CCLXXX. THE ACTION OF PHENYL ISOCYANATE ON INSULIN.

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THE chemistry of insulin has been studied by various methods, and during recent years a considerable amount of information has been obtained about the groupings of the insulin molecule which appear to be necessary for its physiological activity. Inactivation by purified enzyme preparations, acids and alkalis, oxidising and reducing agents, by esterification and methylation and by other mild and vigorous treatments has been extensively studied in attempts to determine whether certain linkages or groupings can be destroyed without loss of the power to cause a fall in the blood-sugar level. In addition, efforts have been made, in a few instances with success, to restore the activity of the inactivated insulin. Full details of these and related investigations are to be found in a very comprehensive review of the chemistry of insulin by Jensen and Evans [1934], and in the papers by Freudenberg and Eyer [1932] and Freudenberg, Weiss and Eichel [1932].

During recent years we have been investigating the chemistry of protein antigens and have studied the action of phenyl isocyanates on various proteins [Hopkins and Wormall, 1933, 1, 2; 1934]. Since this reaction is comparatively mild, and since the earlier work had given some indication as to the groups in the protein which are concerned in the reaction, it was thought possible that the study of the action of phenyl isocyanate on insulin might yield useful information about the chemistry of this substance. The properties of the product of this reaction (phenylcarbamido-insulin) have been studied, and the results are discussed below in the light of the chemical and immunological evidence yielded by the earlier investigations¹.

EXPERIMENTAL.

The insulin activities of the original material and the treated insulin were determined by slight modifications of the physiological assay method of Marks [1926] as described by Burn [1928]. In some instances it was considered unnecessary to use groups of six rabbits, and the tests were made with groups containing three rabbits only. In some of the experiments, sugar determinations were carried out with every blood sample from each individual rabbit, instead of with the pooled samples as in the method of Marks, and in general, the assay method was modified to suit the particular experiment. The blood-sugar determinations were made with duplicate 0.2 ml. blood samples by the Hagedorn and Jensen [1923] method or on pooled samples by the modification used by Marks [1926].

¹ A preliminary report of this work was made in *Nature* (1934), 134, 290.

In the earlier experiments the reaction between phenyl *isocyanate* and insulin was allowed to proceed as in previous investigations, *i.e.* at 5–8° and in the presence of a phosphate buffer, but in later experiments the last-named was replaced by a 0.2 *M* solution of NaHCO_3 . Further details are given with the description of each typical experiment.

In some of the experiments, phenol (to give 0.25 %) was added as antiseptic to the various solutions injected, control tests having shown that this amount of phenol has no influence on the normal blood-sugar curve.

The insulin used for this work was supplied by Messrs Boots Pure Drug Co., Ltd., and had a potency of 19,500 units per g.

The action of phenyl isocyanate on insulin.

In the earlier experiments the conditions were similar to those previously described [Hopkins and Wormald, 1933, 1, 2]. Thus 18.5 mg. of insulin dissolved in 9 ml. of 0.9 % NaCl solution were added to 9 ml. of phosphate buffer of p_H 8, the mixture cooled in ice and treated with 0.75 ml. of phenyl *isocyanate*. The mixture was kept cool, and the reaction was maintained at p_H 7.5–8 by the addition of dil. NaOH . After the mixture had been shaken well for about 1 hour, very little *isocyanate* could be detected by smell, and the solution was centrifuged. The water-clear supernatant solution gave no precipitate on acidification, as do the solutions of the phenylcarbamido-compounds of other proteins so far investigated (serum-proteins, gelatin, caseinogen *etc.*), and indeed it appeared to contain little, if any, protein matter. This solution, and the precipitate which had been removed by centrifuging and which contained a very large proportion of diphenylurea, proved to be inactive as far as insulin activity was concerned.

In other experiments a stronger solution of insulin and less phenyl *isocyanate* were used (41 mg. of insulin, 5 ml. of 0.9 % NaCl , 5 ml. of phosphate buffer, with sufficient dil. NaOH to give a clear solution at p_H 9.5–10.0, and 0.5 ml. of phenyl *isocyanate*). The reaction was allowed to proceed for 1 hour and, as before, the precipitate which formed and the clear supernatant solution were without significant insulin activity. In another experiment with this smaller amount of phenyl *isocyanate*, the reaction mixture was centrifuged after 20 minutes; this precipitate and the faintly opalescent solution were also inactive.

In these tests the amount of solution or suspension injected was such that each rabbit received per kg. the treated material from 5–20 units of insulin, usually the higher amount. From the results of the blood-sugar determinations it could be concluded that in the experiments so far described at least 95 %, and probably more, of the activity of the insulin had been destroyed as a result of treatment with phenyl *isocyanate*.

Blood-sugar changes during 30 hours after the injection of phenylcarbamido-insulin.

One possible explanation of the inability of phenylcarbamido-insulin to lower the blood-sugar of starved rabbits might be that it is rendered very insoluble, and it was considered desirable, therefore, to determine whether large amounts of this material would exert a slow effect after injection. There is also the possibility that adsorption of the insulin or phenylcarbamido-insulin by the solid diphenylurea might decrease the rate of absorption and so cause little alteration of the blood-sugar level during the first few hours.

In order to test these hypotheses, phenylcarbamido-insulin was injected subcutaneously into rabbits, and blood-sugar determinations were made at

intervals over the first 5 or 6 hours and about 24 and 27–30 hours after the injection. The curves were then compared with curves given by suitable controls, in some cases rabbits which had received an injection of insulin only, and in others, animals which had been injected with an insulin solution containing in suspension approximately the same amount of diphenylurea as did the phenylcarbamido-insulin solution.

Table I. *Blood-sugar values of rabbits after the subcutaneous injection of phenylcarbamido-insulin.*

Rabbit No.	Subcutaneous injection of	No. of units per kg.	Blood-sugar (mg. per 100 ml.)					
			Before injection	Time after injection				
				2½ hours	3½ hours	5 hours	23 hours	27 hours
8	Insulin <i>plus</i> diphenyl-urea	0.75	112	75	66	87	109	113
11			107	83	87	79	110	104
13			104	80	86	75	100	106
		Average	108	79	80	80	106	108
9	Phenyl-carbamido-insulin	15	109	104	103	94	97	110
10			107	104	106	99	98	112
12			114	113	110	104	105	105
		Average	110	107	106	99	100	109

EXPERIMENTAL DETAILS.

Phenylcarbamido-insulin. 20.5 mg. insulin were dissolved in 2.5 ml. of 0.9 % NaCl and 2.5 ml. of 0.2 *M* NaHCO₃. One drop of 2 *N* NaOH was added to give a clear solution at *p*_H 8–8.5. The solution was cooled in ice, 0.25 ml. of phenyl isocyanate added and the mixture shaken well for about 1 hour, after which it was kept in the ice-chest until the following morning. No separation of precipitate and solution was made, and the whole mixture, after suitable dilution with 0.9 % NaCl solution, was injected into the rabbits.

Diphenylurea plus insulin. A similar amount of phenyl isocyanate was treated with distilled water and kept slightly alkaline, with constant stirring to break up the gummy mass which formed. On the following day the suspension was diluted to compare with the phenylcarbamido-insulin solution, and the necessary amounts of insulin and NaCl were added.

The results of a typical experiment of this nature are given in Table I, from which it will be seen that the phenylcarbamido-insulin preparation had no significant power to lower the blood-sugar even when observations were made over a much longer period of time. Thus an amount of treated insulin equivalent to about 30 times the dose recommended for standardisation purposes failed to give any very significant fall in the blood-sugar level. The very slight fall recorded after 5 hours is perhaps greater than that observed with most of the other samples of phenylcarbamido-insulin tested, but at the most it would indicate a retention of probably less than 3 % of the original insulin activity.

Experiments have also been carried out to determine the effect of intravenous injection of insulin inactivated by phenyl isocyanate. This treated insulin, and insulin *plus* diphenylurea, were injected intravenously into rabbits (groups of 3 each) in doses equivalent to 10 units and 0.5 unit per kg. respectively and blood-sugar determinations made on each rabbit at intervals during the first few hours. The former again showed no significant insulin activity, and thus it does not appear that the failure of phenylcarbamido-insulin to lower the blood-sugar is due to its slow absorption into the blood-stream.

The possibility that the insulin or phenylcarbamido-insulin is merely inactivated by adsorption on the solid diphenylurea can also be ruled out by these

experiments. A comparison of the blood-sugar-lowering power of insulin on the one hand, and of the insulin-diphenylurea on the other, when injected subcutaneously or intravenously showed that the presence of the solid diphenylurea causes very slight loss of insulin activity, and this slight loss might well be due to the presence of traces of unchanged phenyl isocyanate in the diphenylurea preparation.

Rate of inactivation of insulin by phenyl isocyanate.

Experiments have been carried out to determine how quickly phenyl isocyanate inactivates insulin, the reaction being stopped at intervals by the removal of the excess of phenyl isocyanate by rapid extraction with ether. In the first experiment a solution of 20.5 mg. of insulin in 2.5 ml. of 0.9 % NaCl plus 2.5 ml. of 0.2 *M* NaHCO₃, with dil. NaOH to give p_H 8-8.5, was cooled in ice and 0.25 ml. of phenyl isocyanate added. The mixture was shaken and a sample withdrawn immediately and others after 7 and 15 minutes. These samples were made slightly acid and rapidly extracted six times with about 10 ml. of ether at each extraction. They were then freed from ether by evacuation and neutralised with dil. NaOH. The second and third samples, when tested on rabbits in doses of 2.5-5 units per kg., had no significant insulin activity, but the first sample showed some activity.

In another experiment carried out under similar conditions, the following samples were taken: (a) from the insulin-NaHCO₃ mixture before addition of the phenyl isocyanate, (b) immediately after the mixing of the insulin solution with phenyl isocyanate, and (c) 10 minutes after the mixing. All the samples were treated with ether as before, sample (a) being a control to determine whether this treatment had any marked destructive action on insulin. The results of the animal experiments showed that sample (a) was practically as active as the untreated insulin, sample (c) was inactive, and sample (b) had slight activity only. From these experiments it is concluded that the action of phenyl isocyanate on insulin is quite rapid, and that a short time only is required for a considerable loss of insulin activity.

The action of p-bromophenyl isocyanate on insulin.

p-Bromophenyl isocyanate has been used, as in the previous investigations with other proteins, to determine the extent of the reaction, *i.e.* the number of new groupings introduced into the insulin molecule. The conditions of the experiments were similar to those for the phenyl isocyanate reactions.

Exp. 1. 100 mg. insulin were dissolved in 5 ml. 0.9 % NaCl plus 5 ml. 0.2 *M* NaHCO₃ and dil. NaOH to give p_H 8.5. A solution of 1 g. of *p*-bromophenyl isocyanate in ether was added to the shaken cooled solution, and after 1½ hours the mixture was centrifuged, yielding a slightly opalescent solution and a precipitate. This wet precipitate was treated with absolute alcohol, and after centrifuging, the solution gave on the addition of more absolute alcohol a precipitate (B₁). The original precipitate was extracted with 85 % alcohol, yielding an opalescent solution which gave a precipitate on standing. This last precipitate was washed with absolute alcohol and added to B₁, which was then dried *in vacuo* (wt. 37 mg.). It was then extracted with ether for 25 days in a Soxhlet apparatus, with occasional repowdering of the material to remove any di(*p*-bromophenyl)urea present. This dried product (about 30 mg.) contained 4.82 % Br (Roth).

Exp. 2. A similar experiment was carried out with 150 mg. insulin. The reaction mixture, on being centrifuged, gave a precipitate and an opalescent

solution which, in this but not in the first experiment, gave a precipitate (A_2) on acidification with dil. acetic acid. This last-named precipitate was dissolved in water with the aid of dil. NaOH and reprecipitated with dil. acetic acid, this solution and precipitation being repeated twice more to free the substance as far as possible from NaCl.

The wet precipitate from which the opalescent solution containing A_2 was originally separated was treated with absolute alcohol as in the previous experiment, and from the solution a precipitate (B_2) was obtained by the addition of more absolute alcohol. The residue was then extracted several times with 75 % alcohol, and a cloudy extract was obtained which gave with acetic acid a precipitate (C_2). One-fifth of the solution containing A_2 was kept for physiological tests, and the remainder was precipitated. This fraction and fractions B_2 and C_2 were dried *in vacuo*, the dry weights being 21 mg. (A_2), 56 mg. (B_2) and 78 mg. (C_2). These preparations were then extracted continuously with ether for 30 days, after which micro-determinations on the dried materials by Dr H. Roth gave Br 5.55, 4.97 and 22.2 % respectively. The very high figure for the last-named preparation (C_2) indicates the presence of a fairly considerable amount of di(*p*-bromophenyl)urea, a result anticipated in view of the fairly considerable amount of ether-extractable material originally present in this material. From their mode of preparation it seems justifiable to conclude that B_1 , A_2 and B_2 are *p*-bromophenylcarbamido-insulin preparations. In view of the rather drastic ether extraction no physiological tests were made with these dried compounds, but the solution containing A_2 reserved for tests of this nature showed no insulin activity when injected into rabbits in amounts equivalent to 15 units of original insulin per kg.

Further experiments have been carried out to determine whether any insulin activity is retained in any portion of the mixture when *p*-bromophenyl isocyanate acts on insulin. A solution of 20.5 mg. insulin (*i.e.* 400 units) in 2.5 ml. 0.9 % NaCl and 2.5 ml. 0.2 *M* NaHCO₃ and NaOH to give p_H about 8.5, was cooled and treated with 0.2 g. *p*-bromophenyl isocyanate dissolved in about 1 ml. of ether. The mixture was shaken as before for 1 hour and kept in the ice-chest until the next day when the diluted shaken suspension was injected into rabbits. As in the experiments with phenylcarbamido-insulin, a control experiment was carried out with insulin in a suspension of di(*p*-bromophenyl)-

Table II. *Blood-sugar values of rabbits after the subcutaneous injection of p-bromophenylcarbamido-insulin.*

Rabbit No.	Injection of	No. of units per kg.	Blood-sugar (mg. per 100 ml.)			
			Before injection	Time after injection		
				2 hours	3½ hours	5 hours
1	Insulin	0.75	131	61	78	94
2			117	45	50	79
3			110	70	66	102
		Average	119	59	65	92
5	Insulin <i>plus</i> dibromophenylurea	0.75	110	73	87	98
7			131	62	61	99
8			131	75	82	99
		Average	124	70	77	99
10	<i>p</i> -Bromophenyl-carbamido-insulin	30	130	121	113	126
12			120	120	121	116
14			119	100	115	102
		Average	123	114	116	115

urea, the insulin being added to the suspension just prior to the injection of the mixture.

The results of an experiment of this nature (Table II) show that, as a result of treatment with *p*-bromophenyl isocyanate, insulin loses the whole of its power to reduce the blood-sugar of fasting rabbits. As in the previous experiments, the mere presence of a precipitate, in this instance di(*p*-bromophenyl)urea, does not cause any appreciable loss of activity in an insulin solution.

DISCUSSION.

The experiments described here have shown that phenyl isocyanate has a very marked inactivating action on insulin, as far as the hypoglycaemic function of this hormone is concerned. Insulin behaves, therefore, like other proteins in reacting with this reagent, and from the results of previous investigations [Hopkins and Wormall, 1933, 1, 2; 1934] it is concluded that the phenyl isocyanate reacts with the free amino-groups to form phenylcarbamido-groupings, as follows:

This evidence is based on chemical and immunological studies with the phenylcarbamido-compounds of serum-globulin, gelatin, caseinogen and similar proteins, and with these other proteins the reaction is concerned mainly, if not entirely, with the ϵ -amino-groups of the lysine groupings. In the case of insulin, however, as will be discussed later, the lysine content is only sufficient to account for a fraction of the total free amino-N, and thus phenylcarbamido-insulin contains phenylcarbamido-groups in addition to those attached to lysine.

It is necessary in the first place, with a reaction of this type and when dealing with such a comparatively labile substance as insulin, to establish as definitely as possible that the inactivation is really due to phenyl isocyanate before deductions can be made about the nature of the inactivation. Absolute proof of this is very difficult to obtain, but from the nature of the reaction, and from the evidence previously presented, it appears unlikely that the inactivation can be due to any other agency. The reaction takes place very readily in neutral or faintly alkaline medium (p_H 7–8) and at relatively low temperatures (5–8°), and therefore inactivation due to the action of acid or alkali or to a rise in temperature can be excluded. A rather remote possibility is that the inactivation is brought about by some other substance present in all the samples of phenyl isocyanate used, an impurity which might effect inactivation by oxidation, reduction or some similar process, but this hypothesis appears very unlikely. The inactivating agent is destroyed by water, for the phenyl isocyanate lost its power to inactivate insulin when left in contact with water for some time, *i.e.* sufficient time for the phenyl isocyanate to be converted into diphenylurea. The inactivating agent is ether-soluble and is present in redistilled preparations of another isocyanate (*p*-bromophenyl isocyanate). On these grounds, the conclusion is reached that the isocyanate is the inactivating agent, and it is now possible to consider the changes which such a reagent might effect in the insulin molecule. All our previous investigations have furnished no evidence of any change other than the conversion of free amino- into phenylcarbamido-groups, and it seems probable that this change is the one which causes the inactivation of the insulin. From this it would appear that the free amino-groups of the insulin molecule are essential for its activity, a view which is in agreement with the tentative suggestion made by Jensen [1932].

It is not possible at the present time, partly because of the large amount of expensive material such an investigation would require, to say how many amino-groups of the insulin must be "blocked" before inactivation occurs. Some experiments described in this paper indicate, however, that the process of inactivation is very rapid. An excess of the *isocyanate* was used in these experiments, but it was not anticipated that the insulin would be inactivated so quickly. The reaction was stopped as quickly as possible by repeated ether extractions to remove the remaining *isocyanate*, and even when allowance is made for the fact that after the first, or even the second ether extraction there will still be an appreciable amount of phenyl *isocyanate* in the insulin solution, it appears that a short time only is required for complete loss of insulin activity to occur. Under the conditions of the experiments described here, complete inactivation probably occurs in less than 10 minutes.

The reaction between phenyl *isocyanate* and insulin has also been studied recently by Jensen, Evans and Schock, in an endeavour to obtain more information about the free amino-groups of insulin. These investigations, reported as unpublished results by Jensen and Evans [1934], have yielded results similar to those recorded in this paper. The suggestion was made by these authors that the action of phenyl *isocyanate* might be concerned with the hydroxyl as well as the amino-groups, but no evidence of participation by the former groups, under the normal conditions of the reaction, has been observed in our own investigations. Jensen, Evans and Schock have made further studies with the inactivated material, phenylcarbamido-insulin. From the products of acid hydrolysis the phenylhydantoin of phenylalanine has been separated and identified, and the corresponding naphthylhydantoin has been isolated from naphthylcarbamido-insulin (private communication from Drs Evans and Jensen). These results are of special interest in view of the fact that phenylalanine has not previously been found in crystalline insulin.

This discussion of the significance of the amino-groups of insulin is, of course, incomplete without reference to the action of nitrous acid. This reagent readily inactivates insulin [Scott, 1925; Blatherwick *et al.*, 1927], but it is not definitely established that the inactivation is due to the reaction with free amino-groups. Blatherwick *et al.*, for example, suggest that the destructive action of nitrous acid is probably due to oxidation, and Freudenberg *et al.* [1931] state that the amino-N of insulin can be destroyed by this acid without complete loss of insulin activity.

In the work described in this paper it was considered that some idea should be obtained as to the number of new groups introduced into the insulin molecule, and as in previous studies, use has been made of *p*-bromophenyl *isocyanate*. From the bromine contents of these preparations of *p*-bromophenylcarbamido-insulin (4·8, 5·5 and 5·0 %) it has been calculated that the reaction with the amino-groups is practically complete. With a value of 1·00 % for the free amino-N of insulin [Freudenberg *et al.*, 1931], complete reaction of all the free amino-groups with *p*-bromophenyl *isocyanate* would give a product containing 5·0 % Br. Since lysine is present in insulin to the extent of about 2 % only [Jensen and Wintersteiner, 1932] this amino-acid cannot account, assuming that one amino-group of each lysine molecule is free, for more than about one-fifth of the total free amino-N. From the work of Jensen, Evans and Schock, mentioned above, it seems probable that a much larger amount is present in the phenylalanine groupings, but even this is not sufficient to account for the whole deficiency (private communication from the authors).

SUMMARY.

1. Phenyl *isocyanate* readily destroys the power of insulin to reduce the blood-sugar of fasting rabbits.

2. This inactivation, carried out at p_H 7–8 and at 5–8°, is very rapid and is probably complete in a few minutes.

3. From previous chemical and immunological investigations with other phenylcarbamido-proteins it is concluded that the phenyl *isocyanate* reacts with the free amino-groups of the insulin. These groups appear, therefore, to be essential for the characteristic hypoglycaemic power of insulin.

4. *p*-Bromophenyl *isocyanate* also rapidly inactivates insulin, and the bromine contents of three preparations of *p*-bromophenylcarbamido-insulin confirm the view that the reaction occurs between the *isocyanate* and all the free amino-groups of the insulin.

5. These results are discussed in the light of other investigations on the chemistry of insulin.

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