SUMMARY

1. The persistence of suramin and eleven related compounds in the blood of rabbits has been investigated following their intravenous administration under standard conditions.

2. Marked persistence is a property of polyamides of high molecular weight that contain naphthylaminepolysulphonic acids as end groups.

3. Carboxylic acids of similar molecular complexity are more rapidly eliminated.

4. There is no relation between persistence and therapeutic activity.

5. Suramin is probably not hydrolyzed *in vivo*.

6. Methods of determining each of the twelve compounds in plasma are described.

The author wishes to thank Miss E. France and Miss R. B. Horrocks for valuable technical assistance.

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The Action of Chymotrypsin and Trypsin on Insulin

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Of the various chemical methods used in attempts to elucidate the structure of proteins, the breakdown by mild hydrolysis seems most promising. The constitution of the resulting fragments which can be isolated may then be ascertained separately. This method has been used to some extent by Gordon, Martin & Synge (1941, 1943) who identified a considerable number of small peptide fragments after partial hydrolysis with hydrochloric acid. Whilst the method undoubtedly throws some light on the order of amino-acids in the protein, a complete structural analysis would be a formidable, if not impossible, task, although it is capable of elucidating the structure of the pentapeptide gramicidin S (Synge, 1944, 1945; Consden, Gordon, Martin & Synge, 1946) and is no doubt capable of considerable extension. A more promising method in the case of a protein is hydrolysis by proteolytic enzymes into larger peptides which can then be further examined.

The essential conditions to be satisfied are: (1) The amount of enzyme added shall not be sufficient to contaminate the digest appreciably. This implies that both enzyme and its substrate must not be contaminated with foreign proteins. (2) No rearrangement of the amino-acids must be brought about by the action of the enzyme. Although synthetic actions of papain have been reported by Behrens & Bergmann (1939), there is at present no evidence that proteolysis brought about by animal proteinases such as pepsin and trypsin is a reversible process. We believe that the isolation of a peptide would provide powerful evidence of its existence as a structural component of the protein.

The work reported here is a preliminary examination of the action of the two trypsins on insulin. Numerous studies of the action of preparations of these enzymes on insulin have been made (for bibliographies see Jensen, 1938; Hill & Howitt, 1936), but much of this work is difficult to interpret, owing to the lack of adequate characterization of the enzyme preparations employed. Moreover, when considerable quantities of impure enzyme have been added, as has been done in some of these experiments, it is impossible to tell whether the split-products are derived from insulin or from foreign proteins accompanying the enzyme.

EXPERIMENTAL

Materials. Trypsin and chymotrypsin were prepared from frozen beef pancreas by the methods of Northrop & Kunitz (1932) and Kunitz & Northrop (1935, 1936), the chief modification being that trypsinogen was converted to trypsin and crystallized by the more recent method of McDonald & Kunitz (1946). The trypsin was only once crystallized, the quantity available being rather small. The chymotrypsin was stored in the form of chymotrypsinogen (eight times recrystallized). When required it was activated by the addition of a very small quantity of trypsin ($<0{\cdot}5\%$) and adjusting the pH to 7·6.

Insulin was a crystalline product prepared by Boots Pure Drug Co. Ltd. A portion was recrystallized three times by dissolving 1 g. in water (170 ml.) containing glacial acetic acid (3 ml.). A solution containing 1 mg. of Zn^{++} in the form of its acetate was added, and the pH was adjusted to 6·6 with ammonia. After dilution to 340 ml., the pH was adjusted to 5·9 with glacial acetic acid, and crystallization took place in less than 1 hr. No significant differences were found between the original and the recrystallized insulin, except that the latter contained a rather higher percentage of non-protein N; the material was not dried by washing with ethanol and ether and therefore contained some mother-liquor.

METHODS

Non-protein N (N.P.N.). This was determined by adding 1 ml. of digest to 0·3 N-trichloroacetic acid (5 ml.) at room temperature, allowing to stand for 0·5 hr. and filtering. A micro-Kjeldahl N determination was performed on the filtrate.

Amino N. The sample (1 ml.) was added to 5 ml. of a buffer solution having a pH suitable for arresting enzyme action, i.e. to 0·1 N-acetic acid in the trypsin and chymotrypsin experiments and 0·1 N-phosphate buffer of pH 7·5 in the case of pepsin. The amino N in a portion of the solution was determined by the Van Slyke method in the usual manner. Sanger (1945) has shown that terminal glycyl residues give an abnormally high value for amino N under the conditions used in the Van Slyke method. Since the terminal residues of the peptides are unknown, no correction has been made, but their characterization largely as glycine residues would introduce a significant error in our estimates of the number of peptide bonds broken.

Total N (T.N.). Three hours' digestion with 1 ml. conc. H_2SO_4 containing about 50 mg. of $CuSO_4$, 50 mg. of K_2SO_4 and a few mg. of Na_2SeO_4 was used. With insulin very little change in the T.N. was observed when the period of digestion varied between 2 and 17 hr.

Free amino-acids. These were determined by the ninhydrin method of Van Slyke, Dillon, MacFadyen & Hamilton (1941). Some difficulty was experienced in getting concordant values, the amount of free amino-acid being so small as to give readings of the same order of magnitude as the blanks.

Diffusion coefficient. A cell of the Anson-Northrop type with a sintered-glass membrane was employed. The cell constant, as determined with 0·1-N KCl, was 1·79 cm.^{2} (mean of three results, two before and one after the actual diffusion experiments). The technique used was similar to that described by Mehl & Schmidt (1937). D_{25} (0·1 N-KCl) was taken as 1·46 cm.^{2}/day.

The order of the experiments was as follows. The cell was filled with the digest (18·3 ml.) by suction through the sintered-glass diaphragm. It was suspended in an outer vessel so that the diaphragm just touched the surface of the water (20 ml.) contained therein. The cell was left at the temperature of the experiment for about 24 hr. in order to establish a uniform diffusion gradient in the sintered glass. The water in the outer vessel was then replaced, and after a period of diffusion the concentration of N which had diffused into it was determined. The liquid in the outer vessel was then replaced by 20 ml. of fresh water, and after a further period of diffusion the concentration in the diffusate was again determined. In this way the concentration outside the cell was usually estimated in three successive periods of diffusion. The cell was then opened and the concentration inside estimated. From the analytical results the concentration inside the cell at the beginning of each diffusion period could be ascertained. The diffusion coefficients were calculated by the formula

$$D=\frac{2{\cdot}303V''V'}{Kt\,(V''+V')}\times\log_{10}\frac{C_0''}{C_0''-(1+V'/V'')\,C'},$$

where V', V'' are the volumes of liquid outside and inside the cell, C_0'' the initial concentration in the cell at the beginning of each diffusion run, C' the concentration outside at the end, K is the cell constant and t the time. The measured diffusion coefficient was corrected to 20° by the formula

$$D_{20}=D_x\left(\frac{T_{20}}{T_x}\right)\left(\frac{\eta_x}{\eta_{20}}\right),$$

where η_x, η_{20} are the viscosities of water at $x°$ and 20°. The factor is 1·609 at 3°, 1·557 at 4°, 1·511 at 5°, 1·465 at 6°,

1·302 at 10° and 0·870 at 25°. The molecular weight was determined by the formula

$$\log_{10} D_{20} = -4{\cdot}56 - (1/3)\log_{10} M,$$

which appeared to give the best fit of recorded values of D_{20} for a variety of proteins and other compounds.

Chymotrypsin digests. The solution was usually made by addition of insulin (0·1 g.) to 1·5 ml. of a phosphate buffer (0·5 M, pH 7·6). The pH was then adjusted to about 8·3 by 0·02 N-NaOH (3 ml.) and the volume made up to 15 ml. with water. (This higher pH was chosen because the solubility of insulin is too small at a lower pH; insulin is completely stable in this solution and no change in activity was observed in 5 days at 25°.)

Cystine. The digest fractions were hydrolyzed by refluxing with a mixture of equal volumes of 6 N-HCl and 90 % (w/w) formic acid, usually for 19 hr. The cystine content of the hydrolysate was then determined by the method of Kassell & Brand (1938).

RESULTS

Chymotrypsin

The action of chymotrypsin (Fig. 1) is rapid in the early stages, up to the formation of 50 % N.P.N. After that it slows down considerably and a long time is necessary to complete the digestion; in fact,

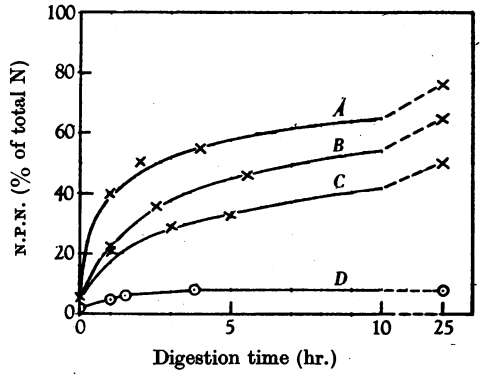

Fig. 1. Action of chymotrypsin and trypsin on insulin. *A*, chymotrypsin, 16×10^{-4} unit/ml.; *B*, chymotrypsin, $3{\cdot}2 \times 10^{-4}$ unit/ml.; *C*, chymotrypsin, $0{\cdot}7 \times 10^{-4}$ unit/ml.; *D*, trypsin, 4×10^{-4} unit/ml.

the N.P.N. only reaches values in the neighbourhood of 100 % when more chymotrypsin is added. The digestion process thus consists of a first stage, in which a rapid liberation of about half the total nitrogen takes place, leaving the remainder in a form still precipitable by 0·3 N-trichloroacetic acid. This is followed by a slow digestion of the remaining peptide bonds. The relation between the liberated N.P.N. and amino N in the earlier stages is linear (Fig. 2), the value of the amino N corresponding to 100 % N.P.N. being about 22 %. This means that the amount of N.P.N. liberated/peptide bond split is approximately the same throughout the reaction, so far as it has been followed. We can estimate this amount in the following way. In the original insulin the free amino N, as determined by the Van Slyke method, is 4·6 % of the total (Chibnall, 1946). Taking the N content of pure insulin as 15·54 %, a 'sub-molecule' of molecular weight 12,000 (which is the average minimal molecular weight required by all the analytical results) contains a total of 133 atoms of N, of which 4·6 % or 6 atoms are in uncombined amino groups. According to Sanger (1945), two of these belong to lysine and the remaining four are terminal α-groups at the free ends of the peptide chains. In the same way, 22 % of amino N in the

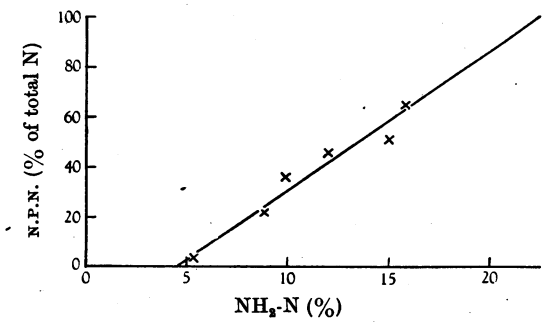

Fig. 2. Action of chymotrypsin on insulin. Relation of amino N to non-protein N.

digest corresponds to 29 amino groups, so that complete digestion with chymotrypsin increases the number of terminal amino groups from 4 to 27. Thus if the breakage of 23 peptide bonds releases 100 % of the nitrogen into the 'non-protein' part, assuming linearity as in Fig. 2, each peptide bond broken releases 4·35 % of the nitrogen, i.e. 5·8 N atoms.

This estimate was confirmed by a direct determination of the amino N in the trichloroacetic acid filtrate. The solution used was obtained from a digest in which N.P.N. was 58·5 %. It was found that the amino N in this filtrate was 22·5 %, i.e. *c.* 1 N atom in 4·5 belongs to a free amino group. After complete hydrolysis with 6 N-HCl for 18 hr., the amino N was 79 %, i.e. it had increased to 3·5 times its original value, so that the original peptide contains on the average 2·5 peptide bonds for each free amino group, and also 1 atom of N which is not potential amino N. These observations also suggest that the average molecule of N.P.N. is a tetra- or pentapeptide.

Biological activity and splitting. It was found in preliminary physiological tests (see Table 1) that the activity disappeared when about 50 % N.P.N. has been formed, i.e. at the end of the rapid reaction, so that the protein-like material precipitated by 0·3 N-trichloroacetic acid, which remains at this point, is biologically inactive. An accurate biological assay of the trichloroacetic acid precipitate was kindly made by Boots Pure Drug Co., Ltd. The dried

Table 1. *Effect of chymotrypsin on the physiological action of insulin*

(The amount injected was equivalent to 0·05 mg. insulin. The results given for pure insulin were the average for 6 rabbits, the remainder obtained by injection of a single rabbit.)

Non-protein nitrogen (%)	0	6	28	33	40·3	50	52	55·2
Average decrease in blood sugar 3 hr. after injection of sample (%)	49·2	55	56	54	51	45	0	0

precipitate from a digest with a N.P.N. value of 58·5 % (see p. 121 for particulars) had an activity of 0·68 unit/mg. (with $P = 0·95$, limits of error 0·56–0·85). On the assumption that the percentage N in the 'protein-like' material was the same as in insulin, the precipitate contained 55 % of 'protein', so that its activity on a weight basis was 5·4 % of that of insulin. It is probable that the protein-like material at this stage of the digestion contained a little unchanged insulin, amounting to *c.* 2 % of the original insulin.

It can be seen (Fig. 3) that it agrees quite closely with the percentage of N precipitated at the isoelectric point,* thus providing a fairly convincing proof that (1) the material precipitated at pH 5·3 is, in fact, unchanged insulin and (2) that trichloroacetic acid precipitates, in addition, material which is not insulin and which does not contribute appreciably to the observed residual activity.

Nature of the split-products. It seemed probable that the initial stage of the reaction consisted of a splitting off of small peptides leaving a compact

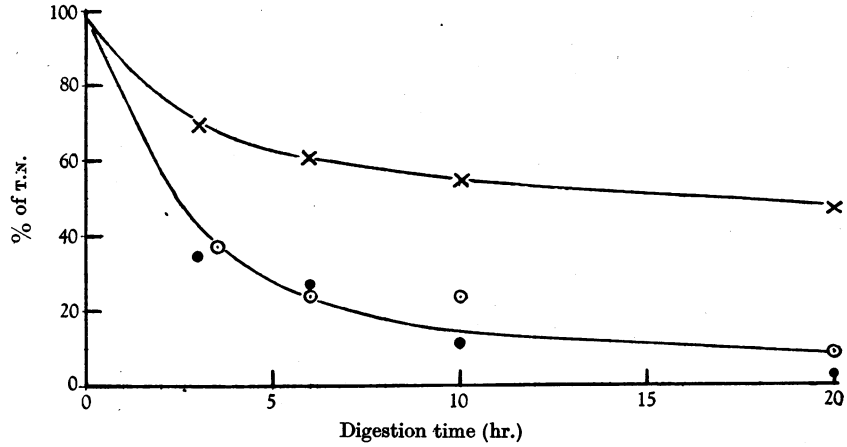

Fig. 3. Action of chymotrypsin on insulin. × Percentage N precipitated by 0·25 N-trichloroacetic acid. ⊙ Percentage N precipitated at the isoelectric point, pH 5·3. ● Percentage of original insulin activity found in trichloroacetic precipitate.

A further determination of the amount of unchanged insulin remaining at various stages of the digestion was made in the following way. Portions (15 ml.) of a chymotrypsin digest were withdrawn at various times and the 'protein' precipitated by trichloroacetic acid at the usual concentration. At the same times samples were added to equal volumes of 0·1 M-acetate buffer of pH 4·7 giving a final pH of 5·3, close to the isoelectric point at which the solubility of insulin is small. The precipitate formed was filtered off after 1 hr. and the N remaining in the filtrate determined. From these data we were able to find the percentage of the original N present in the trichloroacetic precipitate and the percentage of the original N precipitated at pH 5·3 (Fig. 3). The trichloroacetic acid precipitates were dried, weighed and portions assayed for insulin activity by Boots Pure Drug Co. Ltd.

From the results, the total activity of the trichloroacetic acid precipitate was obtained as a percentage of the insulin activity originally present.

protein-like residue. In order to find what proportion of the liberated nitrogen, if any, was derived from free amino-acids, a determination of these latter formed in the first stage was made by the ninhydrin method. The results obtained are given in Table 2. It is evident from these figures that the free amino-acids formed are only a small percentage (about 2 %) of the whole N.P.N. liberated. In the second example shown, the free amino-acid N formed is 1·23 % of the total N; i.e. it is equivalent to 1·6 N atoms per stoicheiometric unit of 12,000.

* The T.N. value of the 10 hr. isoelectric mixture is high because, owing to the time at which this sample was taken, it had to be frozen for a time and filtered about 12 hr. later. After 20 hr. the insulin assay gave a value which was definitely less than that in the isoelectric precipitate, so that in this case the precipitate probably contained material which was not insulin.

Thus between one and two molecules of free amino-acid are formed from the submolecule; i.e. of the 11 peptide bonds broken, one or two may liberate a terminal amino-acid. It is, of course, quite possible that this free amino-acid arises from secondary actions. The greater part of the non-protein fraction must thus consist of peptides with an average chain length, as estimated above, of 4–5 residues.

Table 2. *Products of chymotrypsin digestion*

Digestion period (hr.)	3	30·5
Non-protein nitrogen (% of total N)	31	47·5
Free amino-acid N (% of N.P.N.)	1·8	2·6
Amino N (% of T.N.)	10	12·7
Approx. no. of peptide bonds broken/unit of mol.wt. 12,000	7	11

A further confirmation of this calculation was obtained by determining the molecular weight of the digestion products by the diffusion cell. Insulin (0·5 g.) was digested with chymotrypsin for 17·5 hr. at 25°, the N.P.N. at the end of this period being 58·5%. Trichloroacetic acid was then added to a final concentration of 4%, and after 0·5 hr. the precipitate was filtered off (dry wt. 340 mg.). The filtrate deposited some further precipitate on standing which was removed and the pH was then adjusted to 4·4. This filtrate was then put in the diffusion cell, and after preliminary contact with water to set up the diffusion layer, the amount diffusing out in three successive portions of initially pure water was determined. From these results, and the final concentration in the diffusion cell, the diffusion coefficients were determined and corrected to 20° (Table 3). The values found correspond to a molecular weight of 800 ± 200, which is in good agreement with the figure estimated above. It will be observed that the values of D diminish in successive determinations; this may be due to material of lower molecular weight diffusing out first.

Table 3. *Diffusion coefficient of trichloroacetic acid filtrate*

Temp. (°)	Diffusion time (hr.)	Concentration in 20 ml. diffusate (mg. N/ml.)	$D \times 10^6$	$D_{20} \times 10^6$
5	66·5	0·013	2·05	3·26
5	76·5	0·013	1·87	2·97
4	90	0·013	1·68	2·77

Final conc. in diffusion cell (18·3 ml.) was 0·268 mg. N/ml. Mean D_{20} sec.$^{-1} = 3{\cdot}0 \pm 0{\cdot}3 \times 10^{-6}$.

The trichloroacetic acid precipitate was now dissolved (4 mg./ml.) in 0·02N-NaOH and the pH adjusted to 8·0. This liquid was put in the diffusion cell and after a preliminary contact with water for 24 hr. the amounts of N diffusing out in successive times were determined (Table 4).

Trichloroacetic acid thus divides the digest into a soluble part, consisting of comparatively small peptides of mol.wt. *c.* 800 and an insoluble part of average mol.wt. *c.* 4000. The former makes up about half the molecule. The insoluble part has a molecular weight which agrees quite well with that of a submolecule of mol.wt. 12,000 from which rather more than half the residues (i.e. about 60) have been removed. The action of chymotrypsin therefore appears to be a removal of the outer parts of the insulin molecule leaving a fairly massive nucleus. This process is complete at the end of the first stage of the digestion (50% N.P.N.). In the later slow stage the massive nucleus is decomposed into smaller non-precipitable fragments.

It is surprising that trichloroacetic acid can precipitate molecules with molecular weight as low as *c.* 5000, well below the order of magnitude usually considered as proteins. This finding must cause some revision in our ideas of what constitutes the 'protein' and 'non-protein' parts of a digest. In view of this we determined the effect of varying the trichloroacetic acid concentration on the amount precipitated, with the following results: 1 ml. digest was added to 5 ml. of trichloroacetic acid of concentration shown:

Conc. of trichloroacetic acid (N)	0·15	0·3	0·6	1·0
Percentage of T.N. remaining as N.P.N.	74	51	48	47

There is thus no significant increase in the proportion of N precipitated at concentrations above that usually employed (0·3N). When 1 ml. of the digest was added to 9 ml. of acid tungstate, prepared according to Van Slyke & Hawkins (1928), an even greater percentage, viz. 61·6% of T.N., was precipitated.

When added to an acidified chymotrypsin digest, pepsin produces little further splitting. For example, a chymotrypsin digest which contained 14·2% amino N, was acidified to pH 2 and 9×10^{-4} pepsin unit/ml. was added. After 24 hr. the amino N was 15·0%.

Distribution of cystine and of amino N. Since treatment of the 50% digest with trichloroacetic

Table 4. *Diffusion coefficient of trichloroacetic acid precipitate*

	Temp. (°)	Diffusion time (hr.)	Concentration in 20 ml. diffusate (mg. N/ml.)	$D \times 10^6$	$D_{20} \times 10^6$
Series 1	4	65·5	0·007	1·02	1·68
	4	96	0·006	1·24	2·04
	Final conc. in diffusion cell, 0·223 mg. N/ml.				
Series 2	3	96	0·0066	0·93	1·58
	3·5	96	0·0063	0·91	1·54
	5	123	0·010	1·18	1·88
	Final conc. in diffusion cell, 0·213 mg. N/ml.				

Mean D_{20} sec.$^{-1} = 1{\cdot}74 \pm 0{\cdot}2 \times 10^{-6}$, corresponding to a mol.wt. of about 4000 ± 1500.

acid divides it into two fractions, the distribution of the more important amino-acids between them is of interest. We report below two preliminary experiments on the distribution of cystine. They are not entirely satisfactory, as only about 90 % of the total cystine is accounted for, but this loss can hardly affect the main point which emerges.

Exp. 1. Recrystallized insulin (0·50 g.) in 90 ml. solution, pH 8·2, was incubated with chymotrypsin (3 mg. protein N). After 17·5 hr., N.P.N. was 58·5 %. To 84 ml. of digest was added trichloroacetic acid to 0·24N; the precipitate, which was filtered off after 30 min., amounted to 340 mg. Both precipitate and filtrate were hydrolyzed with the HCl-formic acid mixture for 19 hr. and cystine contents of the hydrolysates were determined (Table 5).

Table 5. *Distribution of cystine in the soluble and insoluble fractions of a partial digest of insulin*

(The figures of T.N. and cystine N are expressed in mg./10 ml. of original digest.)

	Total nitrogen (mg.)	Cystine N (mg.)	$\frac{\text{Cystine N} \times 100}{\text{Total N}}$
Exp. 1:			
Digest	8·05	—	—
Precipitate	3·40	0·504	14·7
Filtrate	4·67	0·114	2·5
Exp. 2:			
Whole digest	7·67	0·618	8·07
Precipitate	4·24	0·476	11·23
Filtrate	3·38	0·151	4·47

In the original solution the N.P.N. was 6 % of the whole, so that the original protein N was 63·5 mg. This should contain 5·95 mg. of cystine N (9·36 %), of which 5·19 mg. (i.e. 87 %) were accounted for.

Exp. 2. In this case the final concentration of trichloroacetic acid was 0·25N; the precipitate which formed was filtered off but not dried. It was prepared after 33·7 hr. digestion (with lower chymotrypsin concentration) when N.P.N. was 44·1 %. The precipitate was dissolved in a little N-KOH and diluted. Digestion with the HCl/formic acid mixture was carried out for 8 hr. The results are given in Table 5. In this experiment the sum of the cystine concentrations in the two fractions agrees with that determined for the original digest, but both are only 90 % of that expected for the total cystine of the insulin.

In both experiments the proportion of cystine in the precipitate is considerably greater than that in the filtrate, and the ratio is greater for the digest which has been digested further.

The proportion of amino N in the precipitate (55 % of T.N.) was found to be 8·7 %, which means an average chain length in the precipitated material, assuming the same average composition as insulin, of 9–10. This also fits in with the general picture that the enzyme has removed about 10–12 peptide fragments averaging 6 residues from the original submolecule of 106 residues in 4 chains, leaving about 40 residues in 4 chains.

Trypsin

Contrary to statements in the literature, trypsin itself had a very slight action on insulin. When 24×10^{-4} unit of trypsin was added to 5 ml. of a solution containing 17 mg. of insulin/ml. at pH 8·2, the increase of N.P.N. was only 4 % in 24 hr. at 25° (Fig. 1). Insulin was recovered from a solution containing 5·5 mg. insulin and $1·4 \times 10^{-4}$ unit of trypsin/ml. after standing at 2·5 hr. at 25° and 16 hr. at 5°. From 14 ml. of this solution which originally contained 77 mg. insulin, 75·4 mg. of insulin were crystallized. An accurate assay of this material was kindly made for us by Boots Pure Drug Co. Ltd., who found that it had 4·03 % moisture and an activity of 22·96 units/mg. corresponding to 23·93 units/mg. of dried crystals. It was therefore indistinguishable from the purest insulin. A much stronger trypsin solution (1 ml.; 0·73 mg. protein N, 800×10^{-4} unit/ml.) was added to 15 ml. of an insulin solution at pH 8·4; this produced about 10 % N.P.N. in 4 hr. and 50 % N.P.N. in 24 hr. Such an action might be produced by the presence of approximately 0·5 % of chymotrypsin in the trypsin. The trypsin was therefore tested by the milk-clotting method as used by Kunitz (1935) and Herriott (1938) standardized with pure chymotrypsin; the activity corresponded to the presence of 0·4 % chymotrypsin. It was therefore concluded that the slight action observed was due to chymotrypsin present and that trypsin itself has no significant action at pH 8·2. In order to find if any action of trypsin occurred at a lower pH, a solution was made containing 1 mg. of insulin/ml. at pH 7·0. Trypsin produced no appreciable change of amino N and no decrease in biological activity.

Trypsin can, however, hydrolyze further the products after digestion of chymotrypsin, if present in considerable amount. A chymotrypsin digest to which trypsin was added at a concentration of 0·05 mg./ml. changed very little in 19 hr.; but at a concentration of 0·5 mg./ml. the amino N rose overnight from 15·8 to 20·8 %, while the increase in the same time without trypsin was only 3 %. It follows that the rate of digestion of insulin by trypsin and chymotrypsin together will be greater than the sum of their separate effects.

SUMMARY

1. The action of crystalline trypsin and chymotrypsin on insulin has been studied. Crystalline trypsin has no appreciable effect on insulin, but is capable of digesting the products formed after chymotrypsin action.

2. The action of chymotrypsin occurs in two stages. The first stage consists of a rapid action leading to the formation of about 50 % non-protein

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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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 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.

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