Observations on the Composition and Activity of Partially Arylated Insulin

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One result of the crystallization of insulin and its identification as a protein hormone has been the stimulation of considerable research into the nature of its action and inquiry into the reasons why this particular molecule should be endowed with its unique physiological properties. It has been suggested as the result of experiments by Fraenkel-Conrat & Fraenkel-Conrat (1950) that although some amino-acid side chains must remain intact if full hormonal activity is to be retained, others may be modified without its destruction. Included in the latter category are the free terminal amino groups and the ϵ -amino groups of lysine. There are, however, certain points of criticism that may be raised against these findings.

In the first place, the amino groups in these experiments were masked by acetylation and, in view of the dynamic state of the proteins of the living organism, it is by no means certain that the acetyl residue could not be removed *in vivo*. Secondly, differently placed groups may be essential in different degrees, which would have the result that inhibition of activity might be a function of the size of the masking group. It is interesting to note in this connexion that Fraenkel-Conrat & Fraenkel-Conrat (1950) report some destruction of activity after coupling with phenyl isocyanate. This was attributed, however, to reaction with some of the essential tyrosine residues.

The suggested non-essential nature of the free amino groups of lysine leads to a third query based on the recent work of Lens (1949), who reported that digestion of crystalline insulin with carboxypeptidase resulted in the removal of about two terminal alanine residues per molecule, leaving a slightly modified insulin which was no longer active. Moreover, the work of Sanger & Tuppy (1951) has shown that the free carboxyl terminals of the pair of so-called *B* chains are derived from alanine residues and that the penultimate units of these chains are lysine. The whole of the lysine present in insulin is accounted for by these two residues. These two investigations seem to show that although the terminal alanine residue must be left intact in order to retain insulin activity, the immediately adjacent residues could be termed unessential. This situation was thought to be sufficiently curious to warrant further study.

In a search for a masking residue which was not only larger than acetyl, but which would form with an amino group a bond less likely to be broken by metabolic processes, a re-investigation has been made of the work of Barger & Tutin (1918). As originally used, this technique involved the reaction of 2:4:5-trinitrotoluene with the protein, when a molecule of nitrous acid was eliminated between any free amino group and a molecule of the reagent. On hydrolysis, coloured amino-acid derivatives analogous to those produced by the well-known method of Sanger (1945) could be isolated. An advantage of this reagent was that the reaction has been found to be slower than that with fluorodinitrobenzene and could be more easily stopped at any desired stage.

This paper describes the results of a preliminary re-examination of the essential nature of the amino groups of insulin and indicates the possible uses of active polynitro aromatic compounds in studies of this kind. Although 2:4:5-trinitrotoluene was the reagent finally selected, several other similar active nitro compounds were tried, specimens of which were liberally provided from the collection of Dr Edward Ward of Leicester Technical College, whose advice has greatly furthered these investigations.

METHODS

Formation of the polynitro derivative of insulin. About 100 mg. of crystalline insulin, prepared by Boots Pure Drug Co. Ltd., were dissolved in about 10 ml. of water to which a few drops of the required buffer had been added. Ethanol was then added to the clear liquid to a final concentration of 70%, followed by about 50 mg. of 2:4:5-trinitrotoluene (2:4:5-TNT). Buffer solution was added dropwise until the mixture was just saturated with salt. It was then allowed to stand with occasional shaking for various periods of time from 3 to 24 hr. at temperatures between 18 and 37°. After the required time, the mixture was diluted with water, the ethanol and excess reagent were extracted with ether and the pH of the aqueous layer adjusted to the isoelectric point. The resulting yellow precipitate was centrifuged off.

Buffer solutions. The pH of the reaction medium was maintained with Sørensen's phosphate buffers of pH 7.6, 7.8 or 8.3, or with dilute NaHCO_3 solution of pH 9.3. The actual pH values of these buffers was increased by solution in 70% ethanol however. The pH values of the ethanolic solutions saturated with buffer were estimated with a glass electrode to be 9.7, 9.2, 9.4 and 10.4 respectively, where the pH in

ethanol has been defined according to Michaelis & Mizutani (1925). It is not clear why the buffer of pH 7.6 gave so high a value when dissolved in the ethanolic medium.

Estimation of the nitro groups introduced. In early experiments this was done by reduction of the intact insulin derivative using TiCl_3 . The blank determinations were rather large, however, so the method was discarded in favour of the analysis of the hydrolysis products of the arylated protein by the method of Mills (1952). Independent measurements on the synthetic dinitrotolyl (DNT) amino-acids showed that their breakdown during acid hydrolysis was not significantly different from that undergone by the corresponding dinitrophenyl derivatives (Porter & Sanger, 1948).

Assay of insulin activity. It was not possible to make very accurate assays as the appropriate facilities were not available. Consequently, the preparations described below were each tested in only two or three rabbits. The fall in blood sugar was measured at hourly intervals for 3 hr. after injection and compared with the values obtained by injection of standard samples of insulin. The concentration of protein in the solutions used was calculated from micro-Kjeldahl estimates of the total N.

RESULTS

It was necessary as a preliminary step to decide whether 2:4:5-TNT reacted appreciably with the phenolic groups of tyrosine. Preliminary qualitative experiments were first made with leucyltyrosine and acetyltyrosine ethyl ester. These substances were both allowed to react separately with 2:4:5-TNT in 70 % ethanol containing pH 7.8 buffer in one set of experiments and bicarbonate in the other. The DNT derivatives were hydrolysed in 6N-hydrochloric acid and examined on a paper chromatogram to discover whether any *O*-DNT-tyrosine had been formed. These tests showed that although little or no masking of tyrosine hydroxyl groups took place at the lower pH, some were destroyed in the more alkaline medium. The fact that some tyrosine remained unattacked even in the presence of bicarbonate was confirmed for the DNT-insulin by paper chromatography of hydrolysates.

In an attempt to obtain more information on this point, 2:4:5-TNT was allowed to react for 18 hr. with an equimolar amount of free tyrosine under the

two sets of conditions already mentioned. The products were analysed chromatographically on a column of kieselguhr according to Mills (1952). In each experiment, two substances were found which were easily separated into yellow bands. The first of these ran at the speed of the diarylated tyrosine and gave no reaction with the Folin phenol reagent. The second band ran more slowly, as would be expected if it were the mono-substituted derivative, and gave a positive phenol reaction. It was concluded that the tyrosine amino group reacted more rapidly than the hydroxyl group, the reaction mixture therefore containing both di-DNT-tyrosine and *N*-DNT-tyrosine. The molecular ratio of mono-substituted tyrosine to di-substituted tyrosine, obtained at pH 10.4, was 1.34. At pH 9.2 the ratio was 13.1. In both experiments 70 % of the tyrosine remained unreacted when the experiment ended. It seemed probable from these experiments that while considerable substitution of the tyrosine residues of insulin was to be expected during reaction in the presence of bicarbonate, at pH 9.2 it was not likely to exceed about 0.5 residue/molecule.

These conclusions have been further examined by the reduction of intact DNT-insulin. If histidine or tyrosine had been masked, the total number of reducible groups found in this way would exceed the total found by the individual estimation of DNT-glycine, DNT-phenylalanine and DNT-lysine after hydrolysis.

Examinations of DNT-insulin made at pH 9.2 as described above (Exps. A/4, A/5 and A/9 of Table 1) showed that under these conditions very little, if any, substitution took place in the tyrosine residues. Reduction of the unhydrolysed protein derivative indicated only small amounts of reducible material in excess of the masked amino groups, but as the blank determinations were rather large, this indicates only that the number of *O*-DNT-tyrosine residues was less than 1 for each molecule of insulin. When Herriot's (1935) method was used to estimate the intact tyrosine of these derivatives, the estimates ranged between 9–10 mol. of tyrosine/mol. of insulin, compared with a value of 9 for the native protein.

Table 1. *The number of tyrosine residues masked in preparations of DNT-insulin made at pH 9.2 and pH 10.4*

Exp.	pH	Total number of amino groups masked/mol.	Tyrosine masked (mol./mol.)	
			Reducible with TiCl_3	By Herriot's (1935) method
A/4	9.2	2.58	<1	—
A/5	9.2	3.19	<1	0
A/9	9.2	2.95	<1	0
D/2	10.4	1.51	7.4	—
D/3	10.4	1.92	2.6	2.4
D/4	10.4	1.77	4.7	6.8
D/5	10.4	1.93	6.0	6.1

The materials of experiments *D/3*, *D/4* and *D/5* (Table 1) were prepared in the following way. About 100 mg. of insulin were allowed to react with 2:4:5-TNT overnight at pH 10.4, the ethanol removed by extraction with ether and the pH adjusted to the isoelectric point. It was found that, after a certain amount of acid had been added and the precipitate (*D/5*) had been removed, more acid could be added without at first causing any further precipitation. However, after the pH had fallen to a lower value, more insulin derivative separated (*D/4*). When this had been centrifuged off, the addition of acid was continued and a third product isolated in a similar way (*D/3*). As can be seen from Table 1, the amounts of *O*-DNT-tyrosine present show a corresponding fall. That some tyrosine remained unmasked was demonstrated by hydrolysis in 6*N*-hydrochloric acid followed by paper chromatography.

Table 2 summarizes the approximate activities found for the treated insulin preparations and the distribution of the three DNT-amino-acids isolated from them. The activity was usually either very high, when it has been called 100 %, or very low, when it has been put as zero.

It is apparent that a change in pH has a marked effect on the distribution of DNT residues among the available amino groups. The fact that the terminal glycine amino group seems to react more readily than that of phenylalanine might be due to either steric hindrance within the insulin molecule, or to a chemical difference in reactivity between these residues when present in a peptide chain. In order to test the last possibility equimolar quantities

of glycine, phenylalanine and 2:4:5-TNT were dissolved in 70 % ethanol saturated with pH 7.8 buffer. After reacting for 18 hr. the DNT-amino-acids were estimated as already described. It was found that phenylalanine had reacted more rapidly than glycine, the molar ratio of the DNT derivatives being 2.58. This experiment was repeated using the amino-acid esters. The phenylalanine derivative again reacted more rapidly, forming 2.64 mol. of DNT compound for every molecule of DNT-glycine ester.

It is noticeable that the amounts of DNT derivatives isolated from the treated insulins are in slightly different proportions even among experiments made under similar conditions (Table 2). The reason for this lack of agreement is not fully understood, but may be due to random variations of room temperature, at which these experiments were made.

The effect of the reaction medium on the activity of insulin was determined by making mixtures for each buffer used, the 2:4:5-TNT being omitted. Assays in rabbits showed that in no case had any detectable inactivation occurred.

DISCUSSION

These results show clearly that, in the first place, sufficiently extensive substitution with 2:4:5-TNT can destroy the activity of insulin, while in the second place, this substitution reaction follows a course dictated by the pH of the medium.

It will be seen from Table 2 that the activity of the partially arylated insulins was dependent on the number of amino groups masked, falling to zero when rather less than three residues had been

Table 2. *The distribution of DNT residues among the free amino groups of insulin, showing mols. of DNT amino-acid isolated/mol. of insulin*

(The pH quoted is an estimate of the value of the buffer in 70 % ethanol solution.)

Exp.	pH	Glycine (mol./mol.)	Phenylalanine (mol./mol.)	Lysine (mol./mol.)	Total of amino groups masked/mol.	Activity as approx. % of original
<i>A/1</i>	9.2	—	—	—	0.3	100
<i>A/2</i>	9.2	1.3	0.5	0.15	1.95	100
<i>A/3</i>	9.2	1.5	0.3	0.12	1.92	100
<i>A/4</i>	9.2	2.0	0.4	0.18	2.58	75
<i>A/5</i>	9.2	2.1	0.7	0.39	3.19	30
<i>A/6</i>	9.2	—	—	—	3.3	10
<i>A/7</i>	9.2	1.6	0.6	0.35	2.55	—
<i>A/8</i>	9.2	2.1	0.6	0.25	2.95	0
<i>B/1</i>	9.4	0.9	0.6	0.18	1.68	100
<i>C/1</i>	9.7	0.7	0.5	0.31	1.51	100
<i>C/2</i>	9.7	0.9	0.4	0.55	1.85	100
<i>C/3</i>	9.7	1.2	0.8	0.80	2.80	10
<i>C/4</i>	9.7	1.2	1.2	0.64	3.04	0
<i>C/5</i>	9.7	0.9	1.0	1.18	3.08	0
<i>D/1</i>	10.4	0.8	0.2	0.87	1.87	0
<i>D/2</i>	10.4	0.6	0.1	0.81	1.51	0
<i>D/3</i>	10.4	0.6	0.2	1.12	1.92	0
<i>D/4</i>	10.4	0.7	0.2	0.87	1.77	0
<i>D/5</i>	10.4	0.8	0.3	0.83	1.93	0

introduced. This seems to indicate that the effect of the masking residue is dependent on its size because, although the amino groups of insulin are unessential when tested with acetic anhydride (Fraenkel-Conrat & Fraenkel-Conrat, 1950), some at least are essential when the reagent is 2:4:5-TNT.

A noticeable feature of the results given in Table 2 is the suddenness of the fall in activity after just over two DNT residues have been introduced into each molecule of insulin. This is consistent with a steric effect which is insufficient to cause inactivation until more than two DNT groups are present. In no case was the amount of DNT-glycine isolated much less than 1 mol./mol. of insulin, and in experiments A/2, A/3 and A/4, it approached the maximum of 2 mol./mol. without marked loss of activity. The terminal glycine-amino groups are therefore relatively unessential, considered by themselves.

The separate effect of masking the phenylalanine and lysine residues cannot be decided from these experiments with any certainty because in preparations where much of either was labelled, considerable amounts of O-DNT-tyrosine were formed as well. Experiment C/2 shows that the lysine residues may not be intimately concerned with insulin activity, since in this case one group in every second molecule had been attacked without destruction of potency. In a similar way, from C/1 and C/2 it appears that the phenylalanine-amino groups cannot be individually connected with the hormone action, since if they were both involved the activity of these preparations would be only 50%. Even if only one of the phenylalanine residues is essential the activity of these derivatives should be no more than 75% of that of the original protein.

The experiments on the relative rates of reaction of 2:4:5-TNT with free glycine and phenylalanine indicate that the more rapid masking of the glycine terminal is unexpected. This is the more so since Schwartz & Lea (1952) have shown that when insulin combines with glucose, the phenylalanine couples more quickly than the glycine residues. However, it is significant that whereas the reaction with 2:4:5-TNT took place in 70% ethanol, Schwartz & Lea used a mixture freeze-dried from water at pH 7. The large increase in rate of reaction of lysine as the pH of the medium was raised may be due to

the increased proportion of the uncharged form of the amino group in more alkaline solutions. A similar explanation would be applicable to the increase in speed of reaction of the tyrosine residues, as a higher pH would favour the dissociation of the hydroxyl group. According to Cohn & Edsall (1943) the pK value for the ionization of the phenolic group is 10.1 while that of the lysine ϵ -amino group is 10.5. The corresponding values for the free amino groups of glycine and phenylalanine are about 8.3 and 7.7 respectively. If this constant is a controlling factor, the phenylalanine should react slightly more rapidly than the glycine, as was found for the amino-acids and their esters. The inverse phenomenon during the reaction between 2:4:5-TNT and insulin suggests that the glycine terminal is sterically more readily available than the phenylalanine, but in view of the findings of Schwartz & Lea this question must remain open.

As no evidence has been found to support the view that the ϵ -amino groups of lysine must remain intact if hormonal activity is to be retained, the essential structure of the carboxyl end of the molecule remains unresolved.

SUMMARY

1. The application of 2:4:5-trinitrotoluene to the preparation of labelled derivatives of insulin has been examined.
2. The glycine amino terminals of insulin have been found to react more quickly than phenylalanine and these more rapidly than lysine.
3. The reaction with all the free amino groups and with the tyrosine phenolic groups was found to be markedly dependent on the pH of the medium.
4. By stopping the reaction after an appropriate time, insulin derivatives have been made which are soluble in water and which retain some or all of the activity of the original protein. The distribution of masked amino groups in such dinitrotolyl derivatives suggests that the free amino groups are not essential to the hormonal action of insulin.

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