CCXXVII. STUDIES IN SYNTHETIC IMMUNOCHEMISTRY

IV. FURTHER INVESTIGATION OF *O*- β -GLUCO- SIDOTYROSYL DERIVATIVES OF PROTEINS

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IN their papers on the preparation and serological properties of the *O*- β -glucosidotyrosyl derivatives of various proteins Clutton *et al.* [1937; 1938] showed that antisera against the gelatin and insulin derivatives precipitated with the corresponding globulin derivative, although they failed to precipitate with the homologous antigens. They also found that antisera against the globulin derivative precipitated both with the homologous antigen and with the gelatin and insulin derivatives. From this they concluded that coupling with glucosidotyrosine converts both gelatin and insulin into full antigens. They also concluded from the similarity in behaviour of globulin derivatives of *O*- β -glucosido-*N*-carbobenzyloxytyrosine and *O*- β -glucosidotyrosine that the hapten function of the carbobenzyloxy group was probably negligible.

In view of the theoretical importance of the points involved it was decided to extend these investigations by the use of other serological techniques, namely complement fixation and bulk precipitation, in addition to the ring precipitation tests and inhibition tests used previously. It was also decided to make a closer study of the carbobenzyloxy group as a possible antigenic determinant, since it seemed surprising that this group should have no influence.

Since an antigen built upon the same (foreign) protein had been used in the earlier experiments for immunization and for testing the antisera, it seemed that the resemblances found originally between the immunological behaviours of *O*- β -glucosido-*N*-carbobenzyloxytyrosyl globulin and *O*- β -glucosidotyrosyl globulin might have been due to the protein moiety. The latter objection was overcome by using the appropriate derivative of rabbit serum proteins as test antigen, thereby avoiding the possibility of the production of antibodies against the protein portion. The preparation of the antigens was otherwise similar to that described in the previous papers, except for the preparation of *N*-carbobenzyloxytyrosyl gelatin, and for slight modifications given below.

EXPERIMENTAL

Preparation of compounds

(1) *O*- β -Glucosido-*N*-carbobenzyloxytyrosyl gelatin (from Gold Label gelatin), *O*- β -glucosido-*N*-carbobenzyloxytyrosyl globulin and *O*- β -glucosido-*N*-carbobenzyloxytyrosyl insulin were prepared as described by Clutton *et al.* [1937; 1938].

The glucose contents (measured by the orcinol method) were, on a nitrogen basis: gelatin compound (average of 5 preparations) 4.6 %; globulin compound (average of 3 preparations) 12 % (the original globulin contained 2.5 %); insulin compound (average of 3 preparations) 12 %.

(2) *O-β-Glucosido-N-carbobenzyloxytyrosyl rabbit serum protein*. Fresh rabbit serum was dialysed against running tap water for 3 days. 25 ml. (containing 1.05 g. protein on a nitrogen basis) were coupled with the azide from 0.7 g. glucosido-*N*-carbobenzyloxytyrosyl hydrazide, the reaction being kept just alkaline to thymolphthalein. The solution was brought to pH 5; next day the precipitate was collected, dissolved in water with the minimum of alkali and dialysed against running tap water for 30 hr. The product contained, on a glucose : nitrogen basis, 7.4 % glucose.

(3) *N-Carbobenzyloxytyrosyl gelatin*. Carbobenzyloxytyrosyl hydrazide was prepared from carbobenzyloxytyrosine ethyl ester and hydrazine hydrate dissolved in alcohol at room temperature. 4.5 g. of the hydrazide were dissolved in 22.5 ml. glacial acetic acid plus 112.5 ml. water, and to the solution were added 13.5 ml. of 5*N* HCl. The mixture was cooled in ice, and 4.5 ml. of 30 % NaNO₂ were added very slowly. The azide formed a white precipitate which was immediately extracted with a little ether; the ethereal solution was washed 4 times with water and added slowly, with stirring, to a solution of 10 g. gelatin in 2 l. of 50 % aqueous alcohol, kept cool and maintained just alkaline to phenolphthalein by addition of the necessary amount of NaOH. Stirring was continued for an hour after the addition was completed. On acidification with acetic acid the compound flocculated; it was separated on the centrifuge, and washed with 70 % and absolute alcohol. Yield, 9.3 g.

Estimation of the tyrosine plus tryptophan content on an alkaline digest by the phenol reagent of Folin & Ciocalteu [1927] showed a tyrosine content of at least 6.5 % (since the untreated gelatin contains none). The compound was negligibly soluble at pH < 6, and on boiling with 5*N* HCl gave a definite odour of benzyl chloride.

(4) *Removal of carbobenzyloxy residues*. In the case of the globulin and the rabbit serum protein compounds the technique was similar to that used by Clutton *et al.* [1938], namely reduction with hydrogen in the presence of palladium black. It was found that after 2 hr. shaking with hydrogen at pH 8–9 the compounds were soluble in comparatively acid solution (pH 5.7), and reduction was therefore continued at this pH with the addition of further palladium black. At the end of the reduction the solution just became cloudy on bringing to pH 5.5. After acidification the CO₂ evolved was about 10 % greater in amount than was theoretically required for the complete removal of the carbobenzyloxy groups, while in the fourth case it accounted for 85 % of these groups. The glucose : nitrogen ratio showed a glucose content of 12.5–14 % for the different samples of coupled horse globulin, and 5.3 % for the coupled rabbit serum.

Apart from the increased acid solubility and the evolution of CO₂ during the reduction no proof was obtained that the carbobenzyloxy groups had been completely removed. Attempts to assess the degree of removal by measurement of the CO₂ evolved on boiling with 5*N* HCl indicated a considerable (80–100 %) removal, but were rendered insufficiently accurate by the fact that the original proteins gave relatively large blanks when treated similarly. In view of the serological results obtained the possibility of a few remaining carbobenzyloxy groups must be borne in mind.

Technique of immunization

Rabbits of 2–3 kg. weight were injected at 2-day intervals. A course of injections comprised three injections of 10 mg., three of 20 mg. and three of 40 mg., the larger doses being given intraperitoneally. Test bleedings were made 8–10 days after the last injection.

The following derivatives were used:

(a) *O*- β -Glucosido-*N*-carbobenzyloxytyrosyl gelatin. Originally three animals were used and received two courses. For repeating the experiments a single animal was used, and received two courses, while for the preparation of strongly precipitating antisera (*v. infra*) three animals were used, and received an extended course totalling 600 mg. per rabbit.

(b) *N*-Carbobenzyloxytyrosyl gelatin (two animals). One course.

(c) *O*- β -Glucosido-*N*-carbobenzyloxytyrosyl globulin (three animals). One course.

(d) *O*- β -Glucosido-tyrosyl globulin (two animals). One course.

(e) *O*- β -Glucosido-*N*-carbobenzyloxytyrosyl insulin. The injections were begun on three animals, using the technique described in the previous paper, but only one rabbit survived to the end of the course. One course.

Serological tests

Complement fixation was carried out at 37° in a volume of 1.25 ml. Complement used was 1½–2 M.H.D. fresh guinea-pig serum, and the haemolysin was standard Burroughs Wellcome anti-sheep corpuscle haemolysin used at 2 M.H.D. 1–2 hr. were allowed for fixation before adding the sensitized cells, and readings were taken at 37° for 1 hr., followed by standing in the cold room. Dilutions given are those of the antigen added to the system; the final concentrations of antigen are 1/5 of these values.

Bulk precipitation was carried out at 37° in a volume of 0.5 ml. consisting of equal volumes of serum and antigen dilution. Initial dilutions of antigen are given in the protocols and should be multiplied by 2 to give final dilutions, except where otherwise stated.

Sera were used in dilutions 1 : 2 to 1 : 4 unless stated otherwise, and were heated at 56° for 30 min. before all complement fixation tests.

Dilutions of antigens were made with normal saline, and all antigens were brought to pH 7 with NaOH before testing.

RESULTS

Gelatin derivatives

A. *Ring tests*. The results of Clutton *et al.* [1938] for antisera to G.C.T.¹ gelatin against G.C.T. globulin were confirmed. We also obtained slight indication of a ring at 1/1000 for G.C.T. gelatin antiserum and the homologous antigen.

Antisera to C.T. gelatin gave no ring test against C.T. gelatin, nor against G.C.T. gelatin. Against G.C.T. globulin no ring appeared at a dilution greater than was given by normal serum.

B. *Complement fixation*. Complement fixation was complicated by two factors. One was that G.C.T. gelatin (but not C.T. gelatin) itself caused haemolysis in the presence of complement when used in 1/50 or 1/100 dilution. This was evident when it was added to a known complement-fixing system containing *Brucella* endoantigen and anti-*Brucella* serum. The other was that

¹ In this section of the paper, G. = *O*- β -glucosido, C. = *N*-carbobenzyloxy, T. = tyrosyl.

certain of the normal sera used as controls fixed complement with uncoupled globulin and globulin derivatives, although never to the same dilutions as the antisera. The results were confirmed with a strong serum from a rabbit which was given a double course of immunization, rising to doses of 70 mg. antigen. The results given in Table I indicate that fixation does occur between antisera to G.C.T. gelatin and C.T. gelatin and the corresponding derivatives, but the reactions are weak.

Table I

Antiserum	Antigen	Degree of fixation of complement
G.C.T. gelatin	G.C.T. gelatin	Complete to 1/200
	G.C.T. globulin	Complete to 1/6000, partial to 1/700,000
	C.T. gelatin	Complete to 1/200
C.T. gelatin	C.T. gelatin	Complete to 1/100, partial to 1/900
	G.C.T. gelatin	Complete to 1/200, partial to 1/400
	G.C.T. rabbit serum	Complete to 1/2700
Normal serum	G.C.T. gelatin	None
	C.T. gelatin	None
	G.C.T. globulin	Complete to 1/1000, partial to 1/20,000
	G.C.T. rabbit serum	Complete to 1/300

C. *Bulk precipitation.* In order to try to confirm the existence of a visible reaction between G.C.T. gelatin and its homologous antiserum, a bulk test was set up with the most strongly fixing serum. After 24 hr. a definite opalescence appeared in the tubes containing antigen in 1/50 and 1/100 dilutions, but not in control tubes with normal serum. However, another group of three rabbits, each receiving a total of 600 mg. antigen per rabbit, yielded sera which gave no visible reaction at all under similar conditions.

Insulin derivative

A. *Complement fixation* (serum 1 in 5). Both the anti-G.C.T. insulin serum and normal serum, although the latter to a less degree, fixed complement with the homologous antigen:

Antiserum fixed complement with G.C.T. insulin up to 1/100,000.

Normal serum fixed complement with G.C.T. insulin up to 1/500.

B. *Bulk precipitation.* Anti-G.C.T. insulin serum in five experiments gave a precipitate up to 1/1600 dilution of the antigen. Two fresh normal sera and two old normal sera gave precipitates up to 1/800, 1/400, 1/200 and nil respectively. Thus there was clearly a considerable formation of non-specific precipitate, which could not be eliminated in the case of the fresh normal sera by varying the concentration of the saline in which the reaction was carried out.

It is difficult to argue from our evidence that a specific reaction between the antiserum and homologous antigen takes place, but taken in conjunction with the evidence from complement fixation tests, we may state that it appears likely to be the case.

Globulin derivatives: the significance of the carbobenzyloxy group

Bulk reactions set up between G.C.T. globulin, G.T. globulin and the antisera to them occurred in an extended zone of precipitation with an optimum at antigen dilution 1/13,000 in each case, thereby bearing out the experience of Clutton *et al.* [1938] with the ring test. When the corresponding derivatives of rabbit serum proteins were used as test antigens, similar results were obtained except that there appeared in each case to be a zone of non-specific precipitation (shown by normal sera also to a lesser degree) up to antigen dilution 1/600, and a second extended zone of less copious precipitation with optima about 1/38,000.

The differences between the sera did not justify any conclusions concerning the effect of the carbobenzyloxy group, and recourse was therefore had to inhibition reactions.

Inhibition reactions. Various possible inhibitors were tried at pH 7.0 using $M/25$, $M/250$ and M/∞ concentrations in saline. 0.25 ml. of inhibitor was incubated with 0.25 ml. of serum (1 in 3) for 2 hr. at 37°; 0.5 ml. of the rabbit serum protein derivative was then added in a concentration serologically equivalent to the antiserum. Equivalence was previously determined by the optimal proportions method of Dean & Webb [1926]. The results after further incubation are shown in Table II.

Table II. *Inhibition of precipitation between antisera to globulin derivatives and similar rabbit serum derivatives*

Antigens	G.C.T.	G.T.	G.C.T.	G.T.	G.C.T.	G.T.	G.C.T.	G.T.
Anti-G.C.T. globulin	—	—	I	i + + +	—	i	I + + +	I + + +
Anti-G.T. globulin	—	—	i + +	i + +	i +	i +	I + +	I + + +
Inhibitors	Tyrosine ethyl ester		<i>N</i> -Carbobenzyl-oxytyrosine methyl ester		<i>N</i> -Acetyltyrosine hydrazide		<i>O</i> - β -Glucosido- <i>N</i> -acetyltyrosine	
Antigens	G.C.T.	G.T.	G.C.T.	G.T.	G.C.T.	G.T.		
Anti-G.C.T. globulin	I + + +	I + + +	i + + +	i + +	i + + +	i +		
Anti-G.T. globulin	I + + +	Not done	i +	—	i +	i +		
Inhibitors	<i>O</i> - β -Glucosido- <i>N</i> -carbobenzyl-oxytyrosyl hydrazide		<i>N</i> -Carbobenzyl-oxyglutamic acid		Glucose			

G.C.T. = G.C.T. rabbit serum. G.T. = G.T. rabbit serum.

I = Inhibition at inhibitor dilution $M/250$.

i = Inhibition at inhibitor dilution $M/25$.

(The + signs indicate degree of inhibition at the particular dilution. + + + = complete inhibition. I or i without a + sign = slight but definite inhibition.)

These results indicated that *N*-carbobenzyloxyglutamic acid (which contains only C. of the G.C.T. hapten group) could inhibit precipitation when, and only when, the C. group was involved. The results in the second column also suggested that the antiserum to G.T. globulin contained antibody to G.C.T. as well. This is a reasonable supposition in view of the mode of preparation of the antigen.

In order to confirm the effect of the carbobenzyloxy group, bulk precipitations were carried out with both sera and the homologous antigens over the whole range of precipitation, using as a check inhibitor G.C.T. hydrazide. The results are given in Table III, and represent 48 hr. readings. Control

Table III. *Anti-G.C.T. globulin serum against rabbit serum as antigen*

Inhibitor	Antigen dilution						
	1/2400	1/4800	1/9600	1/19,200	1/38,400	1/76,800	1/ ∞
Anti-G.C.T. globulin serum against rabbit serum as antigen							
None	+	±	+	+	+	±	—
G.C.T. hydrazide, 1%	±	±	—	—	—	—	—
C.-glutamic acid, 2%	±	+	+	±	±	—	—
Anti-G.T. globulin serum against G.T. rabbit serum as antigen							
None	+	+	+	+	±	±	—
G.C.T. hydrazide, 1%	±	—	—	—	—	—	—
C.-glutamic acid, 2%	+	+	±	+	±	±	—

(Controls were done on the sera and antigens being tested and on normal sera in the presence of the inhibitors.)

mixtures of inhibitor with antisera, normal sera and the antigen solutions remained clear.

The results show that although G.C.T. hydrazide will inhibit both reactions, *N*-carbobenzyloxyglutamic acid only inhibits (though not completely) the reaction between serum and antigen when the carbobenzyloxy group is involved; this group must therefore be significant as a hapten group.

Note on the physiological properties of O- β -glucosido-N-carbobenzyl-oxytyrosyl insulin

This compound retained a considerable degree of physiological activity. Thus, not only was it necessary to give casein, starch and glucose paste by a stomach tube to the rabbits before injections, but even with this precaution the rabbits occasionally went into convulsions 18–20 hr. after injection. With the kind help of Dr Kerr, a test of physiological activity was made on a starving rabbit. It received 0.065 mg. intravenously (equivalent to 1 unit of the original insulin on a N-basis), and the blood sugar fell from 115 to 82 mg./100 ml. during 40 min., taking 3 hr. to return to 105 mg./100 ml.

Having in mind the possibility that insulin with all, or nearly all of its "free" amino groups blocked might be resistant to digestion in the small intestine, one of us (J. H. H.), after fasting 12 hr., received 3.5 mg. of the insulin compound (equivalent to 50 units of the original insulin on a N-basis) by a duodenal tube, whose position was verified by X-ray. The blood sugar curve showed no fall during the next 2½ hr., but only a slight rise. Hence it seems that the compound is not absorbed intact.

DISCUSSION

Complement fixation tests with sera of relatively low antibody content are difficult owing to extraneous effects encountered, such as the occurrence of haemolytic antigen solutions and anticomplementary sera; with direct precipitation tests non-specific precipitates occur which are hard to eliminate and tend to obscure the results. While the work described above confirms the conclusions of Clutton *et al.* 1938 concerning the conversion of insulin and gelatin into full antigens by coupling with *O*- β -glucosido-*N*-carbobenzyloxytyrosine, it appears to contradict their suggestion that the carbobenzyloxy group is not antigenically significant. Only by assuming a hapten function for this group *per se* can we explain the fact that the reaction between *O*- β -glucosido-*N*-carbobenzyloxytyrosyl rabbit serum proteins and the antiserum to *O*- β -glucosido-*N*-carbobenzyloxytyrosyl horse globulin is partly inhibited by *N*-carbobenzyloxyglutamate, whereas with the corresponding derivatives from which the carbobenzyloxy group has been removed there is no inhibition. The evidence of complement fixation between *N*-carbobenzyloxytyrosyl gelatin and homologous antisera is of interest, since in this case gelatin has been converted into a full antigen without the introduction of a carbohydrate group. It is interesting that under the conditions which we have employed we, as others, have failed to obtain any clear evidence for antibodies to a gelatin derivative which will precipitate with the homologous antigen.

In the case of G.C.T. insulin the increased range of precipitation when homologous antiserum is used in place of normal serum is small, but suggestive of a true antigen-antibody effect. This, together with the evidence from complement fixation tests, indicates a probability that the substance can stimulate the formation of specific precipitating antibodies.

In view of a recent paper by Gaunt & Wormald [1939], where results were given showing that treatment of insulin with benzyl chloroformate robs it of its physiological activity, the fact that G.C.T. insulin is still active assumes an added interest, although its significance is at present only a matter for speculation.

SUMMARY

1. The techniques of complement fixation and bulk precipitation have been used to confirm and extend the observations of Clutton *et al.* on the immunological properties of the *O*- β -glucosido-*N*-carbobenzyloxytyrosyl derivatives of gelatin and insulin.

2. Evidence is given for the hapten function of the carbobenzyloxy group, and for the fact that *N*-carbobenzyloxytyrosyl gelatin behaves as a full antigen.

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