CCLXIX. THE ACTION OF PHENYL ISOCYANATE ON INSULIN.

II. FURTHER OBSERVATIONS ON THE CHEMISTRY OF INSULIN AND ITS PHOSPHATE- LOWERING POWER.

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HOPKINS & WORMALL [1934, 2] found that the action of phenyl isocyanate on insulin caused complete, or almost complete, loss of hypoglycaemic activity very rapidly at pH 8 and at 5–8°. Previous chemical and immunological investigations on various phenylcarbamido-protein derivatives [Hopkins & Wormall, 1933, 1, 2; 1934, 1] indicate that phenyl isocyanate probably reacts with free amino-groups and causes no other very drastic change in the protein molecule. The conclusion was reached, therefore, that the free amino-groups of insulin, or at least some of them, are essential for its activity. Jensen *et al.* [1934] had simultaneously reached the same conclusion [cf. also Jensen & Evans, 1935].

Various authors have recorded other changes in the blood of animals following insulin injection. Thus Wigglesworth *et al.* [1923] observed a fall in the blood inorganic phosphate of rabbits, and Briggs *et al.* [1923–24] found a decrease in the blood glucose, potassium and inorganic phosphate and a rise in the blood lactic acid of dogs. Luck *et al.* [1928] have recorded a fall in the blood amino-acid, and other authors have found increases in blood lactic acid and calcium [cf. Peters & Van Slyke, 1931]. Rigo & Frey [1934] found a diminution in blood creatine and creatinine after insulin injection.

Some of these changes, e.g. those in lactic acid and phosphate and, less directly, calcium, appear to be closely connected with the fall in the blood sugar level. (A review of the literature dealing with the relationship between phosphates and carbohydrate metabolism is given by Peters & Van Slyke [1931, pp. 1114–1118], and more recent work is discussed by Cori & Cori [1934, p. 165].)

From this and other evidence it seems probable that the hypophosphataemic capacity of insulin is secondary to its influence on carbohydrate metabolism, and that insulin has no specific direct action on the blood phosphate. Davis *et al.* [1933], however, have recorded that the phosphate-lowering activity of insulin was more resistant to acid alcohol and soft X-radiation than was the sugar-lowering power, and they conclude that insulin owes its activity to a number of "active groups" which vary in stability towards inactivating agents. Savino [1924] found that when glucose is injected to maintain the normal blood sugar level in a fasting sheep after the injection of insulin, there is still a fall in the blood inorganic phosphate.

We have ourselves carried out experiments to determine whether this differentiation could be effected by some other inactivating agent, since confirmation of the results of Davis *et al.* [1933], by some other method, would demonstrate fairly conclusively the independence of these two activities of insulin. For this

purpose, further use has been made of the inactivation by phenyl isocyanate, this reagent being most suitable in view of the mild conditions of the reaction and the relatively specific action of the reagent for one type of grouping.

The action of phenyl isocyanate on insulin and other proteins has been investigated further from the chemical standpoint. In particular, the possibility of a reaction with the hydroxyl group of tyrosine, or with basic groups other than the α -amino-groups of terminal amino-acids and the ϵ -amino-groups of lysine molecules, has been considered. Where the results of these other investigations are applicable to insulin they are discussed below.

EXPERIMENTAL.

In most experiments the inactivation of insulin by phenyl isocyanate and the determination of the hypoglycaemic actions of treated and untreated insulin were carried out as described previously [Hopkins & Wormald, 1934, 2]. Rabbits which had been starved for 24–36 hours were used for all the assays, and the blood inorganic phosphate determinations were made by the method of Fiske & Subbarow [1925]; 0.25 % phenol was generally added to the insulin and NaCl solutions injected. The volume of solution injected into each rabbit was 0.5 ml. per kg.

The insulin was supplied by Messrs Boots Pure Drug Co. Ltd., and had an activity of 19,500 units per g.

Normal fluctuations in the blood inorganic phosphate of rabbits.

In many experiments there appeared to be significant diurnal fluctuations of the blood inorganic phosphate; the level generally rose during the experiment, with a partial return to the original level later in the day and a more or less complete return in 24 hours (cf. Table I).

Table I. *Fluctuations in the blood inorganic phosphate of rabbits.*

		(mg. P per 100 ml. blood.)					
		Time after first bleeding (hours)					
	Rabbit	0	2	4½	24	28	29½
Exp. 1	S 7	2.01	2.27	2.60	2.44	—	3.44
	S 8	2.77	2.66	2.98	3.16	—	3.88
	C	2.68	3.13	3.03	2.65	—	3.31
	D	2.79	3.19	3.08	2.60	—	3.59
	Average	2.56	2.81	2.92	2.71	—	3.55
Exp. 2	Average for 8 rabbits	2.92	3.17	3.38	3.16	3.42	—

During these experiments there was no significant alteration in the blood sugar level, and it has not been found possible as yet to account fully for the fluctuations in the phosphate. As the following experiment will indicate, they do not appear to be related to the water intake of the rabbits during the experiment. One group of four rabbits was allowed water *ad lib.* for 2 days before, and during, the 6 hours of the experiment and another group was given no water during the whole of this period. The water consumed by the rabbits in the first group varied from practically nothing to a considerable volume, but all the rabbits showed very similar fluctuations throughout the day. The blood

inorganic phosphate figures for two experiments were as follows (average values, as mg. P per 100 ml. blood, for each group):

	Time (hours) ...	0	3	6
Exp. 1	Group A	3.67	4.24	3.58
	Group B	2.82	3.28	2.75
Exp. 2	Group A	2.67	2.77	2.80
	Group B	3.16	3.43	3.24
Group A, given water.		Group B, given no water.		

From these figures, and from those of other experiments, the water intake does not appear to influence the blood inorganic phosphate, but it must be emphasized that the rise does not invariably occur. Thus in some experiments only a very slight rise, or even none at all, took place, as will be seen from the second experiment mentioned above and from one of the experiments described below (cf. Table III).

These normal fluctuations must, of course, be considered when the effect of insulin on the blood inorganic phosphate is studied, and in all the investigations reported here a control group of rabbits fed on the same diet and under exactly the same experimental conditions has been examined. In connexion with this fluctuation in the blood phosphate it is of interest to note that Havard & Reay [1925] found that there is often, but not invariably, a rise in the blood inorganic phosphate of man during the day, and on one occasion the midday value was 16% above the morning level. No satisfactory explanation for these variations could be advanced by these authors. In our experiments it seems possible that the withdrawal of 4 ml. of blood from each rabbit at each bleeding might significantly affect the inorganic phosphate level of the blood, but this explanation would not account for the rise observed by Havard and Reay, who withdrew each time less than 1 ml. of blood from the subject. Some observations on the influence of haemorrhage on the blood calcium of rabbits are given by Culhane [1927; 1930] who noted a fall with most of the animals, and it is not inconceivable that this fall in calcium and the rise in inorganic phosphate are in some way interrelated.

The phosphate-lowering power of the phenylcarbamido-derivative of insulin.

A solution of insulin in dilute NaHCO_3 and cooled to $5-8^\circ$ was treated with small amounts of phenyl isocyanate, and the reaction maintained at pH 8-8.5 as previously recorded [Hopkins & Wormall, 1934, 2]. The whole mixture was

Table II. *Influence of phenyl isocyanate on the hypophosphataemic power of insulin.*

(Average values for groups of 4 rabbits—results as mg. inorg. P or sugar per 100 ml. of blood.)

	Control		Insulin (0.75 unit per kg.)		Phenylcarbamido- insulin (15 units per kg.)	
	Time (hours)	Inorg. P	Inorg. P	Sugar	Inorg. P	Sugar
Exp. 1	0	2.82	2.85	97	2.65	101
	2	3.38	2.44	45	3.21	108
	4½	3.38	2.66	—	3.09	—
	24	2.98	3.00	—	2.96	—
	28	3.36	3.66	—	3.31	—
Exp. 2	0	3.22	2.91	102	3.43	90
	3½	3.25	2.25	53	3.52	95
	6	3.29	2.48	71	3.58	112

kept overnight, and was then tested, together with untreated insulin, for hypoglycaemic and hypophosphataemic actions in starved rabbits. The treated insulin was used in amounts equivalent to twenty or thirty times the quantity of unchanged insulin normally used for standardization tests. The experiments made under different conditions and with varying amounts of the treated insulin, gave similar results, and all indicated that where loss of the hypoglycaemic activity occurred, there was also loss of hypophosphataemic activity (Table II). As in previous experiments the control animals received injections of a suspension of diphenylurea, a substance which is present in relatively large amounts in the preparations of phenylcarbamido-derivative of insulin used.

Further experiments have been carried out to determine whether it is possible, by using a smaller amount of phenyl isocyanate or by stopping the reaction at different stages, to distinguish between the hypoglycaemic and hypophosphataemic activities of insulin. Table III records an experiment of this type, in which the amount of isocyanate used was less than previously and in addition the reaction was stopped at certain intervals, as far as this was possible, by extraction with ether to remove the excess of phenyl isocyanate. The results indicated a rough parallelism between the two activities of insulin, and there was no evidence of any preferential destruction of the hypoglycaemic or hypophosphataemic power. In several of these experiments it has been noticed that the injection of phenylcarbamido- or *p*-bromophenylcarbamido-derivatives of insulin has been followed by an unexpected slight rise in the blood sugar, whereas the control animals did not show this phenomenon. This rise may be due to some indirect action of the phenylcarbamido-derivative or to the presence in the original insulin of a hyperglycaemic principle [cf. Bürger & Kramer 1930; Dirscherl, 1931]. This hyperglycaemic substance appears to be more resistant to phenyl isocyanate than is the hypoglycaemic hormone.

Table III. *Influence of insulin, which has been partially inactivated by phenyl isocyanate, on the blood inorganic phosphate of starved rabbits.*

(Average values for groups of 4 rabbits.)

	Time after injection (hours)	Control	Insulin (0.75 unit per kg.)	Phenylcarbamido-insulin	
				1st product (5 units per kg.)	2nd product (15 units per kg.)
Blood inorg. P (mg. per 100 ml.)	0	4.05	3.02	2.68	2.79
	2	4.06	2.32	2.07	2.16
	4½	4.23	2.90	2.24	2.57
Blood sugar (mg. per 100 ml.)	0	—	106	100	96
	2	—	59	67	49
	4½	—	45	58	48

Experimental details. 32.8 mg. insulin (640 units) were dissolved in 4 ml. of 0.9% NaCl plus 4 ml. of 0.2 *M* NaHCO₃. 2 *N* NaOH was added to give pH 8-8.5, and the solution cooled in ice. After a sample (2 ml.) had been withdrawn, 0.08 ml. of phenyl isocyanate was added to the main bulk and the mixture shaken. Another 2 ml. were taken immediately ("1st product" in the above table), and the remainder was shaken well for 15 min., the solution being kept cool and maintained at pH 8-8.5 ("2nd product").

Immediately after it had been taken, each sample was treated with 0.05 ml. of 2 *N* HCl (double quantity for the last sample) and at once extracted with ether to remove the excess of phenyl isocyanate. Six successive extractions with 10 ml. of ether at each extraction were made, and after the addition of 0.05 ml. of 2 *N* NaOH the aqueous solution was freed from ether by gentle warming and evacuation. These solutions were then adjusted to about pH 8, and before being used for injection, phenol was added to them to the extent of 0.25%.

A chemical study of the groups in insulin which might react with phenyl isocyanate.

In previous work [Hopkins & Wormald, 1933, 1; 1934, 1] evidence has been adduced that the main reaction between phenyl isocyanate and proteins is that between the isocyanate and the free amino-groups. With native proteins the reaction is possibly confined to the ϵ -amino-groups of the lysine molecules, but with proteins of the peptone type, whose phenylcarbamido-derivatives were investigated by Raper [1907], or with insulin, the lysine groupings appear to account for a fraction only of the total free amino-nitrogen. From the chemical viewpoint it seems of importance therefore to study (a) the free amino-groups of the insulin molecule which are attacked by phenyl isocyanate, i.e. to find out if possible the amino-acids in insulin which have free amino-groups, and (b) the possibility of a reaction between the isocyanate and groups in insulin other than free amino-groups. It is with the latter problem that we are mainly concerned in this paper.

It is rather difficult to decide, without further investigation, which groups in a protein are likely to react with aryl isocyanates or undergo some modification during the reaction, but evident possibilities are: the hydroxyl group of tyrosine, the disulphide group of cystine, the sulphhydryl group of cysteine, the iminazole group of histidine, the guanidine group of arginine, the pyrrolidine group of proline and the acid-amide groups of asparagine and glutamine. Phenyl isocyanate was caused to react with these amino-acids as far as possible under conditions similar to those employed in the preparation of phenylcarbamido-derivatives of proteins. The amino-acid was dissolved in phosphate buffer at pH 8 or in 0.2M NaHCO₃, and phenyl isocyanate (1.4 mol. for each group in the amino-acid which might conceivably react with the isocyanate), or the corresponding amount of *p*-bromophenyl isocyanate dissolved in ether, was added to the stirred cooled solution. After 2½–4 hours, this period being longer than that allowed for the preparation of phenylcarbamido-proteins and very considerably longer than that necessary for the inactivation of insulin by phenyl isocyanate, the mixture was centrifuged. (This prolongation was made in order to ensure maximum reaction of the isocyanate with the various amino-acids.) In most cases the phenylcarbamido-acid was precipitated from the supernatant solution by dil. H₂SO₄, but in some instances this method had to be modified (see below).

Certain of these phenylcarbamido-derivatives have been converted into the corresponding hydantoins. These hydantoins were prepared in connexion with investigations on the products obtained by the acid hydrolysis of phenylcarbamido-derivatives of insulin and other proteins, and where the analytical data obtained offer confirmatory evidence for the identification of the phenylcarbamido-derivative, a brief description of the preparation is given.

Tyrosine. Gumpert [1885] showed that the reaction between phenyl isocyanate and phenols proceeds to a slight extent in the cold but more completely on the water-bath. Working at higher temperatures, Goldschmidt & Meissler [1890], Dieckmann *et al.* [1904] and Michael [1905] have obtained similar reactions with phenol, phloroglucinol and hydroresorcinol but from the work of these authors no significant reaction of this type appears to take place at ordinary temperatures.

In our experiments there has been no suggestion of any reaction between phenyl isocyanate and the phenolic group of tyrosine, even when a large excess of the isocyanate is used. Under the usual conditions for the preparation of

phenylcarbamido-acids, phenyl and *p*-bromophenyl isocyanates react with tyrosine to give monosubstituted compounds whose N and Br contents agreed with the formulae

[Hopkins & Wormall, 1934, 1].

On account of the low solubility of tyrosine it was not possible to reproduce exactly the same conditions for the preparation of its phenylcarbamido-derivatives as for those of proteins and all other amino-acids used. In the case of tyrosine it is necessary to start with a more alkaline medium, but after the reaction has proceeded for a short time the *pH* is lowered considerably. The yields of the tyrosine derivatives were similar to those obtained in the preparation of the corresponding derivatives of the simple amino-acids (glycine and alanine), and it does not seem probable that any other product is formed in significant amounts.

The phenyl- and *p*-bromophenyl-carbamido-derivatives of tyrosine give well-marked Millon's reactions, and they also give red or black pigments when subjected to the action of tyrosinase, these reactions being indicative of a phenolic grouping [cf. Raper, 1928]. The red pigments produced by the action of the enzyme on the phenyl- and *p*-bromophenyl-carbamido-derivatives are much more stable than is the corresponding pigment from tyrosine, and in this respect these substances appear to behave like tyramine and 3:4-dihydroxyphenylethyl-methylamine (epinine), which give more stable red pigments than does tyrosine [Dulière & Raper, 1930].

In all these enzymic experiments, the *pH* of the medium was controlled, the reactions being carried out at *pH* 6, 7 or 8 in the presence of a phosphate buffer.

These experiments appear to offer satisfactory evidence that in the inactivation of insulin by phenyl isocyanate there is no reaction between the latter and the hydroxyl groups of the tyrosine groups in insulin. This is of special interest in view of the recent work of Harington & Neuberger [1936], in which evidence has been obtained that "the phenolic groups of insulin are of importance in relation to its physiological activity". Jensen *et al.* [1936] also discuss the significance of phenolic groups in reference to the hypoglycaemic power of insulin.

Cysteine and cystine. The cystine derivatives have been made previously [Hopkins & Wormall, 1934, 1] and no evidence was obtained of any change in the disulphide linkage.

The preparation of the cysteine derivatives has presented more difficulty on account of the relative insolubility of their sodium salts. Ultimately, however, by the use of a much larger volume of bicarbonate solution, the phenylcarbamido-derivative of cysteine was made in the following manner: 2 ml. (2.8 mol.) of phenyl isocyanate were added to a cooled solution of 1 g. (1 mol.) of cysteine hydrochloride in 100 ml. of 0.2 *M* NaHCO₃ with the *pH* adjusted and maintained at about 8. The mixture was stirred for about 2 hours and filtered. The clear filtrate was acidified with 2*N* HCl and the precipitated product immediately recrystallized thrice from dilute alcohol, giving needles or long prisms, *m.p.* 135–136° (decomp.). The yield of the pure product was 1.6 g. (Found (Roth): N, 10.86; S, 7.92%. Calc. for C₁₇H₁₇O₄N₃S, C₂H₅OH: N, 10.37; S, 7.90%). After being heated at 104° for 4½ hours the product (*m.p.* 139–140°, decomp.) contained N, 11.90; S, 8.88%. Calc. for C₁₇H₁₇O₄N₃S: N, 11.70; S, 8.91%.

The analytical figures indicated that both the amino-group and the SH group of cysteine had been blocked by phenyl isocyanate. The compound gave a very faint nitroprusside reaction due apparently to the action of the NH_3 , since other tests have shown that the phenylcarbamidothio-linkage is alkali-labile.

In view of the importance attached by many authors to the sulphur-containing groupings of insulin [cf. Jensen & Evans, 1934] other experiments have been carried out to determine whether these groups are acted upon by phenyl isocyanate. In a typical experiment of this type, phenyl isocyanate (2.8 mol.) was added to cooled solutions of cysteine and of cystine (1 mol.) in NaHCO_3 at pH 8. The mixtures were shaken for 2 hours and the nitroprusside test for SH groups was carried out at intervals on small samples of the mixtures and on control solutions of cysteine and cystine in NaHCO_3 . The cystine preparation gave no colour with the nitroprusside at any time: the solution containing cysteine showed rapid diminution of the nitroprusside reaction and after $\frac{1}{2}$ hour nearly all the SH groups had been removed.

This rapid action of phenyl isocyanate on SH groups is interesting, particularly as it takes place under such mild conditions. The authors could find no reference in the literature to such a reaction, although Snape [1885] had prepared the phenyl isocyanate derivative of phenylmercaptan by prolonged heating of a mixture of the two compounds. Further confirmation of this reaction between SH groups and phenyl isocyanate was obtained by a study of the action of the isocyanate on thiolacetic and α -thiolpropionic acids.

α -Thiolpropionic acid (1 mol.) in NaHCO_3 solution at pH 8 was treated with phenyl isocyanate (1.4 mol.) under the normal conditions. After 1 hour, the mixture was filtered and the clear filtrate acidified with conc. HCl to precipitate a white crystalline solid. (Yield 80 %.) The product was recrystallized three times from dilute alcohol, yielding small needles or prisms, m.p. $140\text{--}141^\circ$ (decomp.). (Found (Roth): N, 6.44; S, 14.43 %. Calc. for $\text{C}_{10}\text{H}_{11}\text{O}_3\text{NS}$: N, 6.23; S, 14.22 %.)

The thiolacetic acid compound was prepared in a similar manner in 80 % yield. It was recrystallized from dilute alcohol as long thin prisms, m.p. $144\text{--}146^\circ$ (decomp.). (Found (Roth): N, 6.64; S, 14.84 %. Calc. for $\text{C}_9\text{H}_9\text{O}_3\text{NS}$: N, 6.64; S, 15.18 %.)

Proline. The phenylcarbamido-derivative of proline has been described by Fischer [1901]. In the present investigation, the *p*-bromophenylcarbamido-derivative was obtained by a slight modification of the usual technique, the yield of crude product being about 70 % of the theoretical. On recrystallization from dilute alcohol it gave colourless long plates or prisms, m.p. 169° (decomp.). (Found (Roth): Br, 25.41; N, 9.04 %. Calc. for $\text{C}_{12}\text{H}_{13}\text{O}_3\text{N}_2\text{Br}$: Br, 25.56; N, 8.96 %.)

Histidine. Histidine dihydrochloride (1 mol.) was treated with an ethereal solution of *p*-bromophenyl isocyanate (4.2 mol.) under the usual conditions and the mixture centrifuged after $2\frac{1}{2}$ hours. The precipitate was washed several times with water containing a little Na_2SO_4 and sufficient NaOH to give pH 8, and the washings were added to the supernatant solution previously obtained. This solution was evaporated to small bulk *in vacuo*, and treated with conc. and finally $2N$ H_2SO_4 to give maximum precipitation of a white crystalline material. This preparation was dried *in vacuo*. Yield 1.9 g. from 1.2 g. of histidine dihydrochloride. Recrystallized twice from hot water it gave colourless plates, m.p. $177\text{--}178^\circ$ (decomp.). For analysis, the substance was heated at $102\text{--}103^\circ$ for 4 hours. (Found (Roth): N, 15.56; Br, 22.04 %. Calc. for $\text{C}_{13}\text{H}_{13}\text{O}_3\text{N}_4\text{Br}$: N, 15.87; Br, 22.63 %.)

Arginine. Some difficulty has been experienced in isolating the pure phenyl-

and *p*-bromophenyl-carbamido-derivatives of arginine, and from the results so far obtained it appears probable that when phenyl- and *p*-bromophenyl-isocyanates act on arginine mixtures of mono- and di-derivatives are produced.

Further work is being carried out to determine to what extent, and how rapidly, phenyl isocyanate reacts with the guanidino-group of arginine and with the same group of arginine-containing proteins. The evidence so far available indicates that in the case of arginine even when a large excess of the isocyanate is used, this reaction is less rapid and less complete than that involving the α -amino-group. In the reaction between the isocyanates and insulin (and other proteins), under the conditions used in this and previous investigations, it is believed that no significant change occurs in the guanidino-grouping, but further work will be necessary before this can be definitely established.

Asparagine. Phenyl isocyanate and asparagine under the usual conditions yielded a phenylcarbamido-derivative. Yield (of crude product) 70% of theoretical. On recrystallization from alcohol it gave prisms, m.p. 163° (decomp.). (Found (Roth): N, 16.50%. Calc. for $C_{11}H_{13}O_4N_3$: N, 16.73%.)

When heated on a water-bath with 5*N* HCl for 6 hours, NH_3 was split off and phenylhydantoinacetic acid was obtained, m.p. 231–233°. (Found (Roth): N, 12.13%. Calc. for $C_{11}H_{10}O_4N_2$: N, 11.96%.)

The *p*-bromophenylcarbamido-derivative, recrystallized twice from alcohol as fine needles, contained 1 mol. of alcohol of crystallization, m.p. 175–176° (decomp.). (Found: Br, 21.18%. Calc. for $C_{11}H_{12}O_4N_3Br$, C_2H_6O : Br, 21.25%.) (After being heated at 102° for 2 hours, this product gave N, 12.68% (Roth). Calc. for $C_{11}H_{12}O_4N_3Br$: N, 12.73%.)

On treatment with hot 5*N* HCl, as above, a good yield of *p*-bromophenylhydantoinacetic acid was obtained. This hydantoin was recrystallized twice from alcohol and gave fine needles with m.p. 220°. (Found after heating at 102–103° for 3 hours (Roth): N, 8.94; Br, 24.91%. Calc. for $C_{11}H_9O_4N_2Br$: N, 8.99; Br, 25.53%.)

In these preparations, as in those of the glutamine derivatives, excess of phenyl- or *p*-bromophenyl-isocyanate was used (2.8 mol. of isocyanate per mol. of asparagine or glutamine). Even with this excess, there appears to be no reaction under the conditions of these experiments between the isocyanate and the acid-amide group.

Glutamine. The phenylcarbamido-derivative of synthetic glutamine [Bergmann *et al.*, 1933] was prepared in the usual manner. The washings from the precipitate and the original supernatant solution were combined and concentrated *in vacuo* at the ordinary temperature. The product was precipitated by H_2SO_4 and the yield of crude substance was about 70%. Two recrystallizations from alcohol gave needles, m.p. 161° (decomp.). (Found (Roth): N, 14.50%. Calc. for $C_{12}H_{15}O_4N_3$: N, 15.85%.)

The low N content of this preparation is most probably due to a loss of amide-N during recrystallization.

The corresponding *p*-bromophenylcarbamido-derivative was obtained as small needles after two recrystallizations from alcohol, m.p. 189°. (Found (Roth): N, 12.08; Br, 22.88%. Calc. for $C_{12}H_{14}O_4N_3Br$: N, 12.21; Br, 23.22%.)

On treatment with hot HCl, the phenylcarbamido-derivative yielded phenylhydantoinpropionic acid. Long prisms from water; m.p. 160–161°. (Found (Roth): N, 11.63%. Calc. for $C_{12}H_{12}O_4N_2$: N, 11.29%.)

The hydantoin obtained from the corresponding bromo-derivative gave long prismatic crystals (from alcohol), m.p. 200–201°. (Found (Roth): N, 8.49; Br, 24.57%. Calc. for $C_{12}H_{11}O_4N_2Br$: N, 8.56; Br, 24.43%.)

DISCUSSION.

The primary object of the earlier part of the work described in the present paper was to find out whether differentiation of hypoglycaemic and hypophosphataemic powers of insulin similar to that described by Davis *et al.* [1933] could be obtained by some other method. For reasons mentioned above, phenyl isocyanate was used as the inactivating agent.

Typical results such as are given in Tables II and III, have failed to demonstrate any significant difference between these two activities of insulin. Thus the inactivation by phenyl isocyanate of the hypoglycaemic activity of insulin, an inactivation which appears to involve principally, if not entirely, the free amino-groups of the insulin [Hopkins & Wormald, 1934, 2; Jensen *et al.* 1934], has in all our experiments been accompanied by a corresponding inactivation of the hypophosphataemic power. This parallelism between the two inactivation processes has been observed when varying amounts of phenyl isocyanate have been used and also when the reaction between the insulin and the isocyanate has been stopped, as far as this is possible, at different stages.

These results do not, of course, offer any strong evidence against the view of Davis *et al.* that insulin owes its multiple activity (i.e. power to reduce the blood glucose, phosphate and amino-acid) to a number of "active groups", "centres" or "units" which vary in stability towards inactivating agents. It appears highly probable that even if there are two or more activities associated with different parts of the insulin molecule, differentiation between these activities will not be possible with every inactivating agent. Numerous failures to demonstrate a difference by other methods will not suffice, therefore, to destroy the value of a single well-established differentiation. It seems highly desirable, however, that confirmation of the results of Davis *et al.* should be obtained, either by the use of the same methods which they used, or preferably by some other method of inactivation. As far as the present authors are aware, there is little or no further evidence that the hypophosphataemic action of insulin is due to anything other than the change which the insulin effects in the carbohydrate metabolism of the body.

The previously mentioned observations of Savino [1924] appear to show that the injection of insulin might cause a fall in the blood phosphate even if the normal blood sugar level is maintained by the injection of glucose; this result however might well be explained as follows. The injected insulin causes an acceleration of tissue oxidative and synthetic changes involving glucose, and this leads to the withdrawal of inorganic phosphate from the blood. Under normal circumstances therefore the lowering of the blood sugar and the reduction in blood phosphate following the injection of insulin should be closely related, but the maintenance of the blood sugar at its normal level, by the injection of glucose, would not prevent the reduction in the phosphate value.

The main conclusion which can be drawn from the experiments described in this paper is that phenyl and *p*-bromophenyl isocyanates destroy the hypophosphataemic power of insulin just as readily as they do the hypoglycaemic activity. Inactivation by these reagents appears to be due to interaction with the free amino-groups of the insulin, and thus these groups appear to be essential components of both hypoglycaemic and hypophosphataemic factors or "centres" of the hormone.

In the second part of this paper, further efforts to determine whether the action of phenyl and *p*-bromophenyl isocyanates on insulin and other proteins is limited to the free amino-groups are described. Experiments have been

carried out with various amino-acids to find out whether phenolic groups and basic groups, other than α -amino-groups and the ϵ -amino-groups of lysine, are attacked by these *isocyanates*. In all these tests, a considerable excess of *isocyanate* was used, but no evidence was obtained that, under the conditions used for the inactivation of insulin and for the preparation of other phenylcarbamido-protein derivatives, there is any reaction with the following groups; the hydroxyl group of tyrosine, the acid-amide groups of glutamine and asparagine or the iminazole group of histidine. Experiments made with cystine appear to show that no significant change occurs in the S—S group of this amino-acid. In the case of cysteine however rapid condensation with the SH group occurs, but since insulin gives a negative nitroprusside reaction and does not appear to contain free SH groups [cf. Schock *et al.* 1935; Jensen *et al.* 1936], reaction with this group cannot be a contributory cause of the inactivation of the hormone by phenyl *isocyanate*. The tests made with phenyl *isocyanate* and arginine show that there may be some interaction with the guanidino-group, but this reaction is probably not as rapid or as complete as the reaction with α -amino-groups or the ϵ -amino-group of lysine. This partial reaction with the guanidino-group of arginine offers special interest in view of the marked hypoglycaemic effect of certain guanidine derivatives, and further work is being carried out in this connexion. From the evidence available at the present time, it does not appear probable that a change in the guanidino-group of insulin is the main or even a contributory cause of the inactivation of insulin by phenyl *isocyanate*, a process which is very rapid and complete.

Other amino-acids which are known to react with phenyl *isocyanate* are proline [Fischer, 1901] and hydroxyproline [Fischer, 1902], but since insulin does not contain the latter [Jensen *et al.* 1932], only the former amino-acid need be considered here. Jensen & Evans [1935] obtained evidence of the presence of proline in insulin, but were unable to isolate the phenylhydantoin of this amino-acid from small amounts of hydrolysed phenylcarbamido-insulin. In view of this, and since zein, which contains 9.0% of proline [Osborne & Liddle, 1910] does not react with *p*-bromophenyl *isocyanate* [Hopkins & Wormall, 1933, 1] it seems justifiable to conclude that the formation of phenylcarbamido-protein derivatives does not involve any significant change in the proline grouping.

From these results with amino-acids it does not necessarily follow that similar reactions will occur with natural or derived proteins. One is probably justified however in concluding that if phenyl *isocyanate* does not react with a given group in an amino-acid, there is a strong probability that no reaction will occur with this group when the amino-acid is linked to many others. It is the intention of the authors to carry out a few tests with certain peptides, but there appears to be no strong reason to suspect that the results will differ from those obtained with the simple amino-acids.

In all these later investigations therefore no evidence has been obtained which necessitates modification of the view previously expressed that phenyl *isocyanate* reacts only with the ϵ -amino-groups of lysine and any free α -amino-groups present in proteins. Furthermore, the close relationship between the bromine contents of *p*-bromophenylcarbamido-derivatives of insulin and other proteins and the theoretical values, calculated from the decrease in free amino-N as a result of the action of the *isocyanate* or from the free amino-N content of insulin [Hopkins & Wormall, 1933, 1; 1934, 2], appears to be strong evidence against the view that the reaction involves other groupings of the protein. With the majority of native proteins the lysine probably accounts for the whole of the free amino-N, but this is not true for insulin. Jensen & Evans [1935] have

produced satisfactory evidence that a further portion, but not the whole, of this additional free amino-N of insulin is present in phenylalanine groupings. Further work is being carried out on the phenylcarbamido-derivatives of insulin and other proteins in the hope that more information will be obtained about these important free amino-groups.

SUMMARY.

1. The hypophosphataemic activity of insulin is destroyed by phenyl *isocyanate* in the same way as is the hypoglycaemic power.

2. In these experiments, the destruction of the hypoglycaemic power has been parallel with the destruction of the hypophosphataemic power. This relationship has been found (a) when varying amounts of phenyl *isocyanate* were used and (b) when the inactivation process was stopped at different stages.

3. Davis *et al.* [1933], using acid alcohol and soft X-rays to inactivate insulin, concluded that different "active groups" of the hormone are responsible for the two activities mentioned above. In the work described in this paper, no similar differentiation has been found possible with phenyl *isocyanate* as the inactivating agent.

4. Further investigations have been carried out to determine whether the action of phenyl and *p*-bromophenyl *isocyanates* on insulin and other proteins is confined to the free α -amino-groups. It has been found that these *isocyanates* do not react, under conditions similar to those maintained in the preparation of phenylcarbamido-protein derivatives, with the hydroxyl group of tyrosine, the acid-amide groups of asparagine and glutamine or the iminazole group of histidine, nor is there any significant change in the S—S linkage. The SH group of cysteine (and similar compounds) reacts with phenyl *isocyanate*, but it is not necessary to consider this interaction in the case of insulin which contains no free SH groups.

Phenyl *isocyanate* reacts with the pyrrolidine group of proline and to some extent with the guanidino-group of arginine. From the evidence available, however, it is believed that these reactions, even if they occur when insulin is inactivated by phenyl and *p*-bromophenyl *isocyanates*, do not account for the inactivation of the hormone.

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