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Affinity chromatography of two sets of isomeric antibodies having specificity for different oligosaccharide units of gum arabic*

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ABSTRACT

Two sets of antibodies directed against different carbohydrate units of gum arabic were isolated from the sera of rabbits immunized intramuscularly with gum arabic and Freund's complete adjuvant. The isolation was effected by affinity chromatography on two columns attached in series and containing an adsorbent of AH-Sepharose 4B with ligands of partially hydrolyzed gun arabic in the first column and an adsorbent of AH-Sepharose 4B with ligands of native gum arabic in the second column. The two populations of anti-gum arabic antibodies were obtained and have been designated as Set 1 and Set 2 on the basis of their mobilities on agar diffusion. The antibodies of Set 1 consisted of 4 isomeric antibodies and those of Set 2 consisted of 11 isomeric antibodies. Native gum arabic samples were oxidized with periodate or reduced with sodium borohydride and carbodiimide under standard conditions and the modified samples were totally inactive in the precipitin test. On the basis of methylation data and immunological results it was concluded that terminal disaccharide moieties of the gum having the structure β-D-glucosyluronic acid-(1→6)-D-galactose and α-L-arabinofuranosyl-(1→4)-D-glucuronic acid were the immunodeterminant groups for Set 1 and Set 2 antibodies, respectively.

INTRODUCTION

Antibodies directed at the carbohydrate units of gum arabic have been isolated from the serum of rabbits immunized with the gum and Freund's complete adjuvant by affinity chromatography methods.[1] The antigenicity of gum arabic for pneumococcal antibodies was noted sometime ago by Heidelberger and coworkers.[2] The stimulation of immunocytes to synthesize antibodies specific for gum arabic and the detection of such antibodies has been observed more recently.[3,4] Like other anti-carbohydrate antibodies, the anti-gum arabic antibodies have been found to occur in isomeric forms called isoantibodies.[5]

Two sets (Set 1 and Set 2) of anti-gum arabic isoantibodies have now been separated from the immune serum by a two-column affinity technique. The members of Set 1 are directed at the glucosyluronic acid-galactose units of the gum while the members of Set 2 are directed at the arabinofuranosyl-glucuronic acid units. The immunological reactivity of the gum for Set 2 antibodies is destroyed by mild acid

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hydrolysis. Under mild hydrolytic conditions the arabinofuranyl residues were removed from the antigen. The reactivity of Set 1 antibodies is not destroyed by mild acid hydrolysis but is destroyed by hydrolysis in more-concentrated acid. Affinity adsorbents bearing ligands of native gum arabic or ligands of slightly hydrolyzed gum arabic were used in a two-column affinity chromatography method to separate and isolate the two sets of anti-gum arabic antibodies. Some properties, including molecular weights, inhibition, and isoelectric focusing of the antibody sets have been determined.

Anti-gum arabic antibodies should be useful for studying biosynthetic mechanisms for the generation of isomeric antibodies by immunocytes. Such antibodies should also be useful as analytical reagents for the identification of gum arabic in commercial products and for detection of alterations of gum arabic which may be produced by DNA technology methods.

RESULTS AND DISCUSSION

Fig. 1 shows an agar diffusion plate demonstrating that anti-gum arabic serum possesses two sets of antibodies having specificity for different immunodeterminants of the antigen (Wells S and A). The antibodies for which the precipitin band is located nearer the center well (S) are designated as Set 1. Antibodies that yield a precipitin band further from the serum well (S) are designated as Set 2. Mild acid hydrolysis of the gum removed the arabinofuranosyl units from the gum and the resulting product no longer formed a precipitin band with Set 2 antibodies (Wells 1 and A). Hydrolysis of the gum with more-concentrated acid destroys all of the antigenicity of the gum for both sets of antibodies (Wells 2 and S).

The two sets of antibodies were separated by a two-column affinity chromatography technique as shown in Fig. 2. The first column contained an adsorbent of AH-Sepharose 4B coupled to mildly hydrolyzed gum arabic. The hydrolysis was effected in 0.01N HCl for 10 min at 100° and paper chromatograms showed that only arabinose was released from the gum[1]. The second column contained AH-Sepharose 4B coupled to native gum arabic. While the columns were attached in series, the immune serum was passed through the first column containing the adsorbent with ligands of hydrolyzed gum (0.01N HCl for 10 min at 100°) and then through the second column containing the adsorbent with ligands of native gum. When the non-absorbed protein had passed through the adsorbents, the columns were separated and the antibodies were eluted separately from each column with 0.2M phosphate buffer of pH 5.8 containing 0.5M sodium chloride. The elution patterns are shown in the top portion of Fig. 2. The u.v.-absorbing substances which were eluted are labeled Ab_1 and Ab_2. These substances were collected separately and were mixid with an equal volume of saturated ammonium sulfate. The precipitates that formed on refrigeration overnight were collected and re-dissolved in 0.2M phosphate buffer of pH 7. These solutions were used in additional immunological experiments.

The lower portion of Fig. 2 shows an agar diffusion plate in which the immune serum, the non-adsorbed protein, Set 1 antibodies (Ab_1) and Set 2 antibodies (Ab_2) were

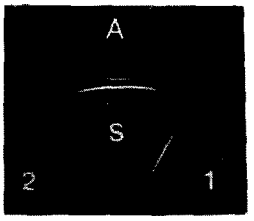


Fig. 1. Agar diffusion pattern for anti-gum arabic serum (S), against native gum arabic (A), mild acid hydrolyzate of gum arabic (1), and concentrated acid hydrolyzate of gum arabic (2).

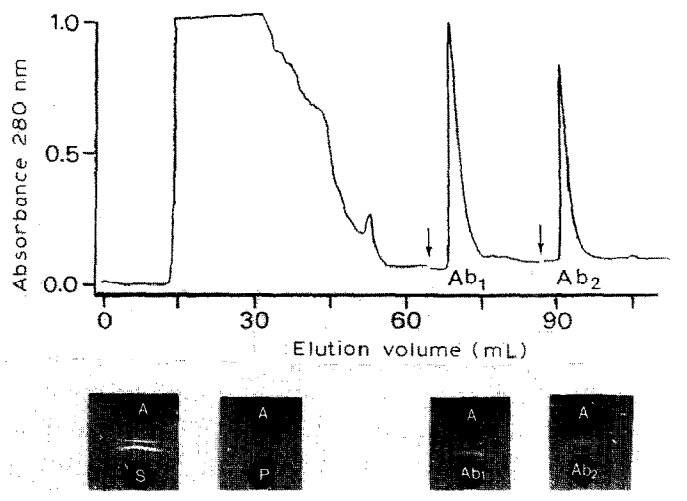


Fig. 2. Two-column affinity chromatography pattern of anti-gum arabic immune serum. Arrows indicate the points of application of phosphate buffer of pH 5.8 containing 0.5 M NaCl. The agar diffusion plate was prepared from native gum arabic (A), immune serum (S), nonabsorbed protein (P), antibodies of Set 1 (Ab_1), and antibodies of Set 2 (Ab_2).

tested against native gum arabic. It may be seen that the original serum (S) contained two groups of antibodies, no antibodies were present in the non-adsorbed protein fraction (P), and the sets of antibodies (Ab_1 and Ab_2) were separated by the affinity chromatography technique.

A number of properties of the two sets of antibodies have been determined. The sedimentation rates on sucrose density-gradient centrifugation of the Set 1 antibodies (B) and reference glucose oxidase (A) are shown in Fig. 3. It may be noted that the antibodies and the glucose oxidase sediment at the same rate. Further, both preparations are homogeneous in molecular size, are free of u.v.-absorbing impurities and are of the same molecular weight. The molecular weight was calculated from these data and an empirical formula[6] to be 1.5×10^5. The antibodies of Set 2 also behaved the same as the antibodies of Set 1 on sucrose density-gradient centrifugation.

In order to determine the hapten units of gum arabic antigen which combine with the antibodies, a number of carbohydrates were tested as inhibitors of the precipitin

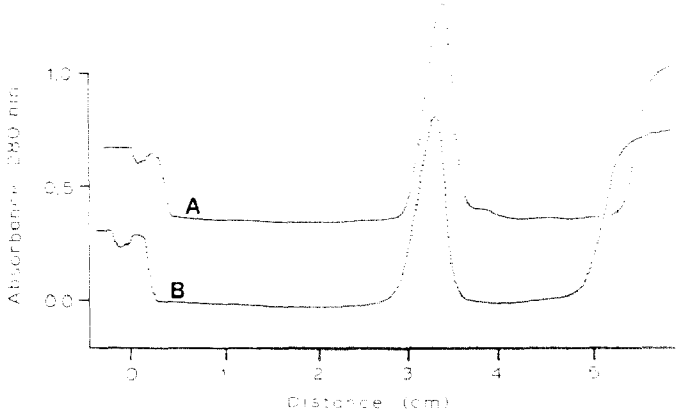


Fig. 3. Sucrose density-gradient ultracentrifugation patterns of glucose oxidase (A) and anti-gum arabic antibodies of Set 1 (B).

reaction by the microdiffusion method[7]. The compounds tested included arabinose, galactose, rhamnose, glucuronic acid, and two oligosaccharides isolated from hydrolyzates of the gum and having the structures arabinofuranosyl-glucuronic acid and glucosyluronic acid-galactose[1]. None of the monosaccharides inhibited the formation of the precipitin complex between the gum and either set of antibodies. However the oligosaccharides did inhibit specific precipitin reactions. Fig. 4 shows the results of the inhibition of the reaction of Set 2 antibodies (Plate A) and native gum by the arabinofuranosyl-glucuronic acid. It maybe noted in the figure that the precipitin bands with the Set 2 antibodies and the inhibitor were less intense than in the control, whereas those with Set 1 antibodies were relatively unchanged. In Plate B it may be noted that the precipitin bands with Set 1 antibodies in the presence of the inhibitor of glucosyluronic acid-galactose were less intense than in the control. The results in Plate B were obtained with mildly acid hydrolyzed antigen and as a result precipitin bands were not formed with antibodies of Set 2. These results indicate that the hapten group for the former is arabinofuranosyl-glucuronic acid and for the latter it is glucosyluronic acid-galactose.

A sample of a mixture of the two sets of anti-gum arabic antibodies was prepared by affinity chromatography of the immune serum on a single adsorbent of AH-Sepharose 4B coupled to native gum arabic. This antibody sample, and the antibodies of the individual sets from the two-column chromatography, were subjected to isoelectric focusing under identical conditions. The gels were stained for proteins by the Coomassie Blue stain. The stained gels were photographed and the photograph is reproduced in Fig. 5.

It may be noted in Fig. 5 that the sample eluted from the adsorbent coupled to native gum arabic contained members of both sets of antibodies (Set 1 and 2) and (as indicated by the arrows) a total of 15 members are present in this sample. The sample eluted from the acid-hydrolyzed gum adsorbent consisted of 4 components (Set 1), and the sample eluted from the native gum arabic adsorbent from the second column consisted of 11 proteins.

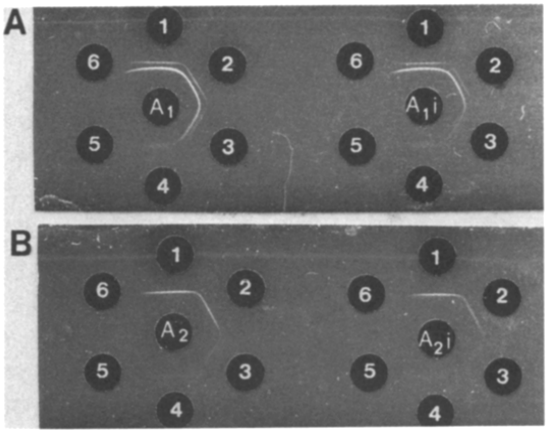


Fig. 4. Agar diffusion patterns showing the inhibition of anti-gum arabic serum by oligosaccharides isolated from hydrolyzates of gum arabic. A_1 and A_2 = anti-gum arabic serum, A_1i = anti-gum arabic serum + Ara-(1→4)-GlcA, A_2i = anti-gum arabic serum + GlcA-(1→6)-Gal. The wells numbered 1 to 6 contained decreasing amounts of antigens of native gum arabic in plate A and decreasing amounts of acid-hydrolyzed gum arabic in plate B.

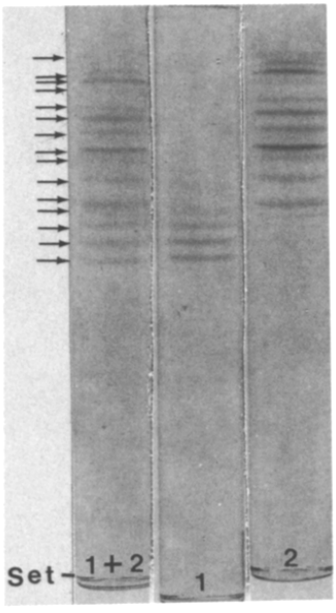


Fig. 5. Isoelectric focusing of purified anti-gum arabic antibodies and the two sets of the antibodies. The arrows mark the components in the preparations.

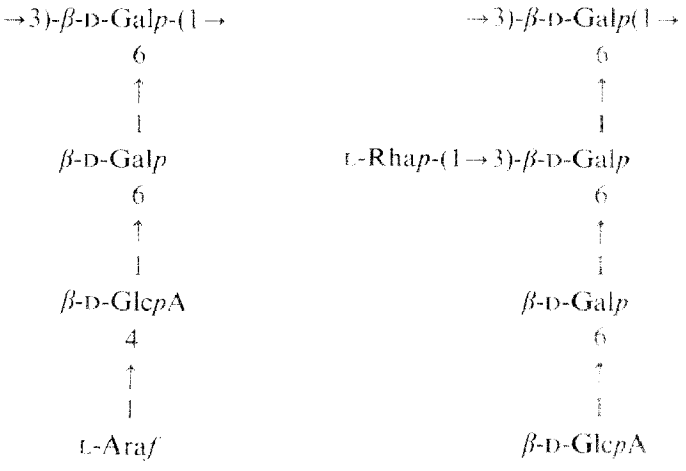
To test whether all of the protein isomers possessed antibody activity, a coupled isoelectric focusing–agar diffusion method was used. The results are shown in Fig. 6. In the figure, A shows the pattern for the mixture of the two sets of antibodies directed against gum arabic. Two bands of precipitin complex were obtained; one band corresponded to Set 2 antibodies and the other to Set 1. The precipitin complex formed

opposite all of the protein components contained in the preparation. Therefore all of the proteins possess antibody activity and such antibodies are termed isoantibodies. Fig. 6 part B shows the results with the Set 1 antibodies. Only one band of precipitin complex was obtained, and all protein bands form the precipitin complex and are therefore antibodies. Fig. 6 part C shows the results with Set 2 antibodies. Only one band of precipitin complex was obtained, indicating uniformity in the immunological reaction and all the components posses antibody activity.

The significant results of this study are that gum arabic stimulates two groups of immunocytes to synthesize two sets of antibodies. The antibodies of each set are different proteins and react with different structural units of the gum arabic. The two sets of antibodies can be separated by a two-column method using acid-hydrolyzed gum arabic as the ligand and native gum arabic as the second ligand. The molecular weight of the two sets of antibodies is the same, namely 1.5×10^5.

Gel isoelectric focusing shows that Set 1 of the antibodies is composed of 4 isoantibodies and Set 2 of the antibodies is composed of 11 isoantibodies. The inhibition results showed that glucosyluronic acid-(1→6)-galactose inhibited the antibodies of Set 1 and hence these antibodies are directed at this oligosaccharide unit of the gum. The inhibition results also showed that arabinofuranosyl-(1→4)-glucuronic acid inhibited the antibodies of Set 2. These antibodies are directed at the arabinose-containing oligosaccharide moiety of the gum.

The structures of the two oligosaccharides that are inhibitors of gum arabic antibodies are shown in the accompanying formulas. The presence of these oligosaccharide moieties in the gum has been established by data from methylation analysis and from the nature of the hydrolytic products liberated by stereospecific enzymes[1].



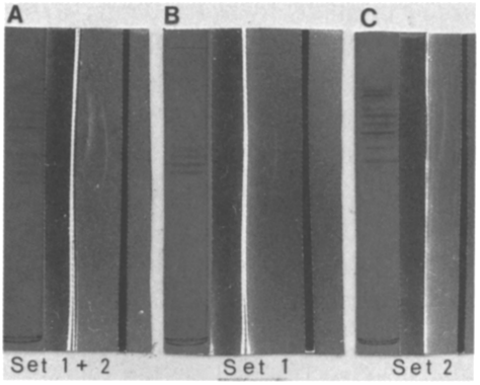


Fig. 6. Isoelectric focusing coupled with agar diffusion of the total anti-gum arabic antibodies (Set 1 + 2) and antibodies of Set 1 and Set 2. The gels and diffusion area are stained gels, unstained gels embedded in agar, troughs of precipitin complex formation, and troughs of antigen solution of gum arabic.

EXPERIMENTAL

Immunological methods — Gum arabic from *Acacia senegal* was obtained from Sigma Chemical Co., St. Louis, MO 63178 (U.S.A.). A solution of gum arabic was prepared by dissolving 320 mg of the gum in 2 mL of sterile phosphate buffer (0.02M phosphate, pH 7) in saline. This solution was then mixed with an equal volume of Freund's complete adjuvant for use in immunization. Samples of 0.4 mL of the gum-adjuvant suspension were injected intramuscularly in the hind leg of a rabbit weekly for a 6-week period. The animal was then allowed to rest for two weeks and the injection 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 conventional methods.

A standard agar-diffusion method was used for detecting precipitin formation and antibody–antigen reactions. Ten μL of the immune serum or of the purified antibodies containing 10–20 μg of antibody protein were placed in the center well of an agar diffusion plate. Ten μL of antigen or other polysaccharide solution containing 10–50 μg of 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 that formed were noted. The plates were also photographed for a permanent record of the results.

A micro method combining inhibition and agar diffusion has been developed in this laboratory for detecting potential inhibitors of the antibody–antigen reactions[7]. This method was used to test for precipitin formation of the antibodies and the gum in the presence of the monosaccharides that are constituents of the gums and oligosaccharides obtained by acid or enzymic hydrolysis of the gum.

Affinity adsorbents and affinity chromatography. — Affinity adsorbents of AH-

Sepharose 4B bearing ligands of native gum arabic or mildly hydrolyzed gum arabic (0.01N HCl for 10 m at 100°) were prepared for use in isolating the antibodies. Three g of AH-Sepharose 4B, 100 mg of the appropriate gum sample, and 250 mg of CMC [1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide metho-*p*-toluenesulfonate] were used for the synthesis of the adsorbents by the procedure described in a Pharmacia bulletin[8]. At the end of the synthesis, the excess reagent and unreacted gum were removed by filtration. The products were then washed sequentially with 500 mL of 0.5M 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 equilibrated with 200 mL of saline and phosphate buffer of pH 7.

The single-column affinity chromatography was performed by the following method. Samples of the immune serum were applied to the adsorbent with ligands of native gum arabic and, after application of the sample, the column was washed with 0.02M phosphate buffer pH 7 containing saline until the unadsorbed proteins were removed. The antibodies were then eluted with M NH_4SCN and the eluate was monitored with a u.v. analyzer. The substance that eluted as a single peak was collected. The solution was mixed with an equal volume of saturated $(NH_4)_2SO_4$. The precipitate that 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 the samples.

A two-column affinity chromatography method was devised and used to separate the antibodies into sets. For the preparation of one adsorbent, a sample of gum arabic was hydrolyzed in 0.01M HCl for 10 min and the hydrolyzate was coupled to AH-Sepharose 4B. The second adsorbent was synthesized by coupling native gum arabic to AH-Sepharose 4B. The columns were attached in a series with the hydrolyzed gum arabic ligand-column preceding the native gum arabic ligand-column. In order to decrease the amount of extraneous protein, the immune serum was treated with an equal amount of saturated $(NH_4)_2SO_4$ for precipitating the globulin fraction. The resulting precipitate was collected by centrifugation and redissolved in phosphate-buffered saline, pH 7. The globulin solution was then applied to the column bearing the hydrolyzed gum arabic ligands and the eluate from this column was passed through the column bearing native gum arabic ligands. After all non-adsorbed proteins had been eluted from the two columns, the columns were separated and each was eluted separately using phosphate buffer of pH 5.8 and containing 0.5M NaCl. The eluates from the columns were monitored for u.v. absorbance and the u.v.-absorbing fractions were collected. These fractions were treated with an equal volume of saturated $(NH_4)_2SO_4$ to precipitate the antibodies. The antibodies were recovered by centrifugation and were dissolved in phosphate buffer and saline of pH 7.

Sedimentation and molecular weights. A sucrose density-gradient centrifugation procedure developed in this laboratory was utilized to determine sedimentation rates and molecular weights of the purified antibody preparations[9]. The antibodies and reference glucose oxidase were centrifuged and analyzed 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[6]. The molecular weights of both anti-gum arabic antibody preparations were 1.5×10^5.

Inhibition by carbohydrates. — The inhibition tests were performed by the coupled inhibition agar-diffusion method[7] with arabinose, galactose, rhamnose, glucuronic acid, a disaccharide isolated from an enzymic hydrolyzate, and a disaccharide isolated from an acid hydrolyzate of the gum. In the coupled inhibition–agar diffusion tests, samples of 0.1 – 5 mg of the potential inhibitior 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. Ten μL of antigen solutions containing decreasing amounts of the antigen (2, 1, 0.5, 0.25, 0.125, and 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 percipitin bands at the different concentrations of antigen on the inhibition plates with the intensity of bands on a plate with control antibody solution.

Isoelectric focusing. — Gel isoelectric focusing of the antiserum and antibody preparation was performed in 10% polyacrylamide gels in ampholine–sucrose solution of pH gradient 5–8. The procedure employed was essentially that described in the literature[10] with minor modifications. The focusing gel was supported in the glass tubes by means of a 6-mm 7.5% acrylamide gel. The focusing gel was poured to a height of 75-mm and polymerized at 4 °C. The gel was overlayed with a 2-mm protective layer containing 5% sucrose and 2% ampholine. A solution of 0.02% H_2SO_4 was employed as the upper anode solution while 0.4% ethylenediamine solution was employed as the lower cathode solution. In the method used, a preliminary isoelectric focusing at a current of 0.7 mA per gel for 0.5 h at 4 °C was used to establish the pH gradient. The antiserum or antibody preparation dissolved in 20% ampholine and 15% sucrose was then introduced onto the gel beneath the protective layer. Isoelectric focusing was performed by applying a constant current of 1 mA per gel until a potential of 215 V was reached. The gels were then run at this constant voltage for 6 h. The apparatus was maintained at 4 °C. The gels were removed from the glass tubes using a water jet, stained with Coomassie Blue, and destained.

A coupled isoelectric focusing and agar-diffusion method[11] developed in this laboratory was also used to detect antibody activity in the purified preparations. In this procedure, duplicate samples of the antibody preparation 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 to allow the antibody to diffuse into the agar. A trough was then cut in the agar ~ 2 cm from the gel and a 1% solution of the antigen (gum arabic) was introduced into the trough. Diffusion of antigen and antibody was allowed to proceed for an additional 24 – 48 h. The plates that developed precipitin arcs were photographed.

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