CI. STUDIES IN SYNTHETIC IMMUNOCHEMISTRY

I. THE SYNTHESIS OF O- β -GLUCOSIDOTYROSINE AND ITS INTRODUCTION INTO THE PROTEIN MOLECULE

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It is still generally accepted that the possession of full antigenic properties is confined to the class of compounds which may be characterized as complete proteins, that is to say proteins which contain a full complement of all the commonly occurring amino-acids and probably also a carbohydrate group.

Certain facts indicate that the aromatic amino-acids, and in particular tyrosine, occupy a position of special importance in relation to the development of antigenic properties; thus proteins containing no tyrosine are non-antigenic, and the specificity of antigenic proteins can be profoundly modified by alterations (e.g. nitration, halogenation etc.) in the tyrosine groups which they contain.

The predominant importance of carbohydrate groups in determining the immunological specificity of bacterial antigens was established by the pioneer work of Avery, Heidelberger and co-workers on the *Pneumococcus* and has been amply confirmed by later work on other organisms. Moreover, the study of artificial carbohydrate-protein compounds has shown that even simple carbohydrate groups such as hexosido residues are capable of inducing a high degree of immunological specificity [Avery & Goebel, 1929].

The artificial carbohydrate-protein compounds which have hitherto been used for such studies have all been prepared by the same general method; this consists in making an aminophenol derivative of the carbohydrate (usually the *p*-aminophenol glycoside), diazotizing the latter and coupling the diazonium compound with the protein in alkaline solution; in this reaction it is to be assumed that two diazonium residues enter the positions *ortho* to the phenolic group of the tyrosine contained in the protein and the further possibility exists that some coupling may occur with the iminazole ring of histidine.

Although the study of the immunological properties of substances prepared in this manner has led to the acquisition of much valuable information, the biological value of the deductions is lessened by the fact that the carbohydrate groups are attached to the protein through a highly artificial linkage and one which certainly does not occur in nature. It has therefore seemed to the present writers that progress in this branch of immunological knowledge might be advanced and conclusions of more secure biological validity might be drawn if artificial compound antigens were available in which the carbohydrate group was attached through a naturally occurring type of chemical linkage.

The ideal would evidently be the preparation of artificial protein-carbohydrate compounds in which the components were combined in the same way as they are in naturally occurring proteins which contain carbohydrate groups. No information is, however, available concerning the nature of the linkage whereby the carbohydrate group in natural proteins is attached and the best that can be done at the present is to have resort to a linkage which, although it may not naturally occur in proteins, is at least a common feature among natural products.

The work described in the present paper represents a first attempt in the direction outlined above and the results described below reveal the possibility of the artificial introduction of simple carbohydrate residues into the protein molecule without gross interference with the structure of the latter and without the interposition of any type of linkage which is not known to occur in nature. It is intended to apply the method to the preparation and immunological study of a number of artificial antigens containing not only carbohydrate groups to be introduced in the manner to be described but also amino-acids of peculiar physiological interest such as thyroxine.

Consideration of the various linkages which might be employed for the purpose in hand and which would come near to fulfilling the conditions indicated above suggested to us as an obvious possibility the *O*-glycosido linkage; it seemed conceivable that simple hexose residues at least might be attached to protein in this way by glycoside formation with the phenolic group of the tyrosine. Direct combination of carbohydrate with the tyrosine already existing in the protein through the glycoside linkage did not indeed seem a practicable procedure; it was hoped however that a derivative of (the hitherto unknown) *O*- β -glucosidotyrosine might be found which could be coupled in peptide linkage with the free amino groups of a protein, and this hope has been realized.

Acetobromoglucose and *N*-carbobenzyloxytyrosine ethyl ester react smoothly in presence of quinoline and active silver oxide to give *O*- β -tetraacetylglucosido-*N*-carbobenzyloxytyrosine ethyl ester (I); this compound can be used (a) for the preparation of *O*- β -glucosidotyrosine (III) and (b) for the introduction of the *O*- β -glucosidotyrosyl residue into a protein.

(a) *O*- β -Glucosidotyrosine. For the preparation of this compound the above ester was shaken for a long period at room temperature with dilute aqueous barium hydroxide; this yielded the sparingly soluble barium salt of *O*- β -glucosido-*N*-carbobenzyloxytyrosine, and the free acid (II) on catalytic hydrogenation in the usual manner, gave the desired *O*- β -glucosidotyrosine.

(b) *O*- β -Glucosidotyrosylgelatin. The introduction of the glucosidotyrosyl residue into a protein has so far only been accomplished with gelatin; the choice of this protein for the first experiments was made for two reasons: (1) in order to simplify conditions as far as possible and (2) because the first aspect of the major problem in which we were interested was the attempt to discover whether antigenic properties could be conferred on the non-antigenic gelatin by the

introduction into its molecule of tyrosine or carbohydrate or both. When *O*- β -tetraacetylglucosido-*N*-carbobenzyloxytyrosine ethyl ester (I) is treated with excess of hydrazine hydrate in alcoholic solution at room temperature the acetyl groups are eliminated and there results *O*- β -glucosido-*N*-carbobenzyloxytyrosylhydrazide (the hydrazide of II); this can be readily converted into the azide, and the latter, when added in dioxan solution to an alkaline aqueous solution of gelatin, couples with part of the free amino-groups of the latter. The product thus consists of *O*- β -glucosido-*N*-carbobenzyloxytyrosylgelatin and the final problem is the removal of the carbobenzyloxy residues. This is a matter of some difficulty, catalytic reduction being excluded by the adverse effect of the protein on the catalyst, and the use of phosphonium iodide or of very dilute hydrogen iodide by the lability of the glycosido linkage towards acid. A solution was finally found in the application of the method of Siffert & du Vigneaud [1935] which consists in reduction with sodium in liquid ammonia solution; under these conditions the carbobenzyloxy residues are removed without rupture of the glycosido linkage and without degradation of the protein molecule. Evidence that the glycosido linkage remains intact is afforded by the unimpaired sugar content of the final product and the absence of significant degradation of the protein molecule may be inferred from the characteristics of the titration curve of the latter.

The titration curve of the final product indicates, as is shown below, that the glucosidotyrosine residues are probably attached to the free α -amino-groups of the original gelatin and not to the ϵ -amino-groups of the lysine.

The final product as well as its carbobenzyloxy derivative have been subjected to preliminary immunological investigation. The results have so far been negative inasmuch as the sera of rabbits subjected to an ordinary short course (3-4 weeks) of immunization with these two products have failed to yield precipitin reactions with the respective substances under test; there is thus no evidence at present that antigenic properties have been conferred on the gelatin by introduction of the glucosidotyrosine residue. The immunological investigation is, however, still incomplete and we desire to reserve final judgement on this matter until it has been more thoroughly studied.

EXPERIMENTAL

A. *Synthesis of O*- β -glucosidotyrosine

O-Tetraacetyl- β -glucosido-*N*-carbobenzyloxytyrosine ethyl ester (I). *N*-Carbobenzyloxytyrosine ethyl ester (3.43 g.) and quinoline (3-4 ml.) were mixed in a mortar with acetobromoglucose (8.22 g.; 2 mol.); quinoline was added to a total of 10 ml. and active silver oxide (5 g.; compare Fischer & Helferich [1911]) was gradually stirred in; the mixture was stirred with a pestle for 15 min. during which time it became warm and viscous. After keeping for 2 hr. in a desiccator the mass was dissolved in glacial acetic acid (60 ml.) and the solution, after treatment with a little dilute nitric acid to coagulate the precipitate, was cleared on the centrifuge. The supernatant liquor was poured into ice-water (300 ml.); after keeping in the ice-chest overnight the precipitate was collected, washed with water and crystallized from alcohol. The product so obtained was slightly discoloured with silver oxide but was sufficiently pure for the next reaction; the yield was 84% of the theoretical. Removal of the last traces of silver was effected by passage H_2S through aqueous alcoholic (90%) solution of the ester. After final recrystallization from alcohol: ether (1:1) the compound formed

colourless needles melting at 108° . (Found: C, 59.3; H, 6.0; N, 2.1%. $C_{33}H_{39}O_{14}N$ requires C, 58.8; H, 5.8; N, 2.1%.)

O- β -Glucosido-*N*-carbobenzyloxytyrosine (II). The above ester (2.5 g.) was finely powdered, sieved (100 mesh) and suspended in a solution of barium hydroxide (7 g. of the octahydrate; 110% excess) in water (110 ml.); the mixture was shaken for 3–4 hr., ether (10 ml.) added and shaking continued for 20 hr. in all. The milky suspension was diluted with water (300–400 ml.), warmed to 50° and saturated with carbon dioxide; barium carbonate was removed by centrifuging and the solution concentrated under diminished pressure. Further barium carbonate separated during the concentration and was filtered off when the volume had reached about 200 ml.; the filtrate was then evaporated to low bulk. During the final concentration the barium salt of the product began to separate; it was redissolved by heating and the hot solution was treated with alcohol (2 vol.). On cooling the barium salt separated slowly in hair-like needles. After 36 hr. at 0° the salt was collected, washed with water and alcohol and dried; it was then dissolved in warm water (50 parts) and treated at 40 – 45° with *N* HCl (1 equiv.); the acid began to crystallize at once; after keeping for a short time at 0° it was collected, washed and dried. The yield was 70% of the theoretical. Recrystallized from water the compound formed fine colourless needles, m.p. 177° . It had $[\alpha]_D -24.2^{\circ}$ ($c=0.455$ in water with 1 equiv. NaOH). (Found: N, 2.7%. $C_{23}H_{27}O_{10}N$ requires N, 2.9%.)

O- β -Glucosidotyrosine (III). *O*- β -Glucosido-*N*-carbobenzyloxytyrosine (1.2 g.) was suspended in 50% aqueous alcohol (35 ml.) containing a few drops of glacial acetic acid and hydrogenated in presence of palladium black (0.3 g.). During the reduction the milky suspension first cleared and crystalline material subsequently separated. When evolution of carbon dioxide had ceased the mixture was diluted with water (100 ml.) which dissolved the crystals, filtered from the catalyst and evaporated to low bulk under diminished pressure. During the evaporation crystals again separated; these were redissolved by warming and the solution treated with alcohol (2 vol.); the product began to separate almost at once and after keeping for a short time at 0° it was collected, washed with 50% and then with absolute alcohol and dried. The yield was 75% of the theoretical. *O*- β -Glucosidotyrosine forms colourless prisms from aqueous alcohol; it has m.p. 282° (decomp. uncorr.) and $[\alpha]_{5461} -77^{\circ}$ (c 1% in 0.1 *N* HCl). It is readily soluble in water but insoluble in alcohol and other organic solvents. It gives a ninhydrin reaction but no Millon reaction. (Found: C, 52.5; H, 6.0; N, 4.1%. $C_{15}H_{21}O_8N$ requires C, 52.5; H, 6.1; N, 4.1%. Glucose (orcinol method) 51.0, 52.8%; calc. 52.5%.)

The glucoside is fairly resistant to hydrolysis by acid. Treatment with 0.1 *N* HCl at 55 – 60° for 3 hr. and with *N* HCl at 38° overnight had no effect (unchanged rotation); boiling with *N* HCl for 2 hr. on the other hand caused a change of rotation from -0.59° to $+0.21^{\circ}$, the latter value remaining constant on further boiling.

The glucoside was hydrolysed by emulsin. A 1% solution at pH 5.0 with an active preparation of emulsin had an initial rotation of -0.91° ; after 7 hr. at 38° this had changed to -0.29° . The solution was left overnight at 28° to complete the reaction and next morning was filled with crystals of tyrosine; after chilling the latter was filtered off and the filtrate, after treatment with ammonia, had $\alpha_D +0.25^{\circ}$; assuming the residual rotation to be due to glucose the theoretical figure would be 0.28° .

B. *Coupling of the O- β -glucosidotyrosine residue with gelatin*

O- β -Glucosido-N-carbobenzyl-oxytyrosylhydrazide. *O- β -Tetraacetylglucosido-N-carbobenzyl-oxytyrosine ester* (5.0 g.) was dissolved in hot ethyl alcohol (30 ml.); the solution was cooled to about 50°, treated with hydrazine hydrate (5.5 ml. of 95%) and left in an incubator at 38°. After 2 hr. some gelatinous material had separated which was brought into solution by warming; the solution was returned to the incubator and after a further hour was again warmed to dissolve a gelatinous precipitate. After 2 hr. more at 38° crystalline material had separated. The mixture was treated with alcohol (10 ml.), brought to the boil on a water-bath and then left in the incubator overnight. Next day 30 ml. alcohol were added, the whole was brought to the boil with stirring and then cooled in the refrigerator. The precipitate was collected, washed with much alcohol and then with ether and dried; the yield was 3.15 g. (86% of the theoretical).

Prepared in this way the hydrazide formed bunches of colourless needles, m.p. 215° (decomp.); it was sparingly soluble in water and all organic solvents tried except pyridine. It had $[\alpha]_D - 37.5^\circ$ ($c = 4$ in pyridine). In recrystallization experiments the compound was occasionally obtained with m.p. 180°; the identity of this product with that melting at 215° was shown by the facts that both were equally satisfactory for the preparation of the azide (see below) and that both had the same rotation; the conditions for interconversion of the two forms were not determined. (Found: C, 56.0; H, 6.3; N, 8.7; hydrazide-N [Meyer, 1931, p. 458], 5.7%. $C_{23}H_{29}O_9N_3$ requires C, 56.2; H, 5.9; N, 8.6; hydrazide-N 5.7%.)

O- β -Glucosido-N-carbobenzyl-oxytyrosylgelatin. *O- β -Glucosido-N-carbobenzyl-oxytyrosylhydrazide* (1.23 g.) was dissolved in hot glacial acetic acid (12 ml.); the solution was cooled to 0° and treated with dilute hydrochloric acid (12 ml. of *N*) and then drop by drop with 30% aqueous sodium nitrite (1 ml.); the azide began to separate in colourless prisms just before the addition of the sodium nitrite was complete. The mixture was diluted with water (25 ml.) and the azide collected, washed thoroughly with water and used directly for the coupling. (The azide is insoluble in water, sparingly so in ether and ethyl acetate but readily soluble in dioxan.)

Gelatin (1.8 g.) was dissolved in warm water (180 ml.) and the filtered solution mixed with alcohol (180 ml.), the whole being made just alkaline to phenolphthalein with sodium hydroxide and chilled to 0°. The azide from the above-described experiment was dissolved in dioxan (10 ml.) and added during about 15 min. to the vigorously stirred aqueous alcoholic solution of gelatin, 2*N* NaOH being run in simultaneously to maintain the reaction just alkaline to phenolphthalein. During this process the solution remained quite clear. After a few minutes 10% acetic acid was added to the point of maximum precipitation; no separation of solid occurred until the point of neutrality was passed. The mixture was kept overnight at 0° and the sticky precipitate was collected at the centrifuge and washed successively with 50, 75% and finally two portions of absolute methyl alcohol.

During the later stages of washing the product hardened and granulated; after drying in a vacuum desiccator it was finally obtained as a light, white granular powder, easily soluble in water. After hydrolysis with boiling 5*N* hydrochloric acid it gave a Molisch reaction and the solution smelt strongly of benzyl chloride (derived from the carbobenzyl-oxy residues).

The product had 14.1% N and 4.6% glucose (orcinol method). The N content

is in good accord with expectation from the N content (15%) of the original gelatin taking into account the increase of molecular weight.

O-β-Glucosidotyrosylgelatin. The difficulty of removing the carbobenzyloxy groups from glucosidocarbobenzyloxytyrosylgelatin has already been referred to in the introductory portion of this paper. The first method attempted was one which had been used with success, in experiments to be published later, on the preparation of tyrosylgelatin, namely, treatment of the protein dissolved in a phenol-cresol mixture with a low concentration of hydrogen iodide. In the present case this method was unsuccessful, since removal of the carbobenzyloxy residues was always accompanied by more or less scission of the glucosido linkage with consequent loss of glucose. Experiments on reduction in aqueous solution both with the aid of a variety of catalysts and with such reducing agents as sodium amalgam at neutral or faintly acid reaction having also proved abortive, an attempt was made to employ the method of Sifferd & du Vigneaud [1935] which consists of reduction with sodium in liquid ammonia. Direct application of this method could not be made owing to the insolubility of the protein in liquid ammonia; it was found however that the solubility could be greatly increased by the addition of various substances and success was finally attained by the use of a mixture of liquid ammonia and ammonium acetate.

O-β-Glucosido-N-carbobenzyloxytyrosylgelatin (3 g.) was finely ground with ammonium acetate (1 g.); the mixture was treated slowly, with stirring, with anhydrous liquid ammonia (30 ml.), the reaction vessel being immersed in a cooling mixture of solid carbon dioxide and acetone. The solution so obtained was vigorously stirred and treated with sodium in small portions (0.2 g. in all). The ammonia was then allowed to evaporate, finally in the vacuum of a water pump and the residue taken up in water (10–15 ml.); the solution was acidified with acetic acid and treated with alcohol (5 vol.) which precipitated the reaction product. The precipitate was collected, washed with alcohol and ether and dried; it was taken up again in water to 1.5% concentration and dialysed for 4 days to remove inorganic salts; finally the protein was reprecipitated by addition of alcohol (5 vol.) and a trace of lithium chloride, collected and dried. The yield was about 70% of the theoretical.

On hydrolysis with 5*N* HCl the product gave a Molisch reaction but no detectable odour of benzyl chloride. It is easily soluble in warm water but forms a gel at room temperature at concentrations of 2% and over.

The nitrogen and glucose contents of the product were 13.6 and 4.6% respectively. In view of the proved removal of the carbobenzyloxy residues (see below) the N content of the final product should have been somewhat higher than that of the carbobenzyloxy derivative; in actual fact it is lower, whilst the glucose contents of the two compounds are the same; these facts are taken to indicate a higher degree of hydration in the case of the deacylated compound; it is however impossible to determine this point with accuracy.

Titration curves

Electrometric titrations were performed on gelatin and its derivatives in the apparatus and according to the technique described by Harington & Neuberger [1936]. The protein solutions before titration were dialysed for 4 days against distilled water. The curves obtained are shown in Fig. 1. Included in this figure are results obtained with tyrosylgelatin, the preparation of which is to be described in a later publication.

The most important deduction to be drawn from the curves is that no appreciable degradation of the protein molecule has occurred; this is shown by

the fact that the glucosidotyrosylgelatin shows no increase in acid binding between pH 3 and the isoelectric point, or in base binding between the isoelectric point and pH 10.5 as compared with gelatin itself. In the case of tyrosylgelatin the increased base binding over the range pH 9–10.5 is in accordance with expectation from the tyrosine content of the protein.

Fig. 1.

The total amino-nitrogen of the original gelatin was 0.9%; from available data on the lysine content of gelatin about two-thirds of this amount would be accounted for by the ϵ -amino groups of this amino-acid. Calculation from the glucose content of the glucosidotyrosylgelatin indicates that the glucosidotyrosyl residue has reacted with approximately 0.3% of the original gelatin amino-nitrogen. At the pH at which the coupling was carried out (8–9) the ϵ -amino-groups of lysine would be ionized whilst α -amino groups would not; under such conditions it is reasonable to expect that coupling would occur predominantly with the α -amino groups. This supposition is supported by the results of electro-metric titration which fail to reveal the increase in base binding over the range pH 7–9 which would result from the replacement of the ϵ -amino-groups of lysine by the α -amino-groups of the tyrosine residue.

Attempts to titrate the glucosidocarbobenzyloxytyrosylgelatin failed owing to the impossibility of obtaining steady potentials; this is doubtless to be ascribed to reaction of the reducible carbobenzyloxy linkage at the hydrogen electrode. The absence of any such difficulty in the case of glucosidotyrosylgelatin is the strongest evidence that removal of carbobenzyloxy residues from this compound was in fact complete.

SUMMARY

1. *O*- β -Glucosidotyrosine has been synthesized by condensation of acetobromoglucose with *N*-carbobenzyloxytyrosine ester followed by hydrolysis of the acetyl and ester groups and reduction of the resulting acid.
2. *O*- β -Glucosido-*N*-carbobenzyloxytyrosylazide, prepared from the condensation product of acetobromoglucose and *N*-carbobenzyloxytyrosine ester, has been coupled with gelatin in alkaline solution.

3. Carbobenzyloxy residues have been removed from the above product to yield an *O*- β -glucosidotyrosylgelatin containing 4.6 % of glucose. The properties of the product are described and evidence is presented that in it the glucosido-tyrosine residues are attached to the α -amino-groups of the original gelatin.

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