CXLVI. STUDIES IN SYNTHETIC IMMUNOCHEMISTRY

II. SEROLOGICAL INVESTIGATION OF *O*-β-GLUCOSIDOTYROSYL DERIVATIVES OF PROTEINS

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IN the first paper of this series [Clutton *et al.* 1937] a method was described by which simple carbohydrate residues could be introduced into a protein in the form of the *O*-β-glycoside of tyrosine, the latter being coupled in peptide linkage with the free amino groups of the protein molecule. Application of this method to the preparation of *O*-β-glucosidotyrosylgelatin was described and it was stated that a preliminary study of the product gave no indication of the possession of antigenic properties.

The present communication deals with the extension of this work to the preparation of *O*-β-glucosidotyrosyl derivatives of other proteins and with a detailed serological investigation of the products; the possession of these new derivatives has made possible a reinvestigation of the serological properties of the gelatin compound itself, as a result of which the previous suggestion that it was non-antigenic has to be modified.

The additional proteins selected for study were (*a*) insulin, (*b*) horse serum albumin and (*c*) horse serum globulin. The case of insulin presents features of general interest in common with that of gelatin. As was pointed out in our previous paper one of our main objects was to try to discover whether the reason for the generally accepted lack of antigenic power of gelatin might be the absence from its molecule of either carbohydrate or tyrosine or both; such a possibility seemed not unlikely in view of the known importance of both tyrosine and carbohydrate groups in immunological reactions. It is, however, doubtful whether it is fair to regard gelatin as a true protein and the possibility remains that the process of its preparation from collagen involves some destructive action which diminishes or abolishes antigenic capacity; in insulin, on the other hand, we have a compound which may be regarded as a complete protein in so far as its molecular weight and amino-acid composition are concerned; as is well known, however, insulin is non-antigenic and it differs chemically from proteins which are known to be antigenic in containing no carbohydrate. It was therefore of particular interest to examine whether the introduction of glucose residues into insulin would yield an antigenic substance.

The derivatives of the serum proteins were prepared with the primary intention of ascertaining how powerfully determinant in an immunological sense the glucosidotyrosyl residue might be, i.e. to what extent the original immunological specificity of the proteins was masked by its introduction. In our previous paper we made a general criticism of earlier work on artificial protein-carbohydrate complexes on the ground that these contained an azo linkage which was foreign

to nature and which might itself affect the immunological reactions; it was hoped that a study of antisera against our glucosidotyrosyl derivatives of serum proteins in comparison with antisera against glucosidophenylazo derivatives of the same proteins might also indicate whether or not our criticism was justified.

As will be seen below, information has been forthcoming on all these points; moreover, the serum protein derivatives have proved unexpectedly useful in elucidating the true situation with regard to the derivatives of gelatin and insulin.

Experimental

Preparation of compounds

(1) O-*β*-*Glucosido*-N-*carbobenzyloxytyrosylgelatin*. This was prepared as described by Clutton *et al.* [1937].

(2) O-*β*-*Glucosido*-N-*carbobenzyloxytyrosylglobulin*. Serum obtained by sedimentation from 8 litres of defibrinated horse blood was diluted with an equal volume of water and the solution treated slowly with an equal volume of saturated aqueous $(NH_4)_2SO_4$; next day the precipitated globulin was separated by decantation and filtration, washed on the filter with half-saturated aqueous $(NH_4)_2SO_4$, suspended in a minimum of water and dialysed exhaustively against running tap water. The necessary amount of NaCl was added to bring the euglobulin into solution and the resulting 15 % solution of protein was stored at 0° with thymol as preservative.

For the coupling, 10 ml. of stock globulin (1·5 g. protein) were made just alkaline to phenolphthalein and diluted to 15 ml. To this solution, cooled in ice and stirred, was added during the course of about 15 min. glucosidocarbobenzyloxytyrosylazide (from 1 g. of the hydrazide: 40 % excess) in dioxane (5 ml.); constant alkalinity was maintained by simultaneous addition of 0·1 *N* NaOH.

The reaction mixture (final vol. 30–40 ml.) was diluted to 150 ml. with water and treated with 10 % acetic acid to *p*H 5·0, together with a little salt; after some hours the precipitate was collected at the centrifuge and dissolved in the minimum of dilute NaOH. The solution was dialysed against running tap water for 18 hr.; traces of insoluble matter were removed by centrifuging at low speed.

The opalescent solution so obtained contained, on a nitrogen basis, an 80–90 % yield of the coupled product; the glucose : nitrogen ratio indicated the introduction of 6 % of glucose. The new product was insoluble in water at *p*H $< 6{\cdot}0$ but readily soluble at *p*H $> 7{\cdot}0$; it could be denatured by heat.

(3) O-*β*-*Glucosido*-N-*carbobenzyloxytyrosylalbumin*. The albumin was crystallized from the $(NH_4)_2SO_4$ mother liquors of the above globulin preparation by adjustment to *p*H 5·2 (approx.) with *N*/5 H_2SO_4 followed by careful addition of solid $(NH_4)_2SO_4$ to the stirred solution until crystals began to appear; the albumin was collected, recrystallized once, again collected, dissolved and dialysed free from salt, yielding a stock solution containing 10 % protein which was preserved at 0° with thymol.

6 ml. of the stock albumin solution were diluted to 20 ml., made just alkaline to phenolphthalein and mixed with 20 ml. of dioxane; the mixture was then treated as above with a dioxane solution of glucosidocarbobenzyloxytyrosylazide from 0·3 g. of the hydrazide; the product was isolated from the reaction mixture exactly as in the case of the globulin derivative.

The yields were not quite so good as in the latter case, but the degree of coupling was more satisfactory, successive preparations having glucose contents of 11·5 and 11·8 % respectively.

The product differs from the globulin derivative in being soluble at *p*H $<3{\cdot}8$; it has a zone of insolubility extending from *p*H 3·8 to 5·5.

(4) *O-β-Glucosido-N-carbobenzyloxytyrosylinsulin.* Crystalline insulin (Boots: 1 g.) was dissolved in water (20 ml.) plus the necessary amount of *N*/10 HCl. *o-β*-Glucosido-*N*-carbobenzyloxytyrosylhydrazide (0·7 g.) was converted into the azide as usual and this, after thorough washing with cold water, was dissolved in chilled dioxane.

The chilled and mechanically stirred insulin solution was then treated with *N* NaOH drop by drop until definitely alkaline to cresol red; the dioxane solution of the azide was added slowly, stirring being continued and alkalinity to cresol red being maintained by addition of NaOH as necessary. Stirring in the cold was continued for about 15 min. after the addition of the reagents. The solution was first diluted with dioxane to make 50 % of the latter and then neutralized; a sticky precipitate separated but the mother liquor still contained much material which was precipitated by dilution with water but was soluble in acid. The whole was therefore diluted with much water, treated with dilute HCl to produce a clear solution, and then with saturated sodium acetate to the point of optimum flocculation.

Numerous attempts were made to crystallize the product by the method employed by Scott [1934] in the case of insulin; these attempts met, however, with no success. The material was therefore purified by re-solution in water with the aid of the minimum amount of ammonia followed by re-precipitation with HCl; optimum flocculation occurred at about *p*H 5·8.

The resulting product was separated at the centrifuge, washed with water and dried in a vacuum desiccator; it formed a cream-coloured amorphous powder having solubilities very similar to those of insulin itself.

The glucose : nitrogen ratio indicated 11·0 % of coupled glucose.

Removal of carbobenzyloxy residues from glucosidocarbobenzyloxytyrosylglobulin. The method of reduction with sodium in liquid ammonia, adopted with success in the case of gelatin, failed with the globulin derivative owing to the insolubility of the latter. Recourse was therefore had to catalytic reduction.

It was first shown that the carbobenzyloxy residue could in fact be eliminated by catalytic reduction in alkaline solution in presence of globulin. Thus a mixture of 5 ml. of a solution containing 0·1 g. globulin and 0·1 g. carbobenzyloxyglutamic acid with 10 ml. of a glycine-NaOH buffer solution of *p*H 9·6 was shaken in an atmosphere of hydrogen with 0·1 g. palladium black for 75 min.; the CO_2 evolved from the reaction mixture on acidification was found by titration to be 13·2 mg. as against a theoretical amount of 14·6 mg.

A sample of the globulin derivative, after reduction by a similar process, yielded 2·2 mg. CO_2 (calc. 3·0 mg.). The best evidence of the removal of the carbobenzyloxy residues, however, is afforded by the results of electrometric titration of the reduced product (see below); the material used in this experiment was prepared as follows. The globulin derivative (0·5 g.) was reduced with hydrogen and palladium black in glycine buffer solution as described above; the catalyst was removed by centrifuging and the protein isolated from the mother liquor by precipitation with acetic acid; re-solution and re-precipitation were repeated several times to remove glycine; finally the solution of the reduction product was dialysed successively against tap water, distilled water and *N*/100 acetate buffer, *p*H 5·2, containing *N*/10 KCl.

The glucose content of the reduction product (5·6 %) was little different from that of the starting material (6·0 %).

The reduction product was titrated electrometrically by the technique of

Harington & Neuberger [1936]; as in the case of glucosidotyrosylgelatin [Clutton *et al.* 1937] the result of the titration indicated that coupling had occurred mainly with the α-amino groups of protein.

Removal of carbobenzyloxy residues from the other protein derivatives was not attempted since, as will appear later, such residues are of little, if any, immunological importance.

Serological experiments

Technique of immunization. Rabbits of 2–3 kg. weight were used, and unless otherwise stated the following injections were given at 2-day intervals:

Week I: 10 mg. intravenously three times.
Week II: 20 mg. intravenously three times.
Week III: 30 mg. intraperitoneally three times.
Week IV: 40 and 50 mg. intraperitoneally three times.

The animals were rested for 10 days and test bleedings were made.

The following derivatives were used:

(*a*) *O*-β-Glucosido-*N*-carbobenzyloxytyrosylgelatin (3 animals).

(*b*) *O*-β-Glucosidotyrosylgelatin (3 animals). An additional course of injections was given consisting of three of 30 mg. intraperitoneally and three of 50 mg. After 10 days' rest a second bleeding was made.

(*c*) *O*-β-Glucosido-*N*-carbobenzyloxytyrosylglobulin (3 animals).

(*d*) *O*-β-Glucosidotyrosylglobulin (3 animals).

(*e*) *O*-β-Glucosido-*N*-carbobenzyloxytyrosylalbumin (2 animals). In this case the last two doses of 40 and 50 mg. respectively were omitted.

(*f*) *O*-β-Glucosido-*N*-carbobenzyloxytyrosylinsulin (3 animals). These animals received a 3 weeks' course consisting of nine doses of 10 mg.

As this derivative has practically the same activity as insulin itself, each rabbit received by stomach tube, before its injection, 50 ml. of a mixture of 10% glucose, 10% casein, 10% starch and 70% water. With this precaution in no case did any of the rabbits exhibit symptoms of hypoglycaemia.

Serological tests. The antisera obtained as above were titrated against the various antigens, the interfacial ring technique with undiluted serum being employed throughout; the specificity of the reactions was studied by means of inhibition tests with appropriate compounds.

Results

Globulin derivatives. Both *O*-β-glucosido-*N*-carbobenzyloxytyrosylglobulin and its reduction product gave strong precipitation reactions up to 1 : 100,000 with the homologous antisera, the original globulin specificity being almost completely masked (Table I A, B).

Table I

	Antigen	Antiserum	Serum no.	Dilutions of antigen 1 : 10^2	1 : 10^3	1 : 10^4	1 : 10^5
A.	G.C.T. globulin*	G.C.T. globulin	1	+ + +	+ + +	+ + +	+ +
	,,	,,	2	+ + +	+ + +	+ + +	+ +
	,,	,,	3	+ + +	+ + +	+ + +	+ +
	Normal globulin	,,	2	+	−	−	−
B.	G.T. globulin*	G.T. globulin	1	+ + +	+ + +	+ +	+
	,,	,,	2	+ + +	+ + +	+ +	+
	,,	,,	3	+ + +	+ + +	+ +	+
	Normal globulin	,,	2	(1/150) +	?	?	?

* In this and succeeding tables G.C.T. = *O*-β-glucosido-*N*-carbobenzyloxytyrosyl, and G.T. = *O*-β-glucosidotyrosyl.

The above precipitations at antigen dilutions of 1 : 10^5 were almost completely inhibited by preliminary incubation of the antiserum with an equal volume of 1 % glucosidocarbobenzyloxytyrosine for 15 min. at 38°.

Albumin derivative. This compound gave a powerful and highly specific reaction with its antiserum, the original albumin specificity being completely masked (Table II A). Inhibition tests with various compounds are shown in Table II B, and discussed below.

Table II

A. Precipitation tests

Antigen	Antiserum	Serum No.	Dilutions of antigen 1 : 10^2	1 : 10^3	1 : 10^4	1 : 10^5
G.C.T. albumin	G.C.T. albumin	1	+ +	+ ±	+ +	+
,,	,,	2	+ +	+ + +	+ +	+
Normal albumin	,,	2	–	–	–	–

B. Inhibition tests

1 vol. antiserum—G.C.T. albumin incubated for 30 min. at 38° with 1 vol. of: *A*, glucosidocarbobenzyloxytyrosine; *B*, glucosidotyrosine; *C*, arbutin; *D*, salicin; *E*, maltose; *F*, saline each in 1 % solution.

Dilution of antigen	Antigen *A*	*B*	*C*	*D*	*E*	*F*	Time of reading min.
1 : 10^3	–	–	.	.	.	+	0
	–	–	.	.	.	+ +	15
	–	–	.	.	.	+ +	30
1 : 10^4	–	–	±	–	+ +	+ +	0
	–	–	±	–	+ +	+ +	15
	–	–	±	–	+ +	+ +	30
	.	.	–	–	+ +	+ + +	45
1 : 10^5	.	.	–	–	±	+	0
	.	.	–	–	+	+	15
	.	.	–	–	+	+ ±	30
	.	.	–	–	+	+ ±	45

Gelatin derivatives. Even after repeated courses of injection the antisera raised against *O*-β-glucosidotyrosylgelatin or its carbobenzyloxy derivative failed to give any sign of precipitation with the homologous antigens. In view of the work of Hooker & Boyd [1933] it was decided to test for precipitin reactions between the anti-gelatin derivative sera and the globulin derivative; Table III A, B, C, shows the positive results which were obtained in this case.

Table III

	Antigen	Antiserum	Serum No.	Dilutions of antigen 1 : 10^2	1 : 10^3	1 : 10^4	1 : 10^5
A.	G.C.T. globulin	G.C.T. gelatin	1	+ +	+	–	–
	,,	,,	2	–	–	–	–
	,,	,,	3	±	±	–	–
B.	G.T. globulin	G.T. gelatin	1	+ ±	+	–	–
	,,	,,	2	–	–	–	–
	,,	,,	3	±	±	–	–
		Antigen controlled with normal serum negative					
C.	G.C.T. globulin	G.T. gelatin	1	+ +	+	–	–
	,,	,,	2	+	±	–	–
	,,	,,	3	+ + +	+ + +	±	–
	,,	Normal serum	Normal serum	–	–	–	–
D.	G.T. gelatin	G.C.T. globulin	1	?	+	+	?
	,,	,,	2	0	+	+	±

Table III D also shows that the reverse cross-reaction can be demonstrated, i.e. that the gelatin derivative will react with antiserum raised against the globulin derivative.

Insulin derivative. Like the gelatin compounds this substance failed to precipitate with its own antiserum; the antiserum however gave precipitation with the corresponding globulin derivative, and the globulin antiserum precipitated with the insulin derivative (Table IV A, B).

It was found, however, that the insulin derivative was capable of inhibiting the cross-reaction of its antiserum with the globulin derivative (Table IV C).

Table IV

	Antigen	Antiserum	Serum No.	Dilutions of antigen 1 : 10^2	1 : 10^3	1 : 10^4	1 : 10^5
A.	G.C.T. globulin	G.C.T. insulin	1	±	±	±	–
	,,	,,	2	+	±	+	–
	,,	,,	3	+	+	±	–
	,,	Normal serum	.	–	–	–	–
B.	G.C.T. insulin	G.C.T. globulin	.	+ + ±	+ +	+ +	+

C. Inhibition tests

Antiserum—G.C.T. insulin incubated 40 min. at 38° with (*a*) 1% insulin, (*b*) 1% G.C.T. insulin.

Inhibitor	Antigen—G.C.T. globulin, 1 : 10^3	Time min.
Insulin	+	10
	+ ±	20
G.C.T. insulin	–	10
	±	20

Discussion

Albumin and globulin derivatives

The first point of importance which emerges from the serological experiments is the powerfully determinant nature of the glucosidotyrosyl residue. This is shown clearly by the experiments recorded in Tables I and II; both globulin and albumin derivatives were strongly antigenic but neither the antiserum against glucosidocarbobenzyloxytyrosylglobulin nor that against the corresponding albumin derivative gave significant precipitin reactions with the original proteins. These results may be contrasted with those of Avery & Goebel [1929] who observed considerable residual globulin specificity when working with glucosidophenylazoglobulin.

The determinant effect of the glucosidotyrosyl residue is shown most clearly in the case of the albumin derivative, as is to be expected from the higher degree of coupling which was attained with this protein; Table II A shows that whilst good precipitin reactions are obtained between the antiserum and the coupled protein up to a dilution of 1 : 10^5 of the latter, no precipitation whatever is obtained with unaltered albumin.

The inhibition reactions shown in Table II B are of considerable significance; it will be observed that almost complete inhibition of the precipitation is obtained with four compounds which possess in common the β-phenolic glucoside linkage; inhibition with maltose on the other hand, which is a β-glucoside of different type, is very slight if indeed it exists at all. The conclusion to be drawn from this is that the determinant effect of the glucosidotyrosyl residue is conditioned entirely by the phenolic glucoside linkage. The predominant character of this

linkage is further emphasized by experiments (not recorded in detail) which have been made with a glucosidophenylazoalbumin derivative prepared by the method of Goebel & Avery [1929]; immunization with this compound yielded an antiserum with a good titre against the homologous antigen but showing marked precipitation with unaltered albumin; it gave good precipitation also with glucosidocarbobenzyloxytyrosylalbumin; the reaction between the antiserum and glucosidophenylazogelatin as well as that between the antiserum and glucosidotyrosylalbumin was completely inhibited by a β-phenolic glucoside (arbutin).

It appears therefore that, at least in the case of powerfully determinant groups, our original criticism of the azo method of coupling with proteins was ill-founded; on the other hand it can be claimed that our "peptide" method has the great advantage of completely masking the original specificity; moreover, the indications are that the carbobenzyloxy residues play little or no part in the immunological reactions.

Gelatin and insulin derivatives

As already stated, no precipitin reactions have at any time been obtained between antisera raised against the gelatin derivatives and the homologous antigens. It is, however, possible to demonstrate indirectly the presence of specific antibodies in such antisera; thus two out of three antisera against glucosidocarbobenzyloxytyrosylgelatin gave good precipitation with the analogous globulin derivative (Table III A) and, of antisera against glucosidotyrosylgelatin, two out of three precipitated with glucosidotyrosylglobulin (Table III B) and all three with glucosidocarbobenzyloxytyrosylglobulin (Table III C). These results are analogous with those recorded by Hooker & Boyd [1933] for a diazoarsanilic acid-gelatin complex.

Furthermore the reverse reaction can be demonstrated; thus from Table III D it is seen that both gelatin derivatives will precipitate with antiserum against glucosidocarbobenzyloxytyrosylglobulin; here again there exists an analogy in the experiments of Hopkins & Wormall [1933] with phenylcarbamido derivatives of gelatin and other proteins.

A precisely similar situation exists with the insulin derivative (Table IV A, B), but here the additional fact has been observed that the insulin derivative is itself able to inhibit the reaction between the anti-insulin derivative serum and glucosidocarbobenzyloxytyrosylglobulin.

Although, as has been pointed out, analogies for each of the separate types of cross-reaction just described are to be found in the literature, the combination of both types is not easy to understand. The failure to obtain direct precipitation between anti-gelatin or anti-insulin derivative serum and the appropriate homologous antigen might be explained by the assumption that the physical properties of these antigens are not such as to enable them to form an insoluble precipitate with the antibody; the validity of this assumption is, however, rendered questionable by the second type of reaction, since here the gelatin (or insulin) derivative does in fact form a precipitate with an antibody which, according to existing theories, should not differ greatly in physical properties from the antibody formed against the corresponding globulin derivative.

Whatever may ultimately turn out to be the explanation of this curious phenomenon there seems to be no escape from the conclusion that both gelatin and insulin, when coupled with glucosidotyrosine, acquire the power of inducing antibody formation, even though this can only be indirectly demonstrated; moreover, the inhibition experiments recorded in Table IV B show that the

reactions of the antibodies formed are specifically determined by the glucosidotyrosyl group which is acting as a hapten. It therefore seems reasonable to regard the glucosidotyrosyl derivatives of gelatin and insulin as complete antigens. Further evidence in favour of the antigenic effect of the insulin derivatives is afforded by the results of (as yet unpublished) anaphylactic experiments carried out by Dr S. D. Elliott in the Department of Pathology, Cambridge.

SUMMARY

1. The preparation is described of *O*-β-glucosido-*N*-carbobenzyloxytyrosyl derivatives of horse serum albumin and globulin and of insulin.

2. The above compounds, together with the corresponding gelatin derivative, have been studied immunologically.

3. The globulin and albumin derivatives are powerful antigens; the specificity of the original protein is entirely masked and the new specificity is conditioned exclusively by the β-phenolic glucoside linkage.

4. Antisera against the gelatin and insulin derivatives fail to precipitate with the homologous antigens; they precipitate, however, with the derivative of globulin, and antiserum against the latter precipitates with the gelatin and insulin compounds themselves. It is concluded that coupling with glucosidotyrosine converts both gelatin and insulin into full antigens.

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