EQUINE ANTIHAPTEN ANTIBODY*

I. 7S β_{2A}- AND 10S γ_1-GLOBULIN COMPONENTS OF PURIFIED ANTI-β-LACTOSIDE ANTIBODY

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Experimental evidence has established that a single animal is capable of producing different molecular forms of antibody (1–3). In early studies, equine antipneumococcal polysaccharide antisera were shown to contain two distinct antibodies, the first with a sedimentation coefficient of approximately 7S and a molecular weight of approximately 160,000, and the second with a sedimentation coefficient of approximately 19S and a molecular weight of approximately 1,000,000 (1, 2, 4, 5). The 7S antibody was a γ-globulin of low electrophoretic mobility, and the 19S antibody was a γ-globulin of higher electrophoretic mobility (2). Numerous examples of comparable forms of antibody have subsequently been described in other animals (3).

More recent investigations have demonstrated that there are also 7S antibodies, migrating on electrophoresis in the γ_1- or β_2-region, which differ in several significant ways from the 7S antibodies of lower electrophoretic mobility. A number of antibodies with these characteristics have been shown to be β_{2A} (γ_{1A})–globulins, an antigenically distinct subclass of the immunoglobulins (6–9). In addition, two antigenically distinct forms of 7S antihapten antibody with different rates of electrophoretic migration, have been isolated from guinea pig antisera (10–12). The biologic activity of the more rapidly migrating (γ_1) guinea pig antibody differs markedly from that of the more slowly migrating (γ_2) antibody (11).

Experimental evidence which established the existence of a group of antibodies with sedimentation coefficients between 7S and 19S has also been presented (13).

Several advantages are afforded by the study of antibody directed against well defined haptenic groups. The present investigations were undertaken in part in an attempt to isolate and characterize multiple molecular forms of anti-

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hapten antibody produced by a single animal. The horse was selected as the experimental animal because of its large blood volume and because of certain unusual properties of equine antipolysaccharide antibody (2). Antibody directed against the haptenic group *p*-azophenyl-β-lactoside (14) has been isolated from equine antiserum. A minimum of 6 antigenically distinct immunoglobulins have been identified in purified antibody preparations. These include β_{2A} (γ_{1A})–globulin, 19S γ-globulin, a 10S γ_1-globulin, and 3 antigenically different 7S γ-globulins. An unambiguous demonstration of the specific hapten-binding activity of the 7S β_{2A}-antibody and of the 10S γ_1-antibody has been obtained.

Materials and Methods

Antigens.—The haptenic group *p*-azophenyl-β-lactoside (Lac) and the azohapten *p*-(*p*-dimethylaminobenzeneazo)phenyl-β-lactoside (Lac dye) used in the present studies have been described in previous publications (14). Antigens were prepared by diazotizing *p*-aminophenyl-β-lactoside and coupling to bovine γ-globulin (Lac-BγG), rabbit γ-globulin (Lac-RγG), human serum albumin (Lac-HSA), and hemocyanin, *Limulus polyphemus*, (Lac-Hy). The Lac-HSA contained from 15 to 20 moles of Lac group per 65,000 gm of human serum albumin, and the Lac-Hy contained from 20 to 25 moles of Lac group per 100,000 gm of hemocyanin (15).

Immunization.—A 500 kg horse was immunized at biweekly or monthly intervals with antigen preparations containing either Lac-BγG, Lac-RγG, or Lac-Hy dispersed in incomplete Freund's adjuvant as described elsewhere (15). Bleedings of 4 liters were spaced at regular intervals between immunizations. The program of immunization and bleeding was continued for a 10 month period. Antisera against normal equine serum, isolated equine serum proteins, and human serum albumin were prepared by immunizing rabbits with the various proteins in complete Freund's adjuvant.

Specific Precipitation of Antibody.—Anti-Lac antibody concentrations were determined by quantitative precipitin analysis (2) with Lac-HSA. Washed precipitates were dissolved in 0.1 N NaOH and the absorption at 287 mμ and 400 mμ was measured with a Zeiss spectrophotometer. In some instances, the N content of the precipitates was determined by micro Kjeldahl analysis (2). The $E_{\lambda 287}^{1\text{ per cent}}$ and $E_{\lambda 400}^{1\text{ per cent}}$ of Lac-HSA and Lac-Hy were measured in 0.1 N NaOH and calculated on the basis of a microKjeldahl analysis. The quantity of antigen in the precipitates was obtained from the optical density at 400 mμ, and a correction was made for its contribution at 287 mμ to allow calculation of the antibody content. Antibody concentration was calculated by using an $E_{\lambda 287}^{1\text{ per cent}}$ of 15 (15).

For the preparation of purified anti-Lac antibody, specific precipitates were formed by the addition of an equivalent amount of either Lac-HSA or Lac-Hy to equine antiserum. In the majority of instances the serum was first absorbed at 37°C with an unrelated antigen-antibody system (BγG-anti-BγG) to remove complement components and other possible co-precipitating factors. Precipitates were washed at least 5 times with cold 0.15 M NaCl and then resolubilized in a solution of 0.5 M lactose, 0.02 M phosphate, pH 7.2.

Column Chromatography.—A chromatographic procedure, a modification of that used by Utsumi and Karush to isolate rabbit anti-Lac antibody (16), was employed to separate antibody from antigen. A column of DEAE cellulose was equilibrated with a solution of 0.5 M lactose, 0.02 M phosphate, pH 7.2 (initial solvent), the same solvent used for solubilization of the antigen-antibody precipitates. The solution of antigen and antibody was applied to the column and the initial solvent was passed through the column until a plateau had been reached following the recovery of a first protein peak. Additional proteins were then eluted from the

DEAE cellulose column with either a stepwise increase of NaCl, or a linear positive gradient of NaCl in the initial solvent. The chromatography was accomplished at room temperature. The effluent was monitored at 254 mμ by a 4701A uvicord recording UV analyzer (Laboratorie och Kemikaliska Produkter, Stockholm, Sweden), and by measurement of the absorption at 280 mμ and 365 mμ of selected fractions. Various fractions were pooled and concentrated by either negative pressure ultrafiltration or lyophilization.

Zone Electrophoresis.—Zone electrophoresis in a starch-supporting medium (17) was carried out in a barbital buffer solution of $\Gamma/2$ 0.05, pH 8.6 in a cold room. The barbital buffer was saturated with lactose when antigen was to be separated from anti-Lac antibody.

Absorption Spectroscopy.—The absorption spectra of antibody preparations and of Lac-HSA and Lac-Hy, in solutions of 0.2 M NaCl, 0.01 M phosphate, pH 7.8, were measured at room temperature in a Cary model 15 recording spectrophotometer. The molar extinction coefficient, ϵ, of the Lac group was taken as 2.5×10^4 at 365 mμ, the wave-length of maximum absorption in neutral solution, on the basis of the extinction properties of the Lac dye (14).

Equilibrium Dialysis.—The technique of equilibrium dialysis employed has been described elsewhere (14). The molar extinction coefficient, ϵ, of Lac dye at the wavelength of maximum absorption, λ 455 mμ, is 2.48×10^4 (14). Protein concentrations were determined by micro-Kjeldahl analysis, done in triplicate, assuming a N content of 16 per cent.

Ultracentrifugation.—Analytical ultracentrifugation was performed in a Beckman model E centrifuge at 52,640 RPM. Sedimentation velocities were determined at 20°C in a solution of 0.2 M NaCl, 0.01 M phosphate, pH 7.8, and corrected for viscosity and solvent density using a partial specific volume of 0.74 for all protein components. A combination of schlieren and absorption optics was used to demonstrate the sedimentation of dye-binding components. A Klett filter (maximum transmission 440 mμ) was placed over the mercury light source of the model E machine, limiting the wavelength of transmitted light to that principally in the region of maximum absorption by Lac dye. Sucrose density gradient ultracentrifugation was accomplished as previously described (3, 13) in a Beckman model L-2 centrifuge at 4–12°C. Samples of 0.10 to 0.20 ml were layered on the continuous gradients and centrifuged in an SW-39 rotor at 35,000 RPM for periods ranging from 12 to 36 hours. The concentration of protein in the fractions was determined by the modified method of Folin-Ciocalteu (18).

Isolation of Normal Equine Serum Proteins.— The 7S γ-globulins of normal equine serum were isolated by zone electrophoresis (17). The proteins from the cathodal region of the γ-peak of the starch block were dialyzed against a solution of 0.015 M phosphate, pH 7.85, and passed over a DEAE cellulose column equilibrated with the same solvent. The 19S γ-globulins of normal equine serum were isolated by a combination of sephadex G-200 gel filtration (19, 20), zone electrophoresis, and density gradient ultracentrifugation.

Agar Diffusion Studies.—Immunoelectrophoresis was accompllshed by the microtechnique (21). For double diffusion studies, agar was layered on microscope slides and antigens were placed in wells cut along the center of the agar strip. Troughs were cut in the agar along both edges and filled with the various antisera.

RESULTS

Isolation of Anti-Lac Antibody.—Antibody was precipitated with either Lac-HSA or Lac-Hy from serum samples obtained during a 10 month period. The anti-Lac antibody reached a maximum concentration of 2.1 mg/ml during the immunization period. The major portion of each of the washed specific precipitates was resolubilized in the solution of 0.5 M lactose, 0.02 M phosphate, pH 7.2, leaving only a small residue of darkly colored insoluble material. The

resolubilized mixture of antigen and antibody was applied to a DEAE cellulose column which had been equilibrated with the initial solvent, and a protein peak (initial solvent peak) which was followed by a trailing shoulder was recovered by passing initial solvent through the column (Fig. 1 *A*, peak I).

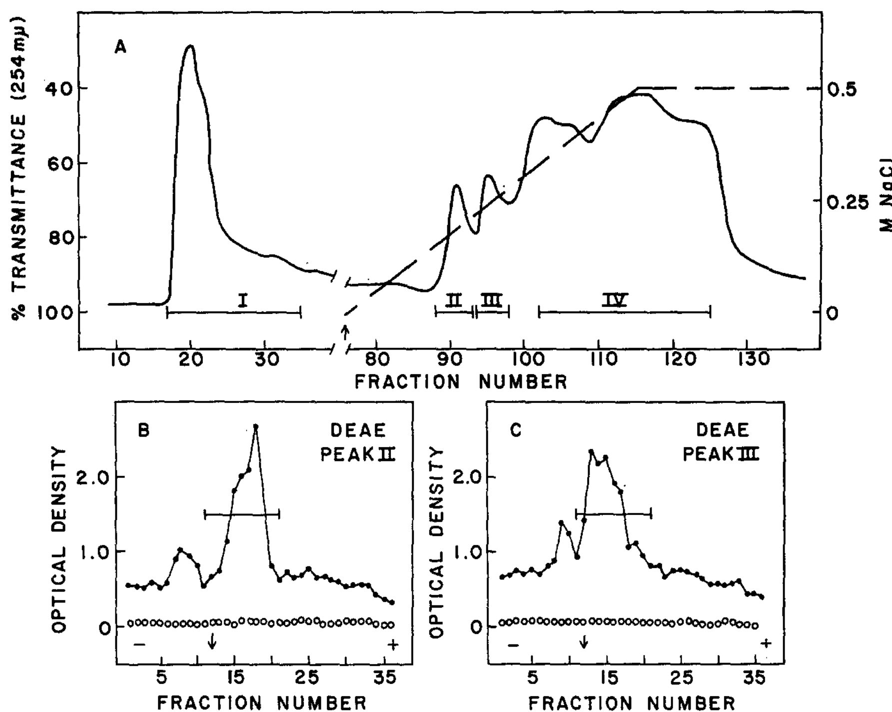

FIG. 1 *A*. DEAE cellulose chromatogram of a mixture of equine anti-Lac antibody and Lac-Hy antigen resolubilized in a lactose solvent. Initial elution with a solvent of 0.5 M lactose, 0.02 M phosphate, pH 7.2, was followed (↑) by a linear positive gradient of NaCl in initial solvent. Significant Lac-Hy antigen did not elute until after the elution of peak III.

FIGS. 1 *B* and 1 *C*. Starch block zone electrophoresis of the proteins of DEAE chromatogram peaks II and III in barbital buffer, pH 8.6, saturated with lactose. Optical density at 280 mμ (●——●) and 365 mμ (○——○), respectively. Anodes to the right.

The proteins contained in various regions of this peak were examined in agar diffusion studies using rabbit antisera which had been prepared against normal equine serum. The antisera either were unabsorbed or had been absorbed with isolated equine 7S γ-globulins. The initial solvent peak contained 3 antigenically distinct 7S γ-globulins (15). This is illustrated by immunoelectrophoresis in Fig. 6 *B*. The trailing shoulder contained, in addition, an antigenically distinct 7S β_2-globulin which was immunologically identical with the 7S β_{2A} (γ_{1A})-antibody to be described presently (Fig. 6 *C*). After the column had been

washed with from 5 to 15 column volumes of initial solvent, additional antibody components were obtained by either stepwise or gradient elution. Stepwise elution with either 0.5 M or 1.0 M NaCl in the initial solvent resulted in fractions which were contaminated with significant amounts of antigen. Elution with a linear positive gradient of NaCl (maximum concentrations either 0.5 M or 1.0 M) in the lactose solvent resulted in the recovery of a considerable amount of antibody before the displacement of antigen. The results of a representative chromatogram are presented in Fig. 1. Two partially resolved protein peaks (Fig. 1 *A*, peaks II and III) were recovered before the elution of antigen. The bulk of the eluted antigen (Lac-Hy) was contained in peak IV (Fig. 1 *A*). Peak II was composed principally of 7S β_{2A}-globulin (Fig. 4 *A*, Fig. 6 *C*). Peak III consisted of 2 antigenically distinct components of which the major one was a 10S γ_1-globulin (Fig. 4 *B*, Fig. 6 *D*) and the minor one a 7S β_{2A}-globulin (Fig. 4 *B*). These findings are described more extensively below.

Antibody fractions which were contaminated with antigen were further purified by zone electrophoresis in a saturated lactose solution. Figs. 1 *B* and 1 *C* present the results of lactose starch block electrophoresis of the proteins of peak II and peak III from the DEAE chromatogram of Fig. 1 *A*. These preparations did not contain enough antigen to result in significant absorption at 365 mμ. The major, more rapidly migrating components shown in Figs. 1 *B* and 1 *C*, were also separated by the electrophoretic procedure from a small amount of 7S γ-globulin which was eluted in this region of the chromatogram. The lactose starch block technique was of particular value when antigen was to be removed from antibody in preparations obtained by stepwise elution of the DEAE cellulose columns. In general, three regions were then obtained. The cathodal region of the block contained antibody which was relatively free of antigen. A mid-region contained both antibody and antigen while the anodal region of the block contained antigen but little antibody. Various starch block fractions were pooled (Figs. 1 *B* and 1 *C*), concentrated by ultrafiltration, and lactose was removed by extensive dialysis at 4°C against saline solutions. The small amount of precipitate which formed was removed by centrifugation.

Purity of Antibody Preparations.—Antibody preparations were shown to be free of antigen by two methods. First, preparations which had been precipitated with Lac-HSA were examined in agar diffusion studies using antisera which reacted strongly with human serum albumin. Although such antisera detected Lac-HSA at concentrations of 0.05 mg/ml or greater, they failed to detect any contaminating antigen in the purified antibody preparations described below, whose protein concentrations were 3 mg/ml or greater.

Secondly, the optical density ratio OD (280 mμ)/OD (365 mμ) of antibody preparations in a solution of 0.2 M NaCl, 0.01 M phosphate, pH 7.8, was measured in the Zeiss spectrophotometer. The values of this ratio for the three antibody preparations described below which contained significant amounts of

the more rapidly sedimenting components were 26.8 (Fig. 3 *B*), 45.6 (Fig. 4 *B*, Fig. 6 *D*) and 94.0 (Fig. 6 *E*). The corresponding ratios of the Lac-HSA and Lac-Hy antigens used in the specific precipitation were measured in the same solvent and were 0.74, 0.57, and 0.91, respectively. Therefore, the maximum

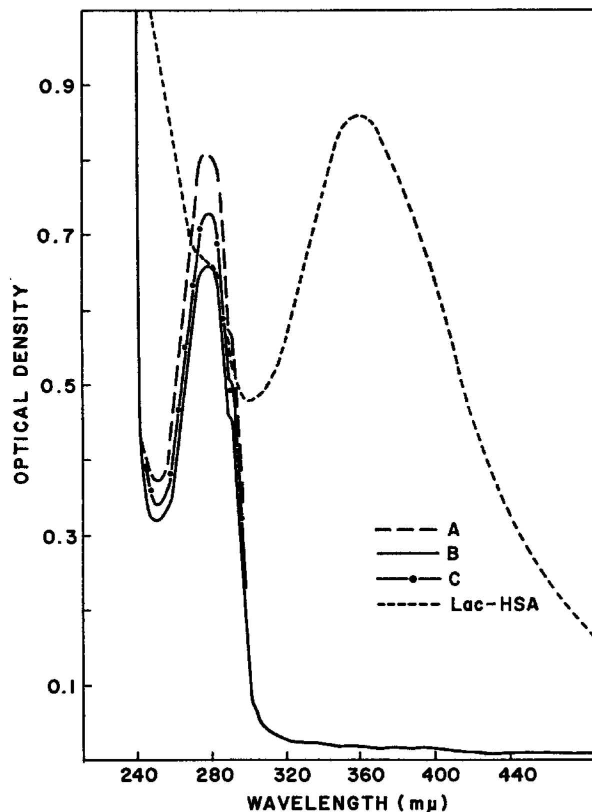

FIG. 2. Absorption spectra of purified equine anti-Lac antibody preparations and of Lac-HSA antigen. *A*, 7S β_{2A}-globulin antibody from peak II of the gradient elution DEAE chromatogram of Fig. 1 *A* (Fig. 4 *A*, Fig. 6 *C*). *B*, Mixture of 7S β_{2A}- and 10S γ_1-globulin antibodies of peak III of the DEAE chromatogram of Fig. 1 *A* (Fig. 4 *B*). *C*, Antibody preparation (Fig. 6 *E*) used for construction of the hapten-binding curve (Fig. 8).

amounts of antigen contained in these three preparations were of the order of 3, 1.2, and 1 per cent, respectively. The absorption spectra of selected antibody preparations were determined by use of the Cary recording spectrophotometer. Fig. 2 illustrates the comparative spectra of three antibody preparations and of Lac-HSA. The 365 mμ peak of the antigen spectrum was absent from the absorption spectra of the purified antibodies. Preparation *A* was the 7S β_{2A}-

antibody of peak II of the DEAE chromatogram illustrated in Fig. 1 *A*, and preparation *B* was the mixture of 10S γ_1- and 7S β_{2A}-antibodies of peak III from the same chromatogram (Fig. 1 *A*, Fig. 4 *B*). Preparation *C* was composed principally of 7S β_{2A}-antibody, but also contained a significant amount of more rapidly sedimenting components (Fig. 6 *E*), and was the preparation used for construction of the hapten-binding curve described below (Fig. 8). The immunologic and ultracentrifugation characteristics of the antibodies of these preparations are described in detail below.

Sedimentation Properties of Antibody Preparations.—Purified antibody preparations were examined in the analytical ultracentrifuge and also used for equilibrium dialysis. Antibody solutions from the binding experiments, containing the Lac dye, were then subjected to analytical ultracentrifugation using combined schlieren and absorption optics. Components with sedimentation coefficients greater than 7S were present in antibody preparations from the second part of the DEAE chromatograms (Fig. 3 *B*, Fig. 4 *B*). Binding of more than one hapten molecule per 150,000 mol wt protein unit in no instance altered significantly the distribution of the variously sedimenting components. The combination of schlieren and absorption optics allowed recognition of the hapten-binding activity of components with different sedimentation velocities. The results of one such experiment are illustrated in Fig. 3. The single hapten-binding component of Fig. 3 *A* had been eluted by DEAE chromatography with initial solvent and had a sedimentation coefficient ($s_{20,w}$) of 6.5S. The components of Fig. 3 *B* had been eluted from the same DEAE cellulose column with 1.0 M NaCl and freed of the antigen, Lac-HSA, by electrophoresis in lactose solution. Preparation *B* bound 1.1 moles of hapten per 150,000 gm of protein at a free Lac dye concentration of 0.9×10^{-5} M. The major hapten-binding component of Fig. 3 *B* had a sedimentation coefficient ($s_{20,w}$) of 9.5S. The corrected sedimentation coefficients of the three other components were 3.5S, 6.8S, and 16.1S (Fig. 3 *B*).

The sedimentation characteristics of the hapten-binding components of the two adjacent protein peaks, II and III (Fig. 1 *A*), of the gradient elution DEAE chromatogram previously described are illustrated in Fig. 4. The major hapten-binding component of peak II (Fig. 4 *A*) had a sedimentation coefficient ($s_{20,w}$) of 6.7S. The agar diffusion studies to be described below established that this was a β_{2A}-globulin (Fig. 6 *C*). Peak III contained two hapten-binding components with different sedimentation velocities (Fig. 4 *B*). The more slowly sedimenting component had a sedimentation coefficient ($s_{20,w}$) of 6.8S. Agar diffusion studies established that it was a β_{2A}-globulin which was antigenically identical with the major component of peak II. The principal hapten-binding component of peak III had a sedimentation coefficient ($s_{20,w}$) of 9.4S. The agar diffusion studies to be described presently (Fig. 6 *D*, Fig. 7) indicated that this more rapidly sedimenting, major hapten-binding component of peak III was a

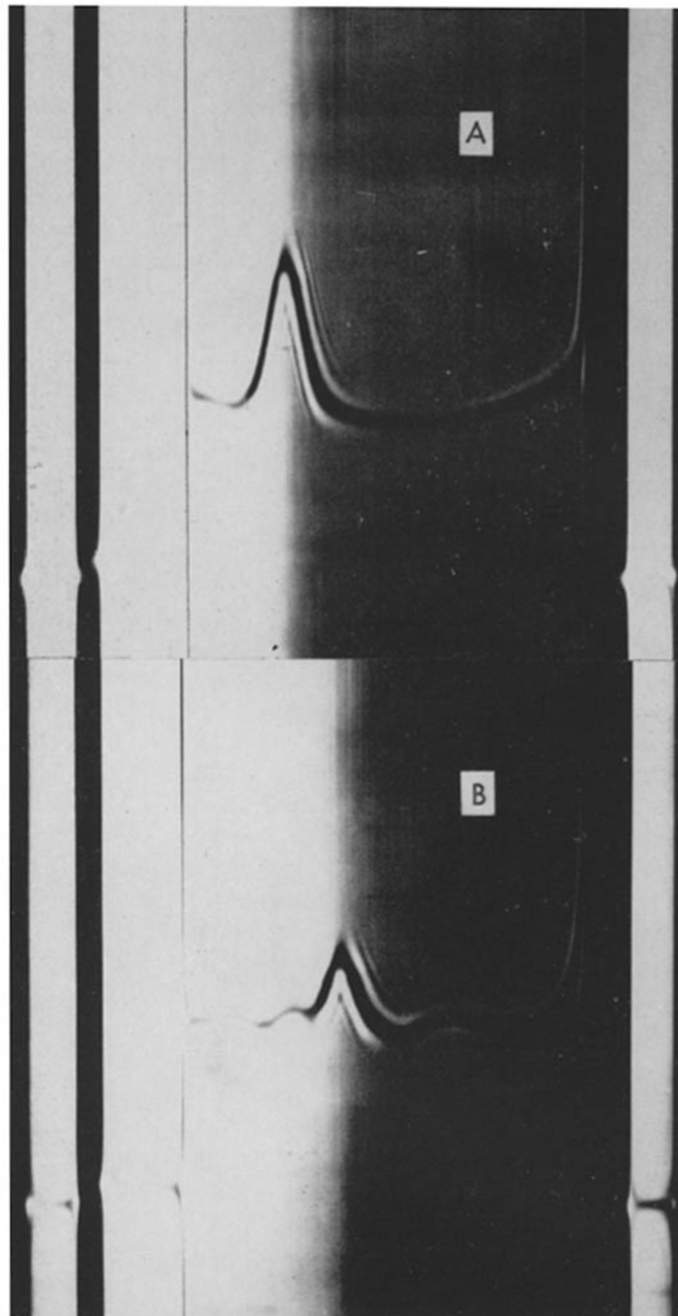

FIG. 3. Analytical ultracentrifugation of equine anti-Lac antibody equilibrated with Lac dye, employing a combination of schlieren and absorption optics. Photographs taken at 52 minutes. Schlieren interference bar angle 45°. *A*, Anti-Lac antibody eluted by DEAE chromatography with a solvent containing 0.5 M lactose, 0.02 M phosphate, pH 7.2. *B*, Anti-Lac antibody eluted from the same DEAE cellulose column with 1.0 M NaCl in initial solvent and freed of antigen, Lac-HSA, by electrophoresis in a solution saturated with lactose.

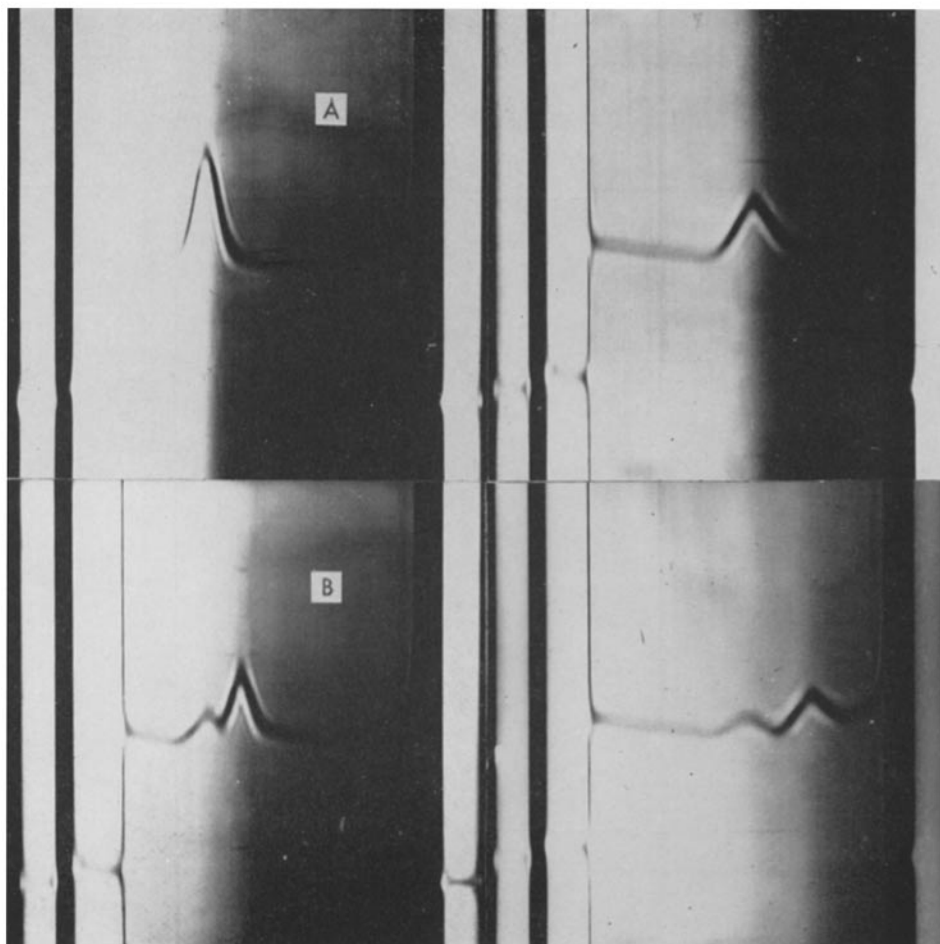

FIG. 4. Analytical ultracentrifugation of equine anti-Lac antibody equilibrated with Lac dye. Photographs taken at 52 and 96 minutes. Schlieren interference bar angle 45°. *A*, The β_{2A}-globulin anti-Lac antibody of peak II of the gradient elution DEAE chromatogram of Fig. 1 *A* (Fig. 6 *C*). *B*, The 7S β_{2A}- and 10S γ_1-globulin antibodies of peak III of the chromatogram of Fig. 1 *A* (Fig. 6 *D*).

γ_1-globulin which differed antigenically from the 7S β_{2A}-globulin components of peaks II and III. When peak III was centrifuged in one compartment of a double sector cell, a base line having been established by placing solvent in the second compartment, it was evident that a more rapidly sedimenting shoulder was also present. The components of peak III (Fig. 4 *B*) bound 1.2 moles of hapten per 150,000 gm of protein at a free Lac dye concentration of 0.6×10^{-5} M.

The extrapolated sedimentation coefficient ($s^0_{20,w}$) of the γ-globulin antibody contained in the initial solvent peak of a DEAE chromatogram, illustrated in Fig. 6 *B*, was 6.8S.

Immunologic Properties of Purified Anti-Lac Antibodies.—The anti-Lac antibody was further fractionated into different molecular species preliminary to

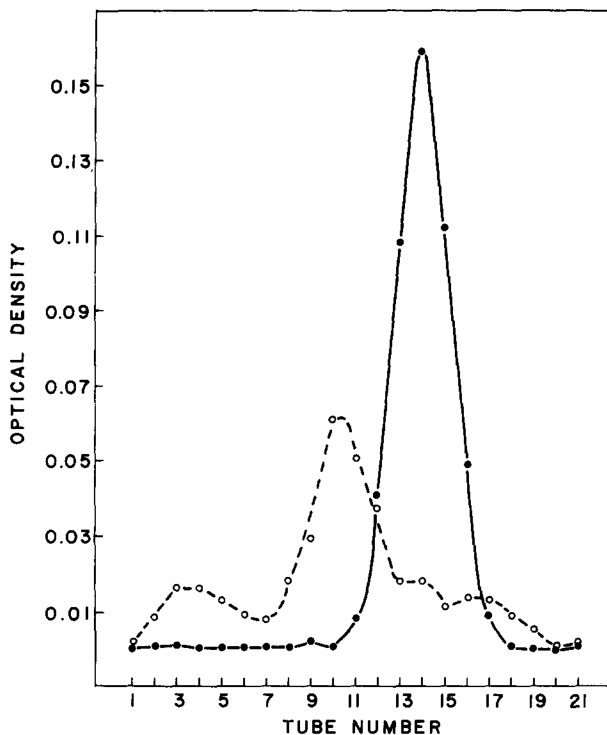

FIG. 5. Sucrose density gradient ultracentrifugation of purified equine anti-Lac antibody. ●——●, anti-Lac antibody of Fig. 3 *A*; ○——○, anti-Lac antibody of Fig. 3 *B*. The more rapidly sedimenting components are to the left.

immunochemical studies. Sephadex G-200 gel filtration (20), DEAE cellulose column chromatography, and zone electrophoresis were employed in certain instances to isolate a less heterogeneous population of antibody molecules. In the majority of instances, sucrose density gradient ultracentrifugation was utilized as the final purification step. The results of a typical density gradient ultracentrifugation experiment are presented in Fig. 5. The two preparations whose analytical ultracentrifugation patterns are illustrated in Fig. 3 had been sedimented in separate centrifuge tubes simultaneously. The change of protein concentration in the serially collected fractions compared closely to the schlieren

pattern seen on analytical ultracentrifugation (Fig. 3). Density gradient ultracentrifugation of the materials of peak II and peak III of the gradient elution DEAE chromatogram (Fig. 1 *A*) also resulted in a curve of protein concentration which corresponded closely to the schlieren pattern seen on analytical ultracentrifugation (Fig. 4). Materials were taken from density gradient tubes containing the peaks of protein concentration and used for immunoelectrophoresis and agar diffusion studies.

A minimum of six antigenically distinct immunoglobulins have been identified in purified equine anti-Lac antibody preparations by these studies. The results obtained by immunoelectrophoresis are summarized in Fig. 6. Rabbit antisera prepared against normal equine serum were placed in the lateral troughs. The central well of Fig. 6 *A* was filled with normal equine serum, and the 7S γ-globulin anti-Lac antibody of the initial solvent peak of a DEAE chromatogram was placed in the central well of Fig. 6 *B*. Three antigenically different 7S γ-globulins (A, B, C), which were distinct from the β_{2A}-, 10S γ_1-, and 19S γ-components described below, were contained in the first protein peak. A more detailed description of the properties of these antibodies will be published elsewhere (15). The 7S β_{2A}-globulin antibody of peak II of the gradient elution DEAE chromatogram (Fig. 1 *A*, Fig. 4 *A*), and the 10S hapten-binding component of peak III (Fig. 4 *B*) from the same DEAE chromatogram, further purified by prolonged density gradient ultracentrifugation, were placed in the central wells of Fig. 6 *C* and Fig. 6 *D*, respectively. The antibody of the preparation used for construction of the hapten-binding curve (Fig. 8) was placed in the central well of Fig. 6 *E*. The central well of Fig. 6 *F* was filled with a 19S component isolated by sephadex G-200 gel filtration, DEAE cellulose column chromatography, and finally density gradient ultracentrifugation from a purified anti-Lac antibody preparation.

Isolated components of the purified antibody preparations and isolated 7S γ- and 19S γ-globulins from normal equine serum were compared in agar diffusion studies, using a variety of anti-equine antisera. Fig. 7 summarizes the results of studies in which the isolated antibody components used for immunoelectrophoresis (Fig. 6) together with isolated normal equine 19S γ-globulin were compared. Four different antisera, prepared against normal equine serum, were used. Antigens were placed in the central wells and two antisera were allowed to diffuse from the opposing troughs cut along the lateral edges of the microscope slide. The 19S component isolated from a purified antibody preparation (19S ab) was antigenically closely related to the 19S γ-globulin obtained from normal equine serum (N19Sγ). The several components of the antibody preparations illustrated in Fig. 6 all showed antigenic individuality. The precipitin line of each component penetrated the precipitin line of each of the others when the proper antiserum was employed. With other antisera, however, there was a lack of prominent spur formation. For

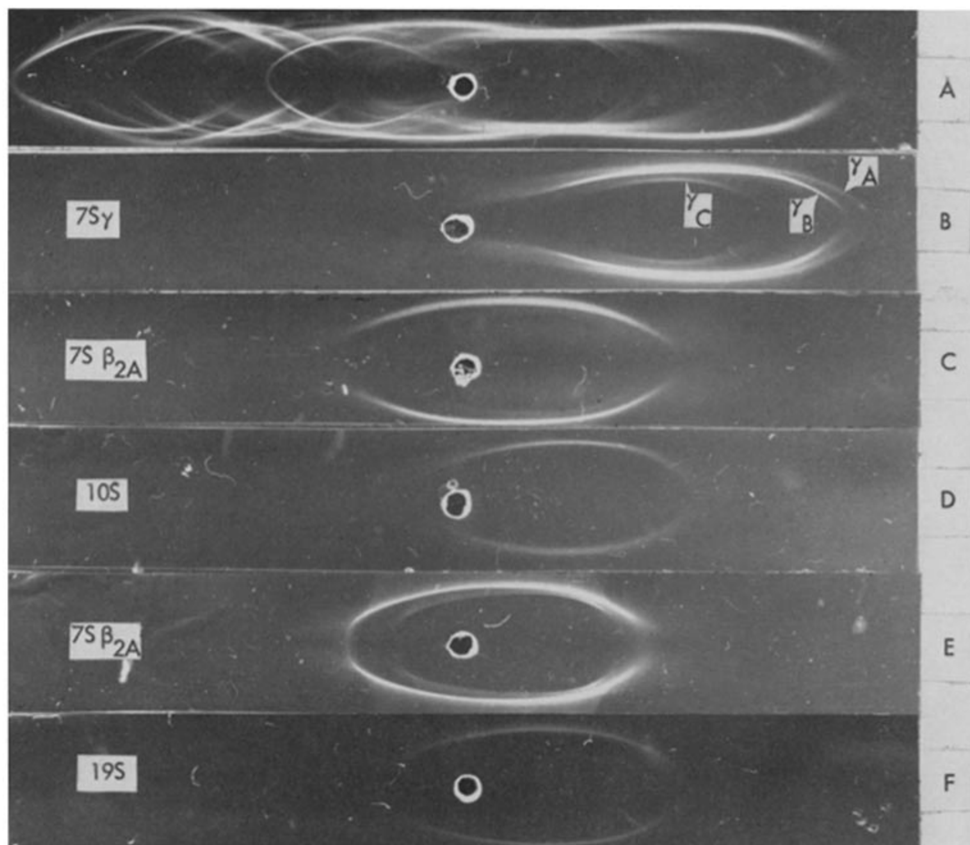

FIG. 6. Immunoelectrophoresis of equine serum and purified equine anti-Lac antibodies. Rabbit antiserum prepared against normal equine serum filled the lateral troughs. *A*, normal equine serum; *B*, 7S γ-globulin anti-Lac antibodies; *C*, 7S β_{2A}-globulin anti-Lac antibody of Fig. 4 *A*; *D*, isolated 10S γ_1-globulin anti-Lac antibody of Fig. 4 *B*; *E*, antibody preparation used for the construction of the hapten-binding curve of Fig. 8; *F*, 19S component isolated from purified equine anti-Lac antibody.

example, a prominent spur of the precipitin line of the 10S γ_1-antibody (10S II) extended through the precipitin lines of the 7S γ-antibodies when antiserum 107 (Fig. 7 *D*) was used but did not develop when antisera 105 and 108 were used (Figs. 7 *C* and 7 *D*). Immunoelectrophoresis and agar diffusion studies which are not illustrated demonstrated that absorption of antisera with an excess of electrophoretically and chromatographically isolated normal equine 7S γ-globulins did not remove all of the antibodies which reacted with the isolated 10S γ_1-antibody. Prominent spurs also formed between the 10S γ_1-antibody (10S II) and the 19S γ-antibody component with antisera 105 and

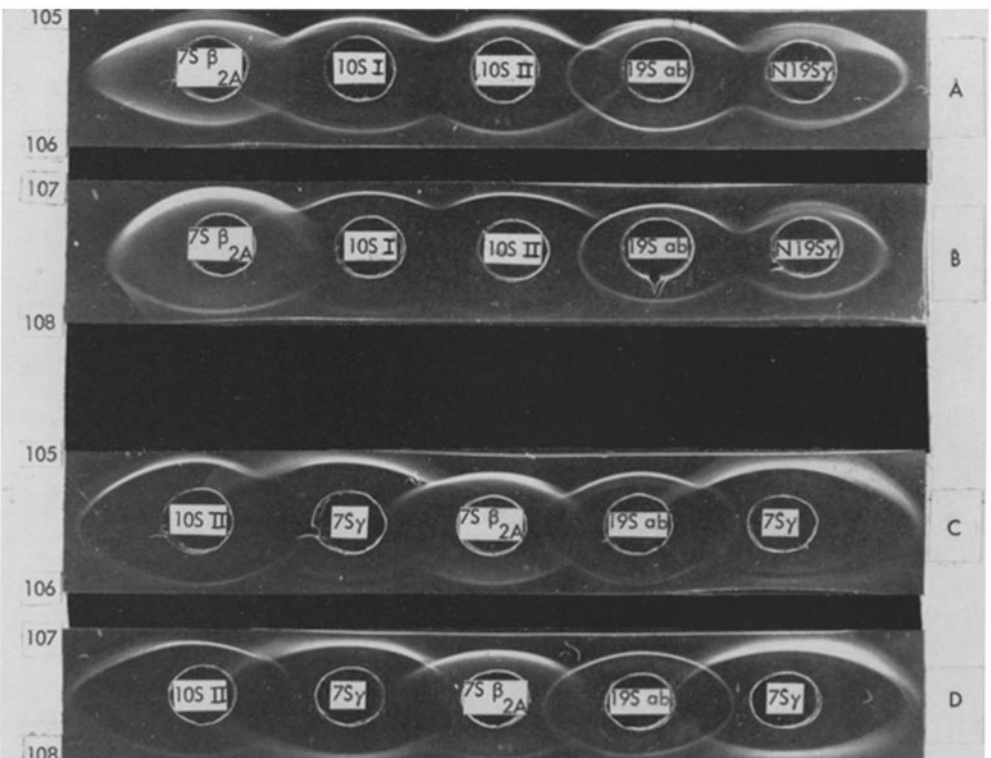

FIG. 7. Double diffusion agar studies with the purified equine anti-Lac antibodies of Fig. 6 and isolated normal equine 19S γ (γ_{1M})-globulin. Four rabbit antisera (105, 106, 107, and 108) prepared against normal equine serum filled the lateral troughs. 7S β_{2A}-*antibody* of Fig. 6 *C*. 10S I–isolated antibody of Fig. 3 *B* originally precipitated with Lac-HSA. 10S II–isolated antibody of Fig. 4 *B* originally precipitated with Lac-Hy (Fig. 6 *D*). 19S ab-γ_{1M}–component isolated from purified anti-Lac antibody (Fig. 6 *F*). N 19S γ-normal equine γ_{1M}-globulin. 7S γ-antibodies of Fig. 6 *B*.

106 (Fig. 7 *A*), and between 10S γ_1-antibody (10S I) and the 7S β_{2A}-antibody with antisera 106 and 107 (Figs. 7 *A* and 7 *B*). The antigenic individuality of the 10S γ_1-antibody and the 7S β_{2A}-antibody was also demonstrated in immunoelectrophoretic experiments in which mixtures of these two components were placed in the central well and separated by electrophoresis. Similar antigenic individuality was evident when the other antibodies were compared in like manner. The precipitin line of the 7S β_{2A}-antibody penetrated the precipitin lines of the 7S γ-antibodies (Figs. 7 *C* and 7 *D*) and also penetrated the precipitin lines of isolated normal equine 7S γ-globulins in experiments which are not illustrated. The spurs which formed between the various antibodies often deviated away from the antigen well and toward the antibody trough. The two isolated 10S γ_1-antibody preparations, the first of which (Fig. 7, 10S I) had been prepared with Lac-HSA (Fig. 3 *B*) and the second (Fig. 7, 10S II) with Lac-Hy (Fig. 4 *B*) were found to be antigenically identical with all of the antisera that were employed.

Isolated normal equine 7S γ-globulins were also found to be antigenically heterogeneous in agar diffusion studies, and contained components which corresponded both antigenically and in electrophoretic mobility to the three components of the 7S γ-globulin anti-Lac antibody (Fig. 6 *B*). These antigenically different components of the 7S γ-globulins were distinct from the β_{2A}-globulins, the 10S γ_1-globulin, and the 19S γ-globulins (Fig. 7). A more detailed account of these findings will be published elsewhere (15). The 3.5S component of Fig. 3 *B* was related to equine 7S γ-globulins but was antigenically relatively deficient.

Hapten-Binding Activity.—A preparation of anti-Lac antibody which was composed principally of 7S β_{2A}-globulin (Fig. 6 *E*) and which was contaminated with a minimum of 7S γ-globulins was used to construct a hapten-binding curve (Fig. 8). The antibody of this preparation was recovered from a DEAE cellulose column by elution with a linear positive gradient of NaCl in lactose solvent as previously described. The concentrated proteins from the chromatogram were dialyzed against a solution of 0.02 M phosphate, pH 7.8, and then rechromatographed on a DEAE cellulose column, eluting now with a linear NaCl gradient in the solution of 0.02 M phosphate, pH 7.8. Analytical ultracentrifugation indicated that the preparation consisted of a major component with a sedimentation coefficient ($s_{20,w}$) of 6.7S and a more rapidly sedimenting shoulder whose principal component had a sedimentation coefficient ($s_{20,w}$) of 9.4S.

Equilibrium dialysis (14) was carried out at 25.0°C in a constant temperature bath. Samples of a protein solution of 3.24 mg/ml were run in duplicate at each of several Lac dye concentrations. The results of these experiments are presented in two ways in Fig. 8 in terms of r and c in which r is equal to the average number of moles of hapten bound per 150,000 gm of antibody at the free equilibrium hapten concentration c. The r *vs.* c plot indicated that 2 moles

of hapten were bound per 150,000 gm of protein at high free Lac dye concentrations. The vertical bars of the r/c *vs.* r plot represent the estimate of uncertainty of the r/c values at lower free hapten concentrations. The duplicate free Lac dye OD readings at the highest r/c value were 0.008 and 0.009.

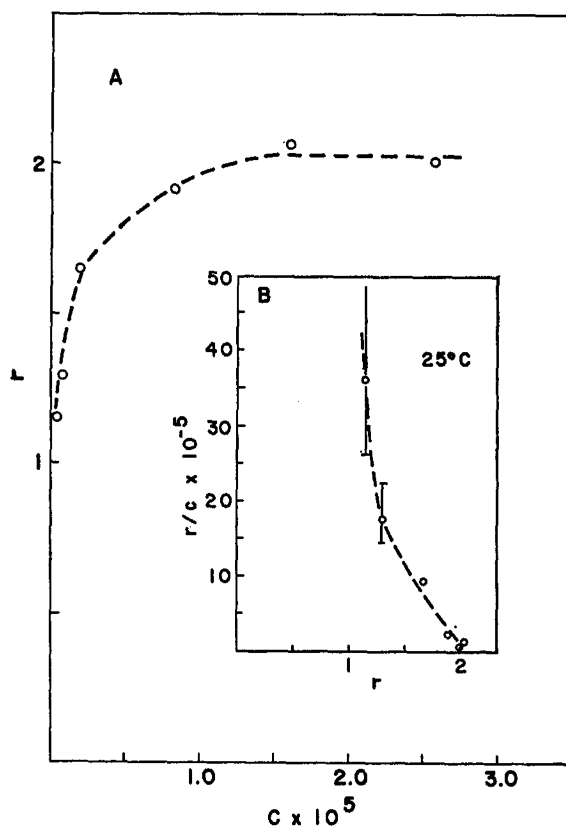

FIG. 8. Hapten-binding curve constructed with the antibody of Fig. 6 *E*. r is the number of moles of hapten bound per 150,000 gm of antibody at the free Lac dye concentration c. Data are represented in 2 different plots in *A* and *B*. Vertical bars of the r/c *vs.* r plot represent the estimated uncertainty of the r/c values at low free hapten concentrations.

DISCUSSION

The antigenically distinct 7S antibody of high electrophoretic mobility which eluted in the second part of the DEAE chromatogram has been referred to as a β_{2A} (γ_{1A})-globulin because of the similarity of its immunoelectrophoretic and physical chemical characteristics to those of the β_{2A}-globulins of other species (6). The antigenic relationship between β_{2A}-globulins and certain myeloma proteins has been of value in the identification of this component of

normal human and mouse serum (6, 22, 23). The availability of human sera which lack only specific β_{2A}–antigenic determinants (24) has also aided in the identification of human β_{2A}-globulins. Such comparative aids, however, are not presently available for the identification of equine β_{2A}-globulins. The T component of hyperimmune equine serum apparently represents an elevated level of equine β_{2A}-globulin (25, 26). The emerging complexity of the antigenic interrelations among the various immunoglobulins (27–32) indicates that the current nomenclature must be applied with the reservation that it may be altered as new evidence appears. Nevertheless, the fact that the precipitin line of this component of purified equine anti-Lac antibody penetrated the precipitin lines of both the 7S γ-globulin anti-Lac antibodies and the electrophoretically and chromatographically isolated normal equine 7S γ-globulins in agar diffusion studies supports the use of the present terminology. β_{2A}-globulin antibodies have been isolated from and identified in the sera of other species (6–9, 33). The relationship between the equine 7S γ_C- and 7S β_{2A}-antibodies and the guinea pig 7S γ_1-antihapten antibody (34) is not clear.

The data presented in the hapten-binding curve (Fig. 8) together with the measured sedimentation coefficient of approximately 7S indicate that the β_{2A}-antibody has a molecular weight of approximately 150,000 (*cf* reference 35) with two combining sites per molecule. The high affinity of the antibody of this preparation precluded an accurate determination of the average association constant (K_A) from the r/c value at r equal to 1 because of the very low concentration of free Lac dye in this region of the binding curve. Nevertheless, an approximate but reasonable estimate of 10^7 liters/mole for K_A may be made from an extrapolation of the binding curve to a value for r of 1. The increased concentration of NaCl in the region of the DEAE chromatogram in which this antibody eluted may have aided in effecting the dissociation of anti-Lac antibody of high affinity from the uncharged Lac group. High concentrations of NaCl have been used effectively to dissociate equine antipneumococcal polysaccharide antibody from antigen (2). The deviation from linearity of the r/c *vs.* r plot indicates that there was a significant heterogeneity of the K_A values of the antibodies of this preparation (14). The data presented in the hapten-binding curve (Fig. 8) provide evidence for the absence (<10 per cent) of any substantial amount of inactive protein in this antibody preparation. This is inferred from the maximum r value of 2 based on a molecular weight of 150,000.

The analytical ultracentrifugation experiments in which a combination of schlieren and absorption optics has been employed (Figs. 3 and 4) establish that the 10S component present in purified antibody preparations specifically bound the Lac dye hapten. The results of the hapten-binding studies by equilibrium dialysis with the preparations of Fig. 3 *B* and Fig. 4 *B* described above indicate that the antibodies of Fig. 3 *B* had an average K_A value of the order

of 10^5 liters/mole and that the antibodies of Fig. 4 *B* had an average K_A value greater than 10^5 liters/mole. In both cases it was evident from the ultracentrifugation experiments (Figs. 3 *B* and 4 *B*) that the principal hapten-binding component was the 10S antibody. These same equilibrium dialysis data indicate that the antibodies of Figs. 3 *B* and 4 *B* had r values greater than 1 per 150,000 gm of protein and, therefore, there was more than 1 mole of sites per 150,000 gm of antibody.

The evidence which establishes that the antibody preparations containing the more rapidly sedimenting components are free of significant antigen eliminates antigen-antibody complex formation as an explanation of the higher sedimentation coefficients. The antibody preparation of Fig. 4 *B*, which was composed principally of the 10S component, contained a maximum of 1.2 per cent Lac-Hy antigen. As it would be anticipated that dialysis of an antigen-antibody complex against hapten would disrupt at least a part of the complex, the fact that binding of more than 1 mole of hapten per 150,000 gm of antibody protein did not alter the distribution of the variously sedimenting components also supports this conclusion. In addition, preliminary experiments have demonstrated that dialysis of the antibody of Fig. 4 *B* against 0.15 M mercaptoethanol followed by dialysis against 0.02 M iodoacetamide reduced the 10S component to subunits with sedimentation coefficients of approximately 7S (36). The 7S subunits retained a major portion of the hapten-binding activity (36).

Of particular interest was the observation that the 10S antibody was not antigenically identical with any of the other anti-Lac antibodies. The prominent spur formation observed between the 10S antibody and each of the other immunoglobulins is evidence that each of these proteins possesses antigenic determinants not shared by the others. The fact that specific rabbit antibodies reacting with the unique antigenic determinants of the 10S antibody were present in rabbit antisera prepared by immunization with normal equine serum indicates that normal equine serum contained a component antigenically similar to the 10S antibody. Immunoelectrophoretic experiments where normal equine serum was placed in the central well and separated by electrophoresis, and then isolated 10S antibody and rabbit anti-equine serum antiserum were allowed to diffuse from the two opposing lateral troughs (37) have also demonstrated the existence of a normal equine serum counterpart of the 10S antibody by the deviation of the 10S antibody precipitin line in the γ_1-region (36). Since the antigenic determinants which distinguish the 7S γ-, β_{2A}-, and the 19S γ-globulins have been found on the H(A) chains (38, 39), the present findings suggest that the 10S antibody has a unique H(A) chain and is representative of a hitherto undescribed class of immunoglobulins. The observation that spur formation was much less pronounced with certain antisera together with the observed deviation of certain spurs from the antigen well toward the antibody

trough is evidence that these several antibody components also share certain common antigenic determinants.

The existence of a group of human antibodies with sedimentation coefficients between 7S and 19S was demonstrated by sucrose density gradient ultracentrifugation experiments (13). Two observations indicated that the intermediate rates of sedimentation were not a function of aggregation of random 7S γ-globulin molecules (13). First, isohemagglutinins with intermediate sedimentation velocities, of relatively high titer, were found in certain sera which lacked 7S γ-globulin antibodies of the same specificity. Secondly, DEAE chromatography fractions which contained the intermediately sedimenting isohemagglutinins showed a marked disproportion between antibody titer and the content of 7S γ-globulins detectable with specific antisera. Filitti-Wurmser, Aubel-Lesure, and Wurmser had measured the rate of sedimentation of the agglutinating activity of human isohemagglutinin sera by centrifugation in partition cells, and concluded that isohemagglutinins with intermediate molecular weights exist (40, 41). However, it was suggested that mixtures of 7S and 19S antibodies could explain these experimental results (42). A large number of the isohemagglutinin sera examined have been found to contain a mixture in various combinations of 7S, 19S, and intermediately sedimenting antibodies (13). It is likely that certain of the isohemagglutinins which were found to elute in an intermediate zone on sephadex G-200 gel filtration by Killander and Högman (43, 44) have intermediate sedimentation velocities although it should be noted that it has also been demonstrated that 7S β_{2A}-globulins are eluted before the 7S γ-globulins upon filtration through sephadex G-200 (45). Human skin-sensitizing antibodies have also been shown to have intermediate sedimentation coefficients (13, 46–49). The human reaginic antibodies in general sediment less rapidly than a number of the human isohemagglutinins with intermediate sedimentation velocities (13, 46), and the possibility that more than a single molecular form of antibody with sedimentation coefficients between 7S and 19S exists must be considered. Additional examples of antibodies with intermediate rates of sedimentation have recently been reported and include human and rabbit antibodies directed against salmonella antigens and rabbit antibodies directed against viral antigens (50–52).

The equine 10S γ_1–anti-Lac antibody described in the present study may be related to the components with unusual sedimentation characteristics which have been observed in certain equine antipneumococcal antibody preparations (4).

SUMMARY

Anti-*p*-azophenyl-β-lactoside (Lac) antibody has been isolated from equine antiserum by specific precipitation with Lac-HSA (human serum albumin) and Lac-Hy (hemocyanin). Antibody was separated from antigen by chroma-

tography on DEAE cellulose and zone electrophoresis in solvents containing lactose. Six antigenically distinct immunoglobulins have been identified in purified equine anti-Lac antibody: 7S β_{2A}-globulin, 19S γ-globulin, a 10S γ_1 globulin, and three antigenically distinct 7S γ-globulins. The specific hapten-binding activity of the 7S β_{2A}-antibody and of the 10S γ_1-antibody has been demonstrated by equilibrium dialysis and by analytical ultracentrifugation using a combination of schlieren and absorption optics. The 10S γ_1-globulin antibody may be representative of a hitherto undescribed class of immunoglobulins.

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