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The Interaction of Glycosides and Saccharides with Antibody to the Corresponding Phenylazo Glycosides

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Previous study of the interaction of a glycosphingolipid, cytolipin H, with anti-tissue antibody showed that this interaction could be inhibited preferentially by lactose in comparison with the other saccharides tested, indicating that a lactosyl residue was present in this molecule, and suggesting that this residue may be responsible for most of the observed interaction (Rapport, Graf & Yariv, 1961). The reactions of antibody directed against various glycosidic residues (lactose, glucose, galactose) thus became relevant for the study of other glycolipids.

Although methods for the preparation of anti-glycoside antibody are available (Avery & Goebel, 1929; Goebel, Avery & Babers, 1934), the estimation of the antibody present in such antisera is difficult because their antibody content is low and a high concentration of precipitating antigen, which is mainly protein, must be used. We therefore developed protein-free precipitating antigens for several of these systems by incorporating phenyl glycosides into a simple polyhaptenic molecule [1,3,5-tri-(*p*-glycosyloxyphenylazo)-2,4,6-trihydroxybenzene]. These precipitating antigens were effective in low concentrations relative to that of antibody, permitting ready measurement of the content of antibody in the precipitate.

The inhibition of these antigen–antibody systems by simple saccharides gave results that could not easily be reconciled with the accepted concept that the reactivity of a free hexose, corresponding to the terminal glycosyl radical of the immunogenic determinant, will account for the interaction of antibody with this residue in the antigen. These findings are discussed and a possible explanation is offered.

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MATERIALS AND METHODS

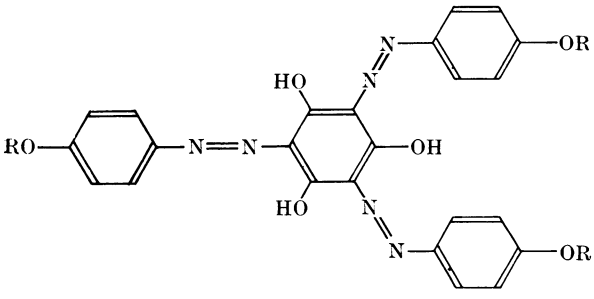
p-Nitrophenyl β-glycosides. *p*-Nitrophenyl β-D-galactoside was prepared from acetobromogalactose (Fischer, 1910) via the *p*-nitrophenyl tetra-acetyl β-galactoside obtained according to the method of Latham, May & Mossetig (1950) as adapted by Beiser, Burke & Tanenbaum (1960). Deacetylation with barium methoxide in hot methanol gave the required product which, after two recrystallizations from methanol, had m.p. 180–181° (decomp.).

p-Nitrophenyl β-D-glucoside was a commercial product (Grade A; California Corp. for Biochemical Research, Los Angeles, Calif., U.S.A.).

p-Nitrophenyl β-lactoside was prepared from acetobromolactose by condensation with *p*-nitrophenol and deacetylation (Bates, 1942) with barium methoxide at 5°. The product had m.p. 260° (decomp.) after recrystallization from aq. 75% (v/v) ethanol.

p-Aminophenyl β-glycosides. The *p*-nitrophenyl β-glycosides were reduced at atmospheric pressure of hydrogen over platinum oxide (Adams catalyst; Amend Drug and Chemical Co., New York, U.S.A.). The galactoside was recrystallized from methanol, m.p. 158–159° (decomp.), the glucoside from ethanol, m.p. 160° (decomp.), and the lactoside from aq. 75% (v/v) ethanol, m.p. 242° (decomp.).

Precipitating antigens. These glycosides have the structural formula (I), where *R* may be a β-galactosyl, β-glucosyl or β-lactosyl residue.



(I)

The galactoside [1,3,5-tri-(*p*-β-D-galactosyloxyphenylazo)-2,4,6-trihydroxybenzene] was prepared as follows: *p*-aminophenyl β-D-galactoside (4 m-moles) was diazotized in 24 ml. of 0·5N-HCl at 0° and added to 1 m-mole of phloroglucinol (Phloroglucin; Fisher Scientific Co., New York, U.S.A.) dissolved in 50 ml. of water, the pH being kept at 9·0 by the addition of 0·5N-NaOH. After about 2 hr. the pH became stable and an equal volume (150 ml.) of methanol was added. The precipitate was collected by filtration, dried and redissolved in 75 ml. of hot water. After the solution had been filtered, the product was precipitated with an equal volume of methanol. The precipitate was collected and dried at 80° over P_2O_5 *in vacuo*; the yield was 430 mg. (Found: C, 51·8, 51·9; H, 5·0, 4·9; N, 8·6, 8·5; Calc. for $C_{42}H_{48}N_6O_{21}$: C, 51·9; H, 5·0; N, 8·6%.) The absorption spectra had the following characteristics: in pyridine, $\lambda_{max.}$ 398 mμ, ϵ $4{\cdot}65\times10^4$; $\lambda_{min.}$ 442 mμ, ϵ $3{\cdot}08\times10^4$; $\lambda_{max.}$ 515 mμ, ϵ $8{\cdot}93\times10^4$; in water, $\lambda_{max.}$ 398 mμ, ϵ $5{\cdot}23\times10^4$; $\lambda_{min.}$ 450 mμ, ϵ $3{\cdot}81\times10^4$; $\lambda_{max.}$ 485 mμ, ϵ $4{\cdot}18\times10^4$. There was no difference in the absorption spectrum in 0·1N-HCl or 0·1M-potassium phosphate, pH 7·0. In 0·1N-NaOH a single band was found, $\lambda_{max.}$ 440 mμ, ϵ $6{\cdot}75\times10^4$.

A sample of the total precipitate obtained on hydrolysis of the galactoside was chromatographed and its absorption spectrum determined. It migrated as a single spot with the same R_F and colour reactions as phloroglucinol triazophenol and also had the same absorption spectrum (see below).

The corresponding glucoside [1,3,5-tri-(*p*-β-D-glucosyloxyphenylazo)-2,4,6-trihydroxybenzene] and lactoside [1,3,5-tri-(*p*-β-lactosyloxyphenylazo)-2,4,6-trihydroxybenzene] were prepared in a similar way. Diazotized *p*-aminophenyl β-D-glucoside (3 m-moles) and diazotized *p*-aminophenyl β-lactoside (4 m-moles) were coupled to phloroglucinol (1 m-mole). Products were precipitated from the reaction mixture by the addition of ethanol and reprecipitated from hot water with ethanol (final concn.: 20%, v/v). The products were dried over P_2O_5 at 25° *in vacuo* to give 500 mg. of the glucoside and 740 mg. of the lactoside. Elementary analyses indicated that the products were not pure. The glycoside content of these products was determined from the absorption spectrum after chemical characterization of the aglycone.

The precipitates obtained by hydrolysis of the glucoside and lactoside were found to be pure phloroglucinol triazophenol (see below). Identity was based on absorption spectrum and behaviour on paper chromatograms (R_F, colour reactions). The absorption spectra (in water) had the following characteristics: phloroglucinol glucoside, $\lambda_{max.}$ 398 mμ, $E^{1\%}_{1\,cm.}$ 405; $\lambda_{min.}$ 450 mμ, $E^{1\%}_{1\,cm.}$ 279; $\lambda_{max.}$ 485 mμ, $E^{1\%}_{1\,cm.}$ 332; phloroglucinol lactoside, $\lambda_{max.}$ 398 mμ, $E^{1\%}_{1\,cm.}$ 300; $\lambda_{min.}$ 450 mμ, $E^{1\%}_{1\,cm.}$ 144; $\lambda_{max.}$ 485 mμ, $E^{1\%}_{1\,cm.}$ 232.

The quantity of material with structure (I) in these products was calculated from the specific absorptions at 398 mμ of the products and of the pure galactoside (for the galactoside $E^{1\%}_{1\,cm.}$ at 398 mμ is 520). The preparation of the glucoside (mol.wt. 973) was 78% pure whereas that of the lactoside (mol.wt. 1458) was 87% pure.

Phloroglucinol triazophenol [1,3,5-*tri*-(p-*hydroxyphenyl*)-2,4,6-*trihydroxybenzene*]. This was obtained from 100 mg. of the corresponding galactoside by acid hydrolysis with 350 ml. of 2N-H_2SO_4 at 100°. When a precipitate began to appear, the solution was repeatedly shaken. When the solution became colourless, the coloured precipitate was collected on a filter and washed repeatedly with water. The precipitate was dried at 100° over anhydrous calcium sulphate *in vacuo* to give 39 mg. of product. (Found: C, 59·3, 59·2; H, 3·9, 3·9; N, 17·0, 16·9. Calc. for $C_{24}H_{18}N_6O_6$: C, 59·5; H, 3·7; N, 17·3%.) The absorption spectrum had the following characteristics: in pyridine, $\lambda_{max.}$ 408 mμ, ϵ $3{\cdot}40\times10^4$; $\lambda_{min.}$ 450 mμ, ϵ $2{\cdot}38\times10^4$; $\lambda_{max.}$ 530 mμ, ϵ $6{\cdot}53\times10^4$.

Chromatography. Descending migration at 20°, on Whatman no. 1 chromatographic paper, with *n*-butylamine–*n*-butanol–methanol–water (4:6:45:45, by vol.) as the developing solvent, was used. The front was allowed to advance 20–25 cm. Phloroglucinol triazophenol moved as a single blue–violet spot with R_F 0·33. On drying, the colour changed to rose–violet. Spraying with NaOH (0·1N) changed the colour to blue; spraying with HCl (0·1N) changed the colour back to rose–violet. A reference substance, resorcinolazosulphanilate (Tropaeolin O; Allied Chemical Corp., New York, U.S.A.) had R_F 0·75.

Sugars. The following commercial preparations were used: D-galactose and lactose (Mann Assayed grades; Mann Research Laboratories Inc., New York, U.S.A.); D-glucose (Dextrose, Certified; Fisher Scientific Co., New York, U.S.A.).

Immunizing antigens. Conjugated proteins were prepared from porcine and bovine γ-globulins (Pentex Biochemicals, Kankakee, Ill., U.S.A.), as described by Karush (1956). *p*-Aminophenyl β-glycosides were diazotized and coupled to the proteins at 0°, the pH being kept at 8·5±0·5 (glass electrode) with 0·5N-NaOH; 0·77 m-mole

of glycoside was added/g. of protein. Dialysed solutions of conjugated protein were adjusted to pH 7 and stored frozen.

Antisera. Rabbits were immunized by nine intravenous injections with conjugated protein given three times weekly, and bled by cardiac puncture 3–4 days after the last injection. Each rabbit received a total of 120–150 mg. of antigen. Reactive sera were selected by preliminary titration with phloroglucinol glycoside. Antisera were dialysed in the cold against two changes of 0·15M-NaCl for 18 hr., and centrifuged in the cold. The clear supernatants were stored frozen.

Hapten-inhibition studies. For antigen–antibody titrations a constant volume of antiserum in 15 ml. centrifuge tubes was mixed with different amounts of antigen dissolved in an equal volume of 0·15M-NaCl. Homologous antigens were used at concentrations of 5, 10, 20, 30, 40 and 50 μg./ml. Controls with 0·15M-NaCl were included. The contents of the tubes were well mixed and kept at 5° for 3 days. After centrifuging (2000 rev./min. at 5°), the precipitates were washed twice with 2 ml. volumes of cold 0·15M-NaCl, dissolved in 0·1 ml. of 0·1N-NaOH and adjusted to 1·0 ml. with a solution of 0·05M-potassium phosphate, pH 8·0, containing the respective sugar for each type of antiserum: 0·5M-galactose; 0·5M-glucose; 0·1M-lactose. The extinction of the redissolved precipitate was measured at 280 and 400 mμ. The extinction at 400 mμ was used to calculate the contribution of the glycoside to that of the whole precipitate at 280 mμ, and hence to obtain the extinction due to antibody. The antibody content was calculated by using the formula of Nisonoff & Pressman (1958) based on the specific extinction at 280 mμ of purified rabbit γ-globulin.

In inhibition studies, 1 vol. of inhibitor dissolved in 0·15M-NaCl was added to 2 vol. of antiserum, and, after mixing, 1 vol. of precipitating antigen (40 μg./ml.) was added. The procedure then followed the one described for antigen–antibody titration. The percentage inhibition at different inhibitor concentrations was calculated as $100 \times (1 - r)$, where r is the ratio of the quantity of antibody precipitated in the presence of inhibitor to the quantity precipitated in its absence.

RESULTS

Precipitation of antibody by phloroglucinol glycosides. Precipitin titrations of anti-(phenyl β-D-galactoside) serum [anti-(phenyl galactoside)] and of anti-(phenyl β-D-glucoside) serum [anti-(phenyl glucoside)] were done with 1·0 ml. serum samples for each point. For anti-(phenyl β-lactoside) serum [anti-(phenyl lactoside)], 0·5 ml. serum samples were used. The precipitin curve of anti-(phenyl lactoside) with phloroglucinol triphenylazo lactoside is shown in Fig. 1. The optimum is broad; since the antigen is poorly soluble, attainment of a concentration that will inhibit precipitation is probably prevented by the formation of aggregates of increased size.

Similar precipitin curves were obtained with the other homologous systems. The antibody content found when homologous and heterologous antigens were used is shown in Table 1.

The absorption spectrum of anti-(phenyl lactoside) antibody is shown in Fig. 2. The precipitate obtained by adding 50 μg. of phloroglucinol triphenylazo lactoside to 2·5 ml. of anti-(phenyl lactoside) serum was dissolved in 0·3 ml. of 0·01N-sodium hydroxide, and 3·0 ml. of 0·05M-potassium phosphate, pH 7·4, 0·1M with respect to lactose, was added. The extinction was determined with a Cary recording spectrophotometer, the base line being set at 700 mμ. The spectrum had maxima at 485, 398 and 279 mμ. The absorption spectrum

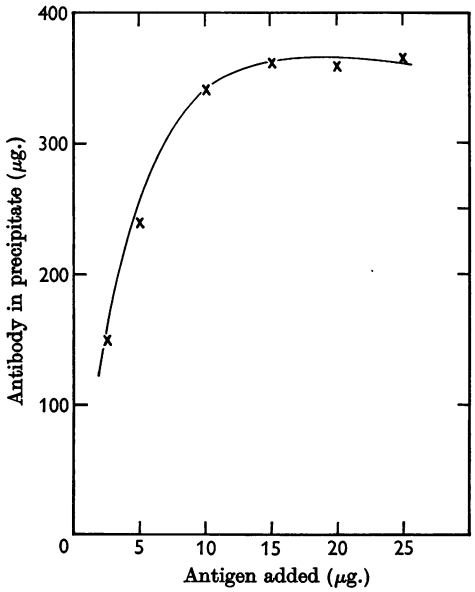


Fig. 1. Precipitin titration curve of phloroglucinol triphenylazo lactoside and anti-(phenyl lactoside) antibody.

Table 1. *Antibody contents of anti-(phenyl glycoside) sera*

Experimental details are given in the text. The symbol 0 indicates either the absence of a visible precipitate or a 'protein-negative' precipitate, i.e. one with an extinction corresponding to less than 0·020/ml. of antiserum.*

Serum	Antibody precipitated with phloroglucinol triphenylazo derivative (μg./ml. of serum)		
	Galactoside	Glucoside	Lactoside
Anti-(phenyl galactoside)	190	0	0
Anti-(phenyl glucoside)	0	268	0
Anti-(phenyl lactoside)	0	0	710

* Phloroglucinol galactoside occasionally gave such precipitates in the heterologous systems. This may have been due to the relative insolubility of this glycoside at low temperatures.

shown in Fig. 2 was obtained by subtracting the absorption of the glycoside from the absorption of the precipitate. The spectrum of anti-(phenyl galactoside) antibody was similar to that shown for anti-(phenyl lactoside).

Inhibition of antibody precipitation. The relations between the inhibitor concentration and the percentage inhibition of the precipitation reactions described above are shown in Fig. 3. The molar concentrations giving 50% inhibition in the respective systems are recorded in Table 2.

Similar concentrations of the homologous *p*-nitrophenyl β-glycosides caused the same degree of inhibition in the respective antigen–antibody systems and were of the same magnitude as the concentration of phenylazo glycoside residues in the respective triglycoside-precipitating antigens. Of the sugars only lactose approached the respective *p*-nitrophenyl glycoside in effectiveness, with a value of the ratio of the equivalent inhibitory concentrations of 15. The interaction of each of the monosaccharides was much weaker, and a significantly higher concentration of D-glucose was required to inhibit the precipitation of anti-(phenyl glucoside) than of D-galactose for the inhibition of anti-(phenyl galactoside). The greater specificity of the interaction of D-galactose with anti-(phenyl

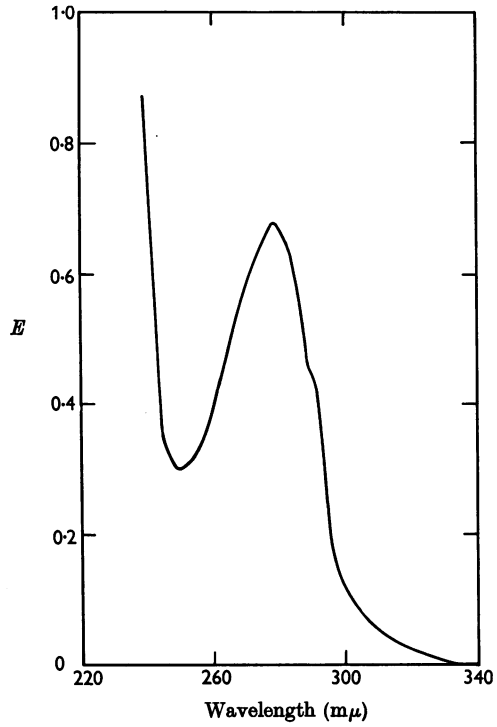


Fig. 2. Absorption spectrum of anti-(phenyl lactoside) antibody. The method of measurement is described in the text.

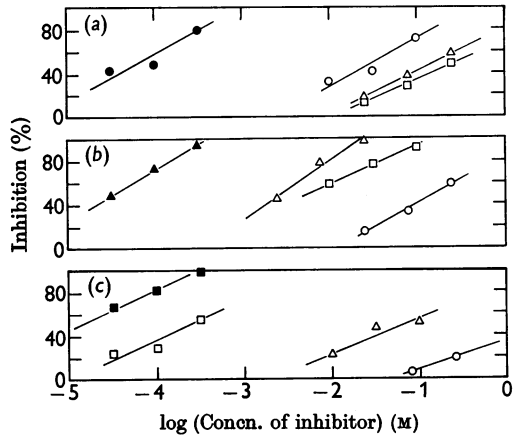


Fig. 3. Reactivity of monosaccharides and glycosides in inhibiting the precipitation of antibody by the corresponding phloroglucinol triphenylazo glycoside. ○, D-Glucose; △, D-galactose; □, lactose; ●, *p*-nitrophenyl β-D-glucoside; ▲, *p*-nitrophenyl β-D-galactoside; ■, *p*-nitrophenyl β-lactoside. The reaction mixtures had the following components: (*a*) Anti-(phenyl glucoside) system: antiserum (1·0 ml.; 268 μg. of antibody) + phloroglucinol glucoside (20 μg.) in a total volume of 2·0 ml.; (*b*) anti-(phenyl galactoside) system: antiserum (1·0 ml.; 190 μg. of antibody) + phloroglucinol galactoside (20 μg.) in a total volume of 2·0 ml.; (*c*) anti-(phenyl lactoside) system: antiserum (0·5 ml.; 340 μg. of antibody) + 10 μg. of phloroglucinol lactoside in a total volume of 1·0 ml.

Table 2. *Inhibition of antibody precipitation*

Experimental details are given in the text. The inhibitor concentrations giving 50% inhibition were calculated from the experimental data in Fig. 3.

	Inhibitor					
	Concn. of homologous *p*-nitrophenyl β-glycosides giving 50% inhibition (mM)			Concn. of sugars giving 50% inhibition (mM)		
Antibody	Glucoside	Galactoside	Lactoside	D-Glucose	D-Galactose	Lactose
Anti-(phenyl glucoside)	0·064	—	—	40·0	180·0	310·0
Anti-(phenyl galactoside)	—	0·040	—	180·0	3·2	6·6
Anti-(phenyl lactoside)	—	—	0·014	> 1000·0	55·0	0·22

galactoside) is also seen with reference to the effect of monosaccharides not present in the determinant [the ratio of equivalent inhibitory concentrations of glucose to galactose for anti-(phenyl galactoside) is 56; of galactose to glucose for anti-(phenyl glucoside), 4·5]. The interaction of lactose with anti-(phenyl glucoside) and anti-(phenyl galactoside) was only slightly less than the interaction of D-galactose.

DISCUSSION

Precipitation of antibody by phloroglucinol triphenylazo glycosides. Pauling, Pressman, Campbell, Ikeda & Ikawa (1942) established that an assembly of three haptenic determinants in one molecule was maximally effective for precipitation of rabbit anti-hapten antibody by simple polyhaptenic molecules. The advantage of using simple polyhaptens in studies concerned with the specificity of the combining region has been noted, and the selective character of such precipitating systems has been applied to the purification of antibody (Pepe & Singer, 1959).

Similar polyhaptens with glycosidic determinants are useful antibody precipitants (Table 1). The added advantage in the use of simple glycosidic dyes for the precipitation of antibody is the ease with which precipitates can be analysed for antibody content: the immune precipitate may be redissolved by the addition of a non-absorbing saccharide inhibitor and the quantity of antibody determined from the ultraviolet-light absorption by subtracting the absorption of the dye from the absorption of the precipitate. The absorption spectra of the precipitants obtained in this way are in good agreement with that of a purified rabbit anti-hapten antibody (Fig. 2; cf. Karush & Marks, 1956).

Since the polyglycosidic molecules described here have a common aglycone, phloroglucinol triazophenol, and differ only in the saccharide substituents, the failure of phloroglucinol triphenylazo lactoside to precipitate anti-(phenyl galactoside) and of the galactoside to precipitate anti-(phenyl lactoside) indicates that two terminal residues of the immunogenic determinant must be present in the test antigen for precipitation to take place. Our results are therefore in agreement with those of Avery & Goebel (1929) and of Goebel *et al.* (1934).

Interaction of sugars with antibody. Previous authors have noted a dissimilarity in the specificity of antibody prepared to various phenylazo glycosides (Goebel *et al.* 1934). They ascribed the differences found to features of saccharide structure of undetermined nature rather than to intrinsic differences in antibody. Marrack & Orlans (1958) pointed out that assignment of the 'C1' conformation to the glycosyl group in α- and β-phenyl glucosides makes it difficult to explain the cross-reactivity found between the respective systems. Our findings suggest that the interaction of a free hexose (corresponding to one incorporated as the terminal residue in a determinant) represents a variable fraction of the interaction of the complete determinant with its antibody (Table 2). The relative interaction of D-glucose with anti-(phenyl glucoside) is less than that of D-galactose with anti-(phenyl galactoside). The ineffectiveness of the interaction of glucose with anti-(phenyl glucoside) is also evident in the reactivity of D-galactose with this antibody. There is only a slight difference in the reactivity of these two hexoses with anti-(phenyl glucoside) (Table 2). This similarity in the relative reactivity of glucose and galactose disappears with the phenyl derivatives of these hexoses (Table 1). These findings may be explained by taking into account the many conformations of pyranose that are possible (Reeves, 1958). Clearly the surface presented by a chair form of a glucose molecule would hinder interaction with an antibody site that is complementary to a boat form. Information is not at hand on the conformation of free sugars and their glycosides in solution which would allow a correlation of the observed reactivity with preferred structures of the glycoside. But present knowledge of the properties of free sugars in solution allows the conclusion that a number of possible ring structures of pyranose may be present, with some conformations predominating at equilibrium (Pigman, 1957). Conversely, a bulky substituent at the anomeric carbon atom could stabilize one particular ring conformation to the exclusion of others.

Since similar considerations have proved fruitful in other areas of biochemical research (Mayer & Larner, 1959), their applicability in immunochemistry is warranted. Such considerations qualify the usefulness of data based on the interactions of free sugars for projection to structural isomers in a glycosidic determinant.

SUMMARY

1. Polyfunctional glycosides of D-glucose, D-galactose and lactose of the following general formula [1,3,5-tri-(*p*-glycosyloxyphenylazo)-2,4,6-trihydroxybenzene] were prepared by coupling a corresponding diazophenyl β-glycoside to phloroglucinol.

2. Antibody prepared in rabbits by the injection of γ-globulin conjugates of phenylazo β-glycosides (glucoside, galactoside, lactoside) was studied by precipitation with each phloroglucinol triphenylazo glycoside.

3. Antibody formed a precipitate only with the homologous phloroglucinol triphenylazo glycoside.

4. A quantitative comparison of the reactivity of sugars and of phenylglycosides with antibody was made by establishing the molar concentration giving 50% inhibition of precipitation in the corresponding antigen–antibody system. The differences so found are considered to be due to different degrees of structural dissimilarity between a hexose in the free state and in glycosidic linkage.

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Phosphorylation of Shikimic Acid by Ultrasonic Extracts of Micro-Organisms

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The role of shikimic acid (I) as a common precursor of the essential aromatic metabolites tyrosine, phenylalanine, anthranilic acid (the precursor of tryptophan), *p*-aminobenzoic acid and *p*-hydroxybenzoic acid in some micro-organisms has been established (see Davis, 1955). Extracts of a phenylalanine auxotroph of *Escherichia coli* have been shown by Levin & Sprinson (1960) to convert shikimic acid 5-phosphate (II), a compound accumulated in the growth medium by some multiple aromatic mutants that do not grow on shikimic acid (Davis & Mingioli, 1953; Weiss & Mingioli, 1956), into 3-enolpyruvylshikimic acid 5-phosphate (III); they suggested that this last compound is the active intermediate in the formation of prephenic acid (IV), the unstable precursor of phenylalanine and probably also of tyrosine (see Davis, 1958). The conversion of shikimic acid 5-phosphate in the presence of L-glutamine into anthranilic acid (V) by extracts of a mutant of *E. coli* blocked in the utilization of this acid (Srinivasan, 1959), and into *p*-aminobenzoic acid (VI) by extracts of baker's yeast (Weiss & Srinivasan, 1959), has been demonstrated.

Shikimic acid 5-phosphate is thus apparently possessed of a multiple intermediary function and a possible metabolic sequence, based on structural considerations and known enzymic reactions, has been suggested by Sprinson (1960). This is illustrated in Fig. 1, X being a possible common intermediate of anthranilic acid, *p*-aminobenzoic acid and, perhaps, *p*-hydroxybenzoic acid (VII). However, as Sprinson (1960) has pointed out, the relationships that emerge on the basis of nutritional requirements and accumulation patterns of multiple aromatic auxotrophs are entirely different, although interpretation of these results may well be complicated by the existence of internal inhibition phenomena. Doy & Gibson (1961), as a result of a study of the formation of 4-hydroxyphenylpyruvic acid and phenylpyruvic acid by tryptophan

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