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Specificity of Bond Fission during the Acid Hydrolysis of Insulin

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The object of the experiments described here has been to determine the nature and number of the bonds broken in the insulin molecule during its digestion with cold, concentrated hydrochloric acid. In this way some information has been gained concerning the relative ease with which various bonds are hydrolysed and the positions of susceptible bonds in the molecule. These data have also been correlated with the loss of activity of the insulin.

Acid digests of insulin have frequently been examined, either in structural studies (Sanger & Tuppy, 1951; Sanger & Thompson, 1953), or in attempts to correlate physiological activity with the structure of the molecule (for example Phillips, 1952). But although the recent work of Desnuelle & Bonjour (1951) on globin, and of Acher, Jutisz & Fromageot (1950) on lysozyme has shown that different peptide bonds may be hydrolysed at greatly differing rates, few accounts have been published of experiments on either proteins or peptides of known structure. The work on globin and lysozyme has demonstrated that the fission of bonds involving a serine or threonine amino group is much more rapid than that of other bonds. Elliott (1952) has further shown that, by treatment with anhydrous acidic reagents, these bonds are transformed from an *N*-acyl to an *O*-acyl form via the intermediate formation of an oxazoline ring. Leach (1953) has reviewed data on hydrolysis rates of peptide bonds.

It is clear from an examination of the previous work that a short period of digestion of insulin in cold acid would bring about the following changes: (1) Some bonds involving serine and threonine amino groups would be attacked. (2) Some ammonia would be removed from glutamine and asparagine residues. (3) According to Partridge & Davis (1950), a few bonds adjacent to aspartic or glutamic acid residues might be broken. (4) Any bonds made peculiarly labile by their position in the molecule would be destroyed. All these effects have been demonstrated under the experimental conditions described here.

After digestion of insulin in 10·5N hydrochloric acid for a prescribed time, as described in the Experimental section, the protein precipitated from the hydrolysate was dialysed for 2 days to ensure that all small peptides were removed. The non-diffusible product will be referred to as H-protein. The free amino groups of the H-protein were labelled by reaction with fluorodinitrobenzene (FDNB) and estimated chromatographically after hydrolysis of the dinitrophenyl protein (DNP-protein). The original terminal amino acids of the intact peptide chains (DNP-glycine and DNP-phenylalanine, Sanger, 1945), were thus always present. Also found were: DNP-serine, DNP-aspartic acid, DNP-glutamic acid and DNP-alanine. DNP-threonine was found only in certain experiments described below.

Sanger (1949*b*) has shown that the only threonine residue present in bovine insulin forms part of the terminal sequence –Thr.Pro.Lys.Ala (using the nomenclature of Brand & Edsall, 1947). The corresponding peptide was isolated, after conversion into its DNP derivatives, from the acid liquor remaining after the H-protein had been filtered off. As this tetrapeptide also accounts for the only lysine residue in insulin, the number of threonine bonds hydrolysed could be determined by estimating the

proportion of lysine residues remaining in the H-protein, and the values quoted in Table 1 have been obtained in this way.

The digestions were continued for increasing times until the hypoglycaemic activity of the H-protein fell to zero. This was found to occur after about 180 hr. and the number of different bonds broken in the H-protein up to this time is given in Table 1. In making these calculations it has been assumed that the molecular weight of the insulin submolecule is 12 000, and that the molecular weight of the H-protein is not significantly different from this.

Clearly, a progressive fission of serine bonds can be brought about, 1·5 being broken after 168 hr. There was a similar fission of threonine bonds, the ratio of liberated serine to threonine amino groups being 1·5 at 168 hr., which compares well with the mean value of 1·8 derived from the measurements of Desnuelle & Bonjour (1951). The H-protein retained intact 79 % of the amide N of the original insulin after 168 hr. digestion, but even after this time only small proportions of the bonds of alanine, glutamic acid and aspartic acid had been broken. No loss of phenylalanine amino or glycine amino end groups was noted, the average recovery being 1·95 and 1·83 residues/molecule of insulin, respectively.

In his study of acid-digested silk fibroin, Elliott (1952) found that the addition of sodium bicarbonate was sufficient to cause the greater number of the serine bonds to return from the *O*-acyl to the *N*-acyl form. As the isolation of the H-protein involved dialysis in an alkaline medium for 2 days, comparative preparations were made (digestion time, 168 hr.) in which the solution was buffered, in two cases at 6·5 and in a further two at 5·1. In the experiments at pH 6·5 the number of DNP-serine residues estimated was 2·1, while at pH 5·1 the number did not rise above 1·5, even though the reaction with FDNB was continued for 10 days. The results at this pH must be regarded as minimum estimates, however, as the H-protein, like insulin, is very insoluble at pH 5·1. At pH 5·1, however, DNP-threonine was isolated in amounts up to 0·2 mole/12000 g. of H-protein.

These experiments suggest that during the formation of H-protein some threonine bonds undergo rearrangement to the *O*-acyl form, and that some of these are then hydrolysed, yielding the tetrapeptide which is found in the acid filtrate. Those that resist hydrolysis are then reconverted into the *N*-acyl form during the dialysis in alkaline solution. On the other hand, most of the serine bonds which are transformed to the *O*-acyl form in acid do not rearrange in alkali.

Sanger & Tuppy (1951) and Sanger & Thompson (1953) have shown that the 'A' chains of insulin contain two serine residues and that the 'B' chains contain one. Samples of H-protein from 168 hr. digests were therefore examined to determine whether all three bonds were attacked during hydrolysis. In the first experiments the DNP derivative of the H-protein was oxidized with performic acid (Sanger, 1949*a*) and the products were separated by paper chromatography. No evidence was found for the existence of small fragments terminating in DNP-serine, and it was concluded that the rearranged serine bonds had not been completely hydrolysed during the acid digestion.

In subsequent experiments the DNP compound of H-protein was first oxidized and then partially hydrolysed by treatment with concentrated hydrochloric acid as described below. By chromatography of the ether extract, ten bands were separated, two of which accounted for at least 95 % of the DNP-serine in the whole fraction. By hydrolysis and paper chromatography these bands were identified as DNP-serine and DNP-serylleucine. The latter can only arise from the 'A' chain in which the sequence –Ser.Leu.Tyr.Glu– occurs (Sanger & Thompson, 1953). When the band containing DNP-serine was chromatographed on paper in *sec.*-butanol:1·5N ammonia (see Experimental), a very

Table 1. *The numbers of insulin peptide bonds of different kinds hydrolysed after treatment in* 10·5N *hydrochloric acid at* 0° *for various times. Values below* 0·1 *bond/molecule were not measured*

Time of hydrolysis (hr.)	Number of groups liberated/molecule insulin (12000)				
	Serine-amino	Threonine-amino	Amide	Alanine-amino	Aspartic + glutamic acid amino
19	0·14	0·08	—	—	—
33	0·30	0·24	0·15	—	—
48	0·51	0·25	0·29	—	—
72	0·63	0·54	0·73	—	—
96	0·91	0·60	0·95	0·08	—
120	1·18	0·73	1·39	0·15	0·10
144	1·28	0·96	1·61	0·19	0·09
168	1·49	1·0	2·49	0·20	0·13
192	1·89	1·25	3·95	0·21	0·13
233	2·13	—	—	0·25	0·19

small band of DNP-Ser.Val separated; its total amount did not exceed about 10% of the DNP-Ser.Leu. The sequence –Ser.Val– also occurs in the 'A' chain.

The yellow peptides from the aqueous layer of the partial digest could be resolved into seven bands on kieselguhr, all the DNP-serine being contained in the first three, which on hydrolysis yielded the same amino acids in similar proportions. It is not clear why there were three bands, but the effect was consistently repeated in several preparations and no evidence of heterogeneity of these peptides could be found by chromatography in other systems. The oxidation of *im*-DNP-histidine may be responsible for this behaviour, as hydrolysates of this peptide material gave DNP-serine, leucine, valine and an unidentifiable spot near leucine on butanol:acetic acid chromatograms. The only sequence in insulin which accounts for this finding is the –Ser.His.-Leu.Val– of the 'B' chains (Sanger & Tuppy, 1951). This interpretation was confirmed by partially hydrolysing the DNP compound of H-protein without first oxidizing it. A water-soluble peptide was then isolated which yielded on hydrolysis DNP-serine, leucine, valine and *im*-DNP-histidine.

To determine whether all the serine bonds of insulin were equally susceptible to hydrolysis, the DNP-peptides just described were quantitatively separated from another similar preparation and the amounts of DNP-serine were estimated. Of the total serine recovered, 12% was accounted for by Ser.His.Leu.Val, 46% as Ser.Leu, 38% as free serine, and about 4% as Ser.Val. The total serine recovered after 168 hr. digestion corresponded to 95% of the free serine amino groups in insulin.

These values show that the bond of the sequence Ser.Leu.Tyr is more easily broken than the others. Owing to the large amount of free DNP-serine isolated, the relative reaction rates for the other two sequences cannot be compared with certainty. In an attempt to resolve this problem a sample of H-protein isolated after 72 hr. digestion of insulin was examined, but the relative amounts of the DNP-seryl peptides were not substantially changed.

From these results it appears that insulin is only slowly hydrolysed in concentrated hydrochloric acid at 0°, about 7·4 bonds out of a total of 110 being broken after 192 hr. The insulin retains some hormonal activity until an average of about 6 bonds have been hydrolysed. It is significant that the distribution of serine peptides is similar in both 72 and 168 hr. digests, indicating that each kind of serine bond is split at a uniform, characteristic rate. On this evidence it is unlikely that the fission of one particular bond is sufficient to destroy the hormonic activity, but that as each bond is hydrolysed the protein becomes progressively less active.

As the hydrolysed bonds are scattered fairly widely along the four peptide chains comprising the insulin sub-molecule, these experiments do not support the hypothesis that there is a small section of the molecule which bears the whole of the hypoglycaemic activity.

The free alanine amino groups liberated must be either those of the sequences Ala.Ser.Val ('A' chains), or Ala.Leu.Tyr ('B' chains), but owing to the small degree of hydrolysis of this bond during the times investigated, exact identification was impossible.

EXPERIMENTAL

Formation of H-protein. About 100 mg. of crystalline ox insulin (Boots Pure Drug Co. Ltd.) were suspended in a few drops of water and on to this were poured 15 ml. of ice-cold conc. HCl. The solution was kept in a bath of melting ice for the prescribed time and then diluted with 3 vol. of water. The precipitate was centrifuged off and in most experiments was redissolved in a saturated $NaHCO_3$ solution and dialysed for 2 days against water. In certain cases, however, the precipitate was suspended in 10 ml. of buffer (0·2M sodium acetate (pH 5·1) or 0·066M Na_2HPO_4-KH_2PO_4 (pH 6·5) buffer and dialysed against this same buffer for 2 days.

Preparation of DNP-H-protein. Where the H-protein had been dialysed from $NaHCO_3$, the reaction with FDNB was carried out according to Sanger (1945). When the preparation was dialysed in buffer, 0·1 ml. of FDNB in 10 ml. of ethanol was added to the 10 ml. of dialysed protein-buffer suspension and the time of reaction was prolonged to ensure that reaction was as complete as possible, since the protein was not soluble at pH 5·1 or 6·5. In the first buffer the greatest time taken was 10 days and in the second buffer the greatest time was 5 days.

Identification of terminal tetrapeptide (Thr.Pro.Lys.Ala). The filtrate from which the H-protein had been filtered off was evaporated to dryness *in vacuo*. The residue was dissolved in 10 ml. of 2% (w/v) $NaHCO_3$ solution and treated with 0·1 ml. of FDNB in 10 ml. of ethanol. The yellow peptide derivatives were acidified and then extracted into ethyl acetate and this solution was evaporated to dryness. On chromatographing on kieselguhr, one outstandingly strong band was found, which was collected, hydrolysed and run on a paper chromatogram. Spots corresponding to DNP-threonine, ε-DNP-lysine, proline and alanine were found.

Hydrolysis of DNP-H-protein and DNP-peptides. About 50 mg. of the derivative were boiled under reflux in 10 ml. of 5N-HCl for 16 hr. The solution was diluted with water, extracted with ether and the aqueous phase was made up to a known volume. The ether layer was evaporated to dryness and the residue was dissolved in the solvent for chromatography.

The DNP-peptides of partial hydrolysates were dissolved in 5N-HCl and heated overnight at 100° in sealed tubes.

Partial hydrolysis of DNP-H-protein. The DNP derivative was first oxidized by the performic acid method of Sanger (1949*a*), the reaction mixture being diluted with several vol. of water after 30 min. and the precipitate of oxidized product centrifuged off. About 50 mg. of the product were dissolved in 15 ml. of conc. HCl and kept at 37° for 5 days. The digest was then thoroughly extracted with ether, and

both the ethereal and aqueous phases were evaporated to dryness.

Chromatographic techniques. For the separation of DNP-amino acids and DNP-peptides, adsorption chromatography on kieselguhr was used (Mills, 1952). For the DNP-peptides of the ethereal extract of the partial digest, no modification was necessary. The DNP-peptides from the aqueous layer were first extracted from their mixture with amino acids by repeatedly washing with ethyl methyl ketone saturated with water. This extract was then evaporated to dryness and redissolved in 60% (v/v) methyl ethyl ketone in $CHCl_3$ prior to chromatography. As a check on resolution, the separated bands were run on paper chromatograms using *sec.*-butanol: 1·5N ammonia (5:2, v/v) as solvent. After the paper had been dried in a current of air, the separate bands were cut out and eluted with 70% (v/v) aqueous ethanol.

The amino acids of hydrolysed peptides were identified by two-dimensional paper chromatography using *n*-butanol: acetic acid: water (5:1:4, v/v) and phenol: water.

Paper chromatograms were also used for separating the oxidation products of DNP-H-protein; the solvent was *sec.*-butanol: aqueous ammonia as above.

Identification of DNP-amino acids. This was done by comparison with the behaviour of authentic DNP-amino acids on kieselguhr columns and, in the case of *im*-DNP-histidine, on paper chromatograms. The presence of DNP-alanine was confirmed as described by Mills (1950).

Determination of amide N. This was done in the manner described by Sanger (1945), but it was found necessary to dialyse the insulin and H-protein against distilled water for 48 hr., as otherwise inconsistent results were obtained.

Determination of protein. The protein corresponding to solutions of H-protein or to preparations of DNP-H-protein was estimated from the amide N content, the relation between different preparations of H-protein and amide N being determined in a separate series of experiments. The total N of insulin digests was estimated by the micro-Kjeldahl method of Levin, Oberholzer & Whitehead (1950).

Determination of DNP-serine distribution. A 168 hr. preparation of H-protein was treated with FDNB, oxidized and partially hydrolysed at 37° as described above. The compounds containing DNP-serine were then separated into ether-soluble and water-soluble fractions and chromatographed on kieselguhr. After complete hydrolysis, the DNP-serine corresponding to each band was estimated according to the method of Mills (1952). A pure sample of DNP-DL-serine was found to undergo 15% breakdown under the conditions of hydrolysis, and this was allowed for in the calculations.

Insulin assay. The hypoglycaemic activities of the various preparations were estimated by determining the fall in blood sugar of rabbits during the 3 hr. following subcutaneous injection of the sample under test. Comparisons were made with the effects in the same rabbits of standard preparations of insulin. The results obtained are summarized in Table 2, where the values represent the average fall in blood-sugar level in several rabbits, referred to an initial starving level of 100. The amount of sample injected was equivalent to 1 unit of the original insulin and the blood sugar was estimated by the method of Hagedorn & Jensen (1923).

Table 2. *The average blood-sugar level of starving rabbits at the stated number of hours after injection of acid-hydrolysed insulin. Initial blood-sugar level taken as* 100

Time of hydrolysis of insulin (hr.)	Blood-sugar level at stated time after injection of sample		
	1 hr.	2 hr.	3 hr.
0	58	55	66
19	57	65	55
33	41	46	44
48	53	40	83
72	69	59	59
96	80	82	91
120	80	76	87
144	89	83	84
168	85	91	100
192	112	106	107
233	109	110	107

SUMMARY

1. Digestion of insulin in 10·5N hydrochloric acid at 0° has been shown to cause preferential fission of peptide bonds involving amino groups of serine and threonine residues and, to a smaller extent, of alanine, glutamic acid and aspartic acid residues.

2. The rate at which the serine, threonine and amide bonds are hydrolysed has been determined.

3. It has been shown that all the three different types of serine bond in insulin undergo hydrolysis, but at different rates.

4. During the acid treatment, a progressive loss of insulin activity takes place. In insulin which has been digested until it is just inactive 1·7 serine bonds, 1·1 threonine bonds and 3·2 amide bonds are hydrolysed.

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