Immunochemical Studies on a Sophorosyl–Azoprotein Conjugate*

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ABSTRACT: Sophorose (2-*O*-β-D-glucopyranosyl-D-glucose) was coupled to bovine serum albumin (BSA) by means of an azophenyl linkage to prepare the synthetic antigen BSA–*p*-phenylazo β-D-sophoroside. The specificity of antisera obtained from rabbits immunized with the β-sophorosyl conjugate was examined by quantitative hapten inhibition. Glucobioses linked through the β-(1→2), β-(1→3), β-(1→4), β-(1→6) positions, the corresponding α isomers, as well as compounds structurally related to sophorose but modified or substituted at various positions, were assayed for their ability to inhibit precipitation by antisophoroside Inhibition data showed antihapten to possess a high degree of specificity directed against the β-sophorosyl grouping, a requirement for the unmodified D-glucopyranosyl ring system, and a difference in the relative contribution to immunochemical reactivity of the configuration of each glucosidic linkage of the *p*-phenylazo-β-D-sophorosyl residue. The ability of various polysaccharides and synthetic glycosylated azoproteins to precipitate with antisophoroside was also examined.

Various disaccharides including maltose (Goebel *et al.*, 1934), lactose (Goebel *et al.*, 1934; Yarif *et al.*, 1962; Karush, 1956, 1957), cellobiose (Goebel *et al.*, 1934; Gleich and Allen, 1965; Goebel, 1939, 1940), gentiobiose (Goebel *et al.*, 1934), and cellobiuronic and gentiobiuronic acids (Goebel, 1939, 1940) have been conjugated to protein carriers by means of an azophenyl or azobenzyl linkage to prepare artificial polyglycosylated antigens. More recently, isomaltonic and isomaltotrionic acids have been introduced as haptenic groups into BSA[1] by use of the mixed-anhydride reaction (Arakatsu *et al.*, 1966). Detailed examination of the reactions of anticarbohydrate produced in response to such synthetic antigens possessing introduced glycosyl groupings of known structure has provided data of immunochemical interest useful in correlating carbohydrate structure with immunochemical specificity (Goebel *et al.*, 1934; Marrack and Orlans, 1958; Landsteiner, 1945).

In the present study sophorose, the β-(1→2)-glucobiose, was coupled to bovine serum albumin *via* the azophenyl linkage to prepare the synthetic antigen BSA–*p*-phenylazo β-D-sophoroside. Rabbits were immunized with the β-sophorosyl conjugate and the specificity and reactivity of the antisera obtained were examined. By use of quantitative hapten inhibition, the isomeric series of glucobioses linked through the β-(1→2), β-(1→3), β-(1→4), and β-(1→6) positions as well as compounds structurally related to sophorose but bearing modifications or substitutions at various positions were assayed for their ability to inhibit conjugate–anticonjugate precipitation. This procedure established an anticonjugate specificity directed against the introduced sophorosyl grouping and also permitted a more precise examination of the relative contribution to hapten binding by: (1) the point of attachment of the interglucosidic linkage; (2) the configuration of each glucosidic bond; and (3) the unmodified D-glucopyranosyl ring. The reactivity of rabbit antisophorose with several naturally occurring, glucose-containing polysaccharides and various synthetic glycosylated azoproteins was also examined.

Materials and Methods

Antisera. Rabbits were immunized with the sophorose–bovine serum albumin conjugate incorporated into complete Freund's adjuvant. A total of 1.2 ml of emulsion containing 2.6 mg of conjugate/ml was administered over the course of 3 weeks by foot-pad injection (Leskowitz and Waksman, 1960). Bleedings taken 21 days after the last injection provided first-course (1C) immune sera. Three months later, a booster series of injections totaling 4 mg of alum-precipitated conjugate was given intravenously over the course of 4 days and bleedings obtained 5 days after the last injection provided second-course immune sera. First-course antisera from five rabbits were sequentially absorbed with whole calf serum and *p*-hydroxyazophenyl-BSA, then pooled to provide antiserum R(1-5)-1C absorbed.

Quantitative precipitin studies were carried out on 0.5-ml aliquots of rabbit anticonjugate by the procedure described by Kabat and Mayer (1961). Antibody nitrogen content of washed specific precipitates was determined by micro-Kjeldahl analysis. Spectrophotometric analysis was also employed to estimate antibody

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† Established Investigator of the American Heart Association.

[1] Abbreviation used: BSA, bovine serum albumin.

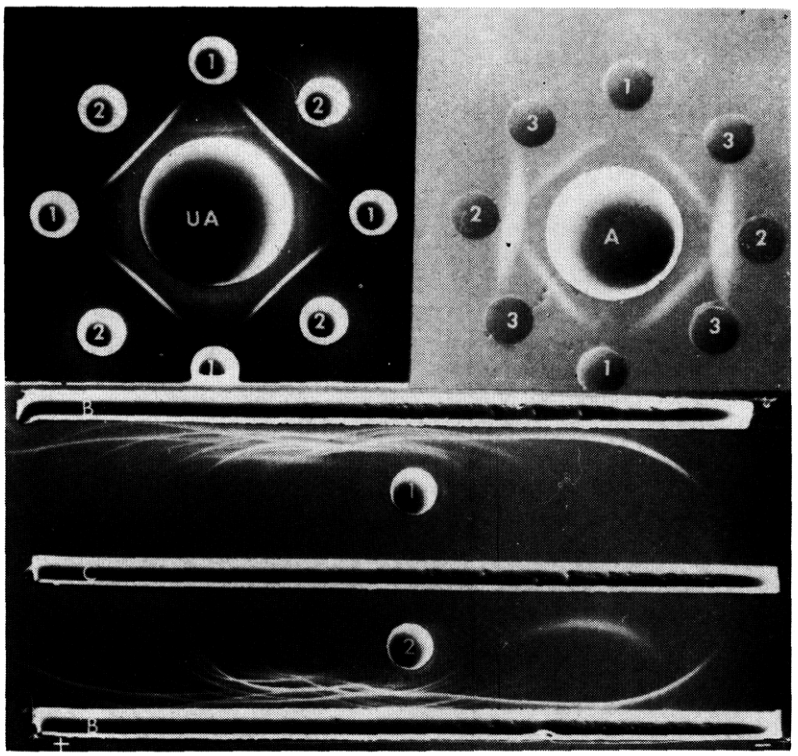

FIGURE 1: Immunodiffusion and immunoelectrophoresis studies. Top: Immunodiffusion with antisophoroside serum R1-1C. Central well (UA) contains unabsorbed antiserum, well (A) contains antiserum absorbed with carrier protein. Peripheral wells contain: (1) BSA, (2) β-sophorosyl–BSA conjugate, (3) β-glucosyl–BSA conjugate. Bottom: Immunoelectrophoresis of antisophoroside R(1-5)-1C absorbed and normal rabbit serum. Troughs labeled B contain goat antinormal rabbit serum, C contains β-sophorosyl–BSA conjugate. Normal rabbit serum and rabbit antisophoroside are placed in wells 1 and 2, respectively.

nitrogen. Washed specific precipitates were dissolved by addition of 75 μl of 0.5 N sodium hydroxide, diluted to 2.5 ml, and the optical density at 277 and 375 mμ was determined. Absorption at 277 mμ was corrected for the antigen contribution and the corrected value was converted to antibody nitrogen.

Quantitative Hapten Inhibition. The scaled-down Folin–Ciocalteu method described by Kabat and Schiffman (1962) was employed to assay haptens for their ability to inhibit precipitation of 5 μg of antisophorose nitrogen. Haptens were added to 100-μl aliquots of a 1:3 dilution of rabbit antisophorose (R(1–5)-1C absorbed) and incubated at 37° for 30 min and 50 μl containing 1 μg of sophorose–BSA nitrogen was then added. The total volume of reaction mixtures was adjusted to 0.4 ml. Tubes were placed in an ice bath at 0°, kept in a refrigerator for 7–9 days, and mixed twice daily.

Immunodiffusion Analysis. Double diffusion in two dimensions was carried out by a modified Ouchterlony method (Kabat and Mayer, 1961). Immunoelectrophoresis was carried out essentially as described by Grabar and Burtin (1960).

Haptens. Methyl β-D-glucopyranoside and *p*-nitrophenyl β-D-glucopyranoside were obtained from California Corp. for Biochemical Research. Glucose was obtained from J. T. Baker and Co. Maltose hydrate was purchased from Pfanstiehl Laboratories, Inc. Laminaribiose, laminaritriose, and maltotriose were gifts from Dr. W. J. Whelan. Preparations of cellobiose, cellotriose, and gentiobiose have been previously described (Allen *et al.*, 1965). Isomaltotriose was purchased from L. Light and Co. Ltd., Colnbrook, England. A sample of 2-*O*-β-D-galactopyranosyl-D-glucose was provided by Professor K. Wallenfels. The preparation and characterization of kojibiose, sophorose, methyl α-sophoroside, methyl β-sophoroside, *p*-nitrophenyl β-sophoroside, *p*-aminophenyl β-sophoroside, methyl 2′,3′,4′,6′-tetra-*O*-acetyl-β-sophoroside, methyl 2′,3′,4′,6′-tetra-*O*-acetyl-α-sophoroside, *p*-nitrophenyl β-laminaribioside, *p*-nitrophenyl β-maltoside, and sophoritol have already been described (Goldstein *et al.*, 1967). A sample of 1,6-anhydrosophorose was supplied by Dr. D. Horton. An additional sample of sophorose as well as preparations of sophorotriose and sophorotetraose were made available through the kindness of Dr. F. W. Parrish.

Polysaccharides and Glycosyl–Phenylazoprotein Conjugates. Oat glucan and laminaran were obtained from Dr. A. S. Perlin. β-(1→2)-Glucan and β-(1→6)-glucan (pustulan) were provided by Dr. F. W. Parrish. Isolichenin was obtained from Dr. W. J. Whelan.

Aerobacter aerogenes A3(S1) polysaccharide was isolated from cultures grown in synthetic media essentially as described by Sandford and Conrad (1966). The final product was, however, dissolved in water, repeatedly emulsified with trifluorotrichloroethane, centrifuged until clear, and then precipitated from the aqueous phase by two volumes of acetone. The preparation and characterization of *p*-phenylazo–bovine serum albumin (BSA) conjugates of α-D-glucose, β-maltose, β-D-galactose, and β-cellobiose employed in the present study have been previously described (Goldstein and Iyer, 1966; Gleich and Allen, 1965). BSA–*p*-phenylazo-β-sophoroside employed as antigen possessed 25 sophorose residues/molecule of BSA and showed an absorption maximum at 3700–3750 A. The antigen was prepared by diazotizing *p*-aminophenyl β-sophoroside and coupling to BSA, employing the conditions described by Westphal and Feier (1956).

Results

Immunodiffusion with Antisophorose Sera. Antisera obtained from five rabbits after a single course of immunization with the sophorose conjugate were examined by immunodiffusion before and after absorption with whole calf serum and *p*-hydroxyphenylazo–BSA. Results obtained with antiserum R1, shown in Figure 1, are identical with findings obtained with all the first-course antisera produced. Unabsorbed serum gave a single band of precipitation with carrier protein (BSA) which disappears upon absorption. The absorbed antisophorose sera failed to give any detectable bands with the *p*-azophenyl–BSA conjugates of α-D-glucose, β-D-galactose, β-maltose, and β-cellobiose. A single intense band of precipitation was obtained with BSA–*p*-phenylazo-β-sophoroside and BSA–*p*-phenylazo-β-glucoside. As shown in Figure 1, the band given by the β-sophorosyl–BSA conjugate spurs over the band given by the β-glucosyl conjugate. Precipitation which occurs with the β-sophorosyl conjugate removes all of the antibody present yielding a supernatant which no longer gives a band in immunodiffusion with e ther the β-sophorosyl or β-glucosyl conjugates. Absorption of antisera with the β-glucosyl conjugate eliminates the band given by this antigen while the band formed by the β-sophorosyl–BSA conjugate persists. After booster stimulation, BSA-absorbed second-course antisera produced by three of the five animals immunized gave a band of precipitation with the β-cellobiosyl–BSA as well as the β-glucosyl and β-sophorosyl conjugate. The band formed by the β-glucosyl conjugate spurred over that given by the β-cellobiosyl conjugate.

Immunoelectrophoretic analysis of rabbit antisophorose was performed employing the β-sophorosyl conjugate to localize specific antibody and a goat antirabbit serum to locate rabbit serum proteins. As can be seen in Figure 1, the arc of specific precipitation given by antisophorose is located in the γ_2-globulin region. Even after booster stimulation none of the rabbits showed any immunoelectrophoretic evidence of an IgA or IgM class of anticonjugate.

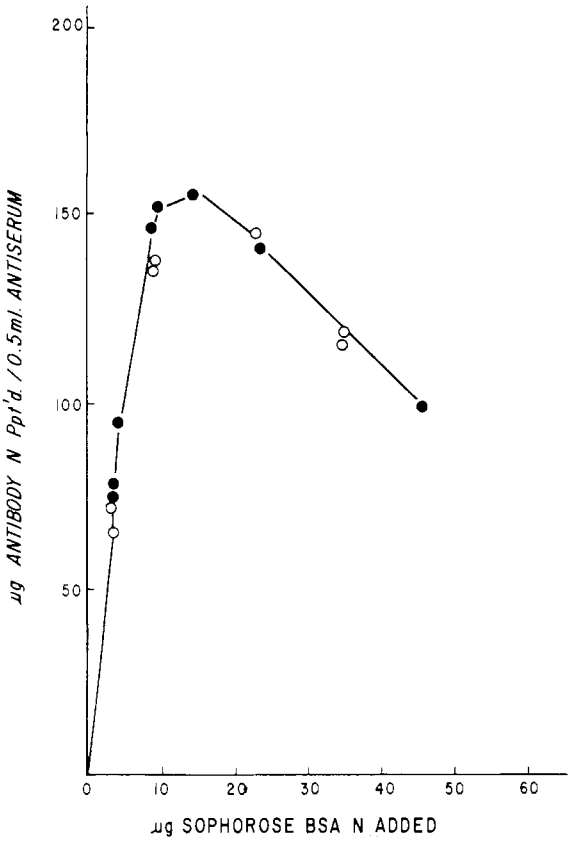

FIGURE 2: Quantitative precipitin curve obtained with rabbit antisophorose serum R(1–5)-1C absorbed. Solid circles estimated by direct nitrogen analysis; open circles determined by spectrophotometric analysis.

Quantitative Precipitin and Hapten Inhibition Studies. A quantitative precipitin curve was obtained on a pool of absorbed first-course antisera (R(1–5)-1C absorbed) employing direct nitrogen estimations by the micro-Kjeldahl method as well as spectrophotometric analysis to determine antibody nitrogen. As shown in Figure 2, both methods give good agreement; the maximum amount of antibody N precipitable from 0.5 ml of antisophorose by the homologous antigen was found to be 155 μg. Supernatants from this reaction gave no precipitate upon addition of the β-glucosyl–BSA conjugate (Table I).

The reactivity of various naturally occurring glucose-containing polysaccharides and glycosylated azoprotein conjugates with antisophorose was examined quantitatively. Of the preparations tested (Table I) the only substance containing β-glucosyl units which showed cross-reactivity in addition to the homologous antigen was BSA–*p*-phenylazo β-D-glucoside which removed 40 μg of antibody N. As indicated in Table I, 114 μg of antibody N could be recovered from the supernatants of this reaction by addition of homologous antigen. In agreement with the immunodiffusion findings on second-course or hyperimmune antiserum R2-2C mentioned above, the β-glucosyl and β-cellobiosyl

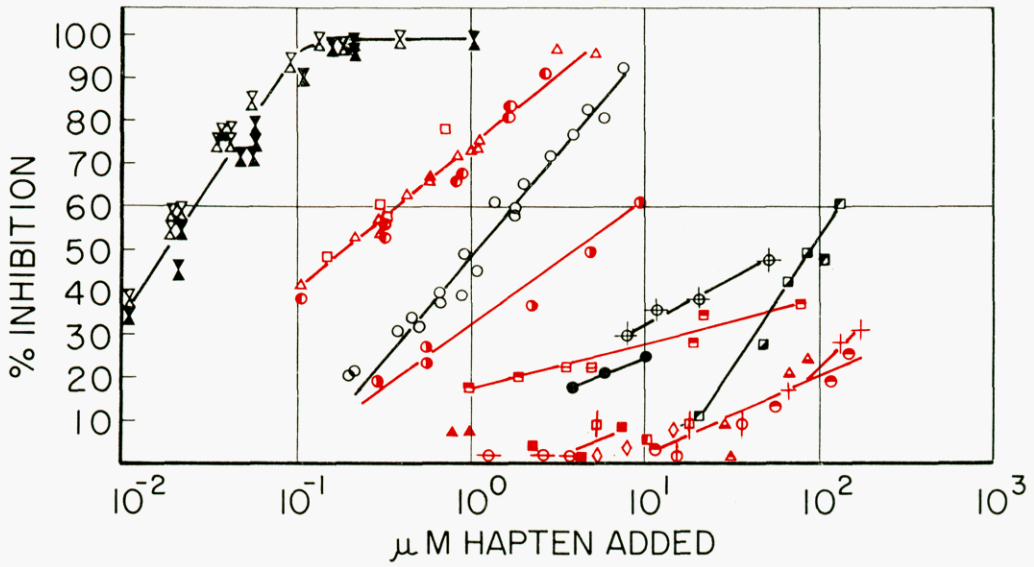

Figure 3: Inhibition of sophorose-antisophorose precipitation by various sugars. ◪, gentiobiose; ◩, cellobiose; ▣, laminaribiose; ⊕, kojibiose; ▲, maltose; △, maltotriose; ⊖, isomaltotriose; +, methyl β-D-glucoside; ⊕, 2-O-methylglucose; ●, 2-O-β-D-galactopyranosyl-D-glucose; ⊟, p-nitrophenyl β-D-laminaribioside; ⊕, p-nitrophenyl β-D-glucoside; ◓, glucose; ○, sophorose; ◐, methyl β-D-sophoroside; ◑, methyl α-D-sophoroside; △, sophorotriose; □, sophorotetraose; ▲, 1,6 anhydrosophorose; ◊, sophoritol; ⧖, p-nitrophenyl β-D-sophoroside; ⧗, p-aminophenyl β-D-sophoroside; ■, Me-2′, 3′, 4′, 6′ tetra-O-acetyl β-D-sophoroside; ▣, Me-2′, 3′, 4′, 6′ tetra-O-acetyl α-D-sophoroside.

conjugates showed reactivity, precipitating 128 and 51 μg of antibody N, respectively. The β-galactosyl conjugate showed a slight reaction by the quantitative precipitin method, removing 17 μg of antibody N.

The specificity of anticonjugate as well as a relative evaluation of the contribution of various portions of the β-linked sophorosyl moiety to interaction with antibody was examined by hapten inhibition. Quantitative hapten inhibition data for the sophorose–antisophorose system are shown in Figure 3. Glucose and methyl β-D-glucoside were relatively poor inhibitors of sophorose–antisophorose precipitation, requiring greater than 100 μmoles to give only 30% inhibition. Oligosaccharides of glucose with an α-(1→2) linkage (kojibiose), α-(1→4) linkage (maltose and maltotriose), or α-(1→6) linkages (isomaltotriose) showed little capacity to inhibit and gave less than 10% inhibition when examined in amounts up to 30 μmoles.

The importance of the point of attachment of the β-glucosidic bond between the two glucose units was examined by comparing the inhibition obtained with various β-linked isomeric glucobioses. Cellobiose (the β-(1→4)-) and gentiobiose (the β-(1→6)-diglucose), while comparable in potency, are poor inhibitors since both required approximately 90 μmoles to give 50% inhibition. Laminaribiose, the β-(1→3) isomer, was the least effective of the β-linked diglucoses, 80 μmoles giving only 36% inhibition. A specificity involving β-(1→2)-linked glucose is evident from the finding that only 1.1 μmoles of sophorose was required to give 50% inhibition. Thus sophorose is at least 90–100-fold more effective as an inhibitor of precipitation

TABLE I: Reactivity of Various Polysaccharides and Glycosylated Azoproteins with Rabbit Antisophoroside.

Substance	Linkages Present	Amount Tested (mg)[a]	Max Antibody N Pptd/0.5 ml (μg)	
			R(1–5) 1C	R2-2C
β-Glucan	β-(1→2)	3.20	1	0
β-Glucan	β-(1→6)	1.41	1	0
Oat glucan	β-(1→4), β-(1→3)	2.94	3	0
Laminaran	β-(1→3), β-(1→6)	6.55	1	0
Isolichenin	α-(1→3), α-(1→4)	1.49	2	2
Aerobacter A3(S1) polysaccharide	NREG[b]	1.22	0	0
BSA–*p*-phenylazo α-D-glucoside		0.412	0	0
BSA–*p*-phenylazo β-D-glucoside		0.041	40[c]	128
BSA–*p*-phenylazo β-maltoside		0.319	0	0
BSA–*p*-phenylazo β-galactoside		0.533	0	17
BSA–*p*-phenylazo β-cellobioside		0.715	0	51
BSA–*p*-phenylazo β-sophoroside		0.010	155[d]	161

[a] Values refer to greatest amount tested, all substances examined over a 100-fold range of concentration. [b] Nonreducing end groups of β-linked glucose, *i.e.*, 6-*O*-β-D-glycopyranosyl residue. [c] Antibody N (114 μg) recoverable from supernatant of this reaction by addition of 4 μg of β-sophorosyl–BSA conjugate. [d] Supernatant shows no antibody N recoverable from supernatant by addition of β-glucosyl–BSA conjugate.

than the β-(1→3)-, β-(1→4)-, and β-(1→6)-glucobioses.

A further increase in potency is seen when the sophorosyl unit possesses the β configuration. The compounds (methyl β-sophoroside, sophorotriose, and sophorotetraose) which possess a terminal β-sophorosyl unit are equipotent in molar inhibitory ability and more effective than sophorose, requiring only 0.2 μmole for 50% inhibition. A further specificity requirement for a β configuration of the sophorosyl residue at the point of attachment to the aglycone is indicated by data obtained with methyl α-sophoroside. This compound is significantly less effective than either methyl β-sophoroside or sophorose and requires 4.4 μmoles for 50% inhibition. Of the compounds examined the most effective inhibitors of precipitation were found to be the *p*-aminophenyl and *p*-nitrophenyl derivatives of β-sophoroside; 1.8×10^{-2} μmole of each gave 50% inhibition.

In an effort to evaluate the relative contribution of various portions of the β-sophorosyl unit to immunochemical reactivity, several derivatives of sophorose-bearing modifications on the reducing or nonreducing ends of the molecule were assayed for inhibitory potency. As can be seen in Figure 3, per-*O*-acetylation of the hydroxyl groups on the terminal glucosyl unit of methyl β-sophoroside to give methyl 2′,3′,4′,6′-tetra-*O*-acetyl-β-sophoroside results in an almost complete loss of inhibitory ability. Thus, while 9 μmoles of methyl β-sophoroside would give 100% inhibition, equimolar amounts of the tetra-*O*-acetyl derivative gave only 7% inhibition. Comparable findings were obtained by per-*O*-acetylation of the hydroxyl groups of the terminal glucopyranosyl unit of methyl α-sophoroside. In this instance, whereas 9 μmoles of the α-sophoroside gave 60% inhibition the same amount of the modified compound gave only 5% inhibition. That less extensive modification of the nonreducing end of the inhibitor molecule may exert a marked influence on reactivity with antibody is shown by a comparison of data obtained with 2-*O*-β-D-galactopyranosyl-D-glucose and 2-*O*-β-D-glucopyranosyl-D-glucose (sophorose). These two disaccharides differ from one another only in the position of the C-4 hydroxyl group of the nonreducing end unit. While 8 μmoles of sophorose gave 93% inhibition, comparable amounts of 2-*O*-β-D-galactosyl-D-glucose gave only 23% inhibition.

Modification at the reducing end of the sophorose molecule may similarly effect interaction with antibody. Thus conversion of the reducing D-glucoside moiety of sophorose into a D-sorbitol residue by borohydride treatment to give sophoritol, or its conversion into an intramolecular glycoside to give 1,6-anhydrosophorose, resulted in a decreased activity; with the greatest amount tested, 14.7 μmoles of sophoritol showed only 8% inhibition. When examined in amounts up to 0.95 μmole, 1,6-anhydrosophorose gave only 6% inhibition compared to 48% inhibition obtained with equimolar amounts of sophorose. The low water solubility of the anhydro sugar did not permit its assay at higher concentrations.

The importance of a β configuration for the (1→2)-glucosidic linkage is evident from a comparison of inhibition data for sophorose with that obtained for kojibiose. Kojibiose (2-*O*-α-D-glucopyranosyl-D-glucose) showed only 8% inhibition when examined at 18 μmoles, the highest concentration tested; comparable amounts of sophorose gave complete inhibition.

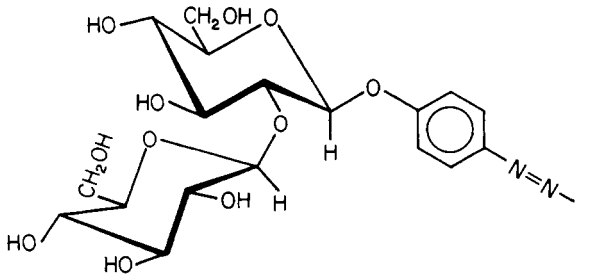

FIGURE 4: Structural formula of the *p*-diazophenyl-β-D-sophorosyl haptenic grouping.

Discussion

Examination of model systems employing synthetic polyglycosylated antigens has provided a body of data of immunochemical interest on the specificity of anticarbohydrate and its relationship to carbohydrate structure (Goebel *et al.*, 1934; Yarif *et al.*, 1962; Karush, 1956, 1957; Gleich and Allen, 1965; Goebel, 1939, 1940; Arakatsu *et al.*, 1966; Marrack and Orlans, 1958; Landsteiner, 1945; Beiser *et al.*, 1960; Beiser and Tanenbaum, 1963; Mage *et al.*, 1963; Tanenbaum *et al.*, 1961; Luderitz *et al.*, 1960; Rude *et al.*, 1966), on the minimal size of an antigenic determinant required for cross-reactivity (Arakatsu *et al.*, 1966), as well as on the size of the combining region and heterogeneity of antiglycosyl antibodies (Arakatsu *et al.*, 1966; Beiser *et al.*, 1960; Mage *et al.*, 1963).

The examination of such systems by the hapten inhibition method and equilibrium dialysis has, moreover, facilitated a more precise evaluation of the effects of stereochemical and configurational changes on hapten–antibody interaction (Karush, 1956, 1957).

In the present study, antisera were prepared against an artificial antigen consisting of a carrier protein (BSA) to which *p*-phenylazo-β-sophorosyl groupings (Figure 4) were coupled. That antihapten antibodies formed have a specificity involving the introduced β-(1→2)-glucobiosyl unit is suggested by immunodiffusion and quantitative precipitin findings. After absorption with BSA to remove antibodies to carrier protein, the absorbed, first-course antisera gave a precipitin reaction with β-sophorosyl and β-glucosyl conjugates (Figure 1 and Table I) but fail to react with α-glucosyl, β-galactosyl, β-maltosyl, and β-cellobiosyl azoprotein conjugates.

A specificity directed against the β-sophorosyl unit is clearly evident from quantitative hapten inhibition data. As can be seen in Figure 3, kojibiose, maltose, and isomaltotriose (oligosaccharides of glucose linked through the α-(1→2), α-(1→4), and α-(1→6) positions, respectively) showed little capacity to inhibit. Glucobioses linked through the β-(1→3), β-(1→4), and β-(1→6) positions are likewise poor inhibitors. A specificity involving β-(1→2)-linked glucose is established by the finding that of the β-glucobioses examined, sophorose was found to be the most potent inhibitor of conjugate–anticonjugate precipitation.

A contribution to specificity by the configuration of the anomeric linkage at the point of attachment to the aglycone is shown by the progressive increase in molar inhibitory ability found for the series methyl α-sophoroside, sophorose, and methyl β-sophoroside. Compounds possessing a terminal β-sophorosyl unit are 22-fold more effective inhibitors of precipitation than methyl α-sophoroside.

While the configurations of both glucosidic bonds of the introduced β-sophorosyl unit contribute to over-all antihapten specificity, a comparison of inhibition data obtained with 2-*O*-β-D-glucopyranosyl-D-glucose (sophorose), 2-*O*-α-D-glucopyranosyl-D-glucose (kojibiose), methyl β-sophoroside, and methyl α-sophoroside permit an evaluation of the relative importance of the configuration of each glucosidic linkage to immunochemical reactivity. The failure of the α-(1→2)-glucobiose to give more than 8% inhibition together with the enhanced potency found for methyl β-sophoroside and its α anomer shows α,β anomerism to play a more critical role in binding to antibody at the terminal position than at the point of attachment to the aglycone. These data are in agreement with and extend earlier observations of Goebel (1934) who found the configuration and point of attachment of the terminal glucosidic bond to play an important role in the serological reactions of maltose, cellobiose, and gentiobiose conjugates.

The finding that tri- and tetrasaccharides of the sophorodextrin series are comparable in molar inhibitory potency to methyl β-sophoroside suggests that the β-sophorosyl unit provides the optimal sugar structure required for interaction with antibody. It is well established that antibodies formed in response to glycosylated azoprotein conjugates may be directed not only against the introduced glycosyl unit but may include the phenylazo group as well as adjacent portions of protein structure to which this group is attached (Yarif *et al.*, 1962; Karush, 1956, 1957; Gleich and Allen, 1965; Beiser *et al.*, 1960; Tanenbaum *et al.*, 1961). In agreement with studies showing phenyl and azophenyl glycosides to be the most potent inhibitors of the anticarbohydrate formed in response to glycosylated azoprotein conjugates, *p*-nitrophenyl and *p*-aminophenyl β-sophorosides were found to be the most effective inhibitors of anti-β-sophoroside precipitation (Figure 3).

Chemical modification of the nonreducing D-glucosyl moiety of the methyl glycosides of sophorose which involves introduction of *O*-acetyl groups into hydroxyls at positions C-2′, C-3′, C-4′, and C-6′ resulted in a loss of inhibitory ability. Of greater significance, perhaps, is the change not involving introduction of multiple large blocking groups but an inversion of the hydroxyl group on C-4′. Substitution of the terminal D-glucopyranosyl unit of sophorose by a D-galactopyranosyl unit results in a marked decrease in potency; thus 2-*O*-β-D-galactopyranosyl-D-glucose shows about 1/30 of the activity of its C-4′ epimer, sophorose. Marked decrease in hapten activity attributable to stereochemical change in the configuration of the

hydroxyl group about a single carbon previously has been shown by assay of epimers in systems involving anti-β-galactosyl (Beiser *et al.*, 1960) and anti-β-lactosyl (Goebel *et al.*, 1934; Karush, 1956, 1957) antibodies.

The requirement for an intact penultimate D-glucopyranosyl moiety is seen from the loss of inhibition which accompanies its transformation into a sorbitol residue to give sophoritol. Similarly, internal glycoside formation involving hydroxyls at C-1 and C-6 renders 1,6-anhydrosophorose inactive as a hapten inhibitor.

A comparison of the reactivity of anti-β-sophorosyl sera obtained in the present study is of interest. After primary immunization, serum obtained from each of five rabbits gave immunodiffusion results identical with those shown in Figure 1 for rabbit 1-1C. All sera failed to react with any of the naturally occurring polysaccharides tested (Table I). The only cross-reaction observed was that obtained with β-D-glucosylated conjugate. Spurring of the band given by the β-sophorosyl conjugate over that given by the β-glucosyl conjugate (Figure 1) indicates the presence of two types of antisophorose. One type of antibody shows a strict specificity for the β-sophorosyl unit and is not precipitable by the β-glucosylated conjugate while the other is reactive with both the β-glucosyl and β-sophorosyl conjugates. Similar findings are apparent from quantitative precipitin data obtained on a pool of first course sera. As shown in Table I, β-glucosylated azoprotein is able to precipitate 25% of the anti-β-sophoroside antibody N.

After booster stimulation, second-course antisera produced by three of five animals immunized showed a broader and more extensive cross-reactivity. Quantitative precipitin data obtained with one of these sera, R2-2C, showed cross-reactivity with the β-galactosyl and β-cellobiosyl as well as the β-glucosyl conjugates (Table I). In immunodiffusion analysis, a band with the β-galactosyl antigen was not detectable; the band given by the β-glucosyl conjugate spurred over that formed by the β-cellobiosyl antigen. This finding suggests the presence of a fraction of antibody directed against a portion of structure present in the β-glucosyl antigen but absent or sterically hindered in the cellobiosyl antigen. An antibody directed against an internal portion of structure, involving the 2-*O*-β-D-glucosylphenylazo unit, would require free hydroxyls on C-3, C-4, and C-6 as well as the phenylazo group for interaction and could accommodate a *p*-phenylazo-β-sophorosyl or -β-D-glucosyl unit but not the *p*-phenylazo-β-cellobiosyl unit. A fraction of anticonjugate possessing such a specificity, however, remains to be isolated and demonstrated.

While synthetic poly-β-glucosylated azoprotein showed extensive cross-reactivity with antisophoroside, *Aerobacter* A3(S1) polysaccharide which possesses one nonreducing β-glucosyl end group per tetrasaccharide repeating unit sequence (Conrad *et al.*, 1966) is completely devoid of cross-reactivity with the rabbit sera employed (Table I). These findings suggest that the 6-*O*-β-D-glucopyranosyl residue may be sterically hindered by the polysaccharide backbone or that multiple isolated β-D-glucosyl end groups do not provide adequate portions of complementary structure required for cross-reactions; an additional structural moiety contributed by the *p*-phenylazo grouping may be required for cross-precipitation with these sera. These data are comparable to the findings of Arakatsu *et al.* (1966) who reported the inability of dextran to cross-react with rabbit antisera to isomaltonic acid coupled to BSA.

An effort to assess the contribution of the glycosyl and aglucone moieties to the precipitation reaction by determinations carried out with BSA–*p*-phenylazo-β-sophoroside before and after treatment with the β-glucosidase of almond emulsin was not successful. As determined by immunodiffusion analysis, the BSA–*p*-azophenyl β-glycosides of glucose, sophorose, and cellobiose are resistant to the action of almond emulsin. The failure of the β-(1→2)-glucan to precipitate antisophoroside is not surprising. Such a failure could arise from the absence of multiple nonreducing sophorosyl end groups required for precipitation or from the absence of the phenylazo group which would provide additional complementary structure for interaction.

It is interesting to note that in its interaction with sophorose and its derivatives, concanavalin A, the phytohemagglutinin of the jack bean differs markedly from the rabbit antisophoroside described in the present study. The combining sites of concanavalin A have been shown to be directed toward the C-3, C-4, and C-6 hydroxyl groups of the α-D-glucopyranosyl or α-D-mannopyranosyl ring system and in fact to form specific precipitates with branched α-glucans and α-mannans by interaction with nonreducing glycosyl residues of the polysaccharide chain ends (Goldstein *et al.*, 1965a,b). In the case of sophorose, however, concanavalin A has been shown to interact with the C-3, C-4, and C-6 hydroxyl groups of the reducing D-glucose moiety (Goldstein *et al.*, 1967).

References

Allen, P. Z., Gleich, G. J., and Perlin, A. S. (1965), *Immunochemistry 2*, 433.

Arakatsu, Y., Ashwell, G., and Kabat, E. A. (1966), *J. Immunol. 97*, 858.

Beiser, S. M., Burke, G. C., and Tanenbaum, S. W. (1960), *J. Mol. Biol. 2*, 125.

Beiser, S. M., and Tanenbaum, S. W. (1963), *Ann. N. Y. Acad. Sci. 103*, 595.

Conrad, H. E., Bamburg, J. R., Epley, J. D., and Kindt, T. J. (1966), *Biochemistry 5*, 2808.

Gleich, G. J., and Allen, P. Z. (1965), *Immunochemistry 2*, 417.

Goebel, W. F. (1939), *J. Exptl. Med. 69*, 353.

Goebel, W. F. (1940), *J. Exptl. Med. 72*, 33.

Goebel, W. F., Avery, O. T., and Babars, F. H. (1934), *J. Exptl. Med. 60*, 599.

Goldstein, I. J., Hollerman, C. E., and Merrick, J. M. (1965a), *Biochim. Biophys. Acta 97*, 68.

Goldstein, I. J., Hollerman, C. E., and Smith, E. E.

(1965b), *Biochemistry* 4, 876.
Goldstein, I. J., and Iyer, R. N. (1966), *Biochim. Biophys. Acta 121*, 197.
Goldstein, I. J., Iyer, R. N., Smith, E. E., and So, L. L. (1967), *Biochemistry 6*, 2373.
Grabar, P., and Burtin, P. (1960), Analyse Immunoelectrophoretique, Paris, Masson et Cie.
Kabat, E. A., and Mayer, M. (1961), Experimental Immunochemistry, 2nd ed, Springfield, Ill., C. C Thomas, pp 72, 85.
Kabat, E. A., and Schiffman, G. (1962), *J. Immunol. 88*, 782.
Karush, F. (1956), *J. Am. Chem. Soc. 78*, 5519.
Karush, F. (1957), *J. Am. Chem. Soc. 79*, 3380.
Landsteiner, K. (1945), The Specificity of Serological Reactions, Cambridge, Harvard University.
Leskowitz, S., and Waksman, B. H. (1960), *J. Immunol. 84*, 58.
Luderitz, O., Westphal, O., Staub, A. M., and Le Minor, L. (1960), *Nature 188*, 556.
Mage, M., Bassett, E. W., Tanenbaum, S. W., and Beiser, S. M. (1963), *J. Immunol. 90*, 318.
Marrack, J. R., and Orlans, E. S. (1958), *Progr. Stereochem. 2*, 228.
Rude, E., Westphal, O., Hurwitz, E., Fuchs, S., and Sela, M. (1966), *Immunochemistry 3*, 137.
Sandford, P. A., and Conrad, H. E. (1966), *Biochemistry 5*, 1508.
Tanenbaum, S. W., Burke, G. C., and Beiser, S. M. (1961), *Biochim. Biophys. Acta 54*, 439.
Westphal, O., and Feier, H. (1956), *Chem. Ber. 89*, 582.
Yarif, J., Rapport, M. M., and Graf, L. (1962), *Biochem. J. 85*, 383.

Optical Rotatory Dispersion and RNA Base Pairing in Ribosomes and in Tobacco Mosaic Virus*

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ABSTRACT: A method for distinguishing base-paired double-stranded ribonucleic acid (RNA) from the single-stranded stacked conformation is discussed. The technique is based on a comparison of the optical rotatory dispersion, in the absorbing region, of RNA in salt-free solutions, where it is single stranded, with that in solutions of moderate or high salt concentration, where it is base paired. The optical rotatory dispersion of the single-stranded stacked conformation is calculated from that of dinucleoside phosphates while the influence of base pairing is estimated from the curves for double-stranded polyadenylic acid (poly A) plus polyuridylic acid (poly U) and polyguanylic acid (poly G) plus polycytidylic acid (poly C). The method is used to demonstrate that the RNA in *Escherichia coli* ribosomes is in a largely base-paired conformation with mainly G–C base pairs. This conformation is found in both the 30S and 50S subunits as well as in the 70S particle, and base pairing is not changed between 10^{-2} and 10^{-4} M Mg^{2+} ion concentration. The results are applicable to ribosomes from other sources, and are at variance with recent proposals that the RNA of ribosomes is in the single-stranded stacked conformation. The technique is also applied to existing optical rotatory dispersion data on tobacco mosaic virus (TMV) where base pairing is impossible in the known virus structure. We conclude that the RNA in TMV is in a rigid single-stranded stacked conformation of the same geometry as dinucleoside phosphates and single-stranded stacked RNA in salt-free solution.

For an ultimate understanding of the mechanism by which ribosomes participate in protein synthesis, it is of interest to know, among other things, the conformation of the RNA in the ribosome. While studies of this question have been reported, they are not all in agreement. From the results of X-ray scattering from wet gels of ribosomes (Klug *et al.*, 1961) and of hypochromicity (Schlessinger, 1960), the RNA of ribosomes has been thought to be in a largely double-stranded base-paired conformation, much the same as RNA is found in solution under appropriate conditions. The base pairing in solution arises from the folding back of the single chain into hairpin loops (Cox, 1966). Also, optical rotatory dispersion studies show a similarity between RNA and ribosomes (Sarkar *et al.*, 1967; McPhie and Gratzer, 1966). However, recent experiments on the binding of acridine orange dyes to ribo-

* From the Department of Chemistry, Cornell University, Ithaca, New York 14850. *Received June 12, 1967.* This work was supported by a research grant (GB-4766) from the National Science Foundation, and by a research grant (GM-14312) from the National Institute of General Medical Sciences of the National Institutes of Health, U. S. Public Health Service.

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