CCXVII. THE ENZYMIC SYNTHESIS OF PROTEINS WITH SPECIAL REFERENCE TO THE ACTION OF PEPSIN.

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(Received October 22nd, 1931.)

THE first enzymic synthesis of protein from substances of lower molecular weight was described by Danilewski, who observed [1886] that, when an extract of stomach was added to a concentrated solution of the products of a peptic digest of protein, a precipitate formed. Okunew [1895] obtained similar results. The phenomenon was further investigated by Sawjalow [1901] who gave the name plastein to the precipitate he obtained. Taylor [1907, 1909] digested protamine sulphate with a glycerol extract of the liver of the Pacific coast clam and, after concentration of the digestion products and further addition of the enzyme, obtained at the end of 5 months' incubation a substance which was identified as protamine sulphate.

Regarding the nature of plastein little was known by the earlier workers. Okunew [1895] stated that his digests contained 2 % more water after plastein formation had taken place. Sawjalow [1901] believed that plastein was a more complex substance than its precursors owing to its greater ease of precipitation by metallic salts. Henriques and Gjaldbak [1911, 1912] found that there was always a decrease in nitrogen titratable in presence of formaldehyde during plastein formation. They determined the ratio of free amino-nitrogen to total nitrogen of purified specimens of plasteins and found the figures to be comparable with those of the native proteins. Wasteneys and Borsook [1925, 2] do not however consider this ratio to be a fair criterion of the complexity of protein substances.

Morrell, Borsook and Wasteneys [1927] demonstrated that the rate of hydrolysis of a protein by pepsin, as measured by the amount hydrolysed in the first half hour, diminishes as the concentration of protein increases. Wasteneys and Borsook [1925, 2] confined their attention to the action of pepsin upon concentrated peptic digests of egg-albumin. They found that plastein formation did not occur if the concentration of the digests were less than 2 g. of nitrogen per 100 cc. These workers [1925, 4] were also the first accurately to determine the optimum p_H for plastein formation. They ob-

tained the greatest yields of plastein from peptic digests of egg-albumin at p_H 4.0 and found that the yield decreased very rapidly on either side of this value.

The present writers have further examined this problem and have extended the investigations to other proteins.

Analytical methods.

The peptic digests were prepared as follows.

To 1000 cc. of water, 25 g. of protein were added followed by sufficient hydrochloric acid to bring the p_H to 1.7. This mixture was incubated for about 6 days at 37° with 0.5 % pepsin. After incubation the whole was placed in a boiling water-bath for 30 minutes to inactivate the enzyme and when cool, filtered. The filtrate was concentrated *in vacuo* at 40° until it had a concentration of 3–4 g. nitrogen per 100 cc.

The amount of plastein formation was determined by the method of Wasteneys and Borsook [1925, 1]. 5 cc. of the digest were diluted to 25 cc. and nitrogen was determined in a 5 cc. sample. To another 5 cc. sample of the digest 15 cc. water were added, then 2.5 cc. of 20 % trichloroacetic acid and finally the whole was diluted to 25 cc. and mixed. The plastein was filtered off and the nitrogen content of 5 cc. of the filtrate was determined. From these two results the percentage plastein formation was calculated.

Influence of hydrogen ion concentration.

Specimens of concentrated peptic digests of egg-albumin, serum-albumin and caseinogen were divided into a series of 10 cc. portions and the p_H of each roughly adjusted by the addition of either dilute HCl or NaOH so that a range from about p_H 2.0 to 6.5 was obtained. The specimens were adjusted to the same volume by the addition of the requisite amount of water. 1 g. of pepsin and a few drops of toluene were added to each. They were stoppered and incubated at 37° for 3 days, being shaken at intervals to facilitate solution of the pepsin. The amount of plastein formation was then determined. The p_H of each specimen was determined electrometrically after the pepsin had dissolved.

The optimum p_H for plastein formation in all peptic digests of the three proteins examined was slightly over 4.0. This is in agreement with the results obtained by Wasteneys and Borsook [1925, 4] for peptic digests of egg-albumin.

The influence of temperature.

Sawjalow [1901] and Henriques and Gjaldbæk [1912] had observed from qualitative experiments that an increase in temperature led to an increase in plastein formation. Robertson [1909] and Wasteneys and Borsook [1925, 3] demonstrated this quantitatively. Plastein formation does not increase indefinitely with temperature, since pepsin is inactivated above 72°. Wasteneys

and Borsook [1925, 3], controlling the p_H and using concentrated peptic digests of egg-albumin, obtained their maximum yield of plastein at 72°, no plastein being formed at 80°.

This phenomenon has been re-investigated, using concentrated peptic digests of egg-albumin and serum-albumin. The digests were adjusted to p_H 4.0 and, before addition of the enzyme, were allowed to come to the temperature at which the reaction was to be studied. Plastein formation was determined after 24 hours' incubation. The results with both proteins were in entire agreement with those of Wasteneys and Borsook [1925, 3].

The effect of enzyme concentration.

The concentration of the enzyme has a marked influence upon the amount of plastein formation. The data obtained are shown in Table I for egg-albumin and serum-albumin.

Table I.

	Concentration of enzyme (g. per 100 cc.)	% plastein formation
Concentrated peptic digest of egg-albumin	0.1	7.9
Total nitrogen—3.94 g. per 100 cc.	0.5	13.2
p_H —4.0	1.0	17.8
Incubated 3 days at 37°	2.0	20.3
	5.0	24.5
	10.0	27.4
Concentrated peptic digest of serum-albumin	0.1	7.9
Total nitrogen—3.75 g. per 100 cc.	0.2	12.8
p_H —4.0	0.5	13.9
Incubated 3 days at 37°	1.0	18.7
	5.0	21.9
	10.0	24.8

Ratio of decrease of amino- and carboxyl-groups.

So far the only criterion of plastein formation which has been considered, is the property ascribed exclusively to proteins of insolubility in 2 % trichloroacetic acid. Other confirmatory evidence therefore seemed essential.

Henriques and Gjaldbak [1912] observed a decrease in nitrogen titratable in presence of formaldehyde during plastein formation. Oda [1926] contested the view that there was any decrease in the free amino-nitrogen. A decrease in the free amino-nitrogen alone cannot however be taken as evidence of the formation of a more complex molecule. It must be shown that there is no simultaneous formation of ammonia such as could be explained by a hydrolysis of amino-groups. Wasteneys and Borsook [1925, 5] found that if either peptic digests of egg-albumin, glycine solutions or solutions of glycine and phosphate were incubated at 37° with high concentrations of glucose, a marked decrease in free amino-nitrogen was observed when the mixtures were more alkaline than p_H 7.0, the effect increasing with increasing alkalinity. They were unable to account for this disappearance of free amino-nitrogen as ammonia, urea or cyanic acid. In the case of the peptic digests, this

diminution in the free amino-nitrogen did not involve any formation of more complex products of protein nature. It must be noted that none of these changes was obtained in the region of p_H 4, the optimum for plastein formation by pepsin.

Steudel, Ellinghaus and Gottschalk [1926] and Steudel and Ellinghaus [1927] state that carboxyl groups are liberated five times as fast as amino-groups during hydrolysis of a protein by pepsin. Felix and Harteneck [1927], however, using histone and Weber and Gesenius [1927] using caseinogen have found that during peptic hydrolysis of these proteins, amino- and carboxyl groups are liberated at equal rates. This was confirmed by Waldschmidt-Leitz and Künster [1927] for egg-albumin, caseinogen, gelatin and histone, and by Sørensen and Katschioni-Walther [1928] for caseinogen, gelatin and gliadin. Cannan and Muntwyler [1930] found that the acid- and alkali-binding powers of gelatin increased at equal rates during peptic digestion.

It is to be expected that during synthesis of a protein, a reversal of the above process will take place, namely, that the concentration of the amino- and carboxyl groups will decrease at the same rate. Rona and Chrometzka [1927] found that although there was a reduction in the free amino-nitrogen during plastein formation there were only indefinite variations in the concentrations of the carboxyl groups. They sought to explain the phenomenon of plastein formation as a deamination, caused by the liberation of amino-groups to form ammonia, which they determined in the digests by aeration with sodium hydroxide or baryta at 70° . Such drastic treatment would however presumably lead to ammonia formation so that the results obtained cannot be relied upon. Rona and Oelkers [1928] indeed re-investigated the problem and found that there was an equivalent decrease of amino- and carboxyl groups during plastein formation, indicating that the latter is actually a synthesis.

Experiments were made by the following procedure to elucidate this point.

The reaction of the digest to be examined was adjusted so that upon addition of pepsin to a concentration of 10 % the mixture had p_H 4. 0.5 g. of finely divided pepsin was weighed into each of two 25 cc. volumetric flasks, followed by 5 cc. of digest. The contents of one flask were diluted immediately to 25 cc. and well shaken until all was dissolved, and the amino-nitrogen, carboxyl groups and ammonia were determined; these values were taken as the initial values before plastein formation. A drop of toluene was added to the contents of the second flask, the flask stoppered and incubated at 37° . After 2 days the contents of this flask were diluted to 25 cc., well mixed and the amino-nitrogen, carboxyl groups and ammonia again determined. The nitrogen insoluble in 2 % trichloroacetic acid was determined upon a sample from the second flask.

Amino-nitrogen was determined by the method of Linderström-Lang [1928]. The error due to separation of peptones *etc.* in presence of acetone being overcome by carrying out a second titration in which sufficient 0.1 N HCl was added prior to the addition of acetone, so that only 1-2 drops of

0.1 *N* acid were necessary to complete the titration. Using this technique consistent results could always be obtained. A blank determination using 1 cc. of distilled water was always carried out, and this correction was allowed for. For carboxyl groups the method employed was that of Willstätter and Waldschmidt-Leitz [1921].

Ammonia was determined as follows: in an 8 × 1 inch pyrex test-tube 1–5 cc. of the solution to be examined were placed. The contents of the tube were diluted if necessary to about 5 cc. with distilled water. 2 cc. of saturated borax solution were added followed by a few drops of liquid paraffin and a glass bead to prevent bumping. A spiral of galvanised iron wire was placed in the tube. A delivery tube was fitted, the end of which dipped under 2 cc. of 0.05 *N* HCl, contained in a test-tube graduated at 25 cc. The ammonia was distilled over by boiling for 4 minutes over a micro-burner. Frothing was prevented by the use of the liquid paraffin and spiral of iron wire. After the distillation, the delivery tube was disconnected and washed into the receiving tube, the contents of which were diluted to about 20 cc. After cooling, the ammonia was determined colorimetrically by Nessler's reagent.

In distilling peptic digests with borax, ammonia from ammonium salts only is liberated, the borax being too weak an alkali to attack the amino-groups. This was proved experimentally with many amino-acids.

The results of these investigations are shown in Table II. The concentra-

Table II.

Total nitrogen (g. per 100 cc.)	Plastein forma- tion %	Amino- nitrogen (cc. <i>N</i> HCl %)	Carboxyl (cc. <i>N</i> KOH %)	Decrease in NH ₂ and COOH		Ratio of de- crease of COOH NH ₂	Concen- tration of ammonia (mg. N per 100 cc.)	Hours
				NH ₂ (cc. <i>N</i> HCl %)	COOH (cc. <i>N</i> KOH %)			
A. Egg-albumin digest. Incubation 2 days at 37°, <i>p</i> _H 4:								
5.139	—	81.0	115.5	—	—	—	36.3	0
5.157	20.7	74.5	109.5	6.5	6.0	0.93	37.1	48
4.419	—	73.0	107.5	—	—	—	48.3	0
4.421	17.2	65.0	98.7	8.0	8.8	1.1	48.7	48
4.160	—	72.0	80.5	—	—	—	25.4	0
4.089	10.5	61.5	69.0	10.5	11.5	1.09	27.1	48
5.198	—	94.3	128.5	—	—	—	30.3	0
5.211	19.7	80.9	115.5	13.4	13.0	0.97	32.6	48
B. Serum-albumin digest. Incubated 2 days at 37°, <i>p</i> _H 4:								
3.516	—	70.2	120.9	—	—	—	56.3	0
3.522	16.4	64.3	115.4	5.9	5.5	0.94	58.1	48
4.144	—	84.3	158.5	—	—	—	49.7	0
4.098	21.4	74.7	148.7	9.6	9.8	1.02	50.3	48
3.917	—	78.0	144.2	—	—	—	61.3	0
4.012	18.7	66.6	133.3	11.4	10.9	0.96	63.1	48
2.961	—	63.4	110.0	—	—	—	27.4	0
3.012	12.8	55.1	102.5	8.3	7.5	0.90	27.8	48
C. Caseinogen digest. Incubated 2 days 37°, at <i>p</i> _H 4:								
3.722	—	87.5	111.5	—	—	—	42.4	0
3.810	10.2	82.1	106.9	5.4	5.6	1.04	43.1	48
3.019	—	80.3	102.4	—	—	—	34.6	0
2.998	8.6	76.1	97.9	4.2	4.5	1.05	34.8	48
4.526	—	90.7	124.6	—	—	—	45.7	0
4.492	11.4	82.4	116.7	8.3	7.9	0.95	46.0	48
4.259	—	86.6	105.4	—	—	—	41.6	0
4.271	9.6	80.1	99.5	6.5	5.9	0.93	41.2	48

tions of the amino- and carboxyl groups are expressed in terms of cc. *N* acid or alkali respectively per 100 cc. digest. The two flasks containing digest and pepsin used for each experiment always contained slightly different amounts of nitrogen, but the amino-nitrogen, carboxyl and ammonia figures of the first flask have been corrected to correspond to the nitrogen figure of the second flask. The corrections, however, were very small, being almost negligible.

It will be seen from the results that in all cases plastein formation is accompanied by an equivalent decrease in the concentration of amino- and carboxyl groups. It appears therefore that plastein formation involves a peptide linking of these groups. These results further strengthen the view that plastein formation is essentially a synthesis. No appreciable formation of ammonia takes place during plastein formation. The amount of plastein formation as determined by the quantity of nitrogen insoluble in 2 % trichloroacetic acid does not appear, however, to run parallel with the decrease in concentration of the amino- and carboxyl groups. This may be due to the fact that in measuring the former, only one phase of the reaction is being determined. Wasteneys and Borsook [1925, 2] have shown that during plastein formation all the fractions of a protein hydrolysate appear to be involved, there being an increase of certain fractions such as proteoses and peptones from molecules of less complexity.

The effect of the degree of enzymic hydrolysis on the process of re-synthesis.

Sawjalow [1901] concluded that the nearer a peptic digest of protein approaches in complexity to the original protein, the more easily does plastein formation take place. Henriques and Gjaldbæk [1912] and Wasteneys and Borsook [1930] controlling the p_H reached similar conclusions. We have re-investigated this point, using serum-albumin.

A peptic digest of serum-albumin was prepared and at intervals of 5, 10, 20 and finally 30 days portions were removed, the active enzyme destroyed by heat, the unchanged protein filtered off and the filtrate concentrated *in vacuo*. The samples were concentrated to approximately the same degree. Pepsin was added to the concentrated digests to 10 % concentration and the mixtures brought to p_H 4. The amount of plastein formation was determined at intervals. The results were in agreement with the observations of the above workers.

The character of the original protein and its relationship to plastein formation.

The amount of plastein obtained under comparable conditions varies considerably according to the protein from which the digest has been prepared. While peptic digests of egg- and serum-albumins gave the highest yields, peptic digests of caseinogen were found to give much lower yields. Wasteneys and Borsook [1930] found that peptic digests of gliadin gave much smaller yields than peptic digests of egg-albumin. So far no investigator has obtained

plasteins from peptic digests of gelatin. The present writers have similarly failed. Wasteney and Borsook [1930] have suggested that this failure may be due to the use of acid in preparing gelatin from collagen since Henriques and Gjaldbak [1911, 1912] found that partial acid or alkaline hydrolysates of proteins yielded no plasteins. With this in view the present writers attempted to form plasteins from a concentrated peptic digest of collagen.

Tendons were first digested with trypsin to hydrolyse proteins other than collagen. The collagen was then washed with water to remove the disintegration products of the foreign proteins. The collagen was digested with pepsin at 37° and p_H 1.7 for several days. The procedure for preparing the concentrated peptic digest of collagen was essentially the same as described earlier in this paper. The concentrated digest of collagen was finally incubated 7 days at p_H 4 with 10 % pepsin, but no evidence of plastein formation was obtained. The results of this experiment do not appear to support the theory advanced by Wasteney and Borsook to account for their failure to produce plastein from concentrated digests of gelatin.

Properties and composition of the plasteins.

The plasteins prepared from the various peptic digests all gave positive protein biuret tests. They were soluble in strong acids and strong alkalis and were coagulated by boiling in alkaline solution, but not in acid solution. The plasteins were all digested rapidly by pepsin at p_H 1.7 and from their peptic digests, primary proteoses could be salted out by half saturation with ammonium sulphate. The sulphur, nitrogen and phosphorus contents of plasteins from various sources were determined and compared with those of the original native proteins. It will be seen from the data (Table III) that the contents

Table III.

Source from which plastein was prepared	Sample	Composition of plastein			Composition of protein from which digest was prepared		
		S %	N %	P %	S %	N %	P %
Egg-albumin	1	1.63	13.41	—	1.62	15.51	—
	2	1.70	13.82	—	—	—	—
	3	1.58	13.56	—	—	—	—
	Average	1.63	13.59	—	—	—	—
Serum-albumin	1	1.91	14.01	—	1.93	15.93	—
	2	1.83	13.89	—	—	—	—
	3	2.10	13.76	—	—	—	—
	Average	1.94	13.89	—	—	—	—
Caseinogen	1	0.81	14.45	0.11	0.78	15.6	0.81
	2	0.83	14.02	0.16	—	—	—
	3	0.76	14.76	0.21	—	—	—
	Average	0.80	14.41	0.16	—	—	—

of these elements in the plasteins and the original proteins are of the same order, with the exception of phosphorus which is almost absent from plasteins derived from caseinogen.

Sulphur was determined by Denis's modification of Benedict's method and phosphorus by the direct weighing of the ammonium phosphomolybdate formed by Neumann's reaction.

In Table IV are shown the cystine content and strength of the Molisch α -naphthol tests both in the plasteins and in the proteins from the digests of which they were derived. The method for the determination of cystine was a modification of the method of Folin and Marenzi [1929] and is described in a subsequent note by one of us [Tompsett, 1931].

Table IV.

	Cystine %	Molisch test
Egg-albumin	2.29	++
Plastein (egg-albumin digest)	3.07	++
Serum-albumin	2.34	++
Plastein (serum-albumin digest)	3.84	++
Caseinogen	0.38	faintly +
Plastein (caseinogen digest)	1.35	++
Pepsin	0.78	++

DISCUSSION.

It has been shown that when peptic digests of egg-albumin, serum-albumin and caseinogen are concentrated to about 3–4 g. nitrogen per 100 cc., upon addition of pepsin and incubation at 37°, an apparent synthesis of protein results, the maximum synthesis being obtained at p_H 4.

The amount of protein or plastein as it has been termed is also influenced by the temperature and the concentration of the enzyme.

Plastein is insoluble in 2 % trichloroacetic acid, gives a positive biuret test and its formation is accompanied by an equivalent decrease in the concentrations of amino- and carboxyl groups without any formation of ammonia. It appears then that plastein formation is actually a synthesis of protein from protein decomposition products. The highest yields were obtained from egg-albumin and serum-albumin digests, caseinogen digests giving much smaller yields, while gelatin digests produced no plastein at all.

The facts that Henriques and Gjaldbæk [1911, 1912] have been unable to obtain plasteins by the action of pepsin upon partial acid or alkaline digests of proteins and that these investigators and also Wasteneys and Borsook were unable to prepare plasteins by the action of trypsin upon tryptic digests of proteins are of great interest. It is curious too that the longer pepsin acts upon a protein during the hydrolytic stage, the lower is the yield of plastein from the subsequently concentrated digest. The possibility arises that pepsin can only unite those products of protein decomposition containing certain groupings in their structure. While little information was derived from the determination of the percentage of sulphur, nitrogen and phosphorus in the plasteins, the figures obtained being of the order of those of the native proteins from which the plasteins were derived, more information was derived from

the analyses of the plasteins for cystine and also by the strength of the Molisch test for carbohydrate. In each case the plasteins contained a higher percentage of cystine than the corresponding native protein. Serum- and egg-albumin both gave strong Molisch tests as did their corresponding plasteins, but the plasteins from caseinogen digests all exhibited stronger tests than the original caseinogen which gave only a faint reaction. Doubt has often been expressed as to whether the protein molecule really contains a carbohydrate or not. Recently, however, Rimington [1929, 1931] and Levene and Mori [1929] have isolated carbohydrate complexes from purified proteins which appear to consist of glucosamine and mannose units and are present to the extent of about 2 %. The question arises as to the importance of cystine and the carbohydrate group of the protein molecule in the formation of plastein. It is suggestive that serum- and egg-albumin contain fairly high percentages of cystine and give strong Molisch tests, while caseinogen contains a very low percentage of cystine and gives a faint Molisch test and gelatin gives no Molisch test and contains no cystine.

SUMMARY.

1. The optimum hydrogen ion concentration for the synthesis of plastein from concentrated digests of egg- and serum-albumin and also caseinogen, incubated at 37°, was p_H 4.
2. The amount of plastein formed from egg- and serum-albumin digests increased rapidly with temperature, reaching a maximum at 70°.
3. Increase in the concentration of enzyme increased plastein formation in concentrated peptic digests of egg- and serum-albumin.
4. The ratio of the decrease in the amino- and carboxyl groups during the process of plastein formation from egg- and serum-albumin and also caseinogen digests was 1 : 1.
5. There was no appreciable liberation of ammonia during formation of plastein.
6. The longer a protein was allowed to digest the less was the yield of plastein.
7. Peptic digests of egg- and serum-albumin gave better yields than those of caseinogen. Gelatin yielded no plastein from the products of digestion.
8. Certain properties of the plasteins are described.

We are much indebted to the Carnegie Trust for a grant in aid of this research.

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