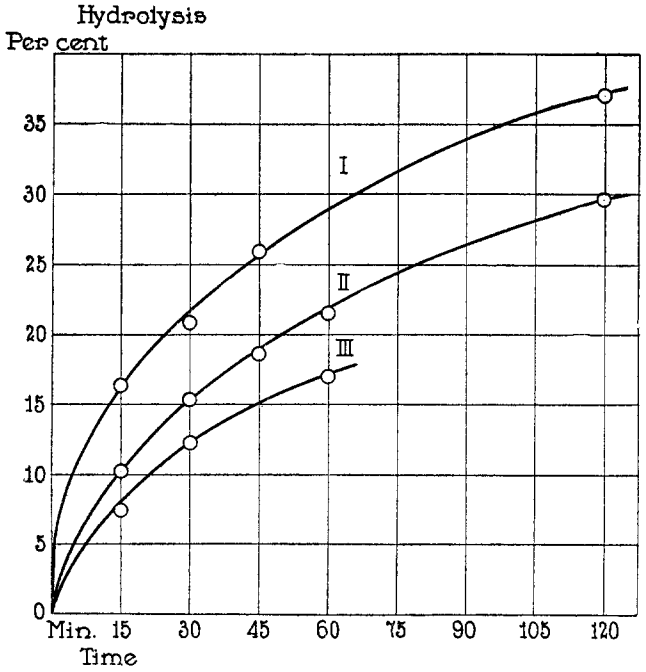

FIG. 2. Relative rates of hydrolysis of albumin and metaprotein. I, albumin; II, mixture of albumin and metaprotein; III, metaprotein.

per cent solution of albumin, acidified with dilute acetic acid, was coagulated by boiling. The coagulate was filtered and washed thoroughly with distilled water. It was then suspended in dilute HCl, and the reaction of the suspension adjusted to pH 1.6. Pepsin was added to a concentration of 0.2 per cent, and the suspension was incubated at 37.7°C. for 3 hours. At the end of this time all of the coagulum had disappeared. Part of the digest was neutralized;

another part was treated with trichloroacetic acid. In the neutralized solution a heavy precipitate appeared. The total nitrogen of the solution was 42 mg. The nitrogen in the filtrate from trichloroacetic acid precipitation was 30.6 mg. indicating that 11.4 mg. of protein nitrogen had not been hydrolyzed. The filtrate of the neu-

TABLE II.

Relation of Acid Metaprotein Formation to the C_{H^+}.

pH	Amount of metaprotein formed
	per cent total N
6.0	0
4.5	0
4.3	0
4.2	0
4.1	0
4.0	0
3.9	3.9
3.5	21.6

TABLE III.

Peptic Hydrolysis of Egg Albumin at Acidities Less than pH 4.0.

Enzyme concentration	pH 4.1		pH 4.2	
	Amount of hydrolysis	Metaprotein formed	Amount of hydrolysis	Metaprotein formed
units	*per cent*		*per cent*	
1	5.3	0	2.8	0
2	6.3	0	—	—
4	6.5	0	—	—
6	8.2	0	3.4	0
8	10.6	0	—	—
10	10.7	0	4.4	0

tralized solution contained 31.9 mg. of nitrogen. The precipitate thrown out on neutralization contained, therefore, 10.1 mg. The nitrogen precipitated by trichloroacetic acid was 11.4 mg., by neutralization 10.1 mg. In view of the difference of the two methods the correspondence is sufficiently close to indicate that the same amount of material was precipitated by neutralization and by tri-

chloroacetic acid. Evidently there is a material produced in the early stages of the hydrolysis of coagulated egg albumin which is soluble in dilute acid, insoluble at the neutral point, and precipitated by trichloroacetic acid. This agrees with the definition of metaprotein. We are led to the conclusion, for the time being, that though metaprotein is not a stage in the peptic hydrolysis of soluble unde-

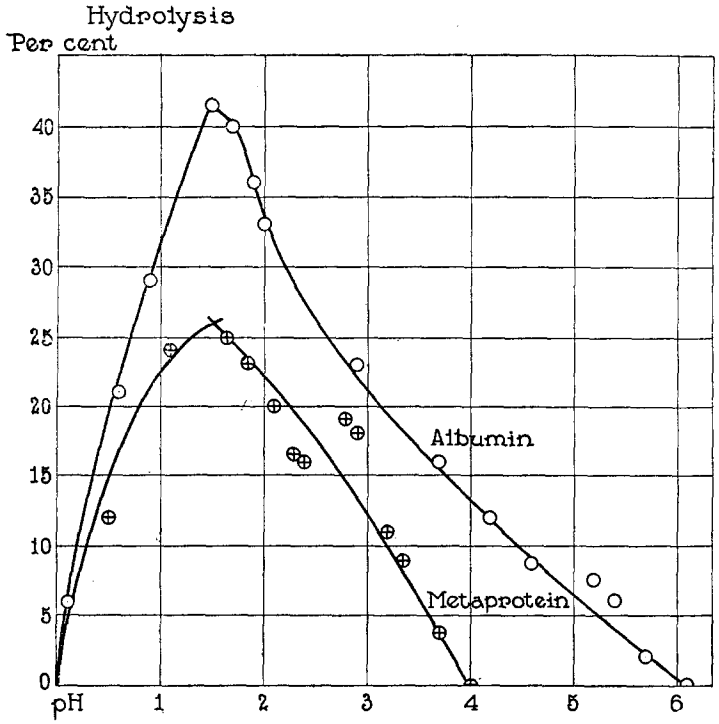

FIG. 3. Relative effect of C_{H^+} on the rates of hydrolysis of albumin and metaprotein.

natured albumin, it is probably the first stage in the hydrolysis of coagulated egg albumin.

The phenomena associated with heat coagulation of albumin have been fully discussed by Wu and Wu (11). This experiment, however, discloses an interesting anomaly. It is ordinarily supposed that in the coagulation of egg white by heat, albumin passes through the

stage of acid metaprotein. On suspending the coagulum in acid so that the reaction of the suspension is pH 1.6, contrary to the behaviour of uncoagulated albumin, no metaprotein is formed. Immediately on adding pepsin, however, metaprotein appears. Either the change from metaprotein to coagulated egg white is a reversible phenomenon with pepsin as a necessary adjuvant for reversion, or else we are dealing with different kinds of metaprotein.

The Stages in the Peptic Hydrolysis of Albumin.

The method of analysis was that described by Wasteneys and Borsook (9) for the fractional analysis of incomplete protein hydrolysates. Two types of experiments were carried out. One was a preliminary survey, in which the pH of the digest was allowed to rise with the progress of the hydrolysis. In the other, the pH was kept approximately constant at 1.6 throughout the course of the experiment by the occasional addition of acid. The findings in both types of hydrolyses were essentially the same, but the latter experiment was more thoroughly controlled, and the results there obtained are taken as the basis for the present discussion.

6 litres of a 5 per cent solution of egg albumin (Merck) were adjusted to pH 1.6 and sufficient solution of scale pepsin (Merck), also adjusted to pH 1.6, was added to make the concentration of enzyme 0.2 per cent.

The following control experiments were carried out. 1000 cc. of the albumin solution at pH 1.6 was precipitated with trichloroacetic acid and analyzed at the same time as the first sample from the digest. A simultaneous analysis was made of another 1000 cc. of the same albumin solution at pH 1.6 which had been in the incubator with the main digest for a week. These two controls determined the effect of the acid alone (pH 1.6) on egg albumin during the period of the experiment (168 hours). The protein N diminished from 96 per cent of the total nitrogen to 94 per cent. The proteose and residual nitrogen fractions remained unchanged. The peptone rose from 2 to 3 per cent and the subpeptone from 0 to 0.5 per cent. The proteolytic effect of the acid is therefore negligible. This is in accord with the conclusions of Frankel (12) who found no significant increase

in the free amino nitrogen of egg white after incubation for 96 hours with 0.2 per cent HCl at 38–40°C.

In the presence of pepsin, as Table IV shows, the hydrolysis of the protein was very rapid. In 4 hours more than two-thirds of the protein was hydrolyzed; in 12 hours 11 per cent of the original total amount of substrate remained. After this point the hydrolysis of the remaining protein proceeded slowly. For the complete hydrolysis of 3 per cent albumin at 37°C. with this enzyme concentration, between 2 and 3 weeks are required.

The proteose fraction attained a maximum value of 55 per cent. Between 12 and 28 hours, the amount of this fraction falls from 55

TABLE IV.

Fractional Analyses of a Peptic Digest of Albumin during the Course of Almost Complete Hydrolysis.

Duration of hydrolysis	Protein N	Proteose N	Peptone N	Subpeptone N	Residual N
	Per cent of original protein N				
3 min.	85	6	6	2	1
12 hrs.	11	55	17	12	5
28 "	5	50	29	15	2
48 "	5	46	29	18	5
72 "	4	45	30	19	5
96 "	5	45	31	17	5
168 "	6	40	27	23	7

per cent to 50 per cent; and in addition there is a further hydrolysis of 6 per cent protein. During this interval the peptone fraction gains 12 per cent, from 17 per cent to 29 per cent, while the subpeptone value increases only 3 per cent. The increase in residual nitrogen is negligible. The relatively greater gain in the peptone fraction is better shown by the curves, Fig. 4. At 12 hours, when the proteose curve has attained its peak, the slope of the subpeptone curve changes from a relatively steep ascent to a very slowly rising straight line. The peptone curve, on the other hand, continues to rise fairly quickly until 28 hours, when it flattens out. After 28 hours the proteose decreases from 50 per cent to 40 per cent; the peptone curve is practically flat, while the subpeptone rises from 15 to 23 per cent.

The peptone fraction rises rapidly to a value of 24 per cent in 28 hours. The increase between 12 and 28 hours is probably due, as discussed above, to the hydrolysis of proteose during this interval. After 28 hours the value of the peptone remains practically constant at approximately 24 per cent. This value probably represents an

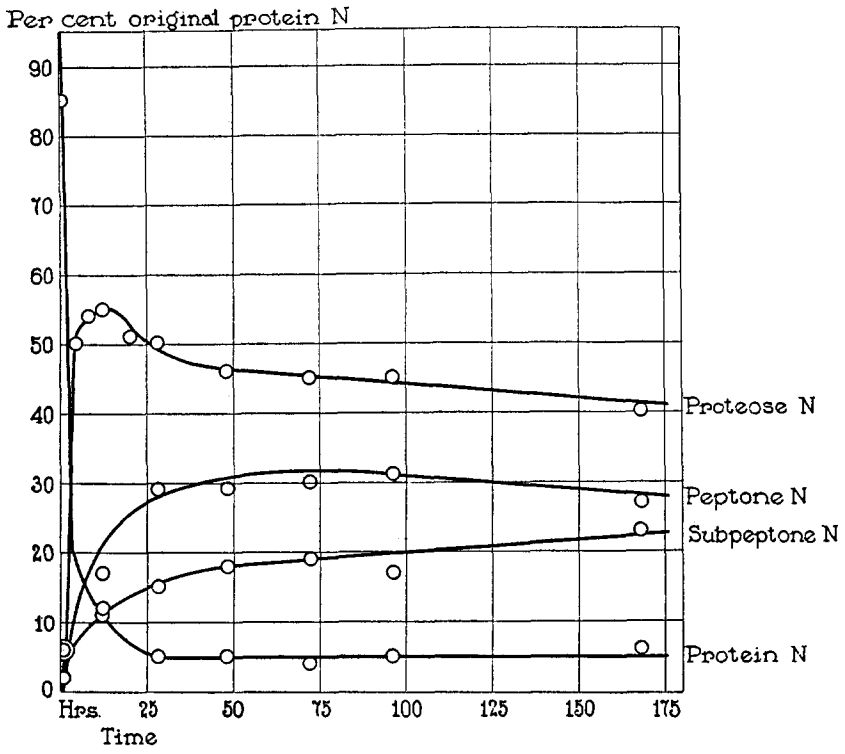

FIG. 4. Fractional composition of the hydrolysate during the course of almost complete peptic hydrolysis of egg albumin.

equilibrium between the amount gained from the hydrolysis of proteose and that lost by secondary hydrolysis of the peptone itself.

The subpeptone rises to a value of 10 per cent in 12 hours. The subsequent increase is slow and becomes significant only in the later periods of the hydrolysis, rising to 19 per cent in 168 hours. There is probably very little, if any, subpeptone hydrolyzed by pepsin. Hence

this fraction continues to increase steadily, gaining, by secondary hydrolysis, from the proteose and peptone.

The increase in residual nitrogen, though small, from 1 to 4 per cent, was obtained in a number of digests, and is larger than can be accounted for as experimental error. As Table IV shows, an increase in residual nitrogen is obtained very early in the hydrolysis.

These results suggest that the secondary hydrolysis is of a serial type, similar to that which the older writers considered to be the nature of the whole hydrolytic process.

In order to study the secondary hydrolysis more closely, a 3.2 per cent solution of egg albumin was digested with pepsin, and samples were removed after 3 minutes and 6, 12, 24, 48, and 72 hours. The protein and proteose were determined at these intervals, and at the same time 500 cc. samples were removed, and the proteose and subproteose fractions isolated from them. The method for the separation of these factions consisted in precipitation of protein with trichloroacetic acid, boiling of the filtrates to decompose trichloroacetic acid, neutralization, and dilution to volume. The solutions were then saturated with sodium sulfate at 33°C. The precipitate was taken as the proteose fraction and the filtrate as the subproteose fraction. The salt was removed from the latter fraction by adding alcohol to 50 per cent. The filtrate from the precipitated salt was evaporated down to drive off alcohol, and the residue was made up to volume.

Both proteose and subproteose fractions were then adjusted to pH 1.6 with 2.0 N HCl. Pepsin to a concentration of 0.2 per cent was added and the increase in formol titrable N was followed at intervals of 3 days and 7 days.

It is generally agreed in the literature that copper acetate is a specific precipitant for primary proteose. Both proteose and subproteose fractions were therefore treated with copper acetate according to the method of Folin (13), at intervals of 3 days and 7 days, simultaneously with the formol titrations.

Considering first the fraction precipitated by copper acetate, it was found to be present in both proteose and subproteose, which makes the specific nature of the copper acetate precipitation doubtful. However, the amount of N so precipitated in the proteose fraction

removed from the earlier stages of the primary hydrolyses was greater than in the fractions removed later, and to an extent that might account for the total falling off in proteose from the maximum attained during the primary hydrolysis. It is significant in this regard that there is no change in the N precipitated by copper acetate in any of the subproteose fractions.

From this it may be inferred that though copper acetate is not a specific precipitant of primary proteose, yet it appears to precipitate all of the proteose which undergoes secondary hydrolysis.

In the second digestion by pepsin the changes in free amino N, as shown by the formol titration, were relatively greatest in the earliest obtained proteose fractions. The later samples of proteose and all the subproteose fractions gave relative increases of approximately equal magnitude.

The conclusions are that secondary hydrolysis takes place in both fractions. It occurs to a greater extent in the earlier proteose fractions coinciding with the fall in the proteose curve. In relation to the main hydrolysis, however, these increases in free amino N, in both fractions, are small, and indicate the minor importance of the secondary hydrolysis in the peptic digestion of albumin.

SUMMARY.

1. Most of the products of the peptic hydrolysis of albumin, about 85 per cent of the total N, are primary in the sense that they arise directly from the protein molecule, and undergo no further hydrolysis.

2. A slow secondary hydrolysis, involving about 15 per cent of the total N, occurs in the proteose and simpler fractions primarily split off.

3. Acid metaprotein in peptic hydrolysis arises as a result of the action of acid. It is not an essential stage in the hydrolysis of undenatured albumin.

4. Acid metaprotein is hydrolyzed by pepsin more slowly under comparable conditions than undenatured albumin.

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