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IMMUNOCHEMICAL STUDIES ON A PANOSYL-AZOPROTEIN CONJUGATE*

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Abstract—The trisaccharide panose, 6-*O*-α-D-glucopyranosyl-4-*O*-α-D-glucopyranosyl-D-glucose, coupled to bovine serum albumin (BSA) via an azophenyl linkage was used as an artificial antigen to immunize rabbits. Antisera rendered specific for the introduced haptenic grouping by absorption with whole calf serum were studied by immunodiffusion, quantitative precipitation and hapten inhibition. Anti-hapten showed a specificity directed against the introduced *p*-phenylazo-β-panoside group. Hapten inhibition assays employing various di- and triglucosides, including two structural isomers of panose, established the relative importance of an α-configuration and a nonreducing terminal (1,6)-(1,4) ordered sequence of glucosidic bonds for optimal interaction with antibody. A heterogeneity in the specificity of antibodies formed in response to β-panosyl-BSA was evidenced from cross reactions obtained between anti-panoside and various sugar azoprotein conjugates.

INTRODUCTION

Immunochemical studies on sugar-protein conjugates involving the relationship of sugar hapten structure to antihapten specificity have been carried out almost entirely with mono and disaccharides as haptens (Avery and Goebel, 1929; Goebel *et al.*, 1934; Goebel, 1939; Beiser *et al.*, 1960; Arakatsu *et al.*, 1966; Allen *et al.*, 1967; Martineau *et al.*, 1969; Allen *et al.*, 1970). Apart from a study by Arakatsu *et al.* (1966), employing a synthetic antigen consisting of isomaltotrionic acid coupled to bovine serum albumin (BSA), conjugates possessing oligosaccharide determinants made up of three or more sugar residues have received little attention. Use of oligosaccharide conjugates as synthetic antigens provides an opportunity for the systematic examination of the role of some more complex structural features of sugar hapten to immunochemical specificity. Thus, for example, immunochemical effects of increase in oligosaccharide

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unit chain length, branching, occurrence of mixed linkage types and their order in a sequence, or the presence of more than a single type of sugar residue may be systematically explored.

In the present study, the specificity and heterogeneity of antihapten antibodies directed against a structurally more complex sugar hapten consisting of a trisaccharide of glucose with mixed types of glucosidic linkages was investigated. Panose, *O*-α-D-glucopyranosyl-(1,6)-*O*-α-D-glucopyranosyl-(1,4)-D-glucopyranose was conjugated to bovine serum albumin through an azophenyl linkage to provide the synthetic antigen BSA-*p*-phenylazo-β-panoside (Fig. 1).

The specificity and reactivity of rabbit anti-β-panosyl sera were examined by immunodiffusion and quantitative hapten inhibition. The use of structural isomers of panose possessing α-(1,6) and α-(1,4) linked glucosyl units in various arrangements, and glucobioses structurally related to portions of the introduced haptenic grouping permitted the establishment of: (1) the specificity of a fraction of anti-hapten antibodies directed against *p*-phenylazo-β-panoside, and, (2) a requirement for interglucosidic linkages in an α-configuration and ordered in a terminal (1,6)-(1,4) sequence, thus giving rise to an immunodominant nonreducing isomaltosyl end group.

Cross reactions of anti-β-panosyl sera with several sugar azoprotein conjugates possessing haptenic groupings related to or comprising a portion of the panose structure were investigated by immunodiffusion. Anti-β-panosyl sera were also examined by immunodiffusion for reactivity with several naturally occurring polysaccharides possessing glucose units in α-(1,6) and α-(1,4) linkage.

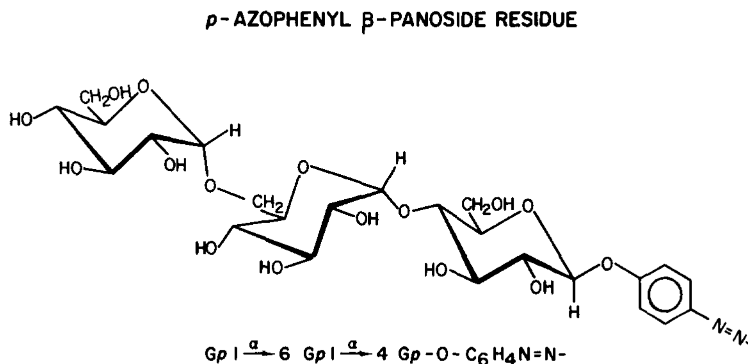

Fig. 1. Structural formula of the *p*-diazophenyl-β-panosyl haptenic grouping.

MATERIALS AND METHODS

Antisera

Four rabbits were immunized with β-panosyl BSA conjugate incorporated into complete Freund's adjuvant. A total of 1·3 ml of emulsion containing 0·97 mg conjugate/ml was administered subcutaneously to each animal in the nuchal region, over the course of 2 weeks. Rabbits bled 3 weeks after the last injection provided first course immune sera (R1-1C, R2-1C, R3-1C, R4-1C). Sera were rendered specific for the introduced haptenic grouping by serial absorption with whole calf serum to remove antibodies to carrier protein. A

slight excess of calf serum proteins, demonstrable by reactivity with rabbit anti-BSA, was present in absorbed anti-β-panosyl sera.

Immunodiffusion and immunoelectrophoresis

Double diffusion in gel and immunoelectrophoresis were carried out essentially as described in earlier studies (Allen *et al.*, 1967) except that the agar for double diffusion was buffered at pH 7·5 with 1·6 mM phosphate.

Quantitative precipitation and hapten inhibition

Quantitative precipitin studies were carried out using the method described by Kabat and Mayer (1961) employing 0·5 ml aliquots of antiserum. Phosphate buffered saline (PBS), 0·01 *M* phosphate pH 7·5 in 0·15 *M* sodium chloride, was used to bring the final volume up to 1·5 ml. Antibody nitrogen content of washed specific precipitates was determined by the Markham micro-Kjeldahl method (Kabat and Mayer, 1961).

The ability of various haptens to inhibit precipitation of 10 μg of anti-β-panosyl-BSA nitrogen from antiserum R1-1C by 1 μg of β-panosyl BSA nitrogen was assayed, using the scaled-down Folin–Ciocalteu method described by Kabat and Schiffman (1962). Hapten was added to 75 μl of antiserum (R1-1C), the mixture allowed to stand for 30 min at 37°C, and 50 μl of a 20 μg N/ml solution of β-panosyl-BSA nitrogen in PBS (pH 7·5) were then added. The volume was brought to 0·5 ml with PBS, tubes were incubated 1 hr at 37°C, and then placed in an ice bath for 1 week. Tubes were mixed twice daily.

Haptens

Isomaltodextrins were obtained by acid hydrolysis of dextran (Grade B, Mann Research Laboratories, Inc.) and isolated by charcoal column chromatography following the method described by Whelan (1962). Isomaltose, isomaltotriose, isomaltotetraose, isomaltopentaose, isomaltohexaose, isomaltoheptaose were further purified by descending paper chromatography employing ethyl acetate–pyridine–water (10 : 4 : 3 v/v) as solvent. Isomaltose was also isolated as a side product from the enzymic synthesis of panose described below. An additional sample of isomaltose was provided by Dr. Allene Jeanes.

Maltodextrins were prepared by acid hydrolysis of starch, separated by charcoal column chromatography according to Whelan *et al.* (1953), and further purified by descending chromatography on Whatman No. 3 MM paper employing ethyl acetate–pyridine–water (10 : 4 : 3 v/v) as solvent.

Panose was obtained from the action of *Aspergilus oryzae* α-amylase (Clarase 900, Miles Chemical Co., Clifton, N.J.) on maltose (Pan, 1962). After removal of D-glucose from the reaction mixture by fermentation with baker's yeast, panose was separated from other reaction products by repeated paper chromatography using the same solvent system employed for malto- and isomaltodextrins.

p-Nitrophenyl-β-panoside was synthesized enzymatically as described by Iyer and Goldstein (1966) while the chemical synthesis of 4,6-di-*O*-(α-D-glucopyranosyl)-D-glucose was performed according to DeSouza and Goldstein (1964). Isopanose was a gift from Dr. W. J. Whelan. *p*-Nitrophenyl-β-maltoside was synthesized according to the procedure of Babers and Goebel (1934), and

phenyl α-maltoside by the method of Helferich and Petersen (1935).

Maltose, cellobiose, melibiose, α,α-trehalose, and galactose were purchased from Pfanstiehl Laboratories, Inc. Gentiobiose was prepared by enzymic synthesis (Goldstein and Whelan, 1962). Methyl-β-maltoside was prepared from β-octaacetylmaltose by a Koenigs–Knorr reaction according to the procedure described by Schoch *et al.* (1942). The compound showed a melting point of 111–113°C (cor.) and $[\alpha]_{D20}+83{\cdot}1$ (C 1·7, H_2O). Maltitol prepared by sodium borohydride reduction of maltose was purified by paper chromatography as described for isomaltose. Methyl-β-D-glucopyranoside, phenyl-β-D-glucopyranoside and cyclohexaamylose were purchased from Mann Research Laboratories, Inc. All commercial samples of haptens were examined by paper chromatography. Where trace contaminants were found, materials were purified by descending paper chromatography. Haptens were dried to constant weight *in vacuo* over P_2O_5 at 55°C.

Polysaccharides and glycosyl-phenylazoprotein conjugates

Dextrans B512(F), B1355-S, B1254-L and B1377 (Jones and Wilkie, 1959; Jeanes *et al.*, 1954) were gifts provided by Dr. Allene Jeanes. Amylopectin (amylose free) was purchased from Calbiochem and glycogen from Pfanstiehl Laboratories, Inc. Clinical dextran (Grade B) was purchased from Mann Research Laboratories, Inc. Type II pneumococcal capsular polysaccharide was provided by Dr. C. M. Helms (1969).

The preparation and characterization of the phenylazo-bovine serum albumin (BSA) conjugates of β-maltose, β-cellobiose, β-sophorose, β-galactose, α and β-glucose have already been described (Allen *et al.*, 1967; Goldstein and Iyer, 1966; Gleich and Allen, 1965). BSA-*p*-phenylazo-β-panoside was prepared by diazotizing *p*-aminophenyl-β-panoside and coupling to BSA using the method described by Westphal and Feier (1956). As determined by anthrone analysis (Gleich and Allen, 1965), the conjugates contained respectively per mole of BSA, 18·2 moles of β-panose, 25 moles of β-sophorose, 16·8 moles of β-cellobiose, 14·6 moles of β-glucose, 14·6 moles of β-galactose, 15·1 moles of β-maltose, and 17·3 moles of α-glucose. The preparation of *p*-hydroxyphenylazo-BSA has been previously described (Gleich and Allen, 1965).

RESULTS

Immunodiffusion analysis with anti-β-panosyl sera

Sera obtained from four rabbits, after the first course of immunization with β-panosyl-BSA were examined by immunodiffusion for the presence of both anti-hapten and anti-carrier protein. Reactions given by serum R1-1C shown in Fig. 2A, are typical of results obtained with each of the four antisera. When diffused against β-panosyl-BSA, anti-β-panosyl serum R1-1C gave an intense band of precipitation which spurs over the band given by carrier protein (BSA).

As shown in Figs. 2B and 2C, following absorption with whole calf serum to remove antibodies to carrier protein, antisera R1-1C and R2-1C no longer react with BSA. The behavior of BSA absorbed anti-β-panosyl sera with a variety of sugar conjugates was examined by immunodiffusion. As seen in Fig. 2B, serum

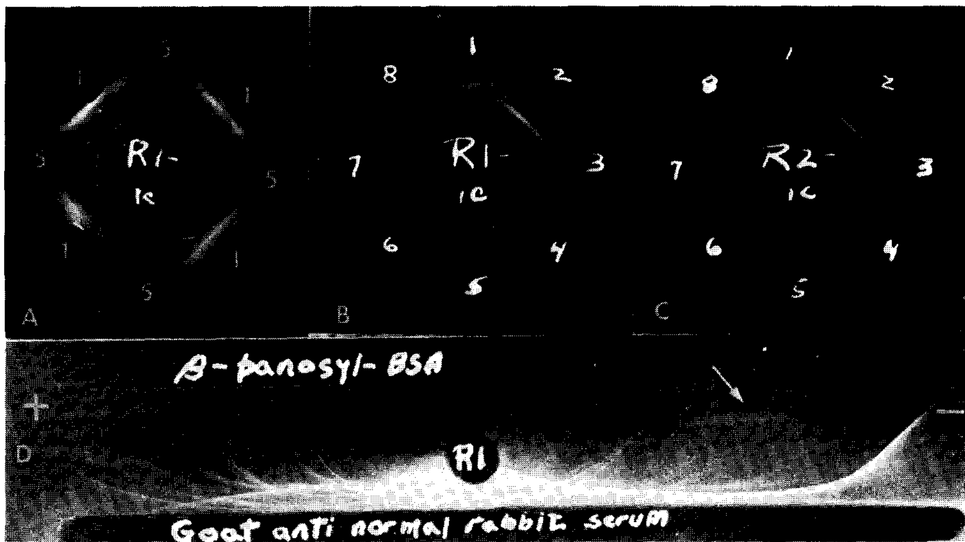

Fig. 2. Immunodiffusion and immunoelectrophoresis of anti-β-panosyl serum. A: Central well contains unabsorbed antiserum R1-1C. Peripheral wells contain: (1) β-panosyl BSA and (5) BSA. B: Immunodiffusion with carrier absorbed anti-β-panosyl serum R1-1C in central well. Peripheral wells contain: (1) β-panosyl BSA, (2) β-maltosyl BSA, (3) β-glucosyl BSA, (4) β-cellobiosyl BSA, (5) BSA, (6) β-sophorosyl BSA, (7) β-galactosyl BSA, and (8) α-glucosyl BSA. C: Immunodiffusion with carrier absorbed anti-β-panosyl serum R2-1C in central well. Peripheral wells as in B. D: Immunoelectrophoresis of anti-β-panosyl BSA serum. Well labeled R1 contains carrier absorbed anti-β-panosyl serum R1-1C; β-panosyl BSA and goat anti-whole rabbit serum placed in lateral troughs. Arrow indicates band of precipitation formed by rabbit anti-β-panosyl BSA serum R1-1C with β-panosyl BSA.

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R1-1C reacted with β-panosyl, β-maltosyl, β-glucosyl, β-cellobiosyl, β-sophorosyl, β-galactosyl and α-glucosyl BSA conjugates. Serum R2-1C however (Fig. 2C) gave a band of precipitation only with the β-panosyl, β-maltosyl, β-glucosyl and β-cellobiosyl BSA conjugates, while BSA absorbed antisera R3-1C and R4-1C (not shown in Fig. 2) reacted only with β-panosyl and β-maltosyl BSA. With each antiserum employed, the band of precipitation given by β-panosyl BSA spurred over the band obtained with β-maltosyl BSA. β-Glucosyl BSA formed a band of precipitate only with sera R1-1C and R2-1C; this band showed partial fusion with and was spurred over by the band formed by β-maltosyl BSA.

Bands given by β-glucosyl BSA, β-cellobiosyl BSA, β-sophorosyl BSA, β-galactosyl BSA, and α-glucosyl BSA with serum R1-1C showed complete fusion with one another. As shown in Figs. 2A, 2B and 2C, multiple bands of precipitation were obtained with β-panosyl BSA and β-maltosyl BSA conjugates. Multiple banding could be induced or exaggerated by variation in the relative concentration of antigen to antibody, and the relative size of the wells, or by allowing one of the reactants to diffuse for several hours before addition of the second reactant.

In order to explore the basis for the reactivity observed with the phenylazo BSA conjugates of α-glucose, β-cellobiose, β-sophorose and β-galactose, carrier absorbed antisera were further absorbed with *p*-hydroxyphenylazo-BSA and reexamined by immunodiffusion. Following absorption, all four anticonjugate sera lost reactivity towards all conjugates tested except β-panosyl and β-maltosyl BSA, the former still spurred over the latter. The disappearance of cross reactivity following absorption with *p*-hydroxyphenylazo-BSA suggests that the basis for the cross reactivity shown by α-glucosyl, β-cellobiosyl, β-sophorosyl, and β-galactosyl-phenylazo BSA conjugates is not related to the sugar moiety but rather to the aglycone portion of the introduced hapten. In an effort to evaluate the contribution of the aglycone moiety to β-panosyl-anti-β-panosyl precipitation, sera were not absorbed with *p*-hydroxyphenylazo-BSA.

Immunoelectrophoresis

Identification of immunoglobulin classes containing anti-β-panosyl precipitins was performed by immunoelectrophoresis using β-panosyl BSA to detect anti-conjugate and goat anti-whole rabbit serum to demonstrate rabbit serum proteins. As seen in Fig. 2D, anti-β-panosyl serum R1-1C abs., gave only one arc of precipitation with β-panosyl BSA, located in the $\gamma 2$ region. Similar findings were obtained with the other anti-β-panosyl sera tested. Immunoelectrophoretic evidence for the occurrence of anti-β-panosyl precipitins in other than the $\gamma 2$ region was not seen with any other sera tested.

Quantitative precipitin determination

Quantitative precipitin determinations were performed on each of the rabbit anti-β-panosyl sera. The course of quantitative precipitation is shown in Fig. 3. Homologous antigen removed 66, 34, 34, and 23 μg of antibody N from 0·5 ml of carrier absorbed antisera R1-1C, R2-1C, R3-1C and R4-1C respectively. The heterologous antigen, β-maltosyl BSA, when tested with anti-β-panosyl serum R2-1C, precipitated 40 per cent of the antibody nitrogen precipitable

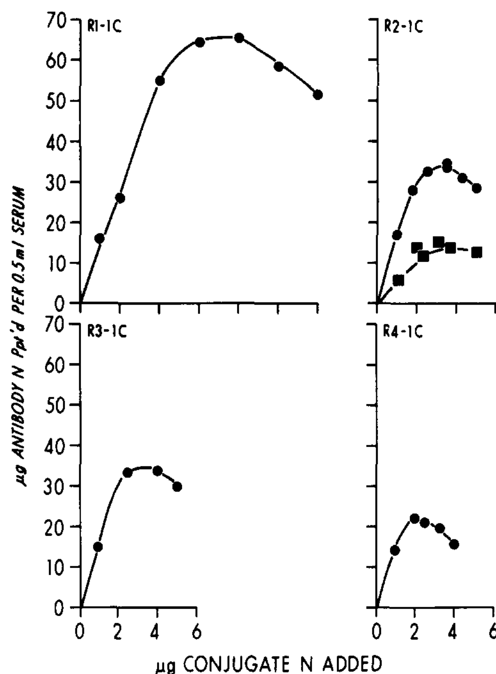

Fig. 3. Quantitative precipitin curves obtained with carrier absorbed rabbit anti-β-panosyl sera R1-1C, R2-1C, R3-1C and R4-1C. ●, Antibody N precipitated by β-panosyl-BSA; ■, antibody N precipitated from serum R2-1C by β-maltosyl BSA.

by β-panosyl BSA. Supernatant fluids from this cross reaction yield the expected remaining antibody nitrogen upon addition of homologous antigen.

Since serum R1-1C contained the highest concentration of anti-β-panosyl precipitins and demonstrated the broadest cross reactivity with the sugar conjugates tested, this antiserum was chosen for further study.

Hapten inhibition determinations

The overall specificity of anti-β-panosyl serum (R1-1C) as well as the relative contribution of various portions of the introduced *p*-azophenyl-β-panosyl haptenic grouping to interaction with antibody was examined by quantitative hapten inhibition. Of the haptens tested, *p*-nitrophenyl-β-panoside was found to be the most potent inhibitor of β-panosyl-anti-β-panosyl precipitation, 2×10^{-2} μM giving 50 per cent inhibition. As shown in Fig. 4, panose, the free sugar moiety lacking the aglycone group shows only one tenth the molar inhibitory ability of *p*-nitrophenyl-β-panoside requiring 0·3 μM to achieve 50 per cent inhibition.

A comparison of the trisaccharide panose with the disaccharide isomaltose which constitutes the nonreducing end of the panose molecule, shows panose to be 18 times more effective as an inhibitor of precipitation than isomaltose which required 5·5 μM for 50 per cent inhibition. Compared to maltose which comprises the reducing disaccharide end of the panose molecule, panose is

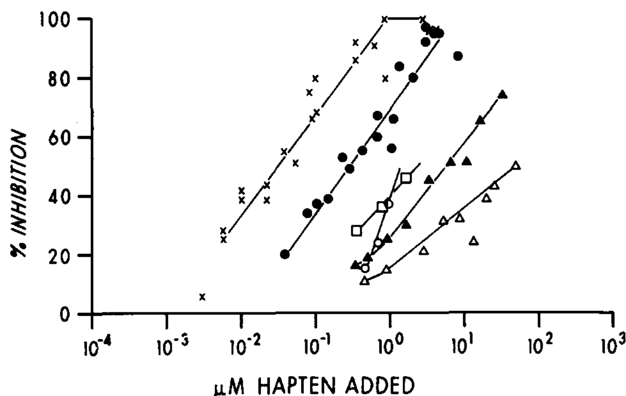

Fig. 4. Inhibition of β-panosyl-anti-β-panosyl precipitation by various sugars. ×, *p*-nitrophenyl-β-panoside; ●, panose; ○, isopanose; □, 4, 6-di-*O*-(α-D-glucopyranosyl)-D-glucose; ▲, isomaltose; △, maltose.

180 times more effective as an inhibitor, since 55 μM of maltose gave only 50 per cent inhibition. D-glucose, not included in Fig. 4, was a relatively poor inhibitor, requiring 400 μM to give 50 per cent inhibition.

To evaluate the relative importance of an α-(1,6)-α-(1,4) ordered sequence of glucosidic bonds for reactivity with anti-β-panoside, two structural isomers of panose were assayed as inhibitors. The trisaccharide isopanose provides the sequence $G_P\alpha$-(1 → 4)$G_P\alpha$-(1 → 6)G_P which is the inverse of the sequence found in panose. While panose provides an isomaltosyl unit at the non-reducing end of the molecule, the isomaltose unit of isopanose is subterminal from the non-reducing end. Isopanose was found to have only one fifth the inhibitory ability of panose, requiring 1·5 μM to give 50 per cent inhibition. Similarly, the branched trisaccharide, 4,6-di-*O*-(α-D-glucopyranosyl)-D-glucose, is also of interest. In this structural isomer of panose, isomaltose and maltose share the same reducing end. Panose was found to be nine times more effective as an inhibitor since 2·6 μM of 4,6-*di-O*-(α-D-glucopyranosyl)-D-glucose were necessary to give 50 per cent inhibition (Fig. 4).

The glucosidic linkages of the trisaccharide panose are of the α-configuration. To evaluate the contribution of this configuration to immunologic specificity, the relative inhibitory ability of isomers differing in the configuration of this anomeric linkage were compared. Isomaltose and maltose were compared to their corresponding β-linked glucobioses, gentiobiose and cellobiose respectively. As summarized in Table 1, at a comparable degree of inhibition, isomaltose, the α-(1,6)-linked glucobiose is nine times more effective than its β-(1,6)-linked isomer, gentiobiose. Similarly, cellobiose, the β-(1,4)-linked isomer of maltose, was also found to have only one ninth the inhibitory ability of its α-linked isomer. A requirement for an α-linked glucose is also evident from a comparison of methyl glucosides as inhibitors. Methyl α-D-glucopyranoside gives 25 per cent inhibition when tested at a concentration of 30 μM while methyl β-D-glucopyranoside failed to show any inhibition of precipitation at this concentration. Although α,α-trehalose shows only one fourth the molar

Table 1. Inhibition of the β-panosyl-anti-β-panosyl BSA precipitation by various sugar haptens

Hapten	μM required for 40% inhibition	μM required for 50% inhibition	Ratio† μM hapten tested / μM isomaltose
Isomaltose	2·8	5·5	1
Gentiobiose	24*		9
Melibiose	100*		36
			μM hapten tested / μM maltose
Methyl-β-maltoside	0·9	2·2	0·06
p-Nitrophenyl-β-maltoside	1·3		0·08
Phenyl-α-maltoside	4·3	12	0·27
Maltose	16	55	1
Maltitol	60	80	4
Cellobiose	140	170	9
			μM hapten tested / μM glucose
Methyl-α-D glucoside	90	170	0·38
D-Glucose	240	400	1
α,α-Trehalose	12*		

*Values obtained by extrapolating hapten inhibition data determined between 10 and 33 per cent inhibition; all other values experimentally determined.

†Ratio at 40 per cent inhibition.

inhibitory ability of isomaltose (Table 1), it is more effective than melibiose, gentiobiose and cellobiose and is comparable to maltose.

That the terminal nonreducing sugar unit makes a significant contribution to immunochemical specificity is further evident from a comparison of the relative molar inhibitory ability of 6-*O*-α-D-galactopyranosyl-D-glucose (melibiose) and 6-*O*-α-D-glucopyranosyl-D-glucose (isomaltose). These two compounds differ only in the configuration of the hydroxyl of C-4 of the nonreducing end. Inversion of the hydroxyl group at this position results in a decrease in molar inhibitory ability and melibiose shows only one thirty-sixth the potency of isomaltose.

A contribution to the specificity by the β-configuration of the anomeric linkage of the sugar moiety at the point of attachment to the aglycone is suggested by a comparison of phenyl-α-maltoside with methyl β-maltoside. While

only 2·2 μM of methyl β-maltoside gave 50 per cent inhibition, 12 μM of phenyl α-maltoside were required to obtain a comparable degree of inhibition.

To evaluate the effect of an increase in the number of α-(1, 6)-linked glucose units on ability to inhibit β-panosyl-anti-β-panosyl precipitation, isomaltodextrins were assayed for their relative molar inhibitory ability. Isomaltotriose, -tetraose, -pentaose, -hexaose and -heptaose were comparable to one another and found to be three to five times more effective than isomaltose in their ability to inhibit the precipitation. Maltodextrins were also assayed as inhibitors. However these assays were complicated by the presence in the rabbit antisera of small amounts of amylase activity (0·2 unit) which readily degrade higher maltodextrins. While maltotetraose, -pentaose and -hexaose were identical in potency to maltose, maltotriose showed only one seventh the inhibitory ability of maltose.

Maltitol and cyclohexaamylose were also used as inhibitors and compared to maltose. Maltitol required in 60 μM amounts to give 40 per cent inhibition, has one fourth the potency of maltose when compared at the same degree of inhibition. Cyclohexaamylose also has only one fourth the inhibitory ability of maltose; this value was obtained by extrapolating inhibition data to 40 per cent inhibition due to the low solubility of this dextrin.

The cross reactivity of anti-β-panosyl BSA antisera with naturally occurring polysaccharides possessing glucose units in α-(1, 6) and α-(1, 4) linkages was examined by immunodiffusion. All antisera failed to react with amylopectin, glycogen and dextrans B1254-L and B1355-S. Only one antiserum, R1-1C, reacted with clinical dextrans B512(F) and B1377, but these cross reactions were not obtained with subsequent bleedings from the same animal. Upon absorption of antiserum R1-1C with β-panosyl BSA, cross reactions with dextrans disappeared. Since a preimmunization bleeding was not obtained on this animal, the significance of this single occurrence of cross reactivity is uncertain.

DISCUSSION

In the present investigation panose was chosen to study the effect upon immunochemical specificity of increasing the structural complexity of a sugar hapten and to compare these effects with structure-specificity interrelationships already established for systems involving di- or trisaccharide haptens with a single type of glycosidic linkage.

BSA-*p*-phenylazo-β-panoside (β-panosyl BSA) was prepared and used as an immunogen. The introduced sugar moiety of this synthetic antigen consists of a glucotriose with α-(1, 6) and α-(1, 4) type linkages. As shown for the β-panosyl structure given in Fig. 1, the glucosidic linkages of the haptenic group are sequentially ordered so as to provide a terminal, nonreducing, α-isomaltosyl group and a subterminal or internal β-maltosyl group. The relative role of terminal and subterminal portions of haptenic structure to the immunochemical reactivity of anti-β-panoside were readily evaluated in the present study, by assay of isomaltose, maltose and structural isomers of panose as hapten inhibitors.

Antisera produced in rabbits following immunization with β-panosyl BSA showed three kinds of specificity directed against distinctive portions of the homologous antigen. One antibody population, directed against BSA, the carrier

protein, could be removed by absorption with whole calf serum (Figs. 2A, 2B and 2C). A second antibody population present in the sera of two of the four rabbits immunized, appears to be directed principally against the aglycone portion of the introduced hapten and was responsible for reactivity of anti-conjugate with the phenylazo-BSA conjugates of α and β-glucose, β-sophorose, β-cellobiose and β-galactose (Fig. 2B). This fraction of antibody could be removed from antisera by absorption with *p*-hydroxyphenylazo-BSA, leaving behind a third population of antibodies specific for the introduced hapten. That carrier absorbed anti-panoside sera R1-1C and R2-1C contain antibodies specific for the introduced haptenic grouping is evident from immunodiffusion data. As shown in Figs. 2B and 2C, all conjugates tested were found to be antigenically deficient with respect to the homologous β-panosyl-BSA antigen.

Although each antiserum shows the presence of a fraction of antibodies specific for the introduced haptenic grouping, the anti-panoside formed is heterogenous in its immunochemical behavior. This is evident from immunodiffusion findings which show a spuring of the β-panosyl BSA band over that given by β-maltosyl BSA.

In agreement with immunodiffusion data, quantitative precipitin determinations (Fig. 3) with carrier absorbed serum R2-1C, also establish the presence of at least two kinds of anti-β-panoside. One fraction (60 per cent of the total anti-panoside) is not cross reactive with β-maltosyl BSA and is precipitable only by β-panosyl BSA. The other fraction of anti-panoside is precipitable, however, by both β-maltosyl and β-panosyl BSA conjugates. The cross reaction of β-maltosyl BSA with anti-panoside is of interest. Despite the absence of a nonreducing α(1,6) linked glucosyl end residue, the maltose conjugate still shows appreciable cross reactivity and precipitates 40 per cent of the total anti-panoside. This cross reactivity cannot, however, be attributed entirely to the maltose portion of the heterologous antigen. In addition to sharing the maltosyl unit as a common structural feature, β-maltosyl and β-panosyl BSA conjugates also share the phenylazo group and may even possess identical phenylazo peptides. The contribution to immunochemical reactivity of the aglycone structure of phenylazo–protein–sugar conjugates has been well established for several other systems (Beiser *et al.*, 1960; Allen *et al.*, 1967, 1970; Gleich and Allen, 1965; Tanenbaum *et al.*, 1961; Karush, 1957) and is similarly involved in the cross reactivity found with β-maltosyl BSA.

That the specificity of anti-panoside is directed against the sugar moiety and involves the aglycone structure as well is established by quantitative hapten inhibition findings. Inhibition data obtained with serum R1-1C (Fig. 4) show that of the compounds tested, *p*-nitrophenyl-β-panoside was the most potent inhibitor of conjugate-anticonjugate precipitation. Panose, the free sugar moiety lacking the aglycone group shows only one tenth the molar inhibitory ability of the aryl panoside.

A trisaccharide constituted of an α-(1,6) followed by an α-(1,4) sequence of glucosidic bonds provides the sugar structure required for optimal reactivity with anti-β-panosyl BSA serum R1-1C. A specificity directed against this ordered sequence is established by hapten inhibition data (Fig. 4). Panose bearing the α-(1, 6)-α-(1, 4) sequence is found to be five times more effective as an inhibitor

than isopanose made up of an α-(1, 4)-α-(1, 6) sequence and 4, 6-di-O-(α-D-glucopyranosyl)-D-glucose where the isomaltosyl and maltosyl moieties share the same reducing end.

Of major importance to the immunochemical specificity of anti-panoside is the presence of the immunodominant, terminal, non-reducing α-(1, 6) isomaltosyl group. This is not only evident from the comparison of the relative molar inhibitory ability of panose and isopanose, but is also shown by the finding that isomaltose is ten times more effective as an inhibitor than maltose (Fig. 4) and that isomaltodextrins are fifteen times more effective than maltodextrins.

The significant contribution of the non-reducing terminal group to the immunochemical specificity of anti-panoside is also seen by the effect on inhibitory ability of a simple inversion of the hydroxyl group at C-4′ of isomaltose (Table 1). Melibiose, 6-O-α-D-galactopyranosyl-D-glucose, which differs from isomaltose in the configuration of the hydroxyl at C-4′ of the non-reducing end, has only one thirty-sixth the molar inhibitory ability of its C-4′ epimer, isomaltose. This finding is comparable to similar effects obtained with glucose, galactose, maltose, gentiobiose, lactose, sophorose and laminaribiose azoprotein-conjugates (Avery and Goebel, 1929; Goebel *et al.*, 1934; Allen *et al.*, 1967, 1970; Karush, 1957).

That both interglucosidic linkages must be in the α-configuration for optimal reactivity with anti-panoside is seen in Table 1, by comparing the α-(1, 6) linked diglucose, isomaltose, to its β-(1, 6) linked isomer gentiobiose and by comparison of the α-(1, 4) linked isomer, maltose to its β-(1, 4) linked isomer, cellobiose. Both α-linked diglucoses were found to be nine times more potent as inhibitors than their corresponding β-linked isomers.

A comparison of methyl α-D-glucoside with its β-isomer, methyl β-D-glucoside, is of interest. When tested at the same concentration (30 μM), methyl α-D-glucoside gave 25 per cent inhibition while no inhibition of precipitation was obtained with methyl β-D-glucoside. Furthermore, α,α-trehalose, containing two α-D-glucosyl groups linked through their reducing C-1 positions, is about eight times more potent than methyl α-D-glucoside. Thus, a specificity involving an α-configuration for glycosyl residues is evident.

The observation that *p*-nitrophenyl-β-maltoside is three times more effective an inhibitor than phenyl α-maltoside (Table 1) suggests that even though removed from the terminal non-reducing end by the length of a trisaccharide unit, the configuration of the anomeric linkage at the point of attachment to the aglycone still makes some contribution to the immunochemical specificity as found in the case of mono- and disaccharide-hapten conjugate systems (Beiser *et al.*, 1960; Allen *et al.*, 1967; Gleich and Allen, 1965; Karush, 1957; Avery *et al.*, 1932). As indicated in Table 1, methyl β-maltoside and *p*-nitrophenyl β-maltoside are better than maltose but comparable to one another in molar inhibitory ability. Thus, substitution of a *p*-nitrophenyl for the methyl group of methyl β-maltoside does not significantly enhance the interaction of the β-maltosyl grouping with anti-panoside.

That an α-linked isomaltosyl end unit provides the optimal structure to binding or interaction by isomaltodextrins is shown by the finding that isomaltodextrins of increasing chain length from isomaltotriose to isomaltoheptaose

were identical in molar inhibitory ability and three to five times more effective than isomaltose. The failure to obtain an increase in inhibitory ability with increase in unit chain length rules out the possibility of bivalency as found with oligosaccharide haptens obtained from enzymic hydrolysates of Type III pneumococcal polysaccharide and antibody to the corresponding capsular polysaccharide (Mage and Kabat, 1963a,b) or oligolysines and polylysine-specific antibodies (Arnon *et al.*, 1965).

The finding that cyclohexamylose, made up of 4-*O*-α-substituted maltosyl units has only one fourth the inhibitory ability of maltose is not surprising. Its poor interaction with anti-β-panosyl antibody may result from the restriction of rotation about its interglucosidic linkages, the presence of bulky 4-*O*-α-substituants on maltosyl units or perhaps its cyclic structure may sterically interfere with a close approach to antibody combining sites.

Maltitol, derived from maltose by transformation of the reducing end into a sorbitol residue, shows only one fourth the molar inhibitory ability of maltose. This comparison suggests that the presence of a glucopyranosyl ring system at the reducing end of maltose makes a contribution to specificity and is required for interaction with anti-panoside.

Panose has been identified in partial acid hydrolysates of amylopectin and glycogen. Although some of the 1, 4, 6-branch points of glycogen may occur at chain ends (Kabat, 1956), no cross reaction was found between the anti-β-panosyl sera tested and glycogen or amylopectin. While anti-β-panosyl sera also failed to react with Type II pneumococcal capsular polysaccharide, Type II antipneumococcal horse serum believed to cross react with glycogen and limit dextrins by virtue of $\alpha(1, 6)$-linked glucose (Heidelberger *et al.*, 1956; Goodman and Kabat, 1960) does however cross react with β-panosyl BSA.

When the panosyl–antipanosyl system was examined by immunodiffusion, multiple banding was observed with certain concentrations of reactants. A recent study by Henney (1970) may have some relevance to this finding and could explain similar observations reported with other sugar azoprotein systems (Allen *et al.*, 1970; Gleich and Allen, 1965). Sera of guinea pigs immunized with DNP-human gammaglobulin show the presence of antibodies whose specificity includes both hapten and part of the carrier termed 'hapten–carrier complex specific', as well as antibodies specific for the hapten itself (Henney, 1970). Double banding could occur if the antiserum used in the present study contained a high concentration of antibodies to panosyl-BSA complex. These antibodies would diffuse rapidly and form a band. Antibodies to β-panoside, if present in smaller amounts, could penetrate this band of precipitation forming a band closer to the antigen well. This process would occur, however, only if antibodies to panosyl-BSA complex masked haptenic determinants necessary for the precipitation of antibodies to β-panosyl hapten.

While internal antigenic determinants have been demonstrated in polysaccharide–antipolysaccharide systems (Uchida *et al.*, 1963; Mage and Kabat, 1963a; Allen *et al.*, 1965), none have been uniquivocally established as yet in systems involving oligosaccharide hapten conjugates. Anti-panoside examined in the present study was found to have a specificity including the non-reducing end group. It would be of interest in this respect to compare the immunochemical

properties of the β-maltosyl BSA cross-reactive fraction of anti-panoside with antibodies produced in response to immunization with β-maltosyl-BSA. Trisaccharide haptens might be too short, perhaps, to permit the synthesis of immunoglobulins whose specificity is directed against an internal portion of the sugar determinant. A critical length longer than a trisaccharide may be required. Results obtained with oligopeptide determinants by Sage *et al.* (1964) and Schechter and Sela (1965) may be in agreement with this notion. The first group used an antigenic determinant carrying an average of 7·6 alanines per chain and obtained antisera with a specificity directed against an internal moiety while the second group dealt with an antigenic determinant bearing an average of 5·1 alanines per chain which resulted in the production of antibodies directed against amino terminal determinants.

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