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The Preparation and Characterization of Antibodies with Specificity for the Carbohydrate Units of Gum Arabic and Gum Mesquite

Frank J. Miskiel* & John H. Pazur‡

Department of Molecular & Cell Biology, Paul M. Althouse Laboratory, The Pennsylvania State University, University Park, Pennsylvania 16802, USA

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ABSTRACT

Antibodies directed against terminal carbohydrate units of gum arabic and gum mesquite were deteced in sera of rabbits immunized intramuscularly with solutions of the gums and Freund's complete adjuvant. The antibodies were isolated by affinity chromatography on adsorbents of AH-Sepharose 4B containing ligands of the appropriate gum. From the sera of animals immunized with gum arabic, two sets of anti-carbohydrate antibodies were isolated and these were shown to have specificity for different disaccharide units at the non-reducing ends of the gum molecule. From the sera of animals immunized with gum mesquite only one set of anti-carbohydrate antibodies with specificity for a terminal disaccharide unit was obtained. Isoelectric focusing coupled with agar diffusion of the purified antibodies showed that all of the sets of antibodies were composed of isomeric proteins with each isomer exhibiting antibody activity. The antibodies of a set are appropriately termed isoantibodies and it is likely that each isomer is synthesized by a different immunocyte of the host. Hapten inhibition studies with oligosaccharides isolated from the gums showed that gum arabic possesses two different immunodeterminants with the structure α-L-arabinofuranosyl-(1→4)-D-glucuronic acid and β-D-glucuronosyl-(1→6)-D-galactose while gum mesquite possessed only one determinant with the structure 4-methyl-β-D-glucuronosyl-(1→6)-D-galactose.

*Present address: Kelco Division of Morck & Co. Inc., San Diego, California 92123, USA. Research Fellow of the Corn Refiners' Association, Washington DC 20036, USA.
‡To whom correspondence should be addressed.

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INTRODUCTION

The main structural features of gum arabic and gum mesquite have been determined and these features are described in detail in comprehensive review articles (Smith & Montgomery, 1959; Aspinall, 1970; Glicksman, 1983). In brief: gum arabic is composed of L-rhamnose, L-arabinose, D-galactose, and D-glucuronic acid; gum mesquite is composed of L-arabinose, D-galactose, and 4-methyl-D-glucuronic acid; and these residues are linked by (1→3), (1→4) or (1→6) glycosidic bonds. The proportion of the monomers varies in different preparations of the gums (Anderson *et al.*, 1968). The configuration of the glycosidic linkages is generally β but recent NMR studies present evidence verifying that α-linkages also occur (Defaye & Wong, 1986). The ring form of the monosaccharide units is mostly pyranose but some of the arabinose units may occur in the furanose form (Aspinall, 1970).

The immunogenic properties of the gums have not yet been completely elucidated. Early studies (Heidelberger & Adams, 1956) showed that gum arabic reacted strongly with antibodies directed against the Type II pneumococcal polysaccharides which possess terminal D-glucuronic acid units as immunodeterminants. More recent reports on the immunogenicity of gum arabic have been contradictory. For example, some investigators claim that the precipitin-forming ability of gum arabic with immune serum was due to protein associated with the gum (Akiyama *et al.*, 1984; Churms & Stephen, 1984; Connolly *et al.*, 1988) while others state that the carbohydrate residues of the gums are responsible for the immunogenicity (Narita, 1985; Pazur & Kelly-Delcourt, 1985).

In the present study gum arabic or gum mesquite in combination with Freund's complete adjuvant have been shown to be immunogenic and induce the synthesis of anti-carbohydrate antibodies. The type of immunocyte stimulation, and the chemical nature and physical properties of the antibodies have been investigated. The antibodies induced by each gum were purified by affinity chromatography on adsorbents bearing gum ligands by methods previously described for the purification of other anti-carbohydrate antibodies (Pazur, 1981). By using a combination of analytical methods, it was found that two sets of antibodies with different carbohydrate specificity were present in the anti-gum arabic serum, one directed at terminal α-L-Ara*f*-(1→4)-D-GlcUA moieties and the other directed at terminal β-D-GlcUA-(1→6)-D-Gal moieties. Gum mesquite induced the synthesis of only one set of antibodies directed at a disaccharide unit with the structure 4-Me-β-D-GlcUA-(1→6)-D-Gal at the terminal non-reducing ends of the gum chains. Since the antibodies

of a set combined with the same type of carbohydrate moiety and since all members of a set were induced by the same carbohydrate determinant group of the antigen, the antibodies are appropriately called anti-carbohydrate isoantibodies. The individual members of a set of isoantibodies are most likely synthesized by different immunocytes stimulated by the same determinant group of the antigen. The biosynthesis of such sets of isomeric antibodies can be studied with polyclonal antibodies because of their diverse structure but not with monoclonal antibodies which are of uniform molecular structure.

EXPERIMENTAL

Materials

Gum arabic, gum karaya, gum locust bean, and gum ghatti were purchased from Sigma Chemical Co. (St. Louis, MO, USA). The proteases used in the study (trypsin, chymotrypsin and Pronase) were also purchased from Sigma. Purified samples of gums from *Acacia karroo* and *Acacia hebaclada* were provided by Dr S. C. Churms of the C.S.I.R. Carbohydrate Research Unit, University of Cape Town, South Africa. Gum mesquite was provided by Dr R. L. Whistler of the Department of Biochemistry, Purdue University, West Lafayette, IN, USA. Lemon gum was obtained in Cyprus and provided by George Papadopoulos of Hammonton, NJ. Cherry and plum gums were obtained from locally grown trees. The latter gums were purified by dissolving the crude gums in distilled water, separating the insoluble impurities by centrifugation and removing the soluble low-molecular-weight impurities by dialysis. Following dialysis, the solutions of the gums were taken to dryness by lyophilization and the resulting residues were used for subsequent experiments.

Immunological Methods

A concentrated solution of gum arabic was prepared for use in immunizations by dissolving 320 mg of the gum in 2 ml sterile phosphate buffer (0·02 M phosphate, pH 7·2) in saline. This solution was then mixed with an equal volume of Freund's complete adjuvant and used to immunize several different rabbits as follows. Samples of 0·4 ml of the gum-adjuvant suspension were injected intramuscularly in the hind leg of each rabbit and the injections were repeated weekly in alternate legs for 6 weeks. The animals were then allowed to rest for 2 weeks and the injec-

tion schedule was repeated. Several cycles of immunization were used to obtain sera of high titer. Blood samples were collected in the second and subsequent cycles and antisera were prepared from the samples by standard methods. Other rabbits were immunized with a solution of gum mesquite and Freund's complete adjuvant and immune sera were obtained from these animals by methods similar to those above.

Affinity chromatography

Affinity adsorbents of AH-Sepharose 4B with ligands of gum arabic or gum mesquite were prepared for use in the purification of the antibodies. Three grams of AH-Sepharose 4B, 100 mg of the appropriate gum sample, and 250 mg of 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide metho-*p*-toluene sulfonate (CMC) from Aldrich Chemical (Milwaukee, WI) were used for the synthesis of the adsorbents by the procedure described in a recent bulletin (Pharmacia, 1986). At the end of the reaction sequence, the excess reagent and unreacted gums were removed by filtration and the gels coupled to the ligands were washed sequentially with 500 ml of 0·5 M NaCl of pH 4·5 and 8·5 followed by 200 ml of distilled water. Finally, the adsorbents were transferred to glass columns (1 × 20 cm) and equlibrated with 200 ml saline and phosphate buffer of pH 7·2. The affinity adsorbents were then used to isolate the antibodies as described below.

The affinity chromatography was performed as follows. Samples of the immune serum were applied to the appropriate adsorbent and, after the adsorption of the sample, the column was washed with 0·02 M phosphate buffer pH 7·2 containing saline until the unadsorbed proteins were removed. The antibodies were then eluted with 1 M ammonium thiocyanate and the eluate was monitored with a UV analyser. The components which eluted as a single peak were collected and mixed with an equal volume of saturated ammonium sulfate. The precipitate which formed on refrigeration overnight was then collected by centrifugation and redissolved in a small volume of phosphate buffer. Agar diffusion tests to detect antibodies were performed on these solutions.

Immunodiffusion assays

A standard agar diffusion method was used for detecting precipitin formation and antibody-antigen reactions. Immune serum (10 μl) purified antibodies (10 μl) containing 10–20 μg antibody protein were placed in the center well of an agar diffusion plate. Antigen or other polysaccharide solution (10 μl) containing 10–50 μg compound were

placed in the outer wells of the agar plate. The diffusion plates were placed in a petri dish on moist filter paper, covered, and maintained in a moist atmosphere at room temperature for periods of 6–30 h. The plates were checked periodically for precipitin formation and the intensities of the precipitin bands which formed were noted. The plates were also photographed for a permanent record of the results. The results by agar diffusion were in agreement with results obtained by a blot method which has been published earlier (Pazur *et al.*, 1986). To check for cross-reactivity of the anti-gum arabic antibodies with other gums which are structurally related to gum arabic, agar diffusion tests were also performed with the antibodies and gum mesquite, gum *A. karroo*, gum *A. hebeclada*, gum ghatti, gum karaya, gum lemon, gum cherry, gum plum, and gum locust bean.

A micro method combining inhibition and agar diffusion has been developed in this laboratory for detecting potential inhibitors of the antibody–antigen reactions (Pazur & Kelly, 1984). This method was used to test a number of carbohydrates and oligosaccharides as potential inhibitors of the precipitin reaction. The carbohydrates included the monosaccharides which are constituents of the gums and oligosaccharides obtained by acid or enzymatic hydrolysis of the gums. Chemically and enzymatically modified gums were also tested with the antibodies for precipitin formation. In the coupled inhibition-agar diffusion tests, samples of 0·1–5 mg of the potential inhibitor were dissolved in 20 μl of the appropriate antibody solution or antiserum and incubated for 2 h. At the end of this time, a 10-μl sample of the incubation mixture was placed in the center well of a diffusion plate. Antigen solutions (10 μl) containing decreasing amounts of the antigen (2%, 1%, 0·5%, 0·25%, 0·125% to 0·0625%) were placed in the outer wells of the agar plate. Diffusion was allowed to proceed for 24 h. The extent of inhibition was determined by comparing the intensity of the precipitin bands at the different concentrations of antigen on the inhibition plates with the intensity of bands on a plate with control antibody solution.

Chemical modification of the gums

Chemical modification of the gums was achieved by oxidation of the monosaccharide residues with periodate and reduction of the carboxyl groups of the uronic acid residues by the carbodiimide–borohydride method. Modification was also effected by acid hydrolysis of the glycosidic linkages of the gums to yield oligosaccharide fragments. The periodate oxidation and subsequent analysis was performed on 0·2 g

samples of the gums by a procedure described in the literature (Pazur & Forsberg, 1978). The reduction of the uronic acid residues of the gums to glucose and 4-methyl-glucose was performed by the carbodiimide (CMC) and sodium borohydride method (Taylor & Conrad, 1972) using samples of 0·25 g of the gums. Analysis of reduced gum arabic by the carbazole method (Dische, 1947) showed that approximately 70% of the glucuronic acid units of gum arabic were reduced in a single reduction. The reduction was repeated twice more and analysis of the products showed that 85% and 97% of the carboxyl groups of glucuronic acid units had been reduced. The reduction of gum mesquite was also carried out three times and analysis showed that 74%, 86% and 96% of the 4-methyl-glucuronic acid moieties of the gum mesquite were reduced in the three reductions.

Acid hydrolysis of the gums was effected at several concentrations of mineral acid and for different time periods. One hydrolysis was conducted in dilute acid (0·01 N HCl) for short periods (10 min). Aliquots of the hydrolysate were removed at various intervals, neutralized with barium carbonate, and analysed for reducing sugars by paper chromatography in the solvent system of *n*-butyl alcohol:pyridine:water (6:4:3 by volume). A portion of each hydrolysate was also used in agar diffusion tests to determine the effects of acid hydrolysis on the antigenicity of the gums. Other samples of the gums were then subjected to hydrolysis in more concentrated acid for longer periods (1·0 N HCl for 2 h). Aliquots of the hydrolysate were analysed by paper chromatography. The hydrolysates in 1·0 N HCl contained the constituent monosaccharides of the gum and several oligosaccharide fragments. The oligosaccharides were isolated from the hydrolysates of both gums by a preparative paper chromatography method (Pazur *et al.*, 1978).

An enzymatic hydrolysis of gum arabic was performed with a fungal extract containing a mixture of enzymes in order to obtain additional oligosaccharide fragments. The enzyme extract was obtained from a fungus collected from decaying maple leaves. The fungus was propagated on sterile nutrient agar at 37°C until sporulation. The spores were then aseptically transferred to fresh media and allowed to germinate. This procedure was repeated until a homogeneous culture was obtained. A 2% solution of gum arabic was added to spores which had been freshly harvested. This mixture was incubated at 28°C for 14 days. The resulting solution was clarified by centrifugation and filtered through sterile glass wool, glass fiber filter and an ultrafilter. The clear filtrate was analysed by paper chromatography and two major arabinose-containing oligosaccharides with R*f* values of 0·15 and 0·10 were present in the enzymatic hydrolysate. These oligosaccharides were isolated by preparative multi-

ple ascent paper chromatography (Pazur *et al.*, 1978). The solutions of the oligosaccharides were taken to dryness by lyophilization. Since preliminary experiments indicated that the oligosaccharide with the R*f* of 0·10 exhibited inhibitory activity, this oligosaccharide was used in the inhibition experiments.

Hydrolytic and methylation analysis

In order to characterize the oligosaccharides derived from acid and enzyme hydrolysates of the gums, the oligosaccharides were further hydrolysed in 0·5 N HCl. The hydrolysates were analysed for monosaccharide composition by paper chromatography to determine constituent monosaccharide units. Other samples of oligosaccharides were reduced by a CMC and borohydride method described earlier (Taylor & Conrad, 1972) which effected a minimal reduction of the reducing groups of the oligosaccharides. The oligosaccharides with the reduced carboxyl groups were then hydrolysed in acid (0·5 N HCl) and the hydrolytic products were separated and identified by R*f* values by paper chromatography. Also, samples of the reduced oligosaccharides and samples of the native gums were subjected to methylation and to GLC–MS analyses.

Methylation of all the compounds was effected by a well-known method (Hakamori, 1964) and analysis of the resulting derivatives was performed by GLC and MS (Bjorndal *et al.*, 1970). The details of the procedure used in this laboratory have been described (Pazur *et al.*, 1976). The partially methylated and acetylated derivatives were separated by GLC at 195°C on a 3% OV-225 80-100 mesh Supelcoport column, detected by flame ionization and subjected to mass fragmentation in a DuPont 21-490 mass spectrometer attached to the gas chromatograph.

Other analytical method

In order to determine isoelectric points of the antibodies, gel isoelectric focusing was performed. In the method a 10% polyacrylamide gel and ampholine–sucrose solution of pH gradient 5–8 was used following a procedure described in the literature (Doerr & Chrambach, 1971). A coupled isoelectric focusing and agar diffusion method developed in this laboratory (Pazur *et al.*, 1987) was also used to detect antibody activity of the components in the purified antibody preparations. In this procedure, duplicate samples of antibody preparations were subjected to identical conditions of gel isoelectric focusing. One finished gel was

stained for protein with Coomassie blue, and the other gel was embedded in liquid agarose. After the agarose had solidified around the polyacrylamide gel, diffusion was allowed to proceed for a period of 12–24 h in a closed, moist chamber at room temperature. Then a trough was cut in the agar about 2 cm from the gel and a 1% solution of the antigen (gum arabic or gum mesquite) was introduced into the trough. Diffusion of antigen and antibody was allowed to proceed for an additional 24–48 h. The plates which developed precipitin arcs were photographed.

The susceptibility of the gum arabic to digestion with proteases with concurrent loss of antigenicity was examined. A 0·1 ml sample of a 10% solution of gum adjusted to pH 7·6 was mixed with 0·1 ml of a 2% solution of trypsin, chymotrypsin or Pronase and the mixtures were incubated at room temperature for 24 h. Samples of each digest and of untreated gum arabic were used in agar diffusion tests with anti-gum arabic serum to determine the effect of protease digestion on the antibody activity.

The immunoglobulin class of the antibodies was determined by the double immunodiffusion method. A 10-μl sample containing purified anti-gum-arabic antibodies or anti-gum-mesquite antibodies was placed in the center well of an agar plate, and goat-anti-rabbit IgA, goat-anti-rabbit IgG, and goat-anti-rabbit IgM antisera (Cappel Labs, Organon Corp., West Chester, PA) were placed in separate outer wells. Diffusion of the samples was allowed to proceed in a moist chamber at room temperature for 24 h, after which time the type of the anti-serum reacting with the gum antibodies was noted.

A density-gradient centrifugation procedure developed in this laboratory (Pazur *et al.*, 1962) was utilized to determine sedimentation rates of the purified antibody preparations. The antibodies and reference glycoprotein (glucose oxidase and glucoamylase) were centrifuged and analysed in an identical fashion. The data were used to check for molecular homogeneity of the antibody preparations and to calculate molecular weights using the empirical formula (Martin & Ames, 1961). The molecular weights of both anti-gum-arabic and anti-gum-mesquite antibodies were $1{\cdot}5 \times 10^5$ in the range of $1{\cdot}48 \times 10^5$ to $1{\cdot}55 \times 10^5$.

RESULTS AND DISCUSSION

The precipitin bands on agar diffusion of gum arabic or gum mesquite with the corresponding immune sera and bands for the cross-reactivity

of the gums and antibodies are shown in Fig. 1. It will be noted in the figure that there are two precipitin bands formed by the reaction of immune serum (well S_1) with gum arabic (well A) showing that two different populations of antibodies occur in this immune serum. For purposes of differentiating the populations of gum arabic antibodies, the set of antibodies responsible for the band closest to the serum well (S_1) has been designated as Set 1 and the other set has been designated as Set 2. Only one set of antibodies was present in the serum (well S_2) reacting with gum mesquite (well M). Some cross-reactions of the antibodies in the sera and the antigens did occur. Thus the anti-gum arabic antibodies of Set 1 reacted with gum mesquite (wells S_1 and M) while the anti-gum-mesquite antibodies reacted with gum arabic (wells S_2 and A). However, the anti-gum arabic antibodies of Set 2 did not cross-react with gum mesquite.

The mixture of anti-gum arabic antibodies in the immune serum cross-reacted with several different gums including gum mesquite, gum *A. karroo*, gum *A. hebeclada*, gum ghatti, and gum lemon. These gums apparently contain terminal oligosaccharide chains of structures similar to those in gum arabic. The participation of protein associated with the gums in the antigen–antibody complex formation is not likely for the following reasons. Karroo gum of very low protein content (less than 2%) and hebeclada gum of very high protein content (over 50%) reacted at comparable rates with the antibodies under comparable conditions. Also, treatment of gum arabic with trypsin, chymotrypsin or Pronase did not modify the ability of the gum to form a precipitin complex with the anti-gum-arabic antibodies.

The determinant of gum arabic inducing the synthesis of Set 2 antibodies is shown to be easily destroyed by mild acid hydrolysis in 0·01 N HCl for 10 min. This result is shown in the top agar diffusion plate of Fig. 2 (wells S_1 and 1). The mildly hydrolysed gum arabic in well 1 no longer forms a precipitin complex with Set 2 antibodies (well S_1) but does form a precipitin complex with Set 1 antibodies. Some of the

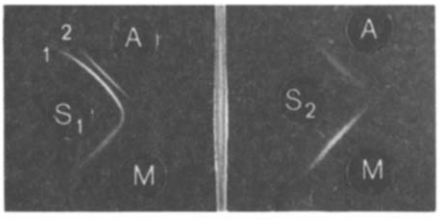


Fig. 1. Agar diffusion patterns for anti-gum arabic serum (S_1) and anti-gum-mesquite serum (S_2) against gum arabic (A) and gum mesquite (M); 1 = Set 1 antibodies, 2 = Set 2 antibodies.

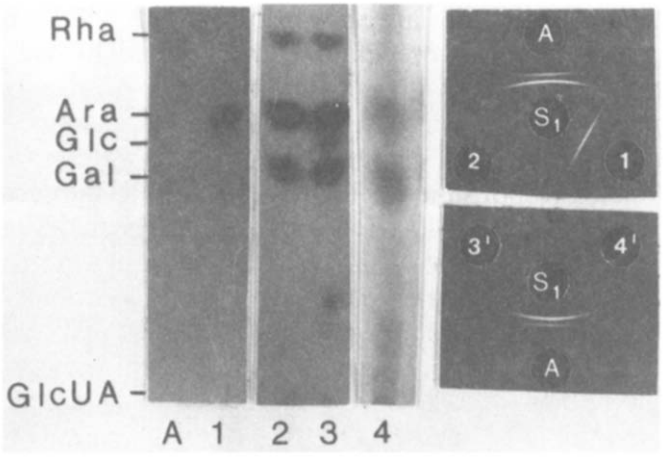


Fig. 2. Paper chromatograms of hydrolysates of native and modified gum arabic and agar diffusion patterns of native and modified gums. A = native gum arabic, 1 = gum hydrolysed in 0·01 N HCl, 2 = gum hydrolysed in 1 N HCl, 3 = gum reduced and hydrolysed in 1 N HCl, 4 = periodate oxidized and hydrolysed gum, 3′ = reduced gum, 4′ = periodate oxidized gum, and S_1 = anti-gum arabic serum.

arabinose units in the native gum are in the furanose ring form (Aspinall, 1970) and such units are readily removed by dilute acid hydrolysis. Paper chromatographic analysis of the native and the dilute acid-hydrolysed gum is also shown in Fig. 2 (lanes A and 1), establishing that arabinose was indeed liberated by mild acid hydrolysis of gum arabic. Therefore, the arabinofuranosyl units are an essential part of the determinant group for antibodies of Set 2.

In order to destroy the determinant groups of gum arabic which combine with the anti-gum arabic antibodies of Set 1, more drastic acid hydrolysis (1·0 N HCl for 2 h) was used. Analysis for monosaccharides in such a hydrolysate (Fig. 2, lane 2) showed that rhamnose, arabinose, galactose, and glucuronic acid were liberated under these conditions. This hydrolysate of gum arabic no longer reacted with Set 1 antibodies nor Set 2 antibodies as shown in Fig. 2, top agar diffusion plate (wells S_1 and 2). The gum mesquite immunodeterminant group was also resistant to dilute acid hydrolysis but was hydrolysed by more concentrated acid with concurrent loss of antigenicity.

Reduction of the carboxyl groups of the gums by a water-soluble carbodiimide and sodium borohydride method causes a loss of reactivity with the antibodies as shown in the lower agar diffusion plate of Fig. 2 (wells S_1 and 3′). This agar diffusion test was performed with the reduced

gum before acid hydrolysis of the sample. In the hydrolysate of the reduced gum, glucose appeared as well as arabinose, rhamnose and galactose while the glucuronic acid disappeared (Fig. 2, lane 3). It should be noted that some oligosaccharide fragments also appeared in the hydrolysate. A similar analysis of reduced gum mesquite showed the appearance of 4-methyl-glucose and the disappearance of 4-methyl-glucuronic acid. The reduced but not hydrolysed gum mesquite did not yield a precipitin band with the anti-gum mesquite antibodies on agar diffusion.

The presence of glucose or 4-methyl-glucose in the hydrolysates of the reduced gums was verified by GLC analysis. A portion of the GLC methylation analysis chart for native and reduced gum arabic is reproduced in Fig. 3. In this portion of the chart the major partially methylated alditol acetates obtained from the hydrolysate of the reduced gum arabic (pattern B) were: 1,3,4-triacetyl-2,5-dimethyl-arabinitol; 1,5-diacetyl-2,3,4,6-tetramethyl-glucitol; 1,5-diacetyl-2,3,4,6-tetramethyl-galactitol; and 1,4,5-triacetyl-2,3,6-trimethyl-glucitol. The two glucosyl derivatives were not present in the analytical patterns of the native gum arabic (pattern A). The trimethyl glucose derivative comes from the internal and reduced glucuronic acid residues of the gum and the tetra-methyl glucose derivative comes from the reduced terminal glucuronic acid residues. Methylations of native gum mesquite and reduced gum mesquite showed that 1,5-diacetyl-2,3,4,6-tetramethyl-glucitol was detected in the hydrolysate of the reduced gum but not the native gum.

Periodate oxidation of the gums also destroyed antigenicity of both gums as evidenced by the inability of the oxidized gums to form precipitin complexes with the purified antibodies. Agar diffusion results with the periodate oxidized but not hydrolysed gum arabic are shown in wells S_1 and 4′ of Fig. 2. Hydrolytic products from the oxidized gum are shown by the chromatogram in lane 4 of Fig. 2. Precipitin bands were not obtained with the oxidized gum (well 4′) but the native gum (well A) yielded two precipitin bands with the antibodies (well S_1). Since the internal moieties of both gum arabic and gum mesquite consist of β-(1→3)-galactosyl residues, such units would not be oxidized by periodate but residues of the terminal chains in which the units are linked by (1→4)- or (1→6)-linkages would be oxidized. The antigenicity of the gums was destroyed by periodate oxidation and therefore the carbohydrate units in the peripheral chains participate in the antigen–antibody reaction.

The anti-gum-arabic and anti-gum-mesquite antibodies were isolated in pure form by affinity chromatography on adsorbents with ligands of native gum arabic or gum mesquite. These adsorbents were synthesized by the carbodiimide method from AH Sepharose and the native gums

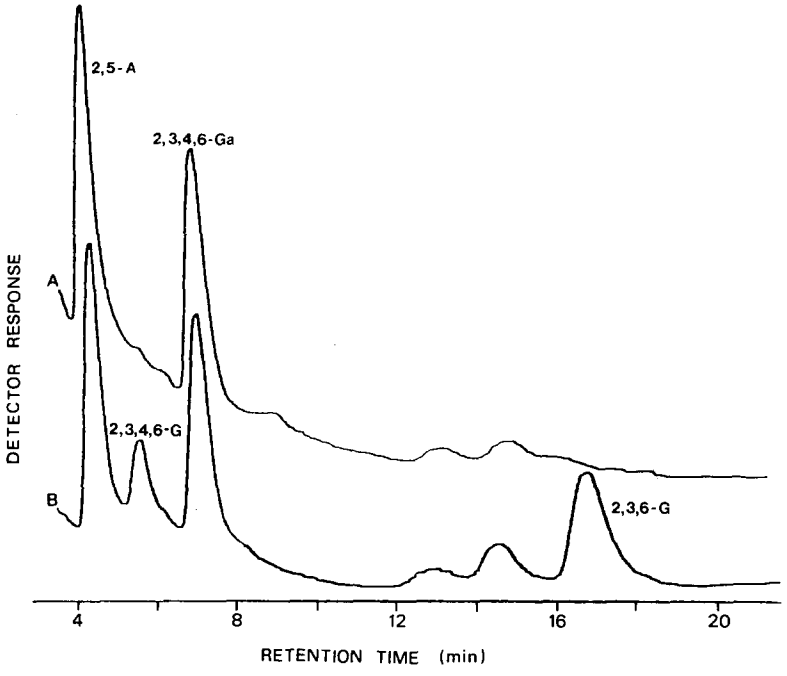


Fig. 3. Comparable portion of the GLC patterns for partially methylated and acetylated derivatives from native (A) and reduced (B) gum arabic. 2,5-A = 1,3,4-triacetyl-2,5-dimethyl-arabinitol; 2,3,4,6-G = 1,5-diacetyl-2,3,4,6-tetramethyl-glucitol; 2,3,4,6-Ga = 1,5-diacetyl-2,3,4,6-tetramethyl-galactitol; 2,3,6-G = 1,4,5-triacetyl-2,3,6-trimethyl-glucitol.

(Pharmacia, 1986). The results of the affinity chromatography experiments are shown in Fig. 4. The upper graph shows the pattern for the chromatography of the anti-gum-arabic antibodies and the lower graph shows the anti-gum-mesquite antibodies. The antibodies were eluted from the respective adsorbents with ammonium thiocyanate. The point of application of the eluting solution to the column is indicated by the arrows. The very symmetrical nature of antibody peaks indicates a homogeneity in molecular types in the antibody preparations.

To verify further that the carbohydrate moiety reacted with the antibodies, several oligosaccharides containing uronic acids were obtained from enzyme and acid hydrolysates of the gums and tested in inhibition experiments. One oligosaccharide was isolated from an enzyme hydrolysate of gum arabic as described in the Experimental section. On acid hydrolysis and paper chromatographic analysis this compound was found to be composed of arabinose and glucuronic acid. The carboxyl

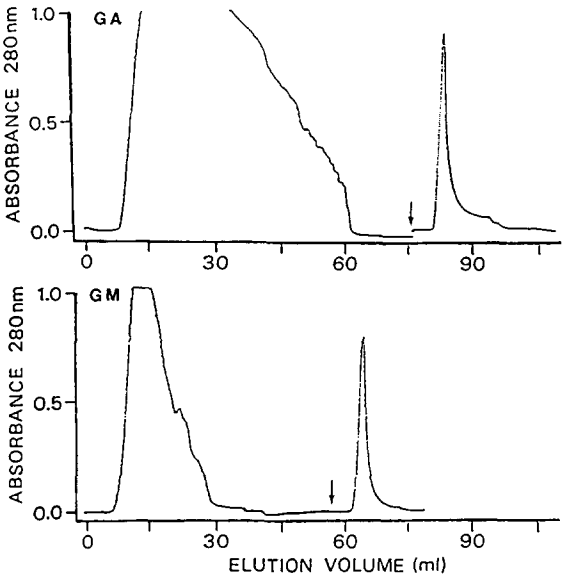


Fig. 4. Affinity chromatography patterns of anti-gum-arabic antibodies (GA) and anti-gum-mesquite antibodies (GM). Arrows indicate the points of application of ammonium thiocyanate.

group of the oligosaccharide was reduced by the carbodiimide and borohydride method and the product was subjected to methylation analysis. The GLC pattern (Frame A) is shown in Fig. 5. The methylation results of Fig. 5 and earlier data (Aspinall, 1970) establish the structure of the oligosaccharide to be α-L-Ara-(1→4)-D-GlcUA. An oligosaccharide was also isolated from the acid hydrolysates of gum arabic. When further hydrolysed, this compound yielded galactose and glucuronic acid. An oligosaccharide from gum mesquite was isolated by similar methods and on hydrolysis this compound yielded galactose and 4-methyl-glucuronic acid. The results of methylation analyses of these oligosaccharides are shown in the GLC patterns in Fig. 5 (Frames B and C). From these results and other available structural information on the gums, the structures of the oligosaccharides isolated from acid hydrolysates are β-D-GlcUA-(1→6)-D-Gal from gum arabic and 4-Me-β-D-GlcUA-(1→6)-D-Gal from gum mesquite.

Hapten inhibition experiments with monosaccharides and oligosaccharides which may be inhibitors of the purified antibodies were conducted by the coupled inhibition-agar diffusion method (Pazur & Kelly, 1984). The inhibition experiments using the constituent monosaccharides showed that single monosaccharides gave no inhibition of

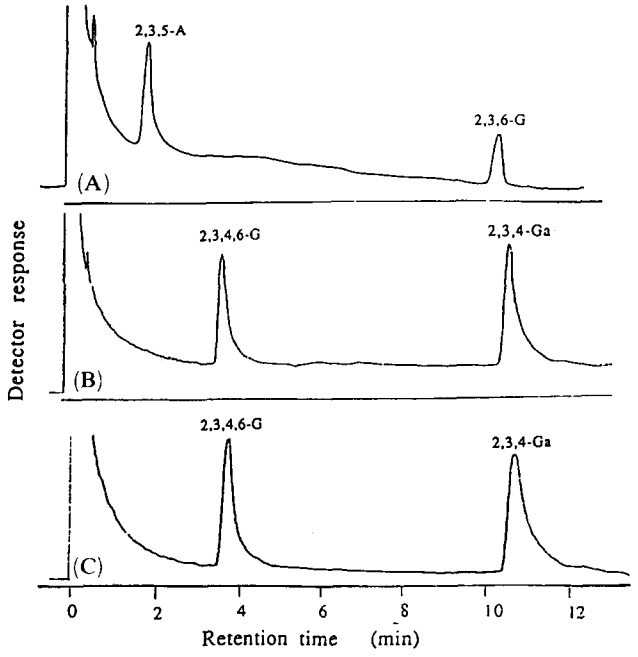


Fig. 5. GLC patterns for partially methylated and acetylated products of the oligosaccharides from enzyme hydrolysed gum arabic (A), from acid hydrolysed gum arabic (B), and acid hydrolysed gum mesquite (C). 2,3,5-A = 1,4-diacetyl-2,3,5-trimethyl-arabinitol; 2,3,6-G = 1,4,5-triacetyl-2,3,6-trimethyl-glucitol; 2,3,4,6-G = 1,5-diacetyl-2,3,4,6-tetramethyl-glucitol; 2,3,4-Ga = 1,5,6-triacetyl-2,3,4-trimethyl-galactitol.

precipitin formation between the antibodies and the gums. Inhibition tests using some of the oligosaccharides prepared from gum arabic and gum mesquite are shown in Fig. 6. The three oligosaccharides show inhibition of the precipitin complex formation. Thus Fig. 6, frame A, shows the inhibition of the reaction of anti-gum-arabic serum with native gum arabic by the enzyme-produced oligosaccharide. Frame B of Fig. 6 shows inhibition of the acid-produced oligosaccharide on the reaction of the anti-gum-arabic antibodies with the dilute acid hydrolysed-gum arabic. Frame C shows the inhibition by the oligosaccharide from gum mesquite of the reaction of anti-gum-mesquite antibodies with gum mesquite as the antigen. The structure for the inhibitor used for the experiments in frame A was α-L-Ara*f*-(1 → 4)-D-GlcUA, in frame B, β-D-GlcUA-(1 → 6)-D-Gal and in frame C, 4-Me-β-D-GluUA-(1 → 6)-D-Gal. In frame A it will be noted that intensities of the precipitin bands with Set 2 antibodies were reduced and that no bands were formed with the antigen in wells 3 and 4 by the arabinosyl-glucuronic acid in comparison to the

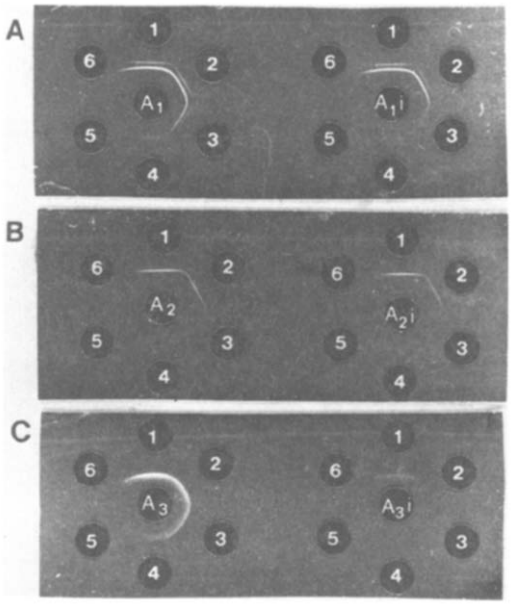


Fig. 6. Agar diffusion patterns showing the inhibition of gum arabic antibodies and gum mesquite antibodies by oligosaccharides prepared from the gums. A_1 = anti-gum-arabic antibodies; A_1i = anti-gum-arabic antibodies + Ara-(1→4)GlcUA; A_2 = anti-gum-arabic antibodies; A_2i = anti-gum-arabic antibodies + GlcUA-(1→6)-Gal; A_3 = anti-gum-mesquite antibodies; A_3i = anti-gum-mesquite antibodies + 4-Me-GlcUA-(1→6)-Gal; numbers 1 to 6 = decreasing amounts of antigens of native gum arabic (A), dilute acid hydrolysed gum arabic (B), and gum mesquite (C).

control which did not contain inhibitor (well A_1). In frame B it is noted that the antibodies of Set 1 incubated with the inhibitor (well A_2i) yielded bands of lower intensity in comparison to the bands for the antibodies with no inhibitor (well A_2). In frame C (patterns A_3 and A_3i) strong inhibition was noted with the oligosaccharide, 4-Me-β-D-GlcUA-(1→6)-D-Gal, on the precipitin reaction of anti-gum-mesquite antibodies and gum mesquite.

Isoelectric focusing experiments with the purified anti-gum-arabic antibodies and the anti-gum-mesquite antibodies showed that both antibody preparations were composed of isomeric proteins (Fig. 7). Gels A in the figure have been stained with a protein stain and these show that a group of protein components with different isoelectric points are present in the gum-arabic and gum-mesquite antibody preparations. The agar diffusion portion of Fig. 7 (B, C and D) shows that all the protein components exhibited antibody activity. Such sets of antibodies have

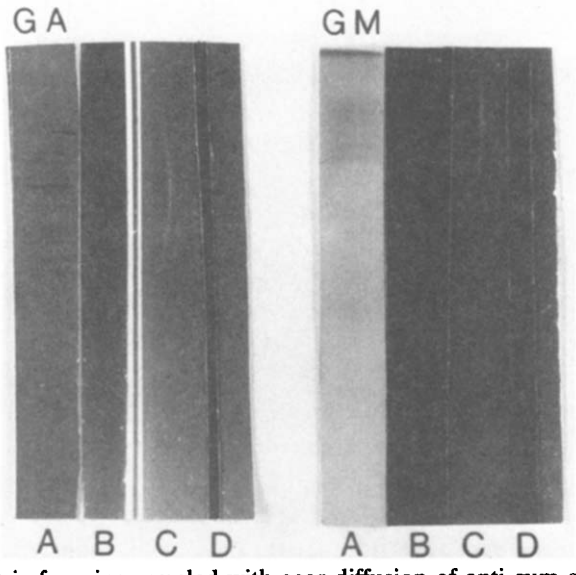
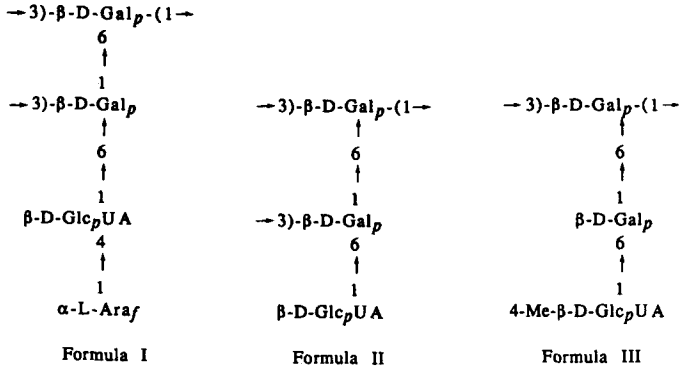


Fig. 7. Isoelectric focusing coupled with agar diffusion of anti-gum-arabic antibodies (GA) and anti-gum-mesquite antibodies (GM); Gel A = stained gels; Gel B = unstained gels embedded in agar; C = area of precipitin complex formation; D = area with antigen solution of gum arabic or gum mesquite.

been termed isoantibodies. Gum mesquite induces the synthesis of six isoantibodies while gum arabic induces the synthesis of 13 isoantibodies. The precipitin results with the latter antibodies confirm that two different sets of isoantibodies were induced by the gum arabic.

Gum arabic and gum mesquite are carbohydrate immunogens and in combination with Freund's adjuvant stimulate immunocytes of a host to produce sets of isomeric anti-carbohydrate antibodies. With gum arabic two sets of antibodies were obtained showing that this immunogen possesses two immunodeterminants for which the structures have been tentatively determined to be α-Ara-(1,4)-GlcUA and β-GlcUA-(1,6)-Gal. These determinant groups stimulate two different groups of immunocytes to produce the different antibodies. Both immunodeterminant groups are located at the non-reducing ends of the branched gum arabic molecule and the structure is shown in Formula I and Formula II. Gum mesquite possesses only one immunodeterminant group and only one set of antibodies was produced on immunization of the host with this immunogen. The immunodeterminant group is also a dissaccharide unit with the structure shown in Formula III.

It is pointed out again that the anti-gum antibodies which are induced in a host by the two different gums occur in sets of isomeric forms as shown by the data from isoelectric focusing and agar diffusion



experiments. Each member of a set is probably synthesized by a different immunocyte stimulated by the same determinant of the antigen. The antibodies are of the IgG type as shown by reaction with goat-anti-IgG serum; they possess molecular weights in the range $1{\cdot}48 \times 10^5$–$1{\cdot}55 \times 10^5$ and do cross-react with other gums with similar determinants. The cross-reactivity of Set I of the anti-gum-arabic antibodies with gum mesquite and of the anti-gum-mesquite antibodies with gum arabic is probably due to the presence of the β-D-glucuronosyl-(1→6)-D-galactose or 4-Me-β-D-glucuronosyl-(1→6)-D-galactose units at the non-reducing ends of gum arabic and gum mesquite, respectively. Although glucuronic acid in gum mesquite is methylated at position 4, cross-reactivity does occur indicating that the substitution at position 4 does not interfere with precipitin complex formation.

These unique sets of anti-carbohydrate isoantibodies will be used to investigate biosynthetic pathways of immunocytes responsible for the production of anti-carbohydrate isoantibodies, to determine the structural differences in sets of isomeric antibodies, and to detect the gums which are incorporated in foods and pharmaceutical products.

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