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ISOMERIC, ANTI-RHAMNOSE ANTIBODIES HAVING SPECIFICITY FOR RHAMNOSE-CONTAINING, STREPTOCOCCAL HETEROGLYCANS

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ABSTRACT

L-Rhamnose (6-deoxy-L-mannose) is a constituent carbohydrate unit of microbial, immunogenic heteroglycans and lipopolysaccharides, and often functions as the immunodeterminant group of such immunogens. Two types of anti-rhamnose antibody have now been isolated by affinity chromatography of immune sera obtained from rabbits immunized with vaccines of *Streptococcus mutans*, strain KI-R, and *Streptococcus pneumoniae*, type 32. The antibodies of one type were directed at a glycan of L-rhamnose, D-glucose, and D-galactose in the cell wall of *S. mutans*, and those of the other type, against a capsular glycan of L-rhamnose and D-glucose from *S. pneumoniae*. The two types of anti-rhamnose antibody were immunologically distinct, and showed no reciprocal cross-reactivity. Additional properties of the two types of antibody were determined; thus, both types of antibody were of the IgG class of immunoglobulins, both possessed molecular weights of 1.45×10^5, and both consisted of multiple or isomeric forms.

INTRODUCTION

Immunogenic heteroglycans in which L-rhamnose is a structural element are present in the cell walls and capsules of many groups and strains of *Streptococci*[1–5]. Antisera with antibodies directed against the L-rhamnose units of these glycans have been obtained from rabbits immunized with vaccines of nonviable cells with the glycans *in situ*[4–6]. Such antisera have been used for a long time in the serological identification of infectious organisms[4,7,8], and more recently have been found to be effective against oral micro-organisms involved in dental-plaque formation[9–11]. Two types of anti-rhamnose antibody have been prepared for the first time from

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sera of rabbits immunized with a vaccine of *Streptococcus mutans,* strain KI-R, serotype d, and with a vaccine of *Streptococcus pneumoniae,* type 32. These antibodies were isolated, and purified, by affinity chromatography of the immune sera on a Sepharose adsorbent bearing L-rhamnose ligands that was synthesized from *p*-aminophenyl α-L-rhamnoside and cyanogen bromide-activated Sepharose[12,13]. Both sets of antibodies occur in isomeric forms, and the biological significance of such sets is considered in this report. In recent years, affinity-chromatography techniques have been extensively used in our laboratory for the preparation of different types of anti-carbohydrate antibodies, some of which have potential value for the detection and treatment of diseases[14].

RESULTS AND DISCUSSION

The cell walls and capsules of different strains of *Streptococci* contain immunogenic heteroglycans composed of unique combinations of monosaccharide residues arranged in characteristic sequences. A triheteroglycan of L-rhamnose, D-glucose, and D-galactose has been isolated from the cell wall of *Streptococcus mutans,* strain KI-R, serotype d, and a diheteroglycan of L-rhamnose and D-glucose, from the culture filtrates of *Streptococcus pneumoniae,* type 32. The monosaccharide constituents of the two glycans were identified by acid hydrolysis followed by paper-chromatographic analysis utilizing appropriate spray-reagents. The R_F values of the monosaccharide constituents and of reference compounds are re-

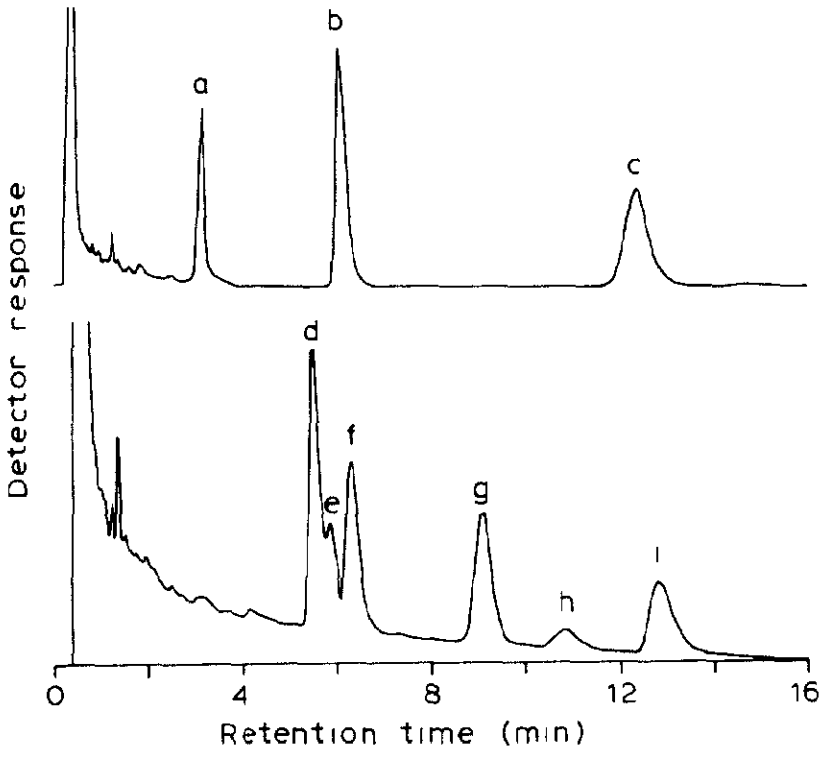

Fig. 1. G.l.c. pattern for the methylated alditol acetates from the methylated triheteroglycan from *S. mutans.* [OV-225 column, temperature 190°; a = reference 1,5 di-*O*-acetyl-2,3,4-tri-*O*-methylrhamnitol, b = reference 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylglucitol, c = reference 1,5,6-tri-*O*-acetyl-2,3,4-tri-*O*-methylglucitol, d = 1,2,5-tri-*O*-acetyl-3,4-di-*O*-methylrhamnitol, e = 1,4,5-tri-*O*-acetyl-2,3-di-*O*-methylrhamnitol, f = same as b, g = 1,2,3,5-tetra-*O*-acetyl-4-*O*-methylrhamnitol, h = 1,3,5-tri-*O*-acetyl-2,4,6-tri-*O*-methylglucitol, and i = 1,4,5-tri-*O*-acetyl-2,3,6-tri-*O*-methylgalactitol.]

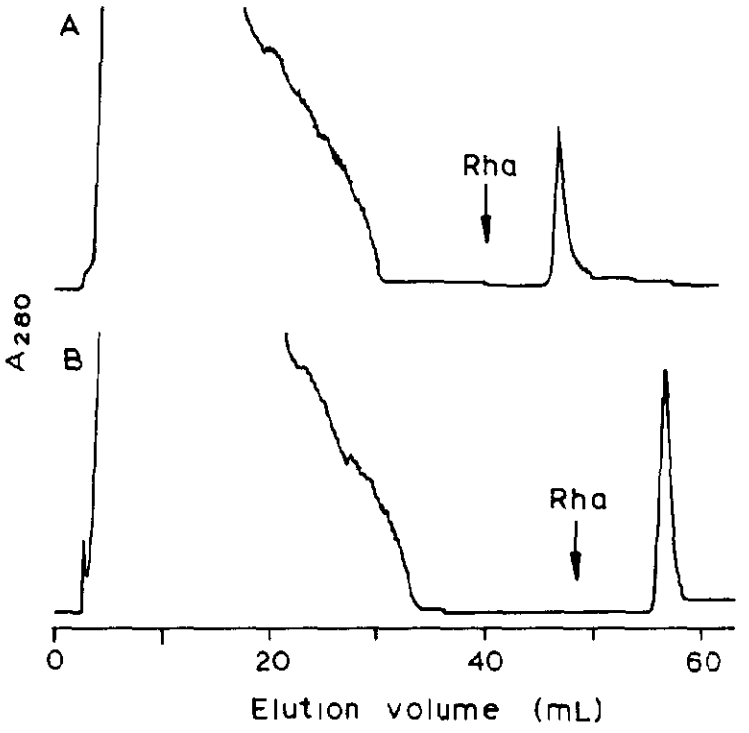

Fig. 2. Elution patterns for anti-rhamnose antibodies from (A) *S. mutans* antiserum and (B) *S. pneumoniae* antiserum from rhamnosyl-Sepharose. (The arrow indicates the point of application of L-rhamnose.)

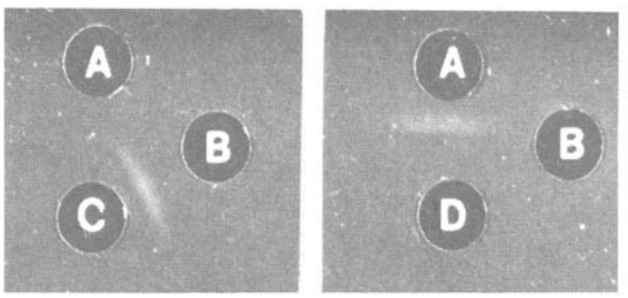

Fig. 3. Agar diffusion patterns of the purified antibodies with the rhamnose-containing glycans from the micro-organisms. [Well A contains *S. mutans* glycan, well B contains pneumococcal glycan, well C contains purified anti-pneumococcal antibodies, and well D contains anti-*S. mutans* antibodies.]

corded in the Experimental section, and these show that the *S. mutans* glycan contains L-rhamnose, D-glucose, and D-galactose, whereas the *S. pneumoniae* glycan contains L-rhamnose and D-glucose. D-Galactose and D-glucuronic acid, previously reported to be present in the pneumococcal glycan[15], were not detected in the present studies.

The *S. mutans* triheteroglycan was further analyzed by methylation, g.l.c., and mass spectrometry[16]. The g.l.c. pattern for the derivatives is reproduced in Fig. 1. It may be noted in this Figure that several L-rhamnose derivatives were produced from the glycan, but that a tri-*O*-methylrhamnose derivative was not. Thus, the L-rhamnosyl residues of the glycan are internal, not terminal, residues. The D-galactosyl units are also internal, but D-glucosyl units are both internal and terminal. The location of the L-rhamnosyl units of the *S. pneumoniae* glycan has been shown to be terminal on the basis of inhibition studies[15]. Preliminary methylation data for this glycan substantiated this observation.

Immunization of rabbits with appropriate vaccines of nonviable cells or glycans yielded antisera that contained antibodies having anti-rhamnose specificity. The anti-rhamnose antibodies were isolated by affinity chromatography on an adsorbent consisting of a rhamnosyl-Sepharose. Details of the affinity-chromatography procedure utilized in our laboratory have been described in earlier studies[17]. The elution of the antibodies from the adsorbent was effected with L-rhamnose solution, and elution patterns for the two types of immune sera are shown in Fig. 2. It should be noted in Fig. 2 that both types of sera yielded a broad peak of u.v.-absorbing material that contained nonadsorbed proteins. In each elution pattern, there was a narrow peak of u.v.-absorbing material that was eluted by L-rhamnose solution. The latter samples contained the anti-rhamnose antibodies.

In agar-diffusion tests, the preparations of anti-rhamnose antibody reacted selectively with the immunogenic glycan from the corresponding organism. These results are shown in Fig. 3, from which it may be seen that cross-reactivities between two antibody preparations and the two L-rhamnose-containing glycans did not occur.

Hapten-inhibition data with the purified antibodies and monosaccharides that are structural units of the antigens are recorded in Table I. L-Rhamnose was the only effective inhibitor of the precipitin reaction of both types of antibody. The inhibition data for the *S. mutans* antibodies and the immune serum were obtained by a standard method, and, for the pneumococcal antibodies, by an agar-diffusion method. It should be noted that the precipitin reaction of the *S. mutans* immune serum and the glycan was inhibited by L-rhamnose, D-galactose, and D-glucose, but the precipitin reaction of the purified antibodies and the glycan was inhibited only by L-rhamnose. The purified antibodies are, therefore, specific for the L-rhamnose units of the glycan, whereas the immune serum also contained antibodies that combine with D-glucosyl and D-galactosyl residues. Antibodies of the latter types had earlier been detected in sera produced with this and other strains of *S. mutans*[18], but anti-rhamnose antibodies have not previously been obtained in purified forms.

The molecular weights of the anti-rhamnose antibodies were estimated from sedimentation rates in density-gradient centrifugation[19]. Results of an ultracen-

TABLE I

PERCENT INHIBITION BY MONOSACCHARIDES OF THE PRECIPITIN REACTION BETWEEN ANTI-*S. mutans* IMMUNE SERUM AND ANTI-Rha ANTIBODIES WITH THE ANTIGENS

Compound	*Anti-S mutans serum*	*Anti-Rha antibodies*	
		S. mutans	S. pneumoniae
L-Rhamnose	65	55	50
D-Galactose	43	8	0
D-Glucose	24	3	0
D-Mannose	4	0	0

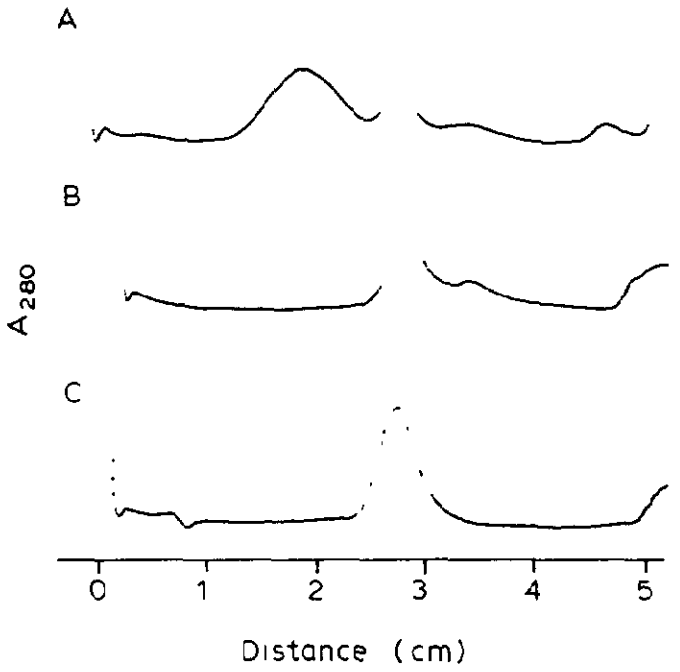

Fig. 4. Density-gradient centrifugation-pattern for (A) the immune serum and (B) the purified anti-rhamnose antibodies against *S. pneumoniae* and (C) against *S. mutans*.

Fig. 5. Isoelectrofocusing patterns for (A) the *S. mutans* anti-rhamnose antibodies and (B) the *S. pneumoniae* anti-rhamnose antibodies. (The arrows indicate the isomeric antibody components in the purified antibody preparation.)

trifugation experiment with *S. mutans* immune serum and the purified antibodies are shown in Fig. 4. The top chart shows the ultracentrifugation pattern of anti-*S. mutans* immune serum, the middle one shows the *S. pneumoniae* anti-rhamnose antibodies, and the bottom, the *S. mutans* anti-rhamnose antibodies. It may be noted that the *S.mutans* anti-rhamnose preparation yielded a symmetrical peak indicative of homogeneity in molecular size. The *S. pneumoniae* anti-rhamnose antibodies gave a major and a minor peak, indicating some polymerization, or slight

contamination. Calculations from the sedimentation rates and an empirical formula[20] yield values of 1.45×10^5 as the molecular weight of the major component of each type of antibody.

Agar-diffusion tests with the two antibody preparations and anti-IgA, anti-IgG, and anti-IgM sera showed that the antibodies reacted only with the anti-IgG serum. Accordingly, both antibody preparations are of the IgG class of immunoglobulins.

Examination of the anti-rhamnose antibody preparations by isoelectrofocusing yielded some interesting results. The gel patterns, stained to detect protein components, are shown in Fig. 5. It may be noted that each preparation of anti-rhamnose antibodies was composed of several protein components, marked by the arrows. The antibodies directed at the glycan from *S. mutans* KI-R consisted of five components, which were separated into sharp bands in the gel, whereas the antibodies directed at the glycan from *S. pneumoniae* consisted of seven protein components, some of which were not well resolved in the gel. Isoelectrofocusing coupled with agar diffusion has been utilized to reveal the components having antibody activity[21]. Results of such experiments showed that all of the components of the antibody preparations possessed anti-rhamnose antibody activity. Antibodies that differ in chemical structure and physical properties, but react with the same determinant group of an antigen, have been called isomeric antibodies, and they represent a special type of heterogeneity[14]. Heterogeneity in antibodies has been attributed to differences in biosynthetic pathways, or to differences in the post-translational modification of a newly synthesized, antibody precursor[22]. The results in the present study have been interpreted as evidence for special biosynthetic pathways for each member of the set, and the pathways are activated in related, but different, immunocytes of the immune system. The net result is the production of unique sets of isomeric antibodies, all of which possess identical biological activity, but differ structurally.

EXPERIMENTAL

Preparation of vaccines and antisera. — The strains of *Streptococci* used in the studies were *Streptococcus mutans,* strain KI-R, and *Streptococcus pneumoniae,* type 32. A lyophilized culture of strain KI-R was provided by Dr. E. E. Smith, Department of Biochemistry, University of Miami, School of Medicine, Miami, FL 33152. The culture was used to prepare the vaccines and the immunogenic glycans. Most of the antisera, and the antigens of *S. pneumoniae,* type 32, were obtained from Dr. K. Amiraian of Health Research Inc., NY State Department of Health, Albany, NY 12237. Type 32 antigen was also provided by Dr. B. Prescott, National Institutes of Health, Bethesda, MD 20205.

In the preparations of vaccines of *S. mutans,* the cells from an 18-h culture (500 mL) were collected by centrifugation, washed thoroughly with 0.02M phosphate buffer and saline, pH 7, and suspended in the phosphate buffer (100 mL).

Formaldehyde was then added to a concentration of 0.2%, and the suspension was stirred for 44 h. The formaldehyde-treated cells were collected by centrifugation, washed thoroughly with buffer, and suspended in the 0.02M phosphate buffer in saline (25 mL). The suspension gave an absorbance reading of unity at 600 nm. Aliquots (0.2–0.3 mL) of the suspension were used as the vaccine for immunizing several New Zealand rabbits by intravenous injection following a schedule outlined earlier[22]. In the secondary immunization period, blood samples were taken weekly, and antisera were prepared from these samples. Antiserum (~50 mL) was obtained from several bleedings of the second and subsequent periods. The samples were pooled, and used for isolating the anti-rhamnose antibodies.

Anti-pneumococcal sera with antibodies directed against the capsular polysaccharide of *S. pneumoniae*, type 32, were prepared by the staff of Health Research Inc. Appropriate samples of the sera were used for isolating antibodies by the affinity-chromatography method.

Immunogenic glycans. — The immunogenic glycans in the cell wall of *S. mutans* KI-R were prepared by extracting wet cells (50 g) with dilute acid[16]. The readily soluble, cell-surface components were removed by heating the cells for a short period at 100° in 0.01M HCl–0.04M KCl (200 mL) at pH 2. The cells were recovered, resuspended in the acid solution (200 mL) and the suspension was heated for 60 min at 100° to solubilize the immunogens in the cell wall. The immunogenic components in the extract were precipitated by addition of ethyl alcohol (5 vol.). A 0.4-g sample of the dried residue was obtained from 50 g of wet cells.

The constituent units of the immunogenic glycans were identified by acid hydrolysis of the glycans, paper-chromatographic analysis by the multiple-ascent method[16], methylation of the native glycan, g.l.c., and mass spectrometry. The R_F values, relative to D-glucose, of the products from the glycans and reference compounds were calculated, and these were, for the reference compounds: Rha, 1.37; Glc, 1.00; Gal, 0.87; and GlcA, 0.15; for the *S. mutans* glycan: Rha, 1.38; Glc, 0.98; Gal, 0.86; and for the type 32 glycan: Rha, 1.35 and Glc, 0.99. Duplicate paper-chromatograms were sprayed with solutions of either D-glucose oxidase[23] or D-galactose oxidase[24] to identify D-glucose and D-galactose. Both monosaccharides were identified in hydrolyzates of the *S. mutans* glycan, but only D-glucose in the hydrolyzate of the type 32 glycan. A methylation analysis was also performed on the unhydrolyzed glycans[16]. Typical g.l.c. patterns for the products from the *S. mutans* glycan and reference compounds are shown in Fig. 1. Mass-spectral data were obtained on the various components, and, together with retention times (see Fig. 1), were used to identify the partially methylated derivatives from the glycan. The reference derivatives were prepared from L-rhamnose, isomaltose, *S. bovis* diheteroglycan[25], and *S. bovis* tetraheteroglycan[16].

A sample of capsular polysaccharide, type 32, provided by Dr. Benjamin Prescott, was further purified by chromatography on a column of DEAE-cellulose equilibrated with 0.1M sodium acetate. The polysaccharide component that was not

eluted with the initial buffer was washed from the column by a discontinuous, salt gradient of 0.2M acetate buffer, pH 4.5, containing 0.1M sodium chloride, followed by the same buffer containing 0.2M sodium chloride. The carbohydrate fractions eluted from the column were collected and combined, and the polysaccharide was recovered by precipitation with 95% ethanol (3 vol.).

The monosaccharides present in an acid hydrolyzate of the glycan were also identified from R_F values in paper chromatography as already described. L-Rhamnose and D-glucose were the constituent units of the glycan. However, D-glucuronic acid and D-galactose were not detectable in the hydrolyzate by the methods employed. To confirm the presence or absence of D-glucose and D-galactose, duplicate chromatograms of the hydrolyzate were sprayed with D-galactose oxidase or D-glucose oxidase solution[24,25]. D-Glucose, but not D-galactose, was detected on these chromatograms.

Affinity chromatography. — The affinity adsorbent used for isolating the anti-rhamnose antibodies was prepared from *p*-aminophenyl α-L-rhamnopyranoside and cyanogen bromide-activated Sepharose 4B (Pharmacia Fine Chemicals, Piscataway, NJ 08854). The glycoside was prepared as described earlier[13], by acetylating L-rhamnose (2 g) with acetic anhydride in pyridine, and fusing the acetate (1.5 g) with *p*-nitrophenol (1.5 g); the yield of product was 0.6 g. This was deacetylated with sodium methoxide, and the product reduced with hydrogen in the presence of platinum oxide catalyst. The *p*-aminophenyl α-L-rhamnoside crystallized from ethyl alcohol; yield 0.2 g; m.p. 165°. A sample of the glycoside (0.05 g) and cyanogen bromide-activated Sepharose 4B (4 g) were used to prepare rhamnosyl-Sepharose[14]. The coupled product was transferred to a column (20 × 1 cm), and washed thoroughly with 0.02M phosphate buffer, pH 7, in saline.

Antibodies were isolated from the immune sera by introducing 1-mL samples of the serum onto the rhamnosyl-Sepharose column, washing with 0.02M phosphate buffer, pH 7, in saline, to remove the unbound protein, and then washing the column with 0.1M L-rhamnose solution to elute the antibodies. The eluates from the column were continuously monitored for u.v.-absorbing components with an ISCO UV analyzer. The fractions containing u.v.-absorbing substances, from the two sera, that were eluted with the L-rhamnose solution were collected separately, and the samples were mixed with an equal volume of saturated ammonium sulfate. On refrigeration overnight, the antibodies precipitated from the solutions, and were recovered by centrifugation. The antibody samples were redissolved in 0.02M phosphate buffer, pH 7, in saline (0.2 mL). Several such samples were obtained, combined, and then concentrated to ~0.5 mL by ultrafiltration. The concentrates constituted the preparations of anti-rhamnose antibody used in subsequent experiments.

Agar diffusion. — Agar-diffusion tests were performed with the antiserum samples, the purified antibodies, and 0.5% solutions of antigens. The diffusion plates that yielded precipitin bands were photographed. The agar-diffusion procedure was also used to establish the class of immunoglobulins to which the anti-

bodies belonged. In these experiments, antisera against IgA, IgG, IgM, and the purified anti-rhamnose antibodies were tested by double diffusion in agar. The anti-IgG serum yielded a positive reaction with both antibody preparations, but the anti-IgA and anti-IgM sera did not.

Hapten inhibition. — Quantitative, inhibition tests were performed with the immune serum, and the anti-rhamnose antibodies directed against *S. mutans,* by methods described previously[26]. In these tests, the concentrations, or amounts, of the materials employed were: 0.1 mL of antiserum or antibody preparation, 40 μg of the antigen, and 36 mg of the potential carbohydrate inhibitor. The precipitin complexes that formed were thoroughly washed, and analyzed for protein by a standard method. In the tests with the anti-rhamnose antibodies directed against the *S. pneumoniae* glycan, a micro procedure utilizing agar diffusion was developed. In these tests, the antibody solution (5 μL) was mixed with 5 μL of a solution containing 180 μg of the potential inhibitor, and kept for 1.5 h at room temperature. This sample was then used in agar diffusion tests with 2 μL of solutions of antigen of concentration of 0.25, 0.05, 0.01, and 0.002%. Plates were also prepared with the original serum, but no inhibitor, and the various concentrations of antigens. Comparisons of the intensity of the diffusion bands were made after diffusion for 24 h. The relative inhibition by the various compounds was estimated from the intensities of the bands.

Ultracentrifugation. — A density-gradient centrifugation-procedure used in this laboratory has been described earlier[19]. This procedure was used to determine the molecular homogeneity and the molecular weight of the anti-rhamnose antibodies. Samples (0.2 mL) of 0.5% solutions of the anti-rhamnose antibodies and of 1 to 10 dilutions of the antisera were placed in separate tubes on sucrose density-gradients (5 to 40%). The tubes were centrifuged at 65,000 r.p.m. for 10 h in a swinging-bucket SW65 rotor in a Beckman L2-65 centrifuge. At the end of the centrifugation, the gradient solutions were fractionated into 0.2-mL fractions by means of an ISCO Density Gradient Fractionator[19]. The u.v. absorbance of the fractions was monitored at 280 nm during fractionation. D-Glucose oxidase was used as a reference protein of known molecular weight, and an empirical formula was used to estimate the molecular weights of the antibodies.

Isoelectrofocusing. — Isoelectrofocusing of the anti-rhamnose antibodies was performed in poly(acrylamide) gels by utilizing a method described in the literature[27]. The pH range of the ampholine solutions used in these experiments was either 5–7 or 4–6. Preliminary isoelectrofocusing was conducted for 30 min at a current of 0.7 mA/gel at 4°. The antibody samples were dissolved in 40% sucrose and 4% ampholine, and the solutions introduced onto the top of the gel, following completion of the preliminary focusing. Isoelectrofocusing was continued for an additional 6 h at a current of 1 mA/gel, until a potential of 215 V had been reached, whereupon the voltage was maintained constant. At completion of the isoelectrofocusing, the gels were removed from the tubes, washed with 10% trichloroacetic acid, and stained with a 0.02% solution of Coomassie Brilliant Blue G-250. Gels that were to be used for agar diffusion tests were not stained.

Because ampholine interferes with the formation of the antigen–antibody complex, a technique utilizing isoelectrofocusing and immunodiffusion was devised for detecting antibodies in poly(acrylamide) gels containing ampholines. In this procedure, duplicate samples of the antibody preparation were subjected to isoelectrofocusing as already described. One finished gel of each set was stained with Coomassie Brilliant Blue, in order to locate the protein components, and the duplicate gel was embedded in fluid agar in a Petri plate. The agar was allowed to solidify, and the plate was placed in a humid chamber. Diffusion of the antibodies was allowed to proceed for 24 h. A trough was then cut in the agar, ~2 cm from the embedded gel. A 0.5% solution of the antigen (~0.3 mL) was placed in the trough, and diffusion was allowed to continue for periods of 24 to 90 h. Plates that developed precipitin bands were photographed. The protein components in both antibody preparations yielded elongated diffusion-bands that corresponded to all of the protein components in the preparations.

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