N as determined by precipitation with 0.25N-trichloroacetic acid. In the second stage, the non-protein N rises slowly to the 100% value. The biological action disappears at about the end of the first stage. Diffusion experiments showed that the 'protein-like' material left here had a molecular weight of only 4000, whilst that of the soluble fraction was about 800. The initial action of chymotrypsin thus involves the cleavage of about 10 or 11 peptides containing on the average 5 or 6 residues from each submolecule, leaving a fairly massive core. The latter is broken down in the second stage of the action.

3. The amino-N content of the 'core' indicates a probable chain length of 10-12 residues. The greater part of the cystine (from 75 to 85%) is in the core.

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REFERENCES

- Behrens, O. K. & Bergmann, M. (1939). *J. biol. Chem.* **129**, 587.
 Chibnall, A. C. (1946). *J. int. Soc. Leath. Chem.* **30**, 1.
 Consden, R., Gordon, A. H., Martin, A. J. P. & Synge, R. L. M. (1946). *Biochem. J.* **40**, xliii.
 Gordon, A. H., Martin, A. J. P. & Synge, R. L. M. (1941). *Biochem. J.* **35**, 1369.
 Gordon, A. H., Martin, A. J. P. & Synge, R. L. M. (1943). *Biochem. J.* **37**, 92.
 Herriott, R. M. (1938). *J. gen. Physiol.* **21**, 501.
 Hill, D. W. & Howitt, F. O. (1936). *Insulin, its Production, Purification and Physiological action*. London: Hutchinson.
 Jensen, H. F. (1938). *Insulin, its Chemistry and Physiology*. New York: The Commonwealth Fund.
 Kassell, B. & Brand, E. (1938). *J. biol. Chem.* **125**, 115.
 Kunitz, M. (1935). *J. gen. Physiol.* **18**, 459.
 Kunitz, M. & Northrop, J. H. (1935). *J. gen. Physiol.* **18**, 433.
 Kunitz, M. & Northrop, J. H. (1936). *J. gen. Physiol.* **19**, 991.
 McDonald, M. R. & Kunitz, M. (1946). *J. gen. Physiol.* **29**, 155.
 Mehl, J. W. & Schmidt, C. L. A. (1937). *Univ. Calif. Publ. Physiol.* **8**, 165.
 Northrop, J. H. & Kunitz, M. (1932). *J. gen. Physiol.* **16**, 267, 295.
 Sanger, F. (1945). *Biochem. J.* **39**, 507.
 Synge, R. L. M. (1944). *Biochem. J.* **38**, 285.
 Synge, R. L. M. (1945). *Biochem. J.* **39**, 363.
 Van Slyke, D. D., Dillon, R. T., MacFadyen, D. A. & Hamilton, P. (1941). *J. biol. Chem.* **141**, 627, 671.
 Van Slyke, D. D. & Hawkins, J. A. (1928). *J. biol. Chem.* **79**, 739.

The Action of Pepsin on Insulin and the Plastein Question

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The course of the action of pepsin on insulin, and the products of the digestion, are quite different from those observed with chymotrypsin. On the whole, the breakdown produced by pepsin is less extensive than that observed with chymotrypsin. The most interesting feature of the action is the diminution, under certain conditions, of the apparent 'non-protein' nitrogen in the digests. This phenomenon appeared to be similar to the process of plastein formation, which has been known for a considerable time. Wasteneys & Borsook (1924, 1925)* reported that when a peptic digest of egg albumin is concentrated, and then incubated with more pepsin at pH 4 (at which pH the proteolytic action of pepsin

* Wasteneys & Borsook give references to earlier observations of this kind.

is slight), the 'non-protein' nitrogen, determined as the nitrogen soluble in the presence of 2% trichloroacetic acid, is diminished. They regarded this as evidence of the synthesis of protein-like material, 'plastein', by the action of pepsin at pH 4, citing as contributory evidence the fact that the plastein, when filtered off, contained an appreciably smaller proportion of amino N than the original digest. They studied the effect of such variables as pH, temperature and concentration, and reported that when boiled pepsin was added instead of the active enzyme, no synthesis was observed. The matter was re-examined by Folley (1932), who concluded that there was no proof of resynthesis and found that plastein formed from egg albumin had a comparatively low molecular weight.

Similar observations were made with a peptic digest of insulin by Fisher & Scott (1933), who used the decrease of material soluble in 3.3% trichloroacetic acid as the criterion of plastein formation during incubation at pH 4. Haddock & Thomas (1942) found that trypsin had a similar action at pH 5.7 on the peptic digest of insulin, as had papain on both peptic and papain digests. The circumstances under which insoluble material is formed in insulin digests have been investigated in some detail in this paper.

EXPERIMENTAL

The pepsin was a crystalline product presented by Parke, Davis and Co. It was kept at -10° as a 2% stock solution in 60% glycerol adjusted to pH 5 with sodium acetate and acetic acid; dilutions were prepared as required. The activity as determined by the method of Anson (1938) was 0.54 unit/ml.

Non-protein nitrogen (N.P.N.). At first the digest was added to boiling trichloroacetic acid (0.3N) in order to inactivate the pepsin, but this was unsatisfactory owing to the uncertain amount of evaporation, and it was found that addition of trichloroacetic acid at the same strength at room temperature gave nearly the same results (Table 1).

The trichloroacetic acid mixture was not allowed to stand too long before filtration, as a slow precipitation of insoluble material might occur. Certain of the pepsin digests which gave no immediate precipitate with trichloroacetic acid gave one after standing for 1–2 hr. The time of standing was standardized at 30 min., which in certain cases of complete digestion gives too low a value for N.P.N.

Table 1. *The determination of non-protein N (N.P.N.) in an insulin digest by trichloroacetic acid (T.C.A.) at room temperature and at the boiling point respectively*

(Insulin (60 mg.) in 12 ml. of 0.05N-HCl; 1 ml. of 1/100 dilution of stock pepsin.)

Time after adding pepsin (hr.)	N.P.N. (mg./ml.)	
	T.C.A. at room temp.	T.C.A. (boiling)
1	0.21	0.21
2.5	0.31	0.29
5	0.62	0.53

Peptic digests. The solution used usually contained 5 mg. of insulin dissolved in 1 ml. of 0.05N-HCl, to which was added the desired amount of pepsin, usually at a dilution of 1:100 of the stock solution.

RESULTS

Extent of the action. The N.P.N. rises smoothly and quickly to values close to 100%, while the amino N reaches and remains at a constant value (with small pepsin concentrations) of about 12–13% (Fig. 1). This may be followed by a very slow action of pepsin on the primary split-products, the amino N reaching 13–14% in 24 hr.; with concentrations of pepsin 5

or 10 times as great the amino N reached 16% in 2 days. The 13% stage is, however, fairly definite and easily reproduced. During this initial action there is a linear relation between the N.P.N. and amino N (Fig. 2), which indicates that the amount of N.P.N. liberated/peptide bond split is the same throughout the action of the enzyme. Thus the hydrolysis of the peptides initially formed is relatively slow.

Fig. 1. Action of pepsin on insulin at pH 2. Change of amino N and N.P.N. with time. Pepsin units/ml. of digest, 3×10^{-4} .

Fig. 2. Action of pepsin on insulin. Relation between amino N and N.P.N.

By the same method of calculation as used in the previous paper (p. 118), it was found that 13% of amino N in the digest corresponds to 17 amino groups, so that the digestion has increased the number of terminal amino groups from 4 to 15. The average peptide chain in the digest has a molecular weight of $12,000/15 = 800$ and thus contains about 7 amino-acid residues.

This gives, of course, no information about the uniformity of the products. The amount of uncombined α -amino-acids in the digest as determined by the Van Slyke ninhydrin method was 0.7% of the

total N. It follows from this that in the digestion product from one unit of 106 residues (mol.wt. 12,000), less than one is present as uncombined amino-acid, i.e. of the ten peptide bonds broken not more than one belongs to a terminal amino-acid. Indeed, the small amount found may be the result of a secondary reaction.

Diffusion coefficient. A peptic digest was prepared by mixing 200 mg. insulin in 40 ml. of 0.05 N-HCl with 3 ml. of a 1:200 dilution of the stock pepsin. After standing overnight at 25° it gave no immediate precipitate with trichloroacetic acid. To destroy the pepsin, the digest was now neutralized to pH 7.2 and heated to 80° for 3 min. The precipitate thus formed was filtered off and when dried amounted to 42 mg., i.e. to about 20% of the original insulin. The nature of this 'heat precipitate' is discussed below. Diffusion coefficients were determined on the filtrate (Table 2).

Table 2. *Diffusion coefficient of digest filtrate*

	Temp. (°)	Diffusion time (hr.)	Conc. in 20 ml. diffusate (mg. N/ml.)	$D \times 10^6$	$D_{20} \times 10^6$
Series 1	25	23.5	0.0075	2.21	1.92
Final conc. inside cell, 0.453 mg. N/ml.					
Series 2	10	49	0.009	1.52	2.05
	5	47	0.006	1.07	1.71
	6	48	0.007	1.32	2.02
Final conc. inside cell, 0.359 mg. N/ml.					
Average $D_{20} \text{ sec.}^{-1} = 1.92 \times 10^{-6}$.					

The average molecular weight of a peptide corresponding to the diffusion coefficient (Table 2) is 3000 ± 1000 , which is significantly higher than that of the average peptide chain in the digest. It follows that the diffusing molecule must contain on the average about 4 peptide chains. These might be held together in various ways, but the disulphide bonds, of which there are only 1.5 in a peptide of mol.wt. 3000, are insufficient by themselves for this purpose.

The plastein question

In the course of our experiments we noticed on one occasion that the N.P.N., which had been nearly 100% after digestion for 5 hr., apparently diminished to approximately 50% after 24 hr. without any diminution of the amino N. This was found to be due to the fact that in the latter determination the trichloroacetic mixture had stood for an abnormally long time before being filtered. The following experiment shows the nature of this action. A series of tubes was made up containing 1 ml. of a fresh peptic digest and 5 ml. of 0.3 N-trichloroacetic acid and the mixtures were allowed to stand at room temperature. Precipitate was first observed after 1 hr. At various intervals the precipitates in the separate tubes were filtered off and the N.P.N. was determined; the results are given in Table 3.

Table 3. *Decrease of non-protein N (N.P.N.) in a peptic digest of insulin after various periods*

Time (hr.)	N.P.N. (mg./ml.)	N.P.N. (% of total N)
1.5	0.57	88.0
3	0.36	55.4
7	0.34	52.5
24	0.29	44.7

The precipitation of some material by trichloroacetic acid can hardly be due to pepsin, since the latter is inactive in such a solution. However, it appeared possible that it was caused by a hydrolytic action of the acid.

It will be recalled (p. 124) that, when a peptic digest is neutralized and heated to 80° for a few minutes, a precipitate is formed which may amount to 20% or more of the original insulin and continues to form in the filtrate at a slower rate at lower temperatures. Indeed, when the filtrate was frozen and stored at -10°, it was usually found that some insoluble matter was present on thawing. It therefore appeared that the peptic digest undergoes some change on being kept at certain acidities, which results in the formation of material which is both insoluble by itself

Fig. 3. Changes in peptic digest at 25° with pH.

and in the presence of trichloroacetic acid. In order to find under what circumstances it is formed, a peptic digest was made which gave no immediate precipitate with trichloroacetic acid. The pH of measured samples of this digest was adjusted to different values. After standing for various times at 25°, samples were added to 0.3 N-trichloroacetic acid, the precipitate formed after 30 min. was filtered off, and the N in the filtrate determined (Fig. 3). Except in the alkaline solution a considerable drop in N.P.N. occurred. The percentage of amino N in the whole mixture was determined by the Van Slyke method (Table 4). There is a slight

Table 4. *Change of amino N and non-protein N with time in peptic digest*

	Original	After standing for 24 hr. at pH			
		1	4.2	7.0	c. 10
Amino N (% of total N)	13.0	13.0	12.7	13.0	13.7
N.P.N. (% of total N)	100	80.8	54.3	60	100

diminution of the amino N in the three solutions in which N.P.N. has diminished, amounting at most to about 1%. This would correspond to the resynthesis of one peptide bond out of the eleven broken; but it is doubtful whether the determinations are sufficiently accurate, since they are made with a mixture of solid and liquid. It is quite possible that under these circumstances small variations would occur with constant reaction time according to the amount of solid. At any rate, the figures do not support the suggestion that any considerable synthesis of peptide bonds, corresponding to the amount of solid formed, has occurred.

In order to find if the changes observed are due to the pepsin still remaining in the solutions, a further series of experiments was made in which the peptic digest was heated to 60° for 5 min. at pH 7.8 before adjusting the pH to its final value. It was shown that the activity of the pepsin was completely destroyed by this treatment. The pH was then adjusted by the addition of HCl to 2.0, 4.0 and 7.0, and after standing for various times at 25°, samples were taken for analysis. The results are shown in Fig. 4.

Fig. 4. Changes in peptic digest at 25° with pH after inactivation of the pepsin.

Fig. 5. Effect of age of peptic digest on amount of precipitate formed on heating.

At pH 4.0 and 7.0 the N.P.N. diminishes steadily, reaching about 50% after 2 days, while there is very little change with time when the pH has been restored to its original value. This experiment shows that the presence of active pepsin has nothing to do with the drop of the N.P.N. observed in our experiments.

In the course of this work it was noticed that the quantity of precipitate obtained on heating appeared to be less

with an old digest than with a fresh one. To test this a peptic digest was prepared in the usual way. After 18 hr. a portion was neutralized to pH 7 and heated for various times. The precipitates formed were removed by filtration and the amounts of N remaining in the filtrates were determined. The same process was repeated with digests 42 and 66 hr. old (Fig. 5). It can be seen that the amount of precipitate obtained diminishes with the age of the digest.

In this experiment the amount of N precipitated by trichloroacetic acid was also determined (Table 5). In nearly every case the amount agrees with or is only slightly less than the amount precipitated by heating, thus confirming the suggestion that the material precipitated by heating is identical with that precipitated by trichloroacetic acid.

Table 5. Insoluble matter formed on heating digest

Age of digest (hr.)	Time of heating (min.)	Percentage of N not precipitated by heat	Percentage of N not precipitated by T.C.A.
18	10	69	69
	20	64	61
	40	59	56
42	10	84	79
	40	71	75
	70	71	65
66	10	85	87
	70	77	74

Properties of the precipitate and filtrate. The precipitate used was obtained from 500 mg. insulin at the usual concentration, digested with 2 ml. of a 1:50 dilution of the stock solution of pepsin for 17 hr. The digest was neutralized and heated to 75° for 10 min. The dried precipitate (6A) amounted to 88 mg. After being frozen for 1 week a further 83 mg. of precipitate (6B) was removed. The total precipitate, allowing for filtrate used in tests, was about 40% of the insulin taken.

The precipitate 6A dissolved slowly (1 mg./ml.) in 0.05N-HCl. Its total N amounted to 15.4% of the dry weight. The precipitate is complex. The following amino-acids have been identified in an acid hydrolysate in a preliminary analysis by paper chromatography: aspartic acid, cystine, tyrosine, glutamic acid, glycine, alanine, leucine. There are almost certainly other amino-acids present, as several spots were not identified. The amino N as a percentage of total N in the precipitate 6A, as determined by Van Slyke's method, was 8.2%; in 6B, 8.7%, and in the filtrate from which both 6A and 6B had been removed, 16.2%.

It can be seen that the process of heating divides the digest into two parts containing respectively a smaller and greater proportion of amino N than the original digest. It is impossible to determine the average chain lengths in the fractions without knowing the distribution of the lysine, etc., but on the assumption that the average composition of each is similar to insulin, the precipitates have a peptide chain length of about 10-12 residues, while the filtrate has an average chain length of about 6. The molecular weight of precipitate 6A was determined in the diffusion cell in the usual way (Table 6).

Table 6. *Diffusion coefficient of heat precipitate*

Temp. (°)	Diffusion time (hr.)	Conc. in 20 ml. diffusate (mg. N/ml.)	$D \times 10^6$
25	41	0.0025	1.35
25	72	0.005	1.58
25	72	0.004	1.32

Original conc. in cell, 0.144 mg. N/ml.

Mean: $D_{25} \text{ sec.}^{-1} = 1.42$, $D_{20} = 1.24 \pm 0.2 \times 10^{-6}$; mol.wt. = $11,000 \pm 4000$.

A certain amount of the high molecular weight precipitate had been removed from the digest used in the previous molecular-weight determination (Table 2). In view of this, it was desirable to determine the molecular weight in a filtrate from which as much as possible of the heat precipitate had been removed. This was done with filtrate 6.

Table 7. *Diffusion coefficient of filtrate*

Temp. (°)	Diffusion time (hr.)	Conc. in diffusate (20 ml.)	$D \times 10^6$
25	48	0.011	2.50
25	48	0.0108	2.55

Final conc. in cell, 0.273 mg. N/ml.

Mean: $D_{25} \text{ sec.}^{-1} = 2.52$, $D_{20} = 2.18 \times 10^{-6}$; mol.wt. = 2000 ± 800 .

It was heated at 80° for 30 min. before use and filtered, but very little additional precipitate formed. The filtrate was introduced into the diffusion cell, and the molecular weight was found to be somewhat lower than the previously determined value, but still clearly higher than the average peptide chain length in the filtrate.

Table 8. *Summary of Results*

	% of total N as		Chain length	Mol. wt. (order of)
	Amino N	Cystine N		
Precipitate (A)	8.2	12.7	10	11,000
(40%) (B)	8.7	13.2		(14,000)
Filtrate (60%)	16.2	7.9	5	2,000 (1800)

DISCUSSION

The idea that a resynthesis of protein-like material takes place in the presence of pepsin under certain conditions was based on the observations that: (1) the proportion of nitrogen not precipitated by trichloroacetic acid (N.P.N.) is decreased, (2) the proportion of amino N in the precipitate is lower than that in the digest, and (3) that the phenomenon was not observed when boiled pepsin was added to the concentrated digests. In our experiments the formation of a precipitate from soluble pepsin digests occurred under circumstances in which the pepsin had been definitely inactivated. The pH and temperature are the main factors conditioning its formation. It is not produced at or about pH 2, but it is formed both in more acid and more alkaline

solutions. The insoluble material appears to be identical with that precipitated by trichloroacetic acid. The fact that the proportion of amino N in the precipitate is less than in the digest as a whole cannot be cited as evidence of any resynthesis of peptide bonds, but merely means that the average chain length of the peptides in the precipitate is greater than that in the whole digest.

The general picture of the peptic digestion which we obtain is the breakage of about 11 peptide bonds in each submolecule of weight 12,000, giving a product which is initially soluble in the solvent in which it is formed and in the presence of trichloroacetic acid. Later, insoluble material is precipitated from this digest over a considerable range of pH and also in the presence of trichloroacetic acid. What is the nature of the reaction involved? The process is accelerated by heating, but occurs slowly even in the frozen state. It might be a hydrolytic reaction giving rise to an insoluble peptide, but there is no evidence that peptide bonds are split and moreover the effect of acidity does not support this explanation. We have also shown that there is probably no significant decrease of amino N during the formation of the precipitate, so that an appreciable resynthesis of peptide bonds is also ruled out.

The phenomenon seems to resemble that of protein denaturation. When insulin solutions are heated, insoluble denatured protein is formed by changes which do not involve the disruption of peptide bonds, but apparently merely a 'disordering' of the peptide chains which brings more hydrophobic material in contact with water and thus decreases solubility. It may be supposed that some of the larger protein fragments produced by pepsin may retain the structural configuration of the original insulin and the particular arrangement of the component groups, presumably with hydrophilic groups pointing outwards and hydrophobic groups inwards. Heating and addition of trichloroacetic acid might then produce a rearrangement or 'disordering' of these larger fragments, similar to that caused by heat on insulin itself, but occurring at a lower temperature. When the digest is exposed to pepsin for a longer time, the amount of insoluble substance which can be formed diminishes. This might be due to the original fragments being further split to smaller molecules which are no longer capable of this change. Table 8 summarizes the characteristics of the material in the precipitate and filtrate, so far as they have been determined. The molecular weight of the precipitated material is of the same order as that of the submolecule of insulin. It must therefore either be a more or less intact submolecule, or must be made up of portions of more than one submolecule. In the former case the percentage of amino N would be the same as insulin. The simplest interpretation of all the facts is that

pepsin, like chymotrypsin, splits off terminal groups. The length of the peptide chains is not much greater than in the case of chymotrypsin, but they are aggregated in some way to give a diffusing molecule containing about three average peptide chains.

The residue of the insulin molecule is soluble when first formed, but undergoes changes on heating, etc., over a wide range of pH, except near pH 2. The residual molecule probably comes from more than one submolecule and may be derived from one half or more of the whole insulin molecule. The cystine is approximately equally divided between precipitated material and the soluble peptides. This possibly means that pepsin eats more deeply into the insulin 'core' than chymotrypsin in the initial stage of its action. Finally, we may observe that the plastein from egg albumin was shown by Folley (1932) to have a comparatively low molecular weight (<1000). It is likely that the mode of formation of this material, which requires more pepsin at pH 4, differs from that of the material from insulin, and the two phenomena are really dissimilar.

SUMMARY

1. The action of pepsin on insulin at pH 2 has been studied. Insulin is quickly and completely transformed into products which are not initially precipitated by trichloroacetic acid.

2. On standing at various pH values the digest undergoes changes whereby material is formed which is insoluble both in the presence of trichloroacetic acid and in the chosen buffer. This precipitate is similar to the so-called 'plastein', the formation of which has been observed in peptic and other digests.

3. The conditions of formation of the precipitate have been studied. No evidence has been obtained that it requires active pepsin for its formation, or that any appreciable resynthesis of peptide bonds occurs. It is suggested that the larger protein fragments originally formed retain much of the order of the protein, and may suffer changes similar to those which occur in protein denaturation.

4. The molecular weight of the precipitated material is of the order of 12,000 (chain length 10-12 residues), while that of the soluble split-products is c. 2000 (chain length about 5 residues). In both cases the diffusing molecule must be an aggregate containing several peptide chains.

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REFERENCES

- Anson, M. L. (1938). *J. gen. Physiol.* **22**, 79.
 Fisher, A. M. & Scott, D. M. (1933). *J. gen. Physiol.* **16**, 741.
 Folley, S. J. (1932). *Biochem. J.* **26**, 99.
 Haddock, J. N. & Thomas, L. E. (1942). *J. biol. Chem.* **144**, 691.
 Wasteneys, H. & Borsook, H. (1924). *J. biol. Chem.* **62**, 15, 633.
 Wasteneys, H. & Borsook, H. (1925). *J. biol. Chem.* **63**, 563, 575.

The Biochemistry of the Gas Gangrene Toxins

3. DEVELOPMENT OF A MEDIUM SUITABLE FOR THE LARGE SCALE PRODUCTION OF THE TOXINS OF *CLOSTRIDIUM WELCHII* TYPE A

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In a previous paper (van Heyningen, 1941b) it was reported that, in order to obtain the toxins of *Clostridium welchii* type A, the organism was grown on a solution of commercial peptone supplemented with a protein-free extract of fresh muscle (Macfarlane & Knight, 1941). This medium has a low protein content which is desirable when the toxins are to be

purified. Later it was necessary to produce the toxins on a scale of several thousand litres of culture filtrate. It was then found that the commercial peptone did not regularly produce good yields of toxin and accordingly another medium was developed. This medium consists essentially of papain digest of horse muscle pre-treated with alkali. In this work the main interest was in the production of the α -toxin (lecithinase).

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