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## The chemistry of insulin

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It is great pleasure and privilege for me to give an account of my work on protein structure and I am deeply sensitive of the great honour that has been done to me in recognizing my work in this way. Since the work on insulin has extended over about 12 years it will be necessary to give a somewhat simplified account and to omit most of the work that did not contribute directly to the main problem, the determination of the structure of a protein.

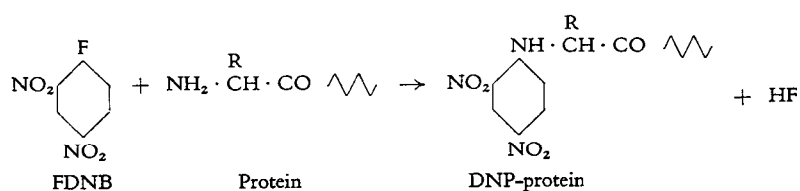
In 1943 the basic principles of protein chemistry were firmly established. It was known that all proteins were built up from amino acid residues bound together by peptide bonds to form long polypeptide chains. Twenty different amino acids are found in most mammalian proteins and by analytical procedures it was possible to say with reasonable accuracy how many residues of each one was present in a given protein. Practically nothing, however, was known about the relative order in which these residues were arranged in the molecules. This order seemed to be of particular importance, since although all proteins contained approximately the same amino acids they differed markedly in both physical and biological properties. It was thus concluded that these differences were dependent on the different arrangement of the amino-acid residues in the molecules. Although very little was known about amino-acid sequence, there was much speculation in this field. The most widely discussed theory was that of Bergmann and Niemann who suggested that the amino acids were arranged in a periodic fashion, the residues of one type of amino acid occurring at regular intervals along the chain. On the other extreme there were those who suggested that a pure protein was not a chemical individual in the classical sense but consisted of a random mixture of similar individuals.

Due largely to the work of Chibnall and his colleagues\* insulin had been studied in considerable detail. It had a somewhat simpler composition than most proteins, in that two of the commonly occurring amino acids, tryptophan and methionine were absent and an accurate analysis was available.

Moreover, using the Van Slyke procedure, Chibnall had shown that insulin was peculiar in having a high content of free  $\alpha$ -amino groups. This indicated that it was composed of relatively short polypeptide chains since free  $\alpha$ -amino groups would be found only on those residues (the N-terminal residues) which were present at one end of a chain. Thus the number of chains could be determined from the number of these N-terminal residues. The nature of one of these N-terminal residues was in fact known. Jensen & Evans<sup>2</sup> had shown that the phenylhydantoin of phenylalanine could be isolated from an acid hydrolysate of insulin that had been treated with phenylisocyanate, thus indicating that phenylalanine was at the end of one of the chains. At that time this was the only case where the position of an amino acid in a protein was known.

There was considerable doubt about the actual molecular weight of insulin and hence the number of amino acid residues present. Values varying from 36,000 to 48,000 were reported by physical methods but it was shown by Gutfreund<sup>3</sup> that these high values were due to aggregation and it was suggested that the real molecular weight or subunit was 12,000. This indicated that there were about 100 residues in the molecule. More recently Harfenist & Craig<sup>4</sup> have shown that the actual value is about 6,000; however during most of our work it was believed to be 12,000.

In order to study in more detail the free amino groups of insulin and other proteins, a general method for labelling them was worked out<sup>5</sup>. This was the dinitrophenyl (or DNP) method. The reagent used was 1:2:4 fluorodinitrobenzene (FDNB) which reacts with the free amino groups of a protein or peptide to form a DNP derivative:

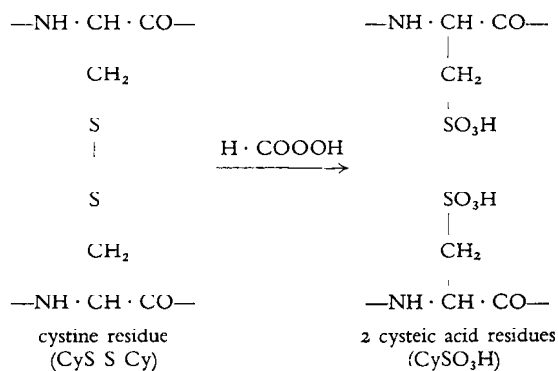


The reaction takes place under mildly alkaline conditions which normally do not cause any breakage of the peptide bonds.

The DNP-protein is then subjected to hydrolysis with acid which splits the peptide bonds in the chain, leaving the N-terminal residue in the form of its DNP-derivative.



cystine residues were converted to cysteic acid residues thus breaking the cross-links:



Performic acid also reacts with methionine and tryptophan residues, the two amino acids which fortunately were absent from insulin.

From the oxidized insulin two fractions could be separated by precipitation methods. One (fraction A) contained glycine and the other (fraction B) phenylalanine N-terminal residues. Fraction A was acidic and had a simpler composition than insulin, in that the six amino acids: lysine, arginine, histidine, phenylalanine, threonine, and proline, were absent from it. It thus had no basic amino acids, which were found only in fraction B. From a quantitative determination of the end groups it was concluded that fraction A contained about 20 residues per chain, four of these being cysteic acid and fraction B had 30 residues, two of which were cysteic acid. Since the yield of each fraction was greater than 50% in terms of the N-terminal residues present and since they appeared to be homogeneous it seemed likely that there was only one type of glycy chain and one type of phenylalanyl chain. This was confirmed by a study of the N-terminal sequences\*.

When the DNP derivative of fraction B was subjected to complete acid hydrolysis, DNP-phenylalanine was produced. If however it was subjected to a milder acid treatment so that only a fraction of the peptide bonds were split, DNP-phenylalanyl peptides were produced which contained the amino acid residues near to the N-terminal end and by an analysis of these peptides it was possible to determine the N-terminal sequence to four or five residues along the chain. The results with fraction B are shown in Table 1. It was concluded from these results that all the N-terminal phenylalanine residues of insulin were present in the sequence Phe · Val · Asp · Glu. This suggested that if there were in fact two phenylalanyl chains, then these two

were identical. Similar results were obtained with fraction A, and it was shown that the N-terminal glycine residues were present in the sequence Gly · Ileu · Val · Glu · Glu.

Table 1.

<i>Peptide</i>	<i>Products of complete hydrolysis of peptide</i>	<i>Products of partial hydrolysis</i>	<i>Structure</i>	<i>Yield from DNP* insulin</i>
B1	DNP-phenylalanine	—	DNP-Phe	13
B2	DNP-phenylalanine Valine	B1	DNP-Phe · Val	16
B3	DNP-phenylalanine Valine Aspartic acid	B1, B2	DNP-Phe · Val · Asp	13
B4	DNP-phenylalanine Valine Aspartic acid Glutamic acid	B1, B2, B3	DNP-Phe · Val · Asp · Glu	30
Other bands giving B4 on partial hydrolysis				20
Total				92

\* Moles peptide as per cent of total N-terminal phenylalanine residues of insulin.

These results, besides giving information about the position of certain residues in the polypeptide chains, showed for the first time that the molecule was composed of only two types of chains and that if the molecular weight was 12,000 as was then believed, then the molecule was built up of two identical halves. The other alternative, which was later shown to be the case, was that the actual molecular weight was 6,000. In any case the structural problem was somewhat simplified since we were now concerned with determining the sequence in two chains containing 20 and 30 residues respectively.

The main technical problem was the fractionation of the extremely complex mixtures that resulted from partial hydrolysis of a protein. However Consden, Gordon, Martin & Syngé<sup>9</sup> had shown that small peptides could be well fractionated by paper chromatography and had determined the sequence in the pentapeptide "gramicidin-S" from the composition of peptides produced on acid hydrolysis.

At this point (1949) I was joined by Dr. Hans Tuppy who came to work in Cambridge for a year. Although we did not seriously envisage the pos-

sibility of being able to determine the whole sequence of one of the chains within a year, it was considered worth while to investigate the small peptides from an acid hydrolysate using essentially the methods that had been applied to "gramicidin-S". Studies were initiated on both the chains at the same time but it soon became clear that there would be more difficulties with fraction A although it was the shorter chain and the work on fraction B progressed so favourably and Tuppy worked so hard that by the end of the year we were virtually able to deduce the whole of the sequence of its 30 residues<sup>10</sup>.

Fraction B was subjected to partial hydrolysis with acid. Since the mixture was too complex for direct analysis by paper chromatography it was necessary to carry out certain preliminary group separations in order to obtain fractions containing 5-20 peptides that could then be separated on paper. This was accomplished by ionophoresis, ion-exchange chromatography, and adsorption on charcoal. These simplified mixtures were then fractionated by two-dimensional paper chromatography. The peptide spots were cut out and the material eluted from the paper, subjected to complete hydrolysis and analysed for its constituent amino acids. Another sample of the peptide was then investigated by the DNP technique to determine the N-terminal residue. Table 2 illustrates the results obtained with a very acidic fraction obtained by ion-exchange chromatography. This contained only peptides of

Table 2. Cysteic acid peptides identified in a partial acid hydrolysate of fraction B.

	CySO <sub>3</sub> H · Gly	CySO <sub>3</sub> H · Gly
	Val · CySO <sub>3</sub> H	Leu · CySO <sub>3</sub> H
	Val · (CySO <sub>3</sub> H, Gly)	Leu · (CySO <sub>3</sub> H, Gly)
	Leu · (Val, CySO <sub>3</sub> H)	
	Leu · (Val, CySO <sub>3</sub> H, Gly)	
Sequences deduced	Leu · Val · CySO <sub>3</sub> H · Gly	Leu · CySO <sub>3</sub> H · Gly

cysteic acid. Since there are only two such residues in fraction B all these peptides must fit into two sequences. The way in which the two sequences Leu · CySO<sub>3</sub>H · Gly and Leu · Val · CySO<sub>3</sub>H · Gly were deduced from the results obtained with the peptides is illustrated in the table.

In this way about 45 peptides were identified in various fractions of the partial acid hydrolysate and the following five sequences were deduced as being present in the phenylalanine chain.

1. Phe · Val · Asp · Glu · His · Leu · CySO<sub>3</sub>H · Gly (N-terminal sequence).
2. Gly · Glu · Arg · Gly.
3. Thr · Pro · Lys · Ala.
4. Tyr · Leu · Val · CySO<sub>3</sub>H · Gly.
5. Ser · His · Leu · Val · Glu · Ala.

These five sequences contain all but four of the amino acid residues of fraction B. It was not possible to determine from the small peptides derived from acid hydrolysates the position of the remaining four residues or how the above five sequences were joined together. There were two reasons for this. Firstly there was considerable technical difficulty in fractionating the peptides containing two or more of the non-polar residues such as tyrosine or leucine. It happened that these residues were grouped together in the chain (see below) and gave rise to a mixture of peptides that moved fast on paper chromatograms and were not well resolved. The second difficulty was due to the great lability to acid of the bonds involving the amino groups of the serine and threonine residue. It was never possible to find a peptide containing this bond and hence to know what residue preceded the serine and threonine.

It was thus necessary to use another method of hydrolysis that would show a different specificity from concentrated acid. Hydrolysates prepared by the action of dilute acid at high temperatures or of alkali were studied but yielded little further information. Much more successful however was the use of proteolytic enzymes<sup>11</sup>. Initially we had refrained from using them since it was considered that they might bring about re-arrangement of the peptide bonds by transpeptidation or actual reversal of hydrolysis. Subsequent work has however shown that this is not a very serious danger and in fact proteolytic enzymes are the most useful hydrolytic agent for studies of amino acid sequences.

Proteolytic enzymes are much more specific than is acid since only a few of the peptide bonds are susceptible. They give rise to larger peptides which in general are more difficult to fractionate by paper chromatography. However there are relatively few of them so that the mixtures are less complex. In this initial work we used essentially the same methods for studying the

enzymic peptides as we had used for the acid ones, depending largely on paper chromatography for fractionation, although more recently it has been shown that better separations can be obtained by ion-exchange chromatography and by ionophoresis.

As an example we may consider a peptide Bp3 obtained by the action of pepsin. It had the following composition Phe (CySO<sub>3</sub>H, Asp, Glu, Ser, Gly, Val, Leu, His) of which the most important components are aspartic acid and serine since they occur only once in the chain. Aspartic acid is present only in the N-terminal sequence 1 and serine is in sequence 5. This shows that all of sequence 1 and at least the N-terminal part of sequence 5 is present in peptide Bp3. That none of the other sequences are present follows from the fact that Bp3 contains no arginine (sequence 2), threonine, proline, or lysine (sequence 3) or tyrosine (sequence 4). One may thus conclude that the two sequences are joined together. By studying other peptides obtained by the action of pepsin, trypsin and chymotrypsin it was possible to find out how the various sequences were arranged and to deduce the complete sequence of the phenylalanyl chain which is shown below:

Phe · Val · Asp · Glu · His · Leu · CySO<sub>3</sub>H · Gly · Ser · His · Leu · Val · Glu  
 · Ala · Leu · Tyr · Leu · Val · CySO<sub>3</sub>H · Gly · Glu · Arg · Gly · Phe · Phe  
 · Tyr · Thr · Pro · Lys · Ala.

In this work many more peptides were studied from both acid and enzymic hydrolysates than were actually necessary to deduce the sequence. This was considered essential since the methods used were new and were qualitative rather than quantitative. The fact that all the peptides fitted into the unique sequence given above added further proof to its validity.

Essentially similar methods were used to determine the sequence of fraction A<sup>12</sup>. Although the shorter of the two chains, the determination of its structure was more difficult. Fraction B contains several residues that occur only once in the molecule and this helps considerably in interpreting the results, whereas fraction A has only a few such residues and these are all near one end. Also fraction A is much less susceptible to enzymic hydrolysis. It is not attacked by trypsin and there is a sequence of thirteen residues which is not split by chymotrypsin or pepsin either. Considerable difficulty was at first experienced with the cysteic acid peptides. Fraction A contains the sequence CySO<sub>3</sub>H · CySO<sub>3</sub>H and this gave rise to a number of very water-soluble peptides which would not fractionate easily by paper chromatog-



raphy. However it was found that by paper ionophoresis at pH 2.75 they could be well separated since they were the only acidic peptides present. At this pH, -COOH groups are uncharged,  $-\text{SO}_3\text{H}$  groups carry a negative and  $-\text{NH}_2$  groups a positive charge. Peptides without cysteic acid were all positively charged, those with one cysteic acid were neutral and could be separated as a group and those with two cysteic acids were negatively charged. If a slightly higher pH (3.5) is used for the ionophoresis, the -COOH groups become slightly charged and all the peptides containing one cysteic acid residue move slowly towards the anode and can be fractionated in this way. This method was found very useful for the separation and identification of cysteic acid peptides. Fig. 1 is a tracing of an ionogram of an acid hydrolysate of fraction A carried out in this way.

The sequence of fraction A was found to be:

Gly · Ileu · Val · Glu · Glu ·  $\text{CySO}_3\text{H}$  ·  $\text{CySO}_3\text{H}$  · Ala · Ser · Val ·  $\text{CySO}_3\text{H}$   
 · Ser · Leu · Tyr · Glu · Leu · Glu · Asp · Tyr ·  $\text{CySO}_3\text{H}$  · Asp.

When a protein is hydrolysed with strong acid, it gives rise not only to amino acids but also to a certain amount of ammonia. This is present in the form of amide groups on some of the aspartic and glutamic acid residues. It was thus necessary to determine the position of these groups<sup>13</sup>. This was done by studying the ionophoretic rates and amide contents of peptides derived from enzymic hydrolysates, since the amide groups are not split off by enzymes, whereas they are by acid. The position of the amide groups are indicated in Fig. 2 by the symbols  $\text{NH}_2$ .

Having determined the structure of the two chains of insulin the only remaining problem was to find how the disulphide bridges were arranged. About this time it was shown by Harfenist & Craig that the molecular weight of insulin was of the order of 6,000, so that it consisted of two chains containing three disulphide bridges, and not of four chains as we had originally thought. The fact that fraction A contained four cysteic acid residues whereas fraction B had only two indicated that two bridges must connect the two chains together and one must form an intrachain bridge connecting one part of the A chain with another part of the same chain.

In order to determine the distribution of the disulphide bridges, it was necessary to isolate from unoxidized insulin peptides containing intact cystine residues. These could then be oxidized to give cysteic acid peptides

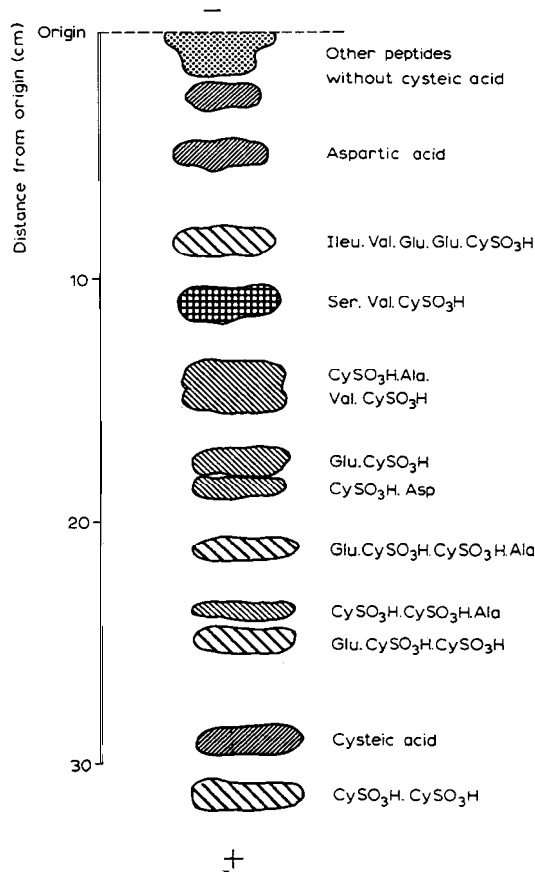
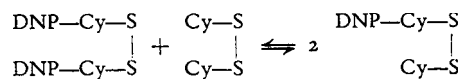


Fig. 1. Ionophoresis of partial acid hydrolysate of fraction A at pH 3.5 showing separation of cysteic acid peptides.

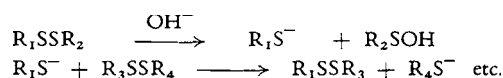
which could be recognized since they had been found in the hydrolysates of the oxidized chains. However an unexpected difficulty arose, in that during hydrolysis a reaction occurred which caused a random rearrangement of the disulphide bonds, so that cystine peptides were isolated which were not actual fragments of the original insulin and it would have appeared from the results that every half-cystine was combined to every other half-cystine residue.

This disulphide interchange reaction could be demonstrated and studied using as a model system a mixture of cystine and bis-DNP cystine, which reacted together to give mono-DNP-cystine

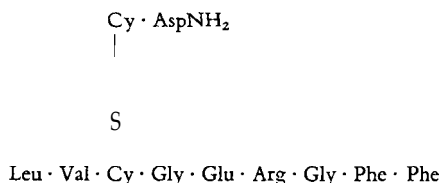


An ether-soluble coloured substance was thus converted to a water-soluble coloured substance and the course of the reaction could be studied by measuring the distribution of colour between ether and water.

It was found that there were two types of disulphide interchange reactions<sup>14</sup>. One took place in neutral and alkaline solution and was catalyzed by -SH compounds. It is probably due to initial hydrolysis of the disulphide which then catalyzes a chain reaction:



In neutral conditions the reaction could be inhibited by -SH inhibitors so that it was possible to use enzymic hydrolysis to obtain cystine peptides<sup>15</sup>. Thus for instance with chymotrypsin a peptide was obtained which on oxidation gave the two cysteic acid peptides  $CySO_3H \cdot AspNH_2$ , and  $Leu \cdot Val \cdot CySO_3H \cdot Gly \cdot Glu \cdot Arg \cdot Gly \cdot Phe \cdot Phe$ . The structure of the cystine peptide was thus:



establishing the presence of a disulphide bridge between the two half-cystine residues nearest the C-terminal ends of the two chains.

It was not, however, possible to determine the positions of the other two disulphide bonds using enzymic hydrolysis, since no enzyme would split between the two consecutive half-cystine residues of the A chain. It was therefore necessary to re-investigate the possibility of using acid hydrolysis.

The disulphide interchange reaction that occurred in acid solution was found to be different from that occurring in neutral and alkaline solution and instead of being catalyzed by -SH compounds, was actually inhibited by them. Not only did this show that a different reaction was involved but it also made it possible to prevent it occurring during acid hydrolysis. Thus when insulin was treated with concentrated acid to which a small amount of thioglycolic acid was added cystine peptides could be isolated which were in fact true breakdown products and from which the distribution of the

remaining two disulphide bonds could be deduced. These are shown in Fig. 2, which shows the complete structure of insulin.

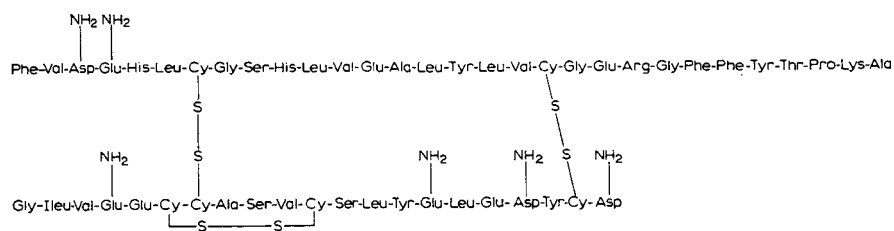


Fig. 2. The structure of insulin.

Of the various theories concerned with protein chemistry our results supported only the classical peptide hypothesis of Hofmeister and Fischer. The fact that all our results could be explained on this theory added further proof, if any were necessary, to its validity. They also showed that proteins are definite chemical substances possessing a unique structure in which each position in the chain is occupied by one and only one amino acid residue.

Examination of the sequences of the two chains reveals no evidence of periodicity of any kind nor does there seem to be any basic principle which determines the arrangement of the residues. They seem to be put together in a random order, but nevertheless a unique and most significant order, since on it must depend the important physiological action of the hormone.

As yet little is known about the relationship of the physiological action of insulin to its chemical structure. One approach to this problem was to study the insulins from different animal species<sup>16,17</sup>. Since all insulins show the same activity it could be concluded that differences would be found only in parts of the molecule that were not important for activity.

All the above results were obtained on cattle insulin. When insulins from four other species were studied by essentially the same methods it was found that the whole of the B chain was identical in all species and the only differences were found in the three amino acids contained within the disulphide ring of the A chain, which in the cattle are Ala · Ser · Val and in the other species are as follows:

Pig	-	Thr · Ser · Ileu
Sheep	-	Ala · Gly · Val
Horse	-	Thr · Gly · Ileu
Whale	-	Thr · Ser · Ileu

These results suggest that the exact structure of the residues in this position is not important for biological activity, but it does not follow that the whole of the rest of the molecule is important.

The determination of the structure of insulin clearly opens up the way to similar studies on other proteins and already such studies are going on in a number of laboratories. These studies are aimed at determining the exact chemical structure of the many proteins that go to make up living matter and hence at understanding how these proteins perform their specific functions on which the processes of Life depend. One may also hope that studies on proteins may reveal changes that take place in disease and that our efforts may be of more practical use to humanity.

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