CHEMO-IMMUNOLOGICAL STUDIES ON CONJUGATED CARBOHYDRATE-PROTEINS

I. The Synthesis of *p*-Aminophenol β-Glucoside, *p*-Aminophenol β-Galactoside, and Their Coupling with Serum Globulin

By WALTHER F. GOEBEL, Ph.D., and OSWALD T. AVERY, M.D.

(From the Hospital of The Rockefeller Institute for Medical Research)

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The problems of the relationship between chemical constitution, physiological effect and biological specificity, which found their origin in the study of the active principles of certain natural drugs, have become so absorbing and so embracing that they have attracted and held the interest of the chemist, the pharmacologist, and the immunologist alike. The question of protein specificity (1), and that of whether it is possible to change specificity by altering the protein molecule through chemical means, have, in particular, engaged the minds of many investigators. On the other hand, the rôle which carbohydrates play in the phenomena of immunity has only recently been disclosed, despite the fact that the presence of these substances in bacteria and yeast has long been known.

Some years ago, Dochez and Avery (2) observed that filtrates of pneumococcus cultures contained a stable substance which reacted specifically with antipneumococcus serum of the homologous type. This observation led to the isolation and identification of these specifically reacting substances from the three specific types of Pneumococcus (3), and from the three types of Friedländer's bacillus (4). Since then, other investigators have isolated similar substances from various other species of bacteria (5).

These type specific substances fall into the class of carbohydrates. They are unusual carbohydrates in that each contains a sugar acid as an integral part of its complex molecule. Immunologically they belong to that important group of specifically reactive but non-antigenic substances which Landsteiner has termed *haptens*.

One of the striking characteristics of these bacterial carbohydrates is their failure to produce antibodies when injected into the animal organism, though in the state in which they occur in the bacterial cell they are not only type specific, but are also antigenic as well. In order that the bacterial polysaccharide may be effective as antigen it is believed, therefore, that it must be combined with another cellular constituent—possibly a protein—to form a complex and easily dissociable antigen. The nature of the substance which confers antigenicity upon the carbohydrate substance of Pneumococcus and the character of its union, are problems which as yet have not been solved.

The products of hydrolysis of these specific carbohydrates have been studied in detail (6). All except the polysaccharide of Pneumococcus Type I yield glucose on hydrolysis. The carbohydrate of Pneumococcus Type III and of Friedländer bacillus Type A yield on hydrolysis isomeric aldobionic acids (glucose-glucuronic acid) in addition to glucose. Evidence has likewise been secured which indicates that the specific carbohydrates of Type II Pneumococcus and of Type B and C Friedländer bacillus also contain other aldobionic acids.

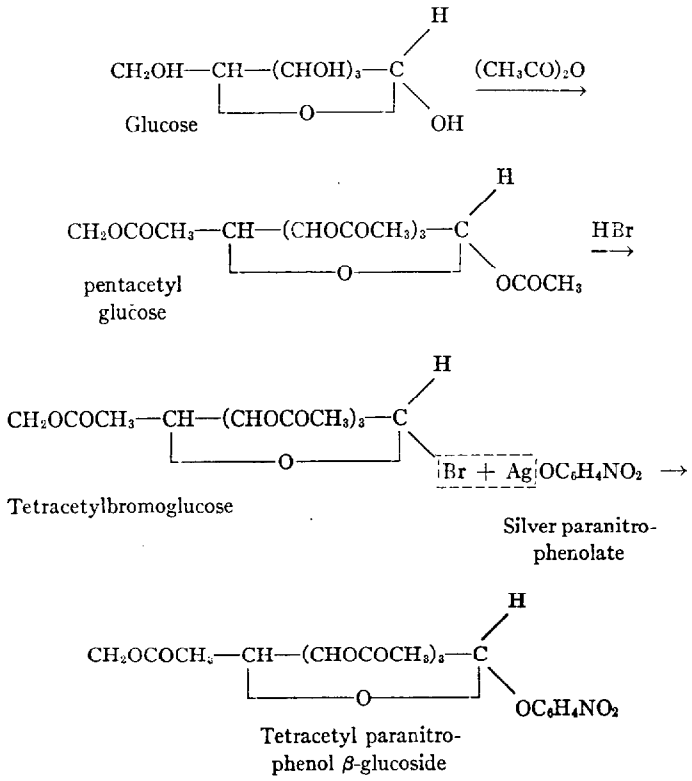
The selective specificity of encapsulated organisms, such as Pneumococcus and Friedländer bacillus, seems to depend primarily on the hapten part of the hypothetical complex antigen. In all of the specific carbohydrates studied thus far, the invariable presence of *isomeric* aldobionic acids seems to indicate that particularly the acid group (carboxyl group) and its stereochemical relationship to other groups of the intact polysaccharide molecule, which in each instance must necessarily be different, has a profound influence in orienting specific response. This question, however, will be dealt with in greater detail in a later communication.

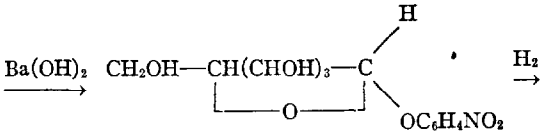
For the purpose of studying the rôle which simple sugars of different spatial configuration might play in altering the specificity of proteins, it was thought that it might be possible to combine these different sugars with a given protein, and to observe specific differences in antigenic properties of the substituted compounds.

The problem thus becomes two-fold, first, to find some means of combining the sugar with the protein, and second, to synthesize the appropriate derivative. Pauly's (7) fundamental method was made use of in combining glucose and galactose to serum globulin.

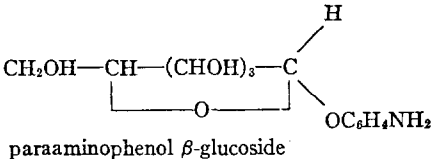
Some years ago Pauly (7) described a color test for the detection of tyrosine and histidine in the hydrolytic products of proteins. This test was dependent upon the formation of colored azo dyes produced by the coupling of diazobenzene sulphonic acid to the aromatic nucleus of amino acids. The coupling of diazobenzene sulphonic acid and other derivatives of phenyldiazonium chloride can be brought about not only with hydrolytic products of proteins, but with the intact protein molecule as well (8). This reaction has been used by Landsteiner (9) in preparing a number of substituted proteins,—"azo proteins,"—and in a series of experiments he studied the immunological reactions of a great variety of differently substituted proteins. By this method he was able to differentiate between the dextro and laevo forms of phenyl-amino-acetic acid. In the same communication (9*f*) he indicated the bearing of his observations on the serological specificity of bacterial carbohydrates.

The second problem, that of preparing a sugar derivative adaptable to this principle, was solved by the following synthesis:



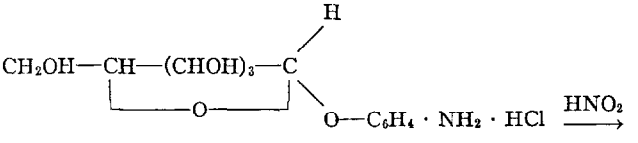


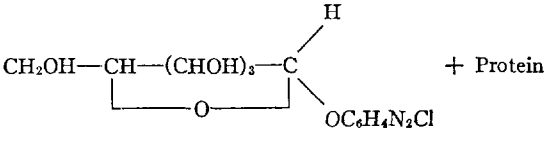
paranitrophenol β-glucoside

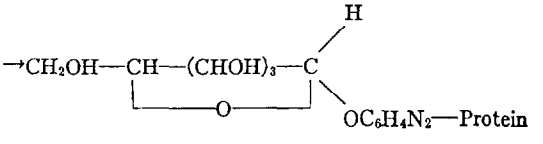


paraaminophenol β-glucoside

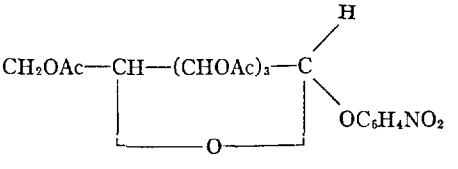
The end product of this series of reactions may then be converted into the corresponding diazonium derivative and coupled with protein in the presence of dilute alkali:







The most difficult step in the synthesis, the coupling of p-nitrophenol with the acetobromo-sugar, was accomplished by shaking a solution of the latter in xylene with the silver salt of p-nitrophenol. In this manner, fair yields of the glucoside



were obtained. The preparation of p-nitrophenol glucoside from this derivative, and the catalytic reduction of this compound to its amino derivative proceeded very smoothly and nearly quantitatively. The coupling of the diazonium phenol hexoside to protein takes place readily and rapidly in the presence of N/100 hydroxide.[1]

Thus with the aid of *p*-aminophenol glucoside and the corresponding galactoside two different hexosides have been attached to the same protein, yielding complexes which exhibit different optical properties and which yield reducing sugars on hydrolysis. It should be possible to attach any aldose or ketose to native protein provided the intermediary glucoside can be synthesized. Owing to the occurrence of isomeric aldobionic acids in specific bacterial polysaccharides, which are believed to play an important rôle in orienting the antigenic specificity of encapsulated bacteria, it would seem to be of even greater interest to couple sugar acids to proteins in order to study the antigenic response of such substituted proteins. We are at present attempting to synthesize such derivatives.

EXPERIMENTAL

1. *Pentacetyl glucose* and *Pentacetyl galactose.* These compounds were prepared by acetylation of the corresponding sugar with pyridine and acetic anhydride at 0°C. Both products were recrystallized from alcohol. The pentacetyl galactose melted at 146°C.

2. *Acetobromo glucose* and *Acetobromo galactose.* These derivatives were prepared by the method of Fischer (12).

3. *Silver p-nitrophenolate.* 1.2 mols of recrystallized dry p-nitrophenol were dissolved in absolute ethyl ether. 1 mol of freshly precipitated dry silver oxide was added. The mixture was shaken for 18 hours at room temperature together with a large quantity of glass beads. At the end of this time a brick red compound separated. This was filtered on a hardened paper, washed repeatedly with

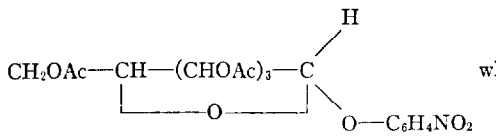
[1] There have recently appeared two papers by Klopstock and Selter (10) wherein they contend that neutral solutions of derivatives of phenyl diazonium chloride do not combine with, but are merely "adsorbed" by protein. This contention seems to have been refuted, however, by the experiments of Heidelberger and Kendall (11). It might be mentioned here again that the presence of alkali is not always necessary for the coupling of phenyl diazonium chloride derivatives to aromatic nuclei. In fact, the formation of some azo dyes is brought about by coupling diazonium chloride derivatives not only in alkaline, but in neutral, and even in acid medium as well.

absolute ether, and dried in a vacuum desiccator over phosphorus pentoxide and paraffin for 4 weeks. The compound is probably a mixture of the true silver salt of p-nitrophenol, $AgOC_6H_4NO_2$, and of its isomeride, $O:C_6H_4:NOOAg$. Two distinct crystalline forms may be seen under the microscope.

Analysis: 0.2000 gms. substance gave 0.1181 gms. AgCl when analyzed by the method of Carius.

Calculated for $C_6H_4NO_3Ag$ — Ag: 43.85 per cent. Found 44.40 per cent.

4. *Tetracetylnitrophenol β-glucoside:* 50 gms. of aceto-bromo glucose were dissolved in 500 cc. of anhydrous xylene. To the mixture was added 10 gms. of the silver salt of p-nitrophenol. After shaking with dry glass beads for 30 minutes a second portion of 10 gms. of the silver salt was added, and finally after another 30 minutes a third portion was added. The mixture was removed from the shaking machine, filtered on a hardened paper, and the precipitate was washed with small portions of xylene. The filtrate was now concentrated to 50 cc. *in vacuo*. 100 cc. of absolute ethyl alcohol were added, and then the mixture was further concentrated. The addition of alcohol, followed by evaporation, was repeated three times. The final concentrate was taken up in 200–300 cc. of absolute alcohol and the flask was placed in the ice box. After 24 hours the glistening pale yellow crystals of the glucoside were filtered from the deeply colored mother liquors. They were dissolved in about 250 cc. of hot absolute alcohol, and boiled with a little norite. The solution was filtered through a hot water funnel. After 24 hours in the cold the nearly snow-white crystals were filtered from the pale yellow mother liquid. A second crystallization yielded about 9 gms. of pure glucoside,



which melted at 172-173°.

$$[\alpha]_D^{25^\circ} = \frac{-\ 0.86 \times 100}{2 \times 1.055} : -\ 40.8^\circ \text{ (in chloroform)}$$

Analysis: 6.595 mgs. substance: 12.370 mgs. CO_2 and 2.940 mgs. H_2O

$C_{20}H_{23}O_{12}N$. Calculated: C = 51.16 per cent, H = 4.94 per cent.

Found:— C = 51.15 per cent, H = 4.98 per cent.

The glucoside is readily hydrolyzed by dilute acid and yields paranitrophenol and tetracetyl glucose. It is slowly hydrolyzed by dilute alkali in the cold. It is insoluble in water, fairly soluble in hot alcohol. It is readily soluble in xylene.

5. *p-Nitrophenol β-glucoside:* 20 gms. of tetracetylnitrophenol β-glucoside were shaken with 60 gms. of recrystallized barium hydroxide, dissolved in 1200 cc. of water, for 24 hours at 4°C. At the end of this time the mixture was nearly

clear. A small amount of unchanged glucoside was filtered off. The filtrate, containing barium acetate and p-nitrophenol β-glucoside was treated with an excess of washed CO_2. The barium carbonate was centrifuged off. The supernatant liquid was concentrated to complete dryness *in vacuo*. The yellow powder was scraped from the flask and dried for two days in a high vacuum over P_2O_5. It was then extracted with 300 cc. of hot absolute alcohol and filtered from the barium acetate by using a hot water funnel. The filtrate was cooled and allowed to stand in the ice box for 2 days. The crystals formed were filtered off and were recrystallized from absolute alcohol. About 8 gms. of p-nitrophenol β-glucoside were obtained as a glistening white product, soluble in alcohol, and, to some extent, in water. This compound was readily hydrolyzed by dilute mineral acid, yielding the reducing sugar and paranitrophenol. It is slowly hydrolyzed by dilute alkali in the cold.

The substance melted at 165°C. $[\alpha]_D^{25°} = \frac{-1.55 \times 100}{2 \times 0.974} = -79.6°$ (in methyl alcohol).

Analysis: 4.305 mgs. substance: 7.555 mgs. CO_2 and 2.030 mgs. H_2O.

$C_{12}H_{15}O_8N$.	Calculated:	C 47.83 per cent	H 5.02 per cent.
Found:		C 47.85 per cent	H 5.27 per cent.

6. *p-Aminophenol β-glucoside.* 5 gms. of p-nitrophenol glucoside were dissolved in 500 cc. of warm 95 per cent redistilled alcohol. To the mixture was added 0.2 gm. of platinum oxide catalyst (13). The substance was hydrogenated under atmospheric pressure in a hydrogenating apparatus. The theoretical amount of hydrogen gas, when corrected for temperature, pressure and vapor tension, was utilized in the reduction of the nitro to the amino glucoside. The reduction took place readily and quickly, and was completed within 30 minutes. The alcohol solution of p-aminophenol β-glucoside was filtered through a quantitative filter paper, and the filtrate carefully evaporated *in vacuo* to dryness. 50 cc. of absolute alcohol were added and the mixture was again evaporated to dryness. The glucoside was finally dissolved in 100 cc. of absolute alcohol and the flask was placed on ice. After 48 hours, the snow-white crystals of p-aminophenol β-glucoside were filtered from the clear mother liquors. 4.0 gms. were recovered. The compound is readily soluble in water. It is soluble in 95 per cent alcohol, though more difficultly soluble in absolute alcohol. It is readily hydrolyzed by dilute mineral acid, yielding the reducing sugar. It is also hydrolyzed by alkali in the cold. If the hydrochloride is treated with nitrous acid and the reaction mixture is poured into an alkaline solution of α-naphthol, a brilliant red dye is produced which precipitates on acidification.

The compound melts at 160–161°C. $[\alpha]_D^{26°} = \frac{-1.11 \times 100}{2 \times 0.867} = -64.1°$ (in methyl alcohol).

Analysis: 5.254 mgs. substance: 10.25 mgs. CO_2 and 2.925 mgs. CO_2.
$C_{12}H_{17}O_6N$. Calculated: C 53.12 per cent, H 6.32 per cent.
Found: C 53.05 per cent, H 6.23 per cent.

7. *Tetracetyl p-nitrophenol β-galactoside.* This compound was prepared exactly as was the corresponding glucoside. The yields were practically the same. It crystallizes from absolute alcohol as long glistening very pale yellow needles melting at 144–145°C.

$$[\alpha]_D^{24^\circ} = \frac{-0.17 \times 100}{2 \times 1.022} = -8.3^\circ \text{ (in chloroform)}$$

Analysis: 4.000 mgs. substance: 7.475 mgs. CO_2 and 1.685 mgs. H_2O.
$C_{20}H_{23}O_{12}N$. Calculated: C 51.16 per cent, H 4.94 per cent
Found C 50.96 per cent, H 4.71 per cent

8. *p-Nitrophenol β-galactoside.* This derivative was prepared exactly as was the glucoside. 25.4 gms. of p-nitrophenol tetracetyl β-galactoside yielded 11.9 gms. of p-nitrophenol β-galactoside. The compound crystallizes from absolute alcohol as colorless needles melting at 181–182°C.

Analysis: 4.100 mgs. substance: 7.175 mgs. CO_2 and 1.825 mgs. H_2O
$C_{12}H_{15}O_8N$. Calculated: C 47.83 per cent, H 5.02 per cent.
Found C 47.72 per cent, H 4.99 per cent.

9. *p-Aminophenol β-galactoside.* The reduction of p-nitrophenol β-galactoside was carried out in the same way as was that of the corresponding glucoside. The reduction does not seem to take place quite as rapidly as does that of the glucoside. The yields are practically quantitative. The compound was isolated from absolute alcohol as a white crystalline product soluble in water. It suffers hydrolysis when boiled with dilute mineral acid. When hydrolyzed solutions of the amino glucoside are boiled with Fehling's solution, a powerful reduction takes place.

The substance melts at 158–159°C. $[\alpha]_D^{25^\circ} = \frac{-74 \times 100}{2 \times 0.914} = -40.5^\circ$ (in methyl alcohol).

Analysis: 5.350 mgs. substance: 10.370 mgs. CO_2 and 3.160 mgs. H_2O.
$C_{12}H_{17}O_6N$. Calculated: C 53.12 per cent, H 6.32 per cent.
Found: C 52.85 per cent, H 6.61 per cent.

10. *Preparation of Serum Globulin.* 1 liter of normal horse serum was poured into 15 liters of distilled water. Dilute acetic acid was added until the maximum turbidity occurred. The mixture was placed in the ice chest for 48 hours. The supernatant liquid was then syphoned off and discarded. The precipitate was centrifuged in the cold. The globulin thus obtained was dissolved in physiological

salt solution and reprecipitated. A final globulin solution of 2 per cent concentration was employed for coupling with the glucosides.

11. *Preparation of Protein-diazophenol glucoside and galactoside.* 100 cc. of 2 per cent globulin solution were placed in a flask and cooled to 0°C. 100 cc. of N/50 NaOH, in physiological salt solution, were also cooled to 0°C. Then 1.00 gm. of p-aminophenol β-glucoside was dissolved in 25 cc. of water. 2 mols of N/1 hydrochloric acid were added. The mixture was cooled to 0°C. The theoretical quantity of sodium nitrite, dissolved in 10 cc. of water, was slowly added. After diazotization was complete and the solution showed no excess of nitrite, it was slowly added to the globulin, which had previously been mixed with the chilled alkali. A yellow color immediately developed which soon deepened to a dark wine-red. After standing for 30 minutes at 0° the mixture was treated with 50 cc. of chilled N/50 HCl in physiological salt solution. A precipitate of highly colored insoluble protein flocculated from the mixture. This protein was centrifuged from the colored supernatant liquid. The precipitate was then thoroughly extracted five times with a total of one liter of 3 per cent salt solution. The final washing was nearly colorless and protein free. The precipitate of protein diazophenol glucoside was suspended in 100 cc. of physiological salt solution and N/20 sodium hydroxide was cautiously added with stirring, until the suspension was completely in solution. The total dry weight of material was determined by evaporation, after dialysis, *in vacuo* to constant weight. The optical rotation was determined in a one-half decimeter tube using the wave length 760.8 mm. which has the same color as the azo-protein. The galactose diazophenol protein was prepared in exactly the same way as was the glucose derivative. It was finally taken up in 200 cc. of physiological salt solution.

Properties of the synthetic sugar proteins

Optical Rotation: A solution of the diazophenol glucoside protein, containing 9.725 mgs. of ash-free solid per cc., rotated the plane of polarized light −0.21° in a 0.5 decimeter tube.

$$[\alpha]^{25°}_{760.8\,\mu\mu} = \frac{-0.21 \times 100}{0.5 \times 0.9725} = -43.2°$$

A solution of the diazophenol galactoside protein, containing 5.200 mgs. of ash-free substance per cc., gave an observed rotation of −0.08° in a 0.5 decimeter tube.

$$[\alpha]^{25°}_{760.8\,\mu\mu} = \frac{-.08 \times 100}{0.5 \times 0.5200} = -30.7°$$

Owing to the difficulty of securing a bright field of vision through these deeply colored solutions, the observed rotations probably suffered from an appreciable error. Six successive readings checked within 0.03°.

Reducing Sugars on Hydrolysis:—2 cc. of the protein solutions of the above concentration were placed in glass tubes together with 2 cc. of N/1 HCl. The tubes were sealed and placed in a boiling water bath. At the end of 3 hours and 5 hours respectively the tubes were removed, cooled, and centrifuged. A sugar analysis was made on 2 cc. of the hydrolysate by the method of Van Slyke and Hawkins (14). The galactose protein yielded approximately 10 per cent of reducing sugars, calculated as glucose. The glucose-protein yielded approximately 17 per cent of reducing sugars calculated as glucose.[2] There was no difference in the reducing value of the samples hydrolyzed for 3 hours when compared with those hydrolyzed for 5 hours.

The two synthetic sugar proteins appear to be different compounds. Animals have been immunized with these synthetic sugar proteins. The immunological results are reported in the following communication.

SUMMARY

1. The synthesis of p-aminophenol β-glucoside and p-aminophenol β-galactoside has been described.

2. These hexosides have been coupled to serum globulin. Two protein sugar complexes with different optical properties have been obtained.

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[2] The writers wish to thank Dr. James Hawkins for carrying out the sugar analyses, and for other helpful suggestions. They also wish to express their thanks to Dr. P. A. Levene for his interest and advice.

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