CXI. IMMUNOLOGICAL AND CHEMICAL PROPERTIES OF CARBOBENZYLOXY- PROTEINS¹

I. SERUM GLOBULIN AND EGG ALBUMIN DERIVATIVES

II. INSULIN DERIVATIVES

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(Received 6 April 1939)

IN previous communications [Hopkins & Wormall, 1933, 1, 2; 1934, 1] the chemical and immunological properties of phenyl- and *p*-bromophenyl-carbamido protein compounds have been described. These compounds were obtained by the condensation of phenyl- and *p*-bromophenyl-isocyanates with various proteins. In other investigations the action of these arylisocyanates on insulin has been studied [Hopkins & Wormall, 1934, 2; Gaunt & Wormall, 1936; cf. also Jensen & Evans, 1934; 1935].

Bergmann and his colleagues [Bergmann & Zervas, 1932; cf. review by Bergmann, 1933] have shown that benzyl chloroformate readily reacts with the amino group of amino-acids in cooled alkaline solution yielding carbobenzyloxy-amino-acids from which the amino-acid can be regenerated by catalytic reduction with hydrogen in the presence of palladium black. There is no direct evidence that benzyl chloroformate reacts with native proteins in this way, i.e. by condensation with the free amino-groups of the protein molecule; it was thought, however, that if such a reaction occurred, it would be suitable for the investigation of the immunological properties of proteins and also the chemistry of insulin. Preliminary experiments were therefore carried out to see whether benzyl chloroformate reacts with serum globulin, egg albumin and insulin. Bergmann's method was modified slightly, the solution being maintained at pH 7.5-8.5 during the reaction in order to minimize the possibility of other changes in the protein molecule taking place. As is described below, this treatment with benzyl chloroformate converts antigenic proteins into acid-precipitable derivatives which have a new antigenic specificity, and in the case of insulin produces a derivative which is devoid of hypoglycaemic power. It was also thought that the new grouping introduced into the protein molecule might be removed by catalytic reduction, with, perhaps, the reproduction of the original immunological specificity in the case of the native proteins and the regeneration of the hypoglycaemic activity in the case of insulin.

¹ The investigations described here were mainly carried out when the authors were members of the Department of Physiology, the University of Leeds.

In the description below, the investigation is divided into two parts, the first part dealing with the preparation and the chemical and immunological properties of carbobenzyloxy-protein compounds, and the second part with the inactivation of insulin by benzyl chloroformate and the chemical and immunological properties of carbobenzyloxy-insulin.

I. SERUM GLOBULIN AND EGG ALBUMIN DERIVATIVES

Preparation of compounds

Carbobenzyloxy-horse serum globulin. The serum globulins were separated from horse serum by means of CO_2 [Hopkins & Wormald, 1933, 1]. 500 ml. of a 2.4% globulin solution were mixed with 400 ml. of phosphate buffer of pH 8. The mixture was cooled in ice and 12 ml. of benzyl chloroformate in 50 ml. of ether added slowly to the well-stirred solution. The reaction was maintained at about pH 8 by addition of 2N NaOH. The globulin compound slowly precipitated out and after 3 hr. the product was centrifuged. The supernatant solution contained no protein and was therefore rejected. The solid material was suspended in water at pH 7.5 and extracted 5 times with 30 ml. of ether to remove the excess of benzyl chloroformate. The ether remaining in the suspension was removed by evacuation. The protein material was washed twice with 400 ml. of 0.9% NaCl solution, and the reaction adjusted to pH 8.0. Finally the carbobenzyloxy-horse serum globulin was ground in a mortar, strained through fine muslin and suspended in 200 ml. of 0.9% NaCl, the reaction being adjusted to pH 7.5.

Carbobenzyloxy-gelatin. 10 g. of gelatin (Coignet's "Gold Label") were dissolved in 300 ml. of water, and 600 ml. of phosphate buffer pH 8 and 900 ml. of water were added. The mixture was cooled, 7.5 ml. of benzyl chloroformate in 100 ml. of ether added slowly and the whole mixture was stirred for 3 hr. at room temperature, the reaction being maintained at pH 8. At the end of 3 hr. the mixture was centrifuged and the supernatant liquid cooled and acidified with 2N HCl to give maximum precipitation of the carbobenzyloxy-gelatin. This product was separated by centrifuging, dissolved in water at pH 8.5 by gentle warming to 36–37° and reprecipitated by HCl. After a third precipitation the product was dissolved in the minimum amount of water (180 ml.) and the pH adjusted to 7.5.

Carbobenzyloxy-egg albumin. 200 ml. of a 1.7% solution of crystalline egg albumin [Adair & Robinson, 1930] were treated as above with 200 ml. of phosphate buffer of pH 8 and 7.5 g. of benzyl chloroformate in 30 ml. of ether. The mixture was stirred at 5° for 3 hr. and then centrifuged. The supernatant solution was acidified with dilute acetic acid to give maximum precipitation of the carbobenzyloxy-egg albumin. This protein derivative was then separated by centrifuging and redissolved in water by adding 2N NaOH to give pH 8. The solution was centrifuged and the protein derivative in the supernatant solution was precipitated by dilute HCl. After two further precipitations the carbobenzyloxy-egg albumin was dissolved in 0.9% NaCl at pH 7.5. A small amount of chloroform was added as a preservative to the solutions of the carbobenzyloxy derivatives of gelatin and egg albumin.

Carbobenzyloxy-amino-acids. For the purposes of inhibition tests a few carbobenzyloxy-amino-acids were made. The method described by Bergmann &

Zervas [1932] for the synthesis of peptides has been used so widely that the carbobenzyloxy-derivatives of most of the naturally occurring amino-acids have been prepared, usually by addition of benzyl chloroformate to a cooled aqueous solution of the amino-acid in the presence of a considerable excess of alkali. For the purposes of the present research it was thought advisable to reproduce in these preparations the conditions obtaining in the production of the carbobenzyloxy-protein derivatives, i.e. to carry out the reaction in the presence of phosphate buffer at pH 8.

Immunization

The carbobenzyloxy-horse serum globulin was prepared as described above and the suspension kept in the ice chest, phenol being added to give a concentration of 0.25% in the suspension. The suspension was injected intraperitoneally into four rabbits at intervals of 7–9 days, each rabbit receiving at each injection a suspension containing 0.25 g. of the protein. The sera were tested 7 days after the third and fifth injections. One rabbit gave a good antiserum after three injections; the others required two further injections.

Precipitin tests

Precipitin tests were carried out as previously described [Hopkins & Wormald, 1933, 1]. Readings were taken after $\frac{1}{2}$, 1 and 2 hr. at 37° and usually after a further 18 hr. at room temperature; those recorded in Tables I and II were obtained after 1 hr. In the case of carbobenzyloxy-gelatin compounds the tests were made at room temperature (15–19°), more pronounced precipitates being obtained in this way [Hopkins & Wormald, 1933, 2].

The extent of precipitin formation is recorded as follows (in increasing degrees of precipitation):

– (no reaction), f.tr. (faint trace), tr. (trace), $\pm$, +, $+\pm$, $++$.

Inhibition tests

Precipitin inhibition tests were carried out under the conditions previously described [Hopkins & Wormald, 1934, 1], the inhibiting substance being in neutralized 0.01 *M* solution. The degree of precipitin formation was noted immediately and after 5 min., $\frac{1}{2}$ and 1 hr. These inhibition tests were carried out at room temperature when carbobenzyloxy-gelatin was used as the antigen, and at 37° in other cases.

The immunological properties of carbobenzyloxy-proteins

The sera obtained by the injection of carbobenzyloxy-horse serum globulin into rabbits were tested against horse serum, carbobenzyloxy-egg albumin and carbobenzyloxy-gelatin. Marked reactions were obtained with the last two antigens (cf. Table I), indicating the possession of good antigenic power by the carbobenzyloxy-globulin. With horse serum the reactions were either feeble or negative, indicating almost complete loss of the original species specificity. The precipitin reactions with carbobenzyloxy-egg albumin and carbobenzyloxy-gelatin show that an immunological relationship exists between carbobenzyloxy-horse serum globulin and the corresponding derivatives of widely differing proteins. This relationship has been undoubtedly conferred on these proteins (e.g. gelatin and egg albumin) by the introduction of the carbobenzyloxy-grouping. The reactions with the gelatin compound are more strongly marked than those with the egg albumin compound. This is particularly interesting since gelatin itself is non-antigenic, whereas egg albumin is a true antigen.

In order to obtain confirmation of the view that the reaction between benzyl chloroformate and proteins involves the free amino groups of the protein molecule a few inhibition tests have been carried out. The results given in Table II show that carbobenzyloxy-glycine inhibits the reaction between carbobenzyloxy-proteins and the antisera to carbobenzyloxy-horse serum globulin, whereas it has no influence at all on the formation of precipitates in other antigen-antibody reactions (e.g. the horse serum anti-horse serum precipitin reaction). The carbobenzyloxy-protein precipitin reaction is partially inhibited by phenyl-carbamido-glycine.

Table I. *Precipitin reactions between carbobenzyloxy-protein derivatives and antisera to carbobenzyloxy-horse serum globulin*

Antigen	Dilution of antigen	Immune serum against carbobenzyloxy-horse serum globulin		
		No. 420	No. 421	No. 422
Horse serum	1:20	tr.	—	f.tr.
	1:100	tr.	tr.	f.tr.
	1:500	±	tr.	±
	1:2500	tr.	—	tr.
Carbobenzyloxy-egg albumin	1:20	f.tr.	—	f.tr.
	1:100	±	+	tr.
	1:500	±	+	+
	1:2500	tr.	±	±
Carbobenzyloxy-gelatin	1:20	tr.	f.tr.	tr.
	1:100	+	+	+
	1:500	+ ±	+ ±	+ ±
	1:2500	tr.	±	±

The dilutions of antigen refer to dilution of a solution which contains 5% of total protein.

Table II. *Inhibition of the precipitin reactions*

Antigen	Antiserum against	"Inhibiting" substance			
		NaCl	Glycine or alanine	Carbobenzyloxy-glycine (or alanine)	Phenyl-carbamido-glycine
Horse serum	Horse serum	++	++	++	++
Carbobenzyloxy-gelatin	Carbobenzyloxy-horse serum globulin	(a) + ±	+ ±	—	+
		(b) +	+	—	±

(a) and (b) give the results with different antisera.

II. INSULIN DERIVATIVES

The effect on insulin of chemical reagents which possess a more or less specific action on certain groups present in proteins has been studied by many authors. By these methods a considerable amount of information has been obtained regarding the groupings in the insulin molecule which may be responsible for its hypoglycaemic activity. A few of these methods which result in the inactivation of the hormone are reversible, i.e. the activity of the original insulin can, at least in part, be regenerated under suitable conditions. Partial reactivation can be effected in insulin inactivated by acetic anhydride, formaldehyde or hot dilute acid [for a review of the literature cf. Freudenberg *et al.* 1930; 1931; Jensen & Evans, 1934], by acid-alcohol [Carr *et al.* 1929; Charles & Scott, 1931],

and by mild reducing agents such as cysteine and glutathione [Freudenberg & Wegmann, 1935]. Perhaps the most satisfactory reactivation of this type is, however, that obtained by Harington & Neuberg [1936]. These authors have shown that iodination of insulin, resulting in the substitution of iodine in the 3:5-positions of the tyrosine groupings, causes a 90–95 % loss of activity. Partial removal of the iodine by catalytic reduction is accompanied by an approximately proportional restoration of the hypoglycaemic activity.

For the reasons stated in the first part of this paper, it seemed likely that benzyl chloroformate would combine with the insulin molecule, possibly with loss of the hypoglycaemic activity of the hormone. Furthermore, it was thought that, if inactivation occurred, it might be found to be reversible, since the carbobenzyloxy-groupings attached to amino-acids can be removed by catalytic reduction. Preliminary experiments showed that benzyl chloroformate reacts with insulin and the properties of the product of this reaction (carbobenzyloxy-insulin) have been studied. A brief report of these investigations has been made [Gaunt *et al.* 1935].

EXPERIMENTAL

The insulin activities of the original material and the treated insulin were determined as previously described [Hopkins & Wormall, 1934, 2]. In practically all the experiments, tests were made with groups containing four rabbits. Duplicate sugar determinations (0.2 ml. blood for each) were carried out with every sample of blood from each rabbit, the method of Hagedorn & Jensen [1923] being used. In the tables the average value for each group is given.

The reaction between benzyl chloroformate and insulin was allowed to proceed at 5–8° and in the presence of 0.2 *M* NaHCO₃ at pH 8–8.5. The use of bicarbonate instead of the phosphate buffer of pH 8 is the only modification of the method employed in the experiments with native proteins as described in the previous part of this paper. The insulin used for this work contained 19,500 units per g.

In all experiments a portion of the insulin solution was removed, before any treatment, for use as a control. This control was usually subjected to the same treatment as the experimental sample without, of course, the addition of benzyl chloroformate. The various solutions injected into the rabbits were treated with phenol (to give 0.25 %).

The action of benzyl chloroformate on insulin

32.8 mg. of insulin were dissolved in 4 ml. of 0.9 % NaCl and 4 ml. of 0.2 *M* NaHCO₃, 2 drops of 0.2 *N* NaOH being added to give a clear solution at pH 8–9; 0.5 ml. of this solution was removed for use as a control in the physiological assay. The remainder of the insulin solution was cooled in ice and treated with 0.8 ml. of benzyl chloroformate, the reaction being maintained at pH 8 by addition of 0.2 *N* NaOH when necessary. After the mixture had been shaken for 2 hr., the excess of benzyl chloroformate was removed by shaking eight times with 10 ml. ether at each extraction. The residual ether was removed by gentle warming and evacuation. The suspension of carbobenzyloxy-insulin was injected in such amounts that each rabbit received per kg. the treated material from 5 to 15 units of insulin. From the results of the blood sugar determinations it could be concluded that probably all the activity of the insulin had been destroyed as a result of treatment with benzyl chloroformate (cf. Table III).

As the insulin compound is insoluble it was thought advisable to determine whether the inability of carbobenzyloxy-insulin to lower the blood sugar of starved rabbits was due simply to its insolubility. If this is true, then the

administration of large amounts of insulin compound might exert a slow effect after injection. Blood sugar determinations carried out up to about 24 hr. after the injection indicate, however, that the carbobenzyloxy-insulin preparations have no power to reduce the blood sugar even when observations are made over a long period of time.

Table III. *Inactivation of insulin by benzyl chloroformate*

Group	Subcutaneous injection of	No. of units per kg.	Blood sugar (mg./100 ml.) Time after injection (hr.)				
			0	2½	4½	5½	23½
A	Insulin	0.75	82	49	55	70	107
B	Carbobenzyloxy-insulin	15	91	97	99	106	96

The rate of inactivation of insulin by benzyl chloroformate

Experiments have been carried out to determine the rapidity with which benzyl chloroformate inactivates insulin. A solution of 41 mg. of insulin in 0.9% NaCl and 0.2 M NaHCO₃ was cooled in ice and treated with 0.5 ml. of benzyl chloroformate, the mixture being well shaken. Samples were removed at intervals of 5, 30 and 60 min. These samples, and the control sample removed before addition of the reagent, were made slightly acid and immediately extracted six times with about 10 ml. portions of ether. The remaining ether was removed by gentle warming and evacuation. The 5, 30 and 60 min. samples, injected into rabbits in amounts equivalent to 4, 10 and 15 units of the original insulin respectively, showed no significant insulin activity (cf. Table IV). The results of this experiment indicate that the inactivation of insulin by benzyl chloroformate is rapid, though it is probable that the results obtained are only of qualitative value, as it is difficult to ensure complete removal of the excess of benzyl chloroformate by extraction with ether.

Table IV. *Time required for inactivation of insulin by benzyl chloroformate*

Preparation	No. of units per kg.	Blood sugar (mg./100 ml.) Time after injection (hr.)				
		0	1½	3½	5½	
Insulin	0.75	103	50	46	47	
Carbobenzyloxy-insulin	5 min.*	4	103	99	94	97
	30 min.*	10	104	101	98	96
	60 min.*	15	94	103	100	101

* These samples were obtained by allowing the benzyl chloroformate to act on the insulin for 5, 30 and 60 min. (see text for full details).

The effect of small amounts of benzyl chloroformate on insulin

It is possible that total inactivation of insulin may occur when only a few of the free amino-groups of the insulin molecule have been blocked by benzyl chloroformate. In order to test this hypothesis experiments were carried out to determine the minimum amount of benzyl chloroformate required to effect this inactivation. For the purposes of these experiments the amounts are expressed in terms of that needed to combine with all the amino-groups of the insulin molecule, based on the assumption that insulin contains 1% of amino-nitrogen.

In the first experiment it was found that 12.5 times the theoretical amount of benzyl chloroformate inactivated insulin completely. In a second experiment of a similar type approximately 5, 3 and 2 times the theoretical amounts of

benzyl chloroformate were allowed to react with insulin. The blood sugar values obtained after the injection of the products of these reactions into starved rabbits are given in Table V. From these figures it will be seen that 5 and 3 times the theoretical amounts of benzyl chloroformate cause complete inactivation of insulin, and twice the theoretical amount destroys at least 95 % of the original hypoglycaemic activity of the hormone.

Further experiments were carried out using the theoretical and half-theoretical amounts of benzyl chloroformate. The results indicate that with the theoretical quantity about 60 % of the original activity is retained, whilst with half the theoretical amount of the reagent over 80 % of this activity remains (Table VI).

Table V. *Effect of different amounts of benzyl chloroformate on insulin*

Amount of benzyl chloroformate allowed to react with insulin	Amount of product injected ("units" per kg.)	Blood sugar (mg./100 ml.) Time after injection (hr.)		
		0	1½	4½
0	0.75	103	57	88
2 × theoretical	2.75	94	92	85
2 × "	0.75	91	97	104
3 × "	0.75	91	100	107
5 × "	1.5	95	100	109

Experimental details. 32.5 mg. of insulin were dissolved in 4 ml. of 0.9 % NaCl and 4 ml. of 0.2 *M* NaHCO₃ at pH 8-8.5. The solution was divided into 4 lots each of 2 ml., all the samples being then cooled in ice. To three of these samples were added 0.96 ml., 0.58 ml. and 0.38 ml. of a 0.5 % solution of benzyl chloroformate in ether. The volume of ether in all four samples was made up to 0.96 ml. The samples were shaken for 2 hr. and then left overnight in the ice chest. The ether was removed by warming and evacuation. The pH of the solutions did not alter during the course of the reaction. After suitable dilution with 0.9 % NaCl the samples were injected into rabbits.

Table VI

Amount of benzyl chloroformate allowed to react with insulin	Amount injected ("units" per kg.)	Blood sugar (mg./100 ml.) Time after injection (hr.)		
		0	1½	6
0	0.6	105	51	68
0	0.3	99	69	97
Theoretical amount	0.6	96	66	78
Theoretical amount	0.3	92	67	78
0.5 × theoretical amount	0.6	88	45	55
0.5 × theoretical amount	0.3	86	62	78

Experimental details. Similar to those for Table V, with smaller amounts of benzyl chloroformate.

Attempts to reduce carbobenzyloxy-proteins catalytically

In view of the ease with which the carbobenzyloxy-group can be removed from carbobenzyloxy-amino-acids by catalytic reduction [Bergmann & Zervas, 1932], it was decided to attempt a similar reduction with the carbobenzyloxy-derivatives of insulin and other proteins. Preliminary experiments were first made to determine the effect of catalytic reduction on untreated insulin. Treatment with hydrogen in the presence of palladium black was found to effect in some experiments, but not in all, appreciable inactivation of insulin. The palladium black undoubtedly adsorbs some of the insulin, but this adsorption (measured by total N determinations on the solutions) was not always sufficient to account for the loss of activity. Freudenberg *et al.* [1931] also experienced this adsorption in their efforts to reduce insulin catalytically, and were unable to overcome the difficulty. Since the experiments described here were completed, Harington & Neuberger [1936] have effected the removal of about two-thirds of

the iodine of iodinated insulin by catalytic reduction with a palladium-barium sulphate catalyst in pyridine-water solution and an atmosphere of hydrogen; this reduction does not appear to have any destructive action on the physiologically active groups of insulin.

In view of the difficulties experienced with insulin, further experiments were made with carbobenzyloxy-gelatin. No significant increase in free amino-N occurred, however, when this derivative was exposed in solution at pH 7 to the action of palladium black and hydrogen for several hours. It was concluded that some other method would have to be devised for the removal of the carbobenzyloxy-groups and in the case of the insulin derivative care would have to be taken to prevent loss of physiological activity due to reduction of the S—S groups.

Since the work described in this paper was completed, Clutton *et al.* [1937] and Clutton *et al.* [1938] have succeeded in removing the carbobenzyloxy-grouping from *O*- β -glucosido-*N*-carbobenzyloxytyrosyl protein compounds. In the case of the gelatin compound it was found impossible to effect this removal by reduction in aqueous solution with a variety of catalysts and reducing agents. However, by reduction with sodium in liquid ammonia the carbobenzyloxy-grouping was removed from the *O*- β -glucosido-gelatin derivative without rupture of the glucosido-linkage and without degradation of the protein. This method, however, could not be used in the case of *O*- β -glucosido-*N*-carbobenzyloxytyrosyl horse serum globulin; here removal of the carbobenzyloxy-grouping was effected by reduction at pH 9.6 in an atmosphere of hydrogen in the presence of palladium black. These workers did not attempt to remove the carbobenzyloxy-grouping from *O*- β -glucosido-*N*-carbobenzyloxytyrosyl insulin and little information is still available regarding the influence of such reducing agents on the insulin molecule.

Numerous authors have observed allergic reactions following the injection of insulin into man, and although in most instances the reactions observed can be attributed to other proteins present in the insulin preparations, there is evidence that pure insulin occasionally produces an allergic response. It is not certain that this hypersensitivity is due to a true antigen-antibody reaction, but the blood of hypersensitive patients has been shown to contain precipitins to insulin [Raynaud & LaCroix, 1925; Karr *et al.* 1933]. Stronger evidence in support of the view that insulin can function as an antigen has been obtained by Lewis [1937]. This author injected relatively large doses of insulin into virgin guinea-pigs, controlling the hypoglycaemia by the administration of glucose, and found that the uteri of these sensitized animals reacted with insulin but not with the pancreas protein or with the serum of the animal from which the insulin was derived. Cross-reactions with hog and beef insulins indicated that insulins from different animal species are immunologically closely related.

The injection of large amounts of insulin into an animal for the production of antibodies is precluded by the intense hypoglycaemic effect of the hormone, although the hypoglycaemia can be partially controlled by the administration of glucose. An alternative method is to inactivate those groups which are responsible for the hypoglycaemic activity by some process which does not destroy the antigenic power of proteins; this inactivated product could then be injected in amounts which are normally needed to incite antibody response to antigenic proteins. From evidence produced in this paper benzyl chloroformate appears to be suitable for this purpose, since it does not appreciably reduce the antigenic power of serum globulins and yet it rapidly destroys the hypoglycaemic activity of insulin. An experiment has therefore been carried out to determine whether

the injection of large amounts of carbobenzyloxy-insulin produces in mice any change in the sensitivity of the animals towards insulin, and whether any serological evidence of the formation of an anti-insulin can be obtained.

A group of 40 mice was given a series of injections of carbobenzyloxy-insulin and the animals subsequently tested for their response to insulin, and their serum for antibodies to carbobenzyloxy-insulin.

0.4 g. of insulin was treated as described previously with five times the "theoretical" amount of benzyl chloroformate, and the final product diluted to 56 ml. with the addition of NaCl (to give 0.9%) and phenol (0.2%); 0.25 ml. of this solution contained the equivalent of 1.8 mg. of insulin. Each mouse received four intraperitoneal injections, each of 0.0125 ml. of this solution per g., at intervals of 2-3 days. During the period of immunization the mice were fed on bread soaked in 10% glucose solution and were given a strong solution of glucose to drink in order to counteract the slight hypoglycaemic action of the carbobenzyloxy-insulin. Any mouse which showed signs of hypoglycaemia was given a subcutaneous injection of 0.25-0.5 ml. of sterile 10% glucose, but in spite of this treatment a few of these mice (and all those which received similar injections of phenylcarbamido-insulin) died during the immunization. A similar control group of mice received injections of a solution containing 0.9% NaCl and 0.2% phenol. At various periods after the last injection (4, 9 and 17 days), these two groups of mice and another group of untreated mice were tested for their response to insulin. The technique employed was that of Trevan & Boock [1926]; the mice received subcutaneous injections of 0.015 (or 0.030) unit per 20 g. and were kept at 38° during the assay. No significant difference between the three groups was observed, and the conclusion was reached that injections of large amounts of carbobenzyloxy-insulin had not led to any detectable change in the response of these mice to subsequent insulin treatment.

The serum of the mice "immunized" against carbobenzyloxy-insulin was tested, in complement-fixation and precipitin tests, against carbobenzyloxy-insulin and carbobenzyloxy-gelatin. The results were all negative.

DISCUSSION

The reaction between benzyl chloroformate and proteins appears to take place mainly if not entirely between the reagent and the free amino-groups of the protein. The reaction is accompanied by the total disappearance of free amino-N, as determined by "formol" titration, and the blocking of the amino-groups is apparently complete. This view is supported by the results of the immunological investigations, for the reaction between carbobenzyloxy-proteins and their antisera is completely inhibited by carbobenzyloxy-amino-acids. To a lesser degree, phenylcarbamido-amino-acids inhibit this precipitin reaction, and this can be attributed to the structural relationship between the carbobenzyloxy- and phenylcarbamido-amino-acid groups. Cross-reactions of this type are quite frequent in serological inhibition tests [cf. Landsteiner, 1936, pp. 122-32].

The results of the immunological experiments described here provide further evidence for the view that substitution in the free amino-groups of antigenic proteins leads to complete, or almost complete, loss of the original specificity and to the acquirement of a new specificity which is characteristic for the introduced grouping. It seems possible that the introduction of any new

group will cause this radical change in the immunological properties of a protein if the new group is sufficiently prominent [cf. Landsteiner, 1936]. The relatively small size of the methylene group might account for the retention of species specificity in formaldehyde-treated proteins; on the other hand, this retention might be due to incompleteness of the reaction between formaldehyde and the free amino-groups, for there is not universal agreement as to the precise nature of this reaction [cf. review by Jordan Lloyd & Shore, 1938].

The action of benzyl chloroformate on insulin also appears to involve the free amino-groups of the protein. The reaction is accompanied by practically complete loss of the hypoglycaemic power of the hormone, similar to, but possibly more complete than, that effected by phenyl isocyanate [Hopkins & Wormald, 1934, 2]. The minimum amount of benzyl chloroformate needed for complete inactivation is about twice the amount required by theory to react with all the free amino-groups. The fact that the "theoretical" amount of the reagent does not effect complete inactivation might be due to action of the reagent on groups other than amino-groups, or, more probably, to loss of the acid chloride by the action of water. The inactivation of insulin is very rapid and takes place at pH 8-8.5 and at ordinary temperatures. The results offer strong support for the view that the free amino-groups of insulin are of significance in connexion with its physiological activity [Hopkins & Wormald, 1934, 2; Jensen & Evans, 1934; 1935].

The attempts to "immunize" mice with carbobenzyloxy-insulin were made in order to determine (a) whether the injection of large amounts of this insulin derivative would influence the response of the animals to ordinary insulin, and (b) whether carbobenzyloxy-insulin would behave as a full antigen. Immunological investigations on insulin are complicated by the hypoglycaemic action of this substance, but this difficulty is overcome by the use of carbobenzyloxy-insulin, which, by analogy with other proteins, should be antigenic if insulin itself has this function. Any antibodies to the inactivated insulin might be expected to show some capacity to react with unchanged insulin, since antisera to carbobenzyloxy-globulin usually give slight precipitation with the untreated globulin. In the experiments described here there was no indication of antibody formation of this type. The sera of the injected mice contained no detectable antibodies to the carbobenzyloxy-derivatives of insulin or gelatin, and furthermore, these animals showed no abnormality when injected with amounts of insulin which are usually sufficient to produce convulsions. It is not easy to forecast exactly what will happen in an animal if an anti-insulin is produced. Small amounts of the antibody could presumably be "neutralized" by insulin with the formation of a complex which might possess some hypoglycaemic activity, but excess of the antibody might be expected to render the animal diabetic. If the animal survives the immunization, perhaps the only evidence of the formation of an anti-insulin might be a change in the response of the animal to insulin injection. Further information on this point might throw some light on problems of insulin-hypersensitivity and insulin-resistance in man.

The antigenic properties of hormones have been studied by numerous authors during recent years, largely stimulated by the observations of Collip and his colleagues. As far as insulin is concerned, the available evidence is rather conflicting. The previously mentioned work of Lewis [1937] suggests that sensitization to insulin can be produced, and Barral [1935] claims to have produced an antigenic and non-hypoglycaemic "aninsulin" by the action of formaldehyde on insulin for 40 days at 60°. Spaccarelli [1936], on the other hand, found that the uteri of insulin-treated virgin guinea-pigs do not react with

insulin, although the uteri of animals injected with a mixture of serum and insulin react to insulin alone. Interesting observations on the antigenicity of insulin have recently been made by Clutton *et al.* [1938]. These workers immunized rabbits against *O*- β -glucosido-*N*-carbobenzyloxytyrosyl insulin and found that although the antisera produced to the insulin derivative failed to precipitate the homologous antigen, they gave precipitates with *O*- β -glucosido-*N*-carbobenzyloxytyrosyl horse serum globulin. Further, the antisera to the globulin derivative gave precipitates with the insulin compound. The fact that the insulin derivative inhibited the cross reaction of its antiserum with the globulin derivative indicated that, despite the failure of the insulin derivative to precipitate its antiserum, a definite antigen-antibody reaction does occur. These authors suggest that the physical properties of the antigen may be such that an insoluble antigen-antibody complex cannot be formed. They conclude that coupling with glucosid $\dot{o}$ tyrosine converts insulin into a full antigen. The question of the antigenicity of insulin itself is, however, still undecided, and, as far as observations on individuals hypersensitive to insulin are concerned, it might be fair to say that they do not as yet offer conclusive evidence of the presence in the blood of true antibodies to pure insulin.

SUMMARY

1. Benzyl chloroformate, the reagent used by Bergmann for the preparation of carbobenzyloxy-amino-acids, readily acts on serum globulin, gelatin and other proteins, at ordinary temperatures and at pH 7.5–8.5. The reaction appears to be concerned mainly with the free amino-groups of the proteins.

2. The injection of carbobenzyloxy-serum globulin into rabbits leads to the production of antibodies which react with the carbobenzyloxy-derivatives of heterologous proteins. The original species-specificity of the serum globulin is almost completely destroyed.

3. The reaction between carbobenzyloxy-proteins and their antisera is completely inhibited by carbobenzyloxy-amino-acids. The immunological y characteristic groups of such proteins are, therefore, the carbobenzyloxy-amino-groups ($-\text{NH}\cdot\text{CO}\cdot\text{O}\cdot\text{CH}_2\cdot\text{C}_6\text{H}_5$).

4. Benzyl chloroformate rapidly destroys the hypoglycaemic activity of insulin. The amount of the acid chloride needed to effect complete inactivation is about twice the amount required by theory to react with all the free amino-groups of insulin, although smaller amounts produce appreciable inactivation. These observations support the view that the free amino-groups of insulin are important for its hypoglycaemic activity.

5. Mice injected with relatively large amounts of carbobenzyloxy-insulin behaved exactly like the control animals when injected with a "convulsive" dose of insulin. These "immunized" mice showed no significant hypo- or hypersensitivity to insulin, and their sera contained no detectable antibodies to carbobenzyloxy-insulin or carbobenzyloxy-gelatin.

The authors are indebted to the Medical Research Council for a personal grant to one of them (W. E. G.) and for a grant which has, in part, covered the expenses of these investigations. Thanks are also tendered to Messrs Boots Pure Drug Co., Ltd., for a generous supply of insulin.

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