

## CHEMO-IMMUNOLOGICAL STUDIES ON CONJUGATED CARBOHYDRATE-PROTEINS

### VI. THE SYNTHESIS OF *p*-AMINOPHENOL $\alpha$ -GLUCOSIDE AND ITS COUPLING WITH PROTEIN

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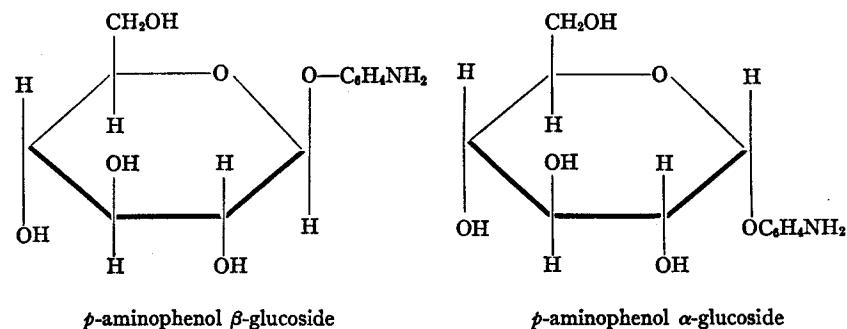
The preparation of synthetic carbohydrate-protein antigens has been described in previous publications from this laboratory (1). It will be recalled that simple hexose derivatives (aminophenol glucosides) have been attached to proteins by means of the diazo reaction. It was shown (2, 3) that when two chemically different carbohydrate derivatives are bound to the same protein, the newly formed substances exhibit a distinct immunological specificity and that this newly acquired specificity is determined by the chemical constitution of the carbohydrate radical attached to the protein molecule. Thus, simple differences in the molecular configuration of the two isomers, glucose and galactose, suffice to orientate specificity when the corresponding beta-aminophenol glucosides of these two sugars are coupled to the same or unrelated proteins.

The opportunity for differences in intermolecular chemical unions appears to be very great in the case of complex carbohydrates, such as the type-specific capsular polysaccharides of bacteria, which are built up of hexose and uronic acid molecules (4). As a result one might not only anticipate, but indeed one actually finds wide differences in immunological specificity among a group of closely related substances. We know but little of the intimate make-up of complex bacterial polysaccharides; still less do we know concerning the factors which orient their specificity. Although intermolecular stereochemical relationships, such as the position of linkage of one hexose molecule to another, exert an influence in orienting immune response, certain

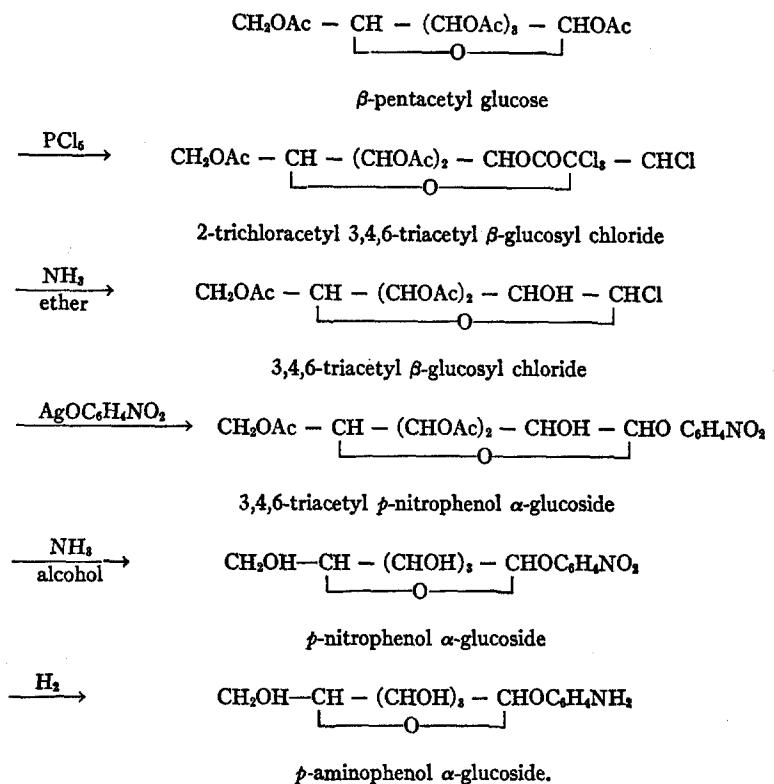
other factors (intramolecular) may assist in determining specificity; factors such as the presence of highly polar groups, stereochemical differences, etc.

The question of whether the type of hexose linkage (*i.e.* an alpha- or beta-glucosidic linkage) could exert an orienting influence on antigenic specificity appears both of interest and of importance. It is a problem approachable from the synthetic view point, and one which could be answered either by studying the immune response elicited by two disaccharides which differ from one another in the type of glucosidic linkage or, more simply, by studying the immunological specificity of two isomeric glucosides of the same hexose attached to the same protein.

We have undertaken the preparation of the alpha-*p*-aminophenol glucoside of glucose, and have coupled it to the globulin of horse serum. Its isomer, the beta-glucoside, has likewise been prepared (1). Thus two synthetic glucoproteins have been synthesized which differ from one another only in the alpha and beta linkage of the glucoside to the protein molecule. These differences in chemical constitution of the two glucosides can best be understood by the graphic formulae:



In the case of the alpha-glucoside, the aglucon (the non-sugar component) lies in a *cis* position in reference to the hydroxyl group in the second carbon atom of the hexose, whereas the aglucon of the beta-glucoside lies in the *trans* position. The method of preparation of the alpha-glucoside was accomplished by the following series of reactions:



## EXPERIMENTAL

(1)  *$\beta$ -Pentacetyl Glucose.*—This compound was prepared in the usual manner by the acetylation of glucose with acetic anhydride and sodium acetate.

(2) *2-Trichloracetyl 3, 4, 6-Triacetyl  $\beta$ -Glucosyl Chloride.*—This product was prepared from  $\beta$ -pentacetyl glucose by the action of phosphorus pentachloride according to the directions of Brügel (5). From 200 gm. of acetyl glucose 80 gm. of the recrystallized end-product were obtained, melting at 140°.

(3) *3, 4, 6-Triacetyl  $\beta$ -Glucosyl Chloride.*—The above compound, when treated at 0° with anhydrous ether saturated with dry ammonia gas (5), gave almost quantitative yields of the desired product. Recrystallization from anhydrous ethyl acetate yielded a product which melted at 154°.

(4) *Silver p-Nitrophenolate.*—This compound was prepared as previously described (1).

(5) *3, 4, 6-Triacetyl p-Nitrophenol α-Glucoside*.—50 gm. of 3, 4, 6-triacetyl β-glucosyl chloride were suspended in 500 cc. of chloroform dried over phosphorus pentoxide. A large number of dry glass beads were placed in the bottle, and to the mixture were added, in three separate portions and at intervals of 10 minutes, 41.5 gm. (1.1 mols) of silver *p*-nitrophenolate. The mixture was shaken mechanically until no more chlorine could be detected in the chloroform solution. The mixture was then filtered by suction, and the precipitate was washed with chloroform. The combined filtrates were now concentrated to a syrup in vacuum. The residue, of dark brown color, was dissolved in 250 cc. of alcohol, and again evaporated to a syrup. After a second solution and evaporation the syrup was dissolved in alcohol and was placed overnight in the ice box. The glucoside crystallized out, it was filtered in the cold and washed with small portions of cold alcohol. 13 gm. of crude glucoside were recovered. The compound was twice recrystallized from alcohol, yielding about 11 gm. of a pure product melting at 148–149°C.

$$[\alpha]_D^{20} = \frac{+ 3.89 \times 100}{2 \times 2.467} = + 78.9^\circ \text{ (in methyl alcohol).}$$

Analysis: 4.659 mg. substance: 8.635 mg. CO<sub>2</sub> and 2.105 mg. H<sub>2</sub>O.

C<sub>18</sub>H<sub>21</sub>O<sub>11</sub>N. Calculated: C 50.58 per cent, H 4.95 per cent.

Found: C 50.54 per cent, H 5.05 per cent.

(6) *p-Nitrophenol Alpha-Glucoside*.—The triacetyl glucoside prepared as described above was dried at 60° in a vacuum oven. 20 gm. of the glucoside were dissolved in 75 cc. of anhydrous methyl alcohol and the solution added to 75 cc. of anhydrous methyl alcohol previously saturated with dry ammonia gas at 0°C. The mixture was placed in the ice box and allowed to stand for 15 hours. At the end of this time a large crystalline mass separated from the solution. Two distinct crystalline forms could be distinguished under the microscope, one form crystallized in needles, the other in prisms. The mixture was now warmed and the crystals redissolved. The solution was concentrated to dryness *in vacuo*, and was dissolved in 150 cc. of 95 per cent redistilled ethyl alcohol. The solution was again concentrated to dryness *in vacuo*. The crystalline mass was now dissolved in 175 cc. of 95 per cent ethyl alcohol, decolorized with a little norit and filtered. After standing overnight at room temperature (22–25°), the pure form of prismatic crystals was separated by filtration. 4.7 gm. were recovered. This compound, unrecrystallized, sintered at 205° and melted at 210–212°. After recrystallization from 60 cc. of ethyl alcohol glistening pale yellow crystals of the *p*-nitrophenol α-glucoside of glucose were recovered which melted at 216–217° sintering slightly at first at 210°. The compound had an optical rotation of

$$[\alpha]_D^{20} = \frac{4.98 \times 100}{2 \times 1.093} = + 227.9^\circ \text{ (in methyl alcohol).}$$

Analysis: 4.692 mg. substance: 8.205 mg. CO<sub>2</sub> and 2.040 mg. H<sub>2</sub>O.

C<sub>12</sub>H<sub>15</sub>O<sub>8</sub>N. Calculated: C 47.83 per cent, H 5.02 per cent.

Found: C 47.68 per cent, H 4.86 per cent.

The original mother liquor from which the  $\alpha$ -glucoside was recovered, deposited on cooling, a mixture of two crystalline forms. When the ethyl alcohol was removed by distillation and the substances subsequently dissolved in 150 cc. of methyl alcohol, and allowed to stand at room temperature, only the needle forms crystallized. This compound was filtered, and dried—8 gm. were recovered. When recrystallized from methyl alcohol glistening white needles melting at 164–165°C. were recovered. The compound had a specific optical rotation of –79.2° in methyl alcohol, and was identified as *p*-nitrophenol  $\beta$ -glucoside after taking a mixed melting point with an authentic sample.

It was stated above that triacetyl  $\alpha$ -nitrophenol glucoside yielded a mixture of  $\alpha$ - and  $\beta$ -nitrophenol glucosides when the former was treated with anhydrous ammonia in methyl alcohol. This interesting phenomenon may possibly be ascribed to a mutarotation of the carbon atom bearing the aglucon group during the process of hydrolysis.<sup>1</sup> As substantiating evidence for this conception, it was observed that a sharp fall in the optical rotation of  $\alpha$ -nitrophenol glucoside occurred when an aqueous solution was permitted to stand in the presence of 0.5 normal ammonia at room temperature; a similar, though much less rapid drop in rotation occurred in 0.1 normal ammonia at 4°C. The substituted  $\alpha$ -nitrophenol glucoside appears, therefore, to be unstable in the presence of hydroxyl ions, and this instability is a function of the temperature and of the concentration of hydroxyl ions.

(7) *p*-Aminophenol  $\alpha$ -Glucoside.—2.0 gm. of *p*-nitrophenol  $\alpha$ -glucoside were dissolved in 100 cc. of warm ethyl alcohol. The substance was reduced catalytically with platinum oxide and hydrogen. From the alcoholic solution 1.4 gm. of the *p*-aminophenol  $\alpha$ -glucoside were isolated. This compound crystallized as a snow white product, readily soluble in water. It melted at 185–186°, and had a specific optical solution of

$$[\alpha]_D^{24^\circ} = \frac{5.35 \times 100}{2 \times 1.378} = + 194.1^\circ \text{ (in methyl alcohol).}$$

A weighed sample, when titrated with standard nitrous acid, utilized the theoretical quantity.

<sup>1</sup> The authors wish to express their thanks to Dr. P. A. Levene for his interest and advice.

Analysis: 4.330 mg. substance: 8.389 mg. CO<sub>2</sub> and 2.495 mg. H<sub>2</sub>O.

C<sub>12</sub>H<sub>17</sub>O<sub>6</sub>N. Calculated: C 53.12 per cent, H 6.32 per cent.

Found: C 52.83 per cent, H 6.44 per cent.

(8) *Serum Globulin*.—Serum globulin was prepared by half saturating horse serum with ammonium sulfate, separating the globulins by centrifugation and again precipitating by half saturation. This process was repeated in all four times. The protein was finally dialysed and redissolved by the addition of solid sodium chloride. Thus pseudo- and euglobulin were separated from serum albumin. This solution of pseudo- and euglobulin was used for the preparation of protein-diazophenol  $\alpha$ -glucoside.

(9) *Preparation of Protein Diazophenol  $\alpha$ -Glucoside and  $\beta$ -Glucoside*.—The alpha-glucoside was coupled to serum protein in the following manner.

120 mg. of  $\alpha$ -aminophenol glucoside were dissolved in 2.5 cc. of water. The solution was cooled to 0°C. and 1.0 cc. of normal HCl added. To this mixture was added 4.62 cc. of N/10 sodium nitrite solution. After standing for 5 minutes at 0°C. the mixture was poured into an ice-cold solution of serum globulin, 300 mg. dissolved in 12 cc. of N/10 sodium carbonate. The mixture was now permitted to stand for 30 minutes at 0°. At the end of this time the solution was carefully acidified to the point of maximum precipitation with 10 per cent trichloracetic acid. The highly colored precipitate of  $\alpha$ -glucoside-azoglobulin was recovered by centrifugation and the supernatant liquid discarded. The precipitate was now suspended in 5 cc. of cold salt solution, stirred, and brought into solution by the cautious addition of N/10 NaOH. The deeply colored solution of sugar-azoprotein was now diluted to 20 cc. with salt solution, and the azoprotein reprecipitated by the careful addition of a few drops of N/10 HCl. The protein was again centrifuged and the supernatant liquid discarded. The precipitate was resuspended in cold saline, dissolved as before, and the final *neutral* solution diluted to 16 cc. One cc. was removed for a nitrogen analysis, and the sugar-azoprotein finally diluted so that each cc. contained 10 mg. of protein. This solution was sterilized by filtration through a Berkefeld candle, and used for immunization experiments, as described in the following paper.

The preparation of the  $\beta$ -glucoside-protein was carried out exactly as described above, except that pure  $\beta$ -*p*-aminophenol glucoside was used to couple with protein.

#### SUMMARY

1. The synthesis of *p*-aminophenol  $\alpha$ -glucoside has been described. This glucoside can be coupled to any protein to yield a synthetic  $\alpha$ -glucoside-protein complex.

2. A synthetic  $\beta$ -glucoside-protein complex has also been prepared.

3. These synthetic sugar-protein complexes have been used as immunizing antigens in order to ascertain whether  $\alpha$ - and  $\beta$ -glucosidic

unions influence the specificity of the immune response in animals. The results of the immunological studies are presented in the following paper.

#### BIBLIOGRAPHY

1. Goebel, W. F., and Avery, O. T., *J. Exp. Med.*, 1929, **50**, 521.
2. Avery, O. T., and Goebel, W. F., *J. Exp. Med.*, 1929, **50**, 533.
3. Tillett, W. S., Avery, O. T., and Goebel, W. F., *J. Exp. Med.*, 1929, **50**, 551.
4. Pryde, J., Recent advances in biochemistry, Philadelphia, P. Blakiston's Son and Co., 1928, 344.
5. Brügel, P., *Z. physiol. Chem.*, 1921, **116**, 1. Brügel, P., and Keppler, H., *Ber. chem. Ges.*, 1926, **59**, pt. 2, 1588. See also Hickinbottom, W. J., *J. Chem. Soc.*, 1928, **50**, 3140; 1929, **51**, 1676; 1930, **52**, 1336.