

Aldonate Coupling, A Simple Procedure for the Preparation of Carbohydrate-Protein Conjugates for Studies of Carbohydrate-Binding Proteins¹

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Aldonate coupling, a new, high-yield method for conjugation of reducing oligosaccharides to proteins is described. It involves oxidation of the oligosaccharides to their corresponding aldonic acids followed by coupling to amino functions of proteins by activation of carboxyl groups with a water-soluble carbodiimide derivative. The immunochemical properties of some of these synthetic glycoproteins were assessed using several plant agglutinins (lectins) as well as antibodies raised to the conjugates. The scope and limitation of the method are discussed.

The preparation of conjugates from carbohydrates and proteins has received considerable attention for nearly half a century. The classical method for their synthesis involves preparation of *p*-aminophenyl or *p*-aminobenzyl glycosides of the sugar followed by coupling to the protein via a diazotization reaction (1-3). The yield in the conjugation can be considerably improved by conversion of the amino function to an isothiocyanate derivative (4). Further methods involve preparation of the 1-(*m*-aminophenyl)-flavazole of the sugar followed by diazotization (5) and use of cyanuric trichloride as the coupling agent (6). The reduction by borohydride of a Schiff base formed between a reducing

sugar and proteins has also been used as a coupling reaction (7). Arakatsu *et al.* oxidized reducing oligosaccharides to aldonic acids and coupled these to bovine serum albumin (BSA)⁶ using the mixed anhydride method (8). The same approach has been applied to the preparation of conjugates of oligosaccharides from human milk and polylysine (9). Recently, Lemieux and co-workers linked a number of 8-carboxy-octyl glycosides (related to human blood-group substances) to BSA by conversion of the corresponding hydrazides to acyl azides via diazotization (10).

The synthetic glycoproteins prepared by the above procedures have been widely used to raise antibodies to defined structural moieties in polysaccharides (8, 10-12) and to study the specificity and cross reactivity of antibody-combining sites (8, 12-14). Carbohydrate-protein conjugates

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⁶ Abbreviations used: BSA, bovine serum albumin; EDC, 1-ethyl-3-(dimethylaminopropyl)-carbodiimide hydrochloride; con A, concanavalin A; RCA₁, lectin from *Ricinus communis*; HEPES, 4-(2-hydroxyethyl-1-piperazine)ethanesulfonic acid; *D.S.*, degrees of substitution, number of sugar residues per molecule of bovine serum albumin; SDS, sodium dodecyl sulfate; T cell, thymus-derived lymphocyte.

have also been used as model glycoproteins in studies of lectin carbohydrate-binding specificities (15, 16). However, several of these conjugates suffer from the disadvantage inherent in the particular kind of carbohydrate-protein linkage present in the conjugate. Notably, aromatic structures appear to give rise to nonspecific interactions in certain lectin systems (15) as well as undesirable antibody specificity toward the conjugate linkage region when these conjugates are used as antigens (12).

In connection with studies of the α -D-galactopyranosyl-binding lectin isolated from the seeds of *Bandeiraea simplicifolia* it was found that melibionate could be conjugated in high yield to aminoethyl Bio-Gel using a water-soluble carbodiimide (1-ethyl-3-(dimethylaminopropyl)-carbodiimide hydrochloride, EDC) (17). This gel was used in affinity columns to isolate the pure lectin. It seemed reasonable to assume that an analogous reaction could be employed to link various oligosaccharides, as their aldonic acids, to proteins. Experiments aimed at synthesis of these conjugates and evaluation of their immunochemical properties are reported in the present communication.

EXPERIMENTAL PROCEDURES

Materials. Bovine serum albumin was purchased from Miles Laboratories, Kankakee, Ill.; ovalbumin from Sigma Chemical Company, St. Louis, Mo.; concanavalin A from Calbiochem, La Jolla, Calif.; and 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride from the Pierce Chemical Company, Rockford, Ill. The *B. simplicifolia* lectin was isolated as described (17) while *Ricinus communis* lectin (RCA₁) was the kind gift of Dr. Marilyn E. Etzler, University of California, Davis, Calif. The carbohydrates used were obtained from Pfanstiehl Laboratories, Waukegan, Ill., or were available in this laboratory.

Analytical methods. Hexose concentrations were determined by the phenol-sulfuric acid assay (18) against blanks of appropriate protein using standards of suitable methyl glycosides. Protein concentrations were determined by the microbiuret assay using BSA as standard (19). Precipitates from precipitation and inhibition studies were digested, and nitrogen was determined by a modification (17) of the ninhydrin procedure of Rosen (20). Electrophoresis on polyacrylamide gels (7.5%) was per-

formed at pH 4.3 in β -alanine-acetic acid buffer (21), and sodium dodecyl sulfate electrophoresis in polyacrylamide gels was performed essentially as earlier described (22). Protein staining was performed with Coomassie brilliant blue.

Immunochemical methods. For precipitation and inhibition studies involving lectins, essentially the same procedures as described previously (17) were used except that all reactions were performed in a total volume of 500 μ l, and all components were dissolved in 0.01 M sodium phosphate-buffered saline (0.15 M) of pH 7.2. Precipitation reactions were incubated for 48 h at room temperature (except for temperature-dependence studies). In volume-dependence studies total volumes of 0.3, 0.5, 1.0, 1.5, and 2.0 ml were used. The digestion of the precipitates was performed as already described (23).

For immunization experiments a group of three adult New Zealand white rabbits was challenged with the melibionate-BSA conjugate containing 34 mol of sugar per mol of BSA. The antigen at a concentration of 1 mg/ml was homogenized in Freund's complete adjuvant (Difco Laboratories, Detroit, Mich.) using a Virtis microblender. This emulsion was injected into each of four foot pads (0.1 ml) and subcutaneously in the back (0.1 ml) for 3 successive weeks. Blood was drawn from the marginal vein of the ears at weekly intervals beginning 1 week after the last injection. The sera obtained, after separation of the blood clot, was stored frozen without addition of preservative. The methods for precipitation reactions and inhibition assays were essentially as outlined by Kabat (24). All assays were performed using 100 or 200 μ l of serum (pool from three bleedings) in a total volume of 500 μ l. Precipitation reactions were allowed to proceed for 1 week at 0°C. Ouchterlony plates were prepared as described (25). None of the rabbits used in this study showed a significant preimmunization titer against immunizing antigen.

Synthetic methods. Aldonic acids (isolated as their potassium salts) were prepared by the procedure of Moore and Link (26).

In a typical coupling experiment, potassium melibionate (1.3 g) was added to a rapidly stirred solution of BSA (400 mg in 4.0 ml of water). The pH was adjusted to 4.75 using 1.0 M hydrochloric acid. A solution of EDC (620 mg in 1.0 ml of water) was added dropwise over 30 min, at room temperature, the pH being maintained at 4.75 by addition of 0.5 M hydrochloric acid. After approximately 40 min the reaction ceased to consume acid. The solution was left for an additional 6 h, during which time the pH gradually dropped to approximately 3.5. Addition of sodium acetate buffer (5 ml, 1 M, pH 5.5) served to quench the reaction, and the modified protein was dialyzed exhaustively against distilled water. Additional experiments were carried out varying the

proportions of sugar to protein. The results of the coupling experiments are summarized in Table I.

Melibiononic acid was conjugated to ovalbumin using the same procedure as used for BSA.

Melibiononic acid was also conjugated to con A in a solution (25 ml) containing 7.5 mg/ml of protein in 1 M sodium chloride–0.1 M methyl α -D-mannopyranoside. Potassium melibionate (975 mg) was added and the pH was adjusted to 4.75. EDC solution (465 mg in 2.0 ml of water) was added over 15 min with pH control. After 30 min the reaction was quenched by addition of an excess of acetate buffer, and the solution was dialyzed exhaustively against 0.15 M sodium chloride. The yield of soluble protein was 80%.

Culture methods. Several strains of mice were used to test the mitogenic properties of melibionate-con A: B10.Br(*H-2^k*), B10.K(*H-2^k*), B10.A(3R)-(*H-2^g*), Balb/c(*H-2^d*), and athymic, homozygous nude Balb/c (*nu/nu*) mice. The mice were maintained in Dr. Niederhuber's colony at The University of Michigan, and in each experiment were matched for age and sex.

Spleen cells were dispersed by teasing in serum-free RPMI 1640 medium (Microbiological Associates, Bethesda, Md.) supplemented with 3 ml of HEPES (1 M) and 50 μ g/ml of gentamycin per 100 ml. Quadruplicate cultures of 5×10^6 viable lymphoid cells per well were incubated in multiwell Linbro plates (Linbro Chemical Co., New Haven, Conn.) for 72 h. Cultures were stimulated with a dose range of con A or melibionate-con A. Final volume in each well was 0.3 ml, and culture plates were incubated at 37°C in a humidified atmosphere of 5% CO₂.

During the last 18 h of culture, 0.2 μ Ci of [³H]thymidine (2Ci/mmol) was present in the cultures. Cultures were harvested and assayed for the

incorporation of [³H]thymidine into DNA using a multiple sample harvester (Otto Hiller Co., Madison, Wis.). The acid-precipitable DNA was collected on glass-fiber filters and counted in a liquid scintillation counter using a toluene-based scintillation fluid.

Thymus-derived lymphocytes were prepared from whole spleen using nylon wool (LP-1 Leuko-pak leukocyte filter, Fenwal Laboratories, Morton Grove, Ill.), as described by Julius *et al.* (27). Such cells are 80–90% Thy-1 positive.

RESULTS AND DISCUSSION

The Coupling Reaction

The aldonic acids (as potassium salts) of reducing sugars used in this study were prepared by oxidation with alkaline iodine (26). The yield in this reaction is essentially quantitative and the preparation does not require further purification.

The coupling reaction involves activation of the carboxyl group of the sugar derivative by reaction with a water-soluble carbodiimide (EDC) to give an *O*-acylisourea (28). The latter reacts with accessible nucleophiles in the protein, e.g., ϵ -amino groups of lysyl residues, forming amide linkages (peptide bonds). In the procedure used, an EDC solution is added over 30 min to a rapidly stirred water solution of the protein and the aldonic acid. During this time the acidity of the reaction mixture is carefully controlled by addition

TABLE I
PROPERTIES AND YIELDS OF ALDONATE-BOVINE SERUM ALBUMIN CONJUGATES

Aldobiononic acid	Molar ratio of aldonic acid to protein in reaction mixture	Molar ratio of aldonic acid to lysyl residues in reaction mixture	Molar ratio of sugar to protein in product (<i>D.S.</i>)	Yield ^a (%)	Yield ^a (%)	Yield ^c (%)
Melibiononic acid	1300	22	32	93	54	2.5
Melibiononic acid	540	9	34	104	58	6.4
Melibiononic acid	300	5	22	83	37	7.4
Melibiononic acid	120	2	12	83	20	10
Melibiononic acid	60	1	7	88	12	12
Gentiobiononic acid	540	9	48	105	81	9
Lactobiononic acid	540	9	37	105	63	7
Maltobiononic acid	540	9	45	91	76	8.4
Maltotrionic acid	540	9	33	92	56	6.2

^a Yield of recovered protein according to microbiuret assay.

^b Yield in coupling reaction assuming 59 lysyl residues per BSA molecule (29).

^c Based upon amount of starting sugar present in the reaction solution.

of acid. After standing for 6 h at room temperature, the reaction is quenched by addition of an excess of acetate ions, and the conjugate is dialyzed free of reagents. The carbohydrate content of the conjugates was determined by the phenol-sulfuric acid assay (18). The yields and reaction conditions used for the synthesis of several conjugates of BSA are presented in Table I. As seen in the table, the highest degrees of substitution (*D.S.*, number of sugar residues per BSA molecule) was obtained when the molar ratio of sugar to BSA in the reaction mixture was ≥ 500 . The yields obtained compare well with the best procedures described. The isothiocyanate coupling procedure (4) gives a maximum *D.S.* of 54 whereas the diazotization procedure of Lemieux and co-workers (10) gives *D.S.* of about 35, both to BSA. Furthermore, it is apparent that the *D.S.* could be readily controlled by varying the molar proportions of sugar to protein in the reaction mixture. All the BSA conjugates appeared as multiple bands on polyacrylamide electrophoresis at pH 4.3. This demonstrates that the conjugate preparations comprise mixtures of populations of BSA molecules bearing different numbers of sugar residues on the amino functions. In contrast, the conjugates appeared as a single band upon SDS-gel electrophoresis, showing that no appreciable aggregation had occurred.

The aldonate coupling reaction was also used to covalently link melibionate to ovalbumin and to concanavalin A (see below) and it is readily apparent that the method can be used for the coupling of virtually any aldonic acid prepared from natural and synthetic oligosaccharides to various protein carriers. However, an obvious limitation of the procedure involves loss of the reducing sugar residue which becomes the conjugation "arm." It should also be possible to link other carbohydrates containing carboxyl groups, e.g., uronic acid containing sugars or glycosides of aglycones containing carboxyl groups, in an analogous fashion.

Evaluation of the Immunochemical Properties of the Conjugates Using Lectins

The interaction of the aldonate-BSA

conjugates with lectins was investigated. Of the different systems studied the properties of the melibionate-BSA conjugates in their interaction with the α -D-galactopyranosyl-binding lectin from *B. simplicifolia* (17) was investigated most thoroughly.

Figure 1 presents the quantitative precipitation curves obtained when the *B. simplicifolia* lectin interacted with melibionate-BSA conjugates of *D.S.* 34, 22, and 7. About 80-90% of the added protein could be precipitated by the conjugates of *D.S.* 34 and 22. It is apparent from the early stages of the precipitation curves that the order of reactivity of the conjugates is related directly to the number of melibionate units present on the protein. The solubility of the lectin melibionate-BSA (*D.S.* 34) precipitate was determined by performing the precipitation reaction at a point in the "equivalence zone" in five different volumes ranging from 0.3 to 2.0 ml. Virtually the same amount of precipitate was formed in all instances. The influence of temperature on the reaction was assessed by incubation of the precipitating system at 37, 23, and 0°C. No significant difference was observed between the 23 and 0°C experiments but at 37°C only about 85% of the amount of precipitate formed. These results closely parallel those obtained from similar investigations (17) on the *B. simplicifolia* lectin-guaran system and indicate that the lectin-conjugate precipitate has a low solubility. Several glycosides and disaccharides were tested for their

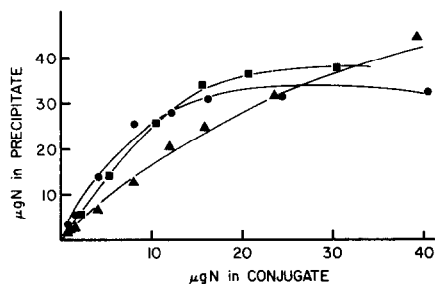


FIG. 1. Comparison of precipitating power of melibionate-BSA conjugates containing varying amounts of melibionate residues; (\blacktriangle), 7 melibionate residues/mol of BSA; (\blacksquare), 22 melibionate residues/mol of BSA; (\bullet), 34 melibionate residues/mol of BSA. *B. simplicifolia* lectin, 22.8 μ g of nitrogen per tube.

ability to inhibit the precipitation reaction. The results are summarized in Table II. The concentrations of sugars required for 50% inhibition of the *B. simplicifolia* lectin-melibionate-BSA system are somewhat lower than the corresponding values for the same lectin interacting with guaran (17). Nevertheless, the ratios of inhibitor concentrations are comparable in both systems. The results indicate that the interaction between the lectin and the BSA conjugate, although highly specific, is somewhat weaker than in the guaran system. However, the interaction appears to involve solely the carbohydrate-binding site of the lectin; no hydrophobic interactions appear to be involved as is true for carbohydrate-protein conjugates containing azophenyl linkages (15). This is apparent from the fact that the precipitin reaction was completely inhibited by several sugars. Furthermore, a lactobionate-BSA conjugate failed to precipitate the *B. simplicifolia* lectin; polysaccharides and glycoproteins containing terminal β -D-galactopyranosyl groups also fail to precipitate this agglutinin (17).

The maltobionate-BSA conjugate (*D.S.* 45) was investigated next. Quantitative precipitation studies (Fig. 2) with concanavalin A (con A) demonstrate that only about 50% of the total added protein was precipitated in the "equivalence zone." This indicates a relatively weak conjugate-lectin interaction. Inasmuch as α -D-glycopyranosyl groups are among the structures most complementary to the binding site of con A (30), these results

appear somewhat surprising. The weak lectin-conjugate interaction was confirmed by hapten-inhibition studies. The concentrations of several sugars necessary to produce 50% inhibition of the precipitation reaction are given in Table III. The values obtained compare with those obtained in studies of the con A-levan system which shows a considerably weaker interaction than, for example, con A and dextrans (31). As in the *B. simplicifolia* lectin-melibionate-BSA system, complete inhibition was achieved indicating that nonspecific binding does not occur. As expected a gentiobionate-BSA conjugate failed to precipitate with con A; it is known that β -D-glucopyranosyl groups bind only weakly if at all to the lectin.

The maltotrionate-BSA conjugate contained 33 residues of maltotrionate groups per mole of protein. As a precipitant of con A, the maltotrionate-BSA conjugate containing two intact α -D-glucopyranosyl

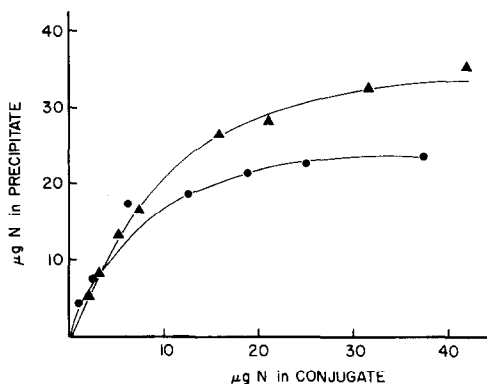


FIG. 2. Quantitative precipitin curves of maltobionate-BSA conjugate (●) and maltotrionate-BSA conjugate (▲) with con A. Con A, 19.5 and 16.3 μ g of nitrogen per tube, respectively.

TABLE II

HAPTEN INHIBITION OF THE *B. simplicifolia* LECTIN-MELIBIONATE-BSA SYSTEM

Sugar	Concentration for 50% inhibition (mM)
Methyl α -D-galactopyranoside	0.48
Melibiitol	0.58
Melibiose	0.60
D-Galactose	1.6
Methyl β -D-galactopyranoside	3.1
Lactose	15% inhibition at 20 mM
Methyl α -D-mannopyranoside	0% inhibition at 20 mM

TABLE III

HAPTEN INHIBITION OF THE CON A-MALTOBIONATE-BSA SYSTEM

Sugar	Concentration for 50% inhibition (mM)
Methyl α -D-mannopyranoside	0.20
Methyl α -D-glucopyranoside	0.85
Maltose	2.6
Methyl β -D-glucopyranoside	21.0
D-Galactose	13% inhibition at 200 mM

units is superior to the maltobionate-BSA conjugate containing but a single α -D-glucopyranosyl group (Fig. 2). Under the conditions employed, the maltotriionate conjugates required 0.27 mM methyl α -D-mannopyranoside for 50% inhibition.

The interactions between the *Ricinus communis* lectin (RCA₁) and the melibionate-BSA (D.S. 34) and lactobionate-BSA (D.S. 37) conjugates were investigated. The quantitative precipitation curves are shown in Fig. 3. This lectin specifically binds D-galactopyranosyl groups; β -glycosides are bound somewhat more strongly than the α -anomers (32). The amounts of lectin-conjugate complex precipitated by the two conjugates reflect this specificity inasmuch as the lactobionate conjugate is considerably more effective than the melibionate conjugate.

The results discussed above indicate that BSA conjugates of aldonic acids are suitable agents for studies of lectins, e.g., in evaluation of their carbohydrate-binding specificity.

Preparation and Investigation of a Carbohydrate-con A Conjugate

Melibionic acid was linked to con A using the same procedure as for the synthesis of the BSA conjugates, although a shorter reaction time was used and the lectin was "protected" by the presence of methyl α -D-mannopyranoside. The melibionic-con A conjugate contained approximately six sugar residues per mole of protein. This conjugate showed approximately 50% of the activity of native con A

when assayed by the quantitative precipitation reaction against dextran B-1355. The *B. simplicifolia* lectin reacted with the melibionate-con A conjugate generating a typical precipitin curve (Fig. 4). In this experiment the incubation solution contained methyl α -D-mannopyranoside in order to abolish the reactivity of melibionate-con A against *B. simplicifolia* lectin since the latter is a glycoprotein containing a carbohydrate moiety with which con A reacts (17).

The con A conjugate was also tested for its capacity to stimulate mouse spleen cells and nylon wool-purified splenic T lymphocytes, as measured by incorporation of [³H]thymidine into DNA. The dose-response curve is given in Fig. 5 together with that obtained from native con A under the same conditions. The curve obtained for the con A conjugate resembles those reported for acetyl- and succinyl-con A (33, 34).

The melibionate-con A gave similar stimulation with nylon wool-purified splenic T cells but no stimulation was obtained using splenic B cells from congenitally athymic Balb/c (*nu/nu*) mice. The con A conjugate, therefore, retained the properties of a T-cell mitogen. The mitogenic activity could be inhibited by methyl α -D-mannopyranoside at the same concentration as for native con A.

The experiment reported above justifies the conclusion that carbohydrates can be conjugated to certain biologically active

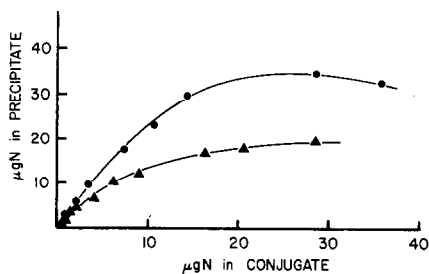


FIG. 3. Quantitative precipitin curves of melibionate-BSA conjugate (▲) and lactobionate-BSA conjugate (●) with *Ricinus communis* (RCA₁) lectin. *Ricinus communis* lectin, 19.5 μ g of nitrogen per tube.

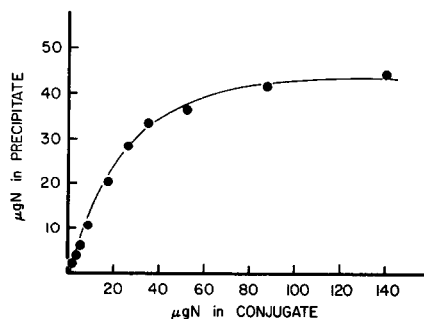


FIG. 4. Quantitative precipitin curve of melibionate-con A conjugate with *B simplicifolia* lectin in the presence of 50 mM methyl α -D-mannopyranoside. *B. simplicifolia* lectin, 26.4 μ g of nitrogen per tube.

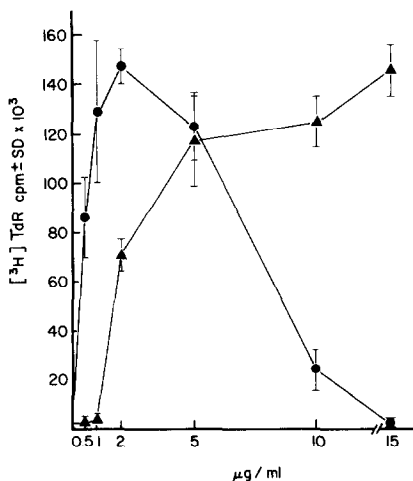


FIG. 5. Dose-response curves obtained on stimulation of mouse spleen cells with con A (●) and melibionate-con A (▲).

proteins by the present method with retention of activity.

Evaluation of Immunochemical Properties of the Conjugates Using Hapten-Specific Antibodies

The melibionate-BSA conjugate (*D.S.* 34) was used as antigen in immunization experiments with rabbits. A set of three rabbits was sensitized with the antigen in Freund's complete adjuvant. For evaluation of the hapten-specific response a second conjugate, namely, melibionate-ovalbumin (*D.S.* ~ 5), was synthesized. This conjugate contains the homologous hapten linked to a heterologous carrier, thus abolishing any precipitation of antibodies against the BSA carrier in precipitation reactions. Two-dimensional immunodiffusion of various conjugates against an antiserum raised against melibionate-BSA is shown in Fig. 6. The expected spur formation between BSA and melibionate-BSA as well as between the latter and melibionate-ovalbumin is clearly discernible. The quantitative precipitation curves obtained from the individually pooled antisera of three different rabbits from three successive bleedings are presented in Fig. 7. The antibody titers are quite different in the three animals. Nevertheless, it is apparent that the conjugates give rise to hapten-specific antibodies and that the titers com-

pare well with those obtained employing other types of conjugates.

Kabat and colleagues have made a careful evaluation of the hapten-specific antibody response in rabbits to closely related antigens (8). In their study, isomaltonic and isomaltotronic acid conjugates of BSA (*D.S.* approximately 10) were used to assess the size of the antibody-combining site. The isomaltonate-BSA conjugate gave antibodies which were incapable of

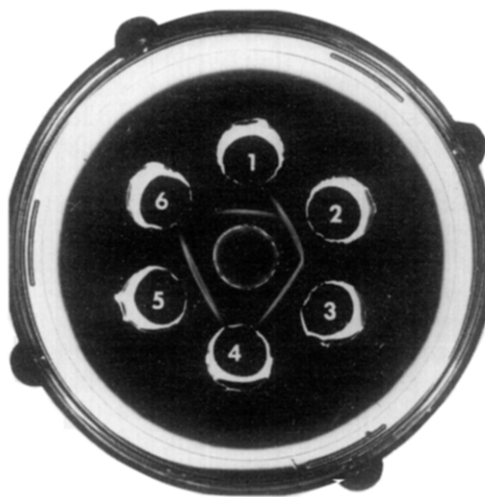


FIG. 6. Ouchterlony double diffusion in agar. Center well, melibionate-BSA antiserum; well 1, melibionate-ovalbumin conjugate; well 2, melibionate-BSA conjugate; well 3, BSA; well 4, saline control; well 5, melibionate-BSA conjugate; well 6, gluconate-ovalbumin conjugate. All samples were 0.5 mg/ml in saline.

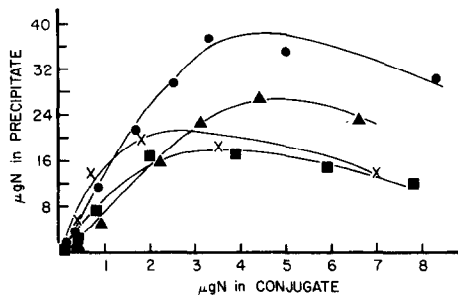


FIG. 7. Quantitative precipitin curves of melibionate-ovalbumin conjugate with melibionate-BSA antiserum. (●), Rabbit 1, 100 µl of serum; (■), rabbit 2, 100 µl of serum; (▲), rabbit 3, 200 µl of serum. (×), Quantitative precipitin curve of gluconate-ovalbumin with melibionate-BSA antiserum (200 µl) from rabbit 1.

precipitating dextrans. Our results confirm this finding inasmuch as none of the antisera raised to melibionate-BSA conjugate was capable of precipitating guaran or blood-group B substance. These results show that a single glycosyl residue does not generate antibodies capable of precipitating a heterologous substance containing this residue as the only structural feature in common with the immunizing antigen. This is in accord with the general view which requires that the antibody-combining site be complementary to at least two sugar units in order to form precipitates with a heterologous polysaccharide (8, 35). The quantitative hapten inhibition studies presented in Fig. 8 lend further support to this view. It is apparent that potassium melibionate is by far the most potent inhibitor. Despite the fact that methyl α -D-galactopyranoside and melibiose both contain nonreducing α -D-galactopyranosyl groups, an approximately 500 times higher concentration of these substances was required to produce 50% inhibition of the precipitation system. A β -glycoside, e.g., methyl β -D-galactopyranoside, is essentially noninhibitory, demonstrating the importance of the anomeric center. Melibiose parallels methyl α -D-galactopyranoside as an inhibitor. Potassium gentiobionate is comparable to methyl α -D-galactopyranoside, and this together with the fact that potassium melibionate is so potent an inhibitor justifies the conclusion that the gluconate "arm" plays an

important role in the binding specificity of the antibody-combining site. Indeed, antiserum from one rabbit precipitated a gluconate-ovalbumin conjugate as illustrated in Figs. 6 and 7. This could, however, be due to a population of antibodies directed exclusively to the gluconate residue and not to antibodies complementary to melibionate. Thus, the majority of antibodies raised by the melibionate-BSA conjugate have a combining site complementary to the galactosyl residue plus the gluconate moiety, i.e., the melibionate group. The relative importance of the glyconate moiety can be expected to decrease in conjugates prepared from larger oligosaccharides.

These studies indicate that aldonic acid-BSA conjugates prepared by the aldinate coupling technique are potent immunogens. Most of the antibodies raised to an aldobionate conjugate appears to include the glyconate "arm" in the combining site which tends to limit the utility of the method for preparation of antibodies against a specific nonreducing monosaccharide structure. However, the aldinate coupling method should be of interest in the preparation of antibodies to larger oligosaccharides. Furthermore, oligosaccharide conjugates can be prepared and used in evaluating the cross reactivity and carbohydrate specificity of antisera prepared by immunization with "natural" carbohydrate antigens.

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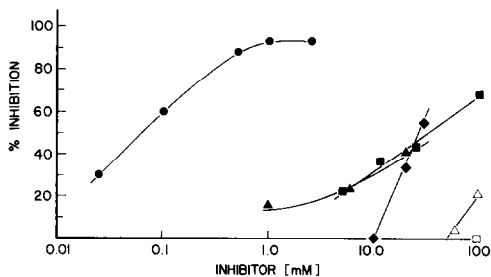


FIG. 8. Inhibition of melibionate-BSA antiserum (100 μ l from rabbit 1) precipitation of melibionate-ovalbumin conjugate by low molecular weight sugars. (●), Potassium melibionate; (▲), melibiose; (■), methyl α -D-galactopyranoside; (◆), potassium gentiobionate; (△), methyl β -D-galactopyranoside; (□), methyl α -D-glucopyranoside.

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