EQUINE ANTIHAPTEN ANTIBODY*

The Subunits and Fragments of Anti-β-Lactoside Antibody

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A large body of experimental evidence has established that there is extensive variation in the structure of the proteins which comprise the immunoglobulin systems of a number of species. Heterogeneity of equine immunoglobulins was described in early studies (1–3), and an increasing body of evidence has accumulated which indicates that the horse is capable of synthesizing a multiplicity of molecular forms of antibody (4–12). Six antigenically unique immunoglobulins were identified in purified equine anti-β-lactoside antibody isolated from the serum of a single horse: γGa-, γGb-, γGc-, γA-, 10Sγ$_1$- and γM-globulins (4, 6).[1] The several forms of anti-β-lactoside antibody have been found to differ in their molecular size, electrophoretic mobility, chromatographic behavior, ability to fix complement and precipitate antigen, and in their affinity for hapten (4–7). The observation that a single animal is capable of forming a multiplicity of distinct immunoglobulins with specificity for a single haptenic grouping is relevant to considerations of the relationship between the heterogeneity of the structure of the immunoglobulins and the mechanism of acquisition of immunological specificity. The submolecular localization of common and unique structural features of the equine anti-β-lactoside antibodies has been further defined in the present investigations.

Materials and Methods

Isolation of Antibody.—The preparation and characterization of the antigens, and the immunization of the horse have been described previously (4, 6). Anti-Lac antibody was isolated by specific precipitation with Lac-HSA (human serum albumin) (4, 22) or by coprecipitation with Lac-Hy (hemocyanin, *Limulus polyphemus*) (7).

Column Chromatography.—DEAE-cellulose column chromatography at 4°C was used to

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[1] The nomenclature assigned to the equine immunoglobulins has been selected to conform to the system of nomenclature proposed for human immunoglobulins by a committee organized by the World Health Organization (13).

prepare subfractions of equine anti-Lac antibody and immunoglobulins (4, 6). Purified anti-Lac antibody, equine serum, and serum proteins precipitated with 50% saturated ammonium sulfate were equilibrated with 0.005–0.02 M sodium phosphate buffer, pH 8, and passed over DEAE-cellulose (Whatman DE 11, 1.0 meq/g) columns equilibrated with the same solvent. Retained components were eluted with either a stepwise increase or a positive linear gradient of sodium chloride.

Zone Electrophoresis.—Zone electrophoresis was performed in either a starch-supporting medium (16) or a Pevikon® (Fosfatbolaget, Stockholm, Sweden)—supporting medium (17), in a barbital-buffered solution of $\Gamma/2$ 0.05, pH 8.6, at 4°C.

Enzymatic Digestion.—Immunoglobulins at a concentration of 1–10 mg/ml were incubated in 0.2 M sodium acetate buffer, pH 4.5, with 1–2% (w/w) pepsin (2 × crystallized, Worthington Biochemical Corp., Freehold, N. J.) at 37°C for 4–96 hr (8, 10, 18). The low molecular weight products of peptic digestion were separated from the pepsin 5S Fab_2' fragments either by exhaustive dialysis, or by Sephadex G-100 gel filtration. Immunoglobulins at a concentration of 1–10 mg/ml were incubated in 0.2 M sodium acetate buffer, pH 5.6, 0.01 M cysteine, 0.002 M EDTA (ethylenediaminetetraacetic acid) with 1–2 % (w/w) papain (2 × crystallized, Worthington Biochemical Corp.) at 37°C for 4–48 hr (19, 20).

Preparation of Heavy and Light Polypeptide Chains.—Equine immunoglobulins in 0.2 M Tris-HCl (Tris(hydroxymethyl)aminomethane), pH 8, 0–10 M urea (3 × recrystallized) at concentrations of 5–15 mg/ml, were incubated at room temperature under a stream of nitrogen with 0.1–0.4 M 2-mercaptoethanol for 1–6 hr. A 10–20% molar excess of iodoacetamide (3 × recrystallized) was added without exposure to atmospheric oxygen and alkylation was allowed to progress at room temperature for 30 min. The heavy (A, H) and light (B, L) polypeptide chains were separated by Sephadex gel filtration either in 1.0 N propionic acid at 4°C (21), or in 0.04 M sodium decyl sulfate, 0.002 M EDTA, 0.01 M sodium phosphate buffer, pH 7.5, at room temperature (22). The sodium decyl sulfate was generously supplied by Dr. F. Karush. Three columns (4 × 60 cm) of either Sephadex G-100, G-150, or G-200 were prepared in the appropriate solvent and connected in series. The void volume (V_0) of each column set was taken as the volume at which Blue Dextran 2000 (Pharmacia, Uppsala, Sweden) was recovered. Reduced and alkylated proteins were initially mixed with detergent before application to the columns of Sephadex in decyl sulfate. Soluble preparations of reduced and alkylated proteins were applied directly to the Sephadex columns in cold propionic acid. Polypeptide chains were concentrated and dialyzed against 0.01 M Tris-HCl or 0.01 M sodium phosphate–buffered solvents of pH 6.5–8.5 containing 0–0.2 M sodium chloride at 4°C. Detergent micelles were removed by Sephadex G-100 gel filtration, and sodium decyl sulfate was eliminated by dialysis against a quaternary amine anion exchange resin (Rexyn RG 1, Fisher Scientific Company, Fair Lawn, N. J.) (22).

Equilibrium Dialysis.—The interaction of antibody with the hapten dye *p*-(*p*-dimethylaminobenzeneazo)-phenyl-β-lactoside (Lac dye) was measured by equilibrium dialysis at 4°, 25°, and 37°C. Lac dye concentrations were determined optically by employing a molar extinction coefficient, ϵ, at the wave length of maximum absorption in neutral solvents, 455 mμ, of 2.48×10^4 (14). Tritiated Lac dye synthesized with generally labeled dimethylaniline (23) was also used for equilibrium dialysis. The Lac dye and the tritiated Lac dye were generously supplied by Dr. F. Karush. Radioactivity was measured in a liquid scintillation spectrometer employing the scintillation solution described by Bray (24). Protein concentrations were determined optically from the absorbancy at 280 mμ. The quantity of Lac dye or tritiated Lac dye absorbed to the dialysis cell components, as a function of the final free hapten concentration, was determined by equilibrium dialysis in an identical manner excepting that protein solutions were replaced by buffer.

Fluorescence Quenching.—The association of Lac dye with antibody also was determined by

fluorescence quenching (25). Protein fluorescence was measured with a Turner Model 110 null-balance fluorometer (G. K. Turner Associates, Palo Alto, Calif.) (26). A Baird-Atomic A21 interference filter (Baird-Atomic, Inc., Cambridge, Mass.) with a peak wave length transmission region of 230–340 mμ was used as the primary filter. A Corning 7–60 glass filter (Corning Glass Works, Corning, N. Y.) with a peak transmission of 365 mμ and a Polaroid *1/10* neutral density filter (Polaroid Corporation, Cambridge, Mass.) were used as the secondary filter. The temperature of the holder housing the quartz cell was maintained at either 25° or 37°C by a flow of water from a thermostatically controlled bath. Lac dye at concentrations of 10^{-6}, 10^{-5}, or 10^{-4} M was added in 5–25 μl fractions with a microburet syringe. The data obtained with anti-Lac antibody were corrected for dilution and other factors by employing data obtained in an identical manner with equine γG-globulins isolated from the serum of a nonimmunized horse. Intrinsic association constants (K_0) (47) were calculated from the corrected data by assuming that an average of one mole of sites had been occupied by hapten per mole of antibody at that amount of added Lac dye which had resulted in one-half of the total obtainable reduction of corrected antibody fluorescence. Excitation and fluorescence spectra of equine immunoglobulins were determined at room temperature in 0.2 M sodium chloride, 0.01 M sodium phosphate buffer, pH 8, with an Aminco-Bowman spectrophotofluorometer (American Instrument Co., Inc., Silver Spring, Md.).

Absorption Spectroscopy.—The absorption spectra of equine immunoglobulins, antigens, and Lac dye were determined with either a Zeiss PMQ II spectrophotometer or a Cary Model 15 recording spectrophotometer at room temperature, in either 0.1 N NaOH or neutral solvents. Extinction coefficients were calculated on the basis of micro-Kjeldahl analysis, done in triplicate, assuming 16% N.

Ultracentrifugation.—Analytical ultracentrifugation was performed in a Beckman Model E centrifuge, equipped with a constant temperature control, at 20°C. Sedimentation coefficients were measured in 0.2 M sodium chloride, 0.01 M sodium phosphate buffer, pH 8, and corrected for solvent viscosity and density by use of a conversion factor of 1.048. A partial specific volume of 0.74 (41) was assumed for all proteins. A combination of schlieren and absorption optics (4) was used to demonstrate the sedimentation of Lac dye specifically bound to antibody. Polypeptide chains of anti-Lac antibody were sedimented in 0.0125 M sodium decyl sulfate, 0.2 M sodium chloride, 0.002 M EDTA, 0.01 M Tris-HCl, pH 8 (22). A conversion factor of 1.067 was used to convert sedimentation coefficients to values in water. Sucrose density gradient ultracentrifugation was accomplished as described previously (27). Samples were centrifuged either at 36,000 or 47,000 rpm in a SW 39 or SW 50 rotor, respectively, for periods of 12–36 hr in a Beckman Model L-2 centrifuge at 4–5°C.

Amino Acid Analysis.—The amino acid composition of the subunits of anti-Lac antibody was determined by the Piez and Morris modification (28) of the method of Spackman, Stein, and Moore (29). Samples were hydrolyzed for either 24 or 72 hr at 110°C. Amino acid analysis was performed in duplicate for each hydrolysate with a Technicon amino acid analyzer using a single column (0.9 × 122 cm, Technicon chromo-bead Type A resin). Norleucine was incorporated into each analysis as an internal standard. Conversion factors were determined from analyses of a standard mixture of amino acids performed at intervals between analyses of hydrolysates of antibody subunits.

Carbohydrate Analysis.—Hexose was determined by the method of Momose and coworkers (30) and hexosamine by the Elson-Morgan method as described by Belcher and coworkers (31). D-glucose (Fisher Scientific Company Certified Reagent) and D-glucosamine (A grade, Calbiochem, Los Angeles, Calif.) were used as standards.

Agar Diffusion Studies.—Immunoelectrophoresis was accomplished by the microtechnique of Scheidegger (32). The modified technique of immunoelectrophoresis in which antigen and antiserum are allowed to diffuse from two laterally placed troughs after electrophoresis of a

centrally placed protein preparation (33) was used to define antigenic relations between isolated proteins and the distinct components of the equine immunoglobulins, and to obtain a measure of the concentration of minor immunoglobulin components in antibody preparations. A range of concentrations of an isolated immunoglobulin was placed in one of the lateral troughs of a series of immunoelectrophoresis slides and allowed to diffuse for a standard time period against a selected antiserum in the opposing trough. The component of interest was identified by the fusion of its precipitin line with that of an identifiable component in the centrally placed, electrophoretically separated equine immunoglobulins. The concentration of a contaminating immunoglobulin in an unknown was determined by comparing the distance of its precipitin line from the antiserum trough with the distances which had been obtained when different concentrations of the purified protein had filled the lateral trough. Double diffusion studies were performed in agar layered on microscope slides and glass plates.

RESULTS

Isolation of Antibody.—The maximum concentration of anti-Lac antibody in precipitating sera, determined by quantitative precipitin curve analysis (4), was 2.1 mg/ml during the 2nd month of immunization. After the 10th month, the antisera contained sufficient nonprecipitating anti-Lac antibody to prevent formation of a specific precipitate with Lac-HSA (5, 7) and anti-Lac antibody was then isolated by coprecipitation with Lac-Hy. A maximum recovery of anti-Lac antibody of 1.5 mg/ml was obtained from the serum of the 11th month of immunization by the coprecipitation method. The recovery of antibody from sera obtained from the 12th to the 15th month remained near this level and thereafter progressively fell to a low of 0.2 mg/ml at 22 months, in spite of continued immunization.

Purified preparations of anti-Lac antibody were examined by double diffusion agar analysis and by immunoelectrophoresis. These studies defined a minimum of eight antigenically distinct immunoglobulins: γGa-, γGb-, γGc-, γA-, γM-, 10Sγ_1-globulins (4, 6), a second antigenically distinct component of γA-mobility (Fig. 1) and an additional component migrating on immunoelectrophoresis less rapidly than the γGc-globulins but more rapidly than the γGab-globulins (Fig. 2).

Distinct populations of anti-Lac antibodies were isolated by DEAE-cellulose column chromatography. γGab-globulins were obtained by passing antibody preparations over a column equilibrated with 0.005–0.010 M sodium phosphate buffer, pH 8. γGc-globulins were obtained from the trailing shoulder of the DEAE chromatograms eluted with 0.02 M sodium phosphate buffer, pH 8. Additional γGab- and γGc-globulins were obtained from the intermediately recovered DEAE chromatography fractions by zone electrophoresis. Preparations rich in γA-globulins were obtained by elution of the washed DEAE-cellulose columns with either a 0.5–1.0 M solution of sodium chloride or a positive linear gradient of sodium chloride. Preparations of γA-globulins were also subjected to zone electrophoresis to remove small amounts of γG-globulins of low electrophoretic mobility contained in the chromatographically isolated

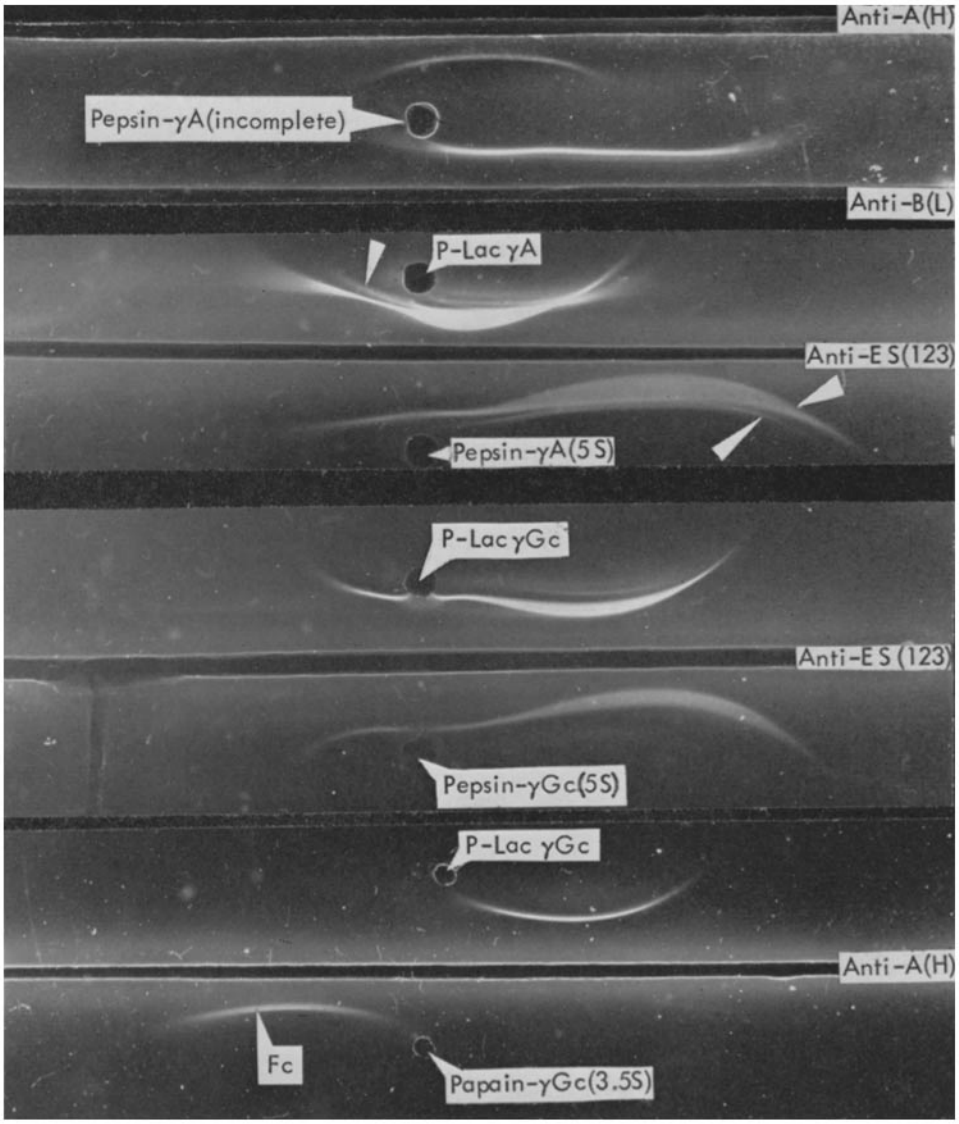

FIG. 1. Immunoelectrophoresis of γA (P-Lac γA) and γGc (P-Lac γGc) anti-Lac antibodies and the products of peptic and papain digestion. Pepsin-γA (incomplete), incompletely digested γA-antibody. Pepsin-γA (5S) and pepsin-γGc (5S), pepsin 5S Fab_2' fragments. Papain-γGc (3.5S), mixture of papain Fab and Fc fragments; anti-A (H), anti-γG heavy (A, H) chain antiserum; anti-B (L), anti-light (B, L) chain antiserum; and anti-ES (123), anti-equine serum antiserum.

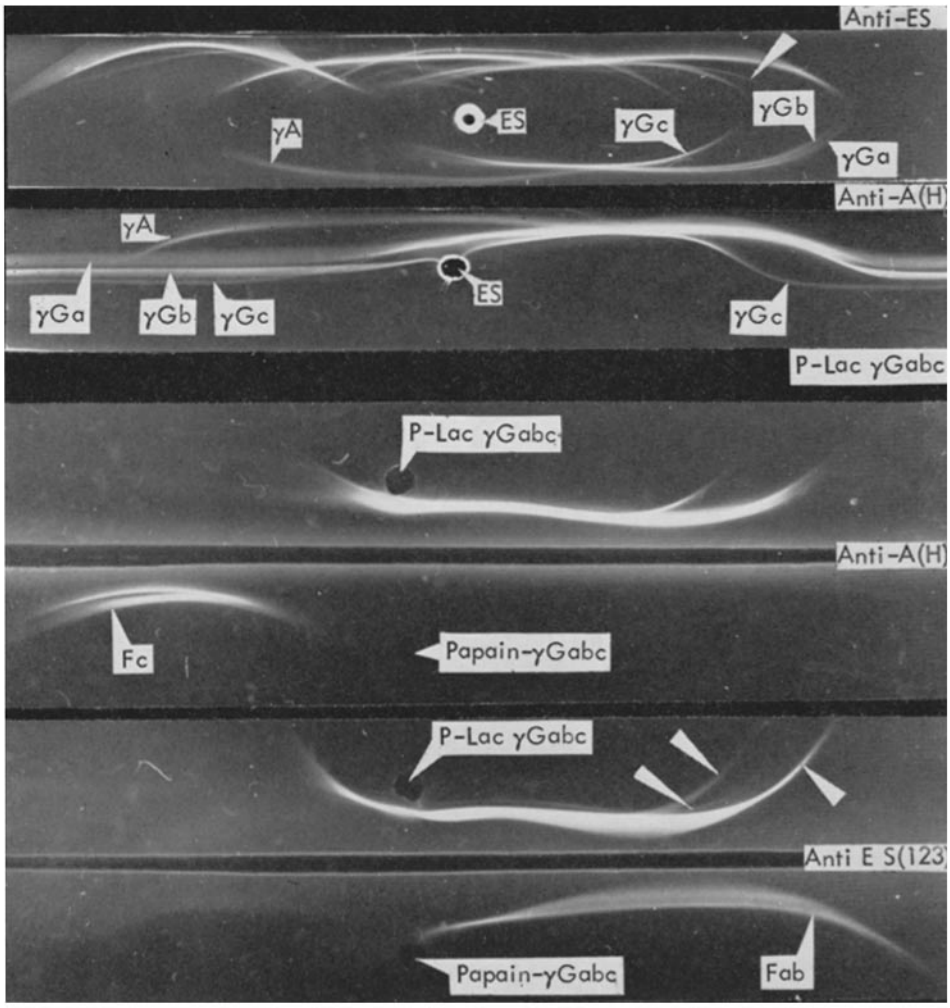

FIG. 2. Immunoelectrophoresis of equine serum (ES), γGabc-anti-Lac antibody (P-Lac γGabc) and the products of papain digestion of γGabc-antibody. Anti-Lac antibody (P-Lac γGabc) was placed in the lower trough of second slide. Anti-ES, anti-equine serum antiserum and anti-A (H), anti-γG heavy (A, H) chain antiserum. Anti-ES demonstrated an additional component (white pointer) migrating less rapidly than the γGc- but more rapidly than the γGab-globulins. Anti-A (H) demonstrated unique γGa-, γGb-, and γGc-antigens which were absent from the γA-globulins.

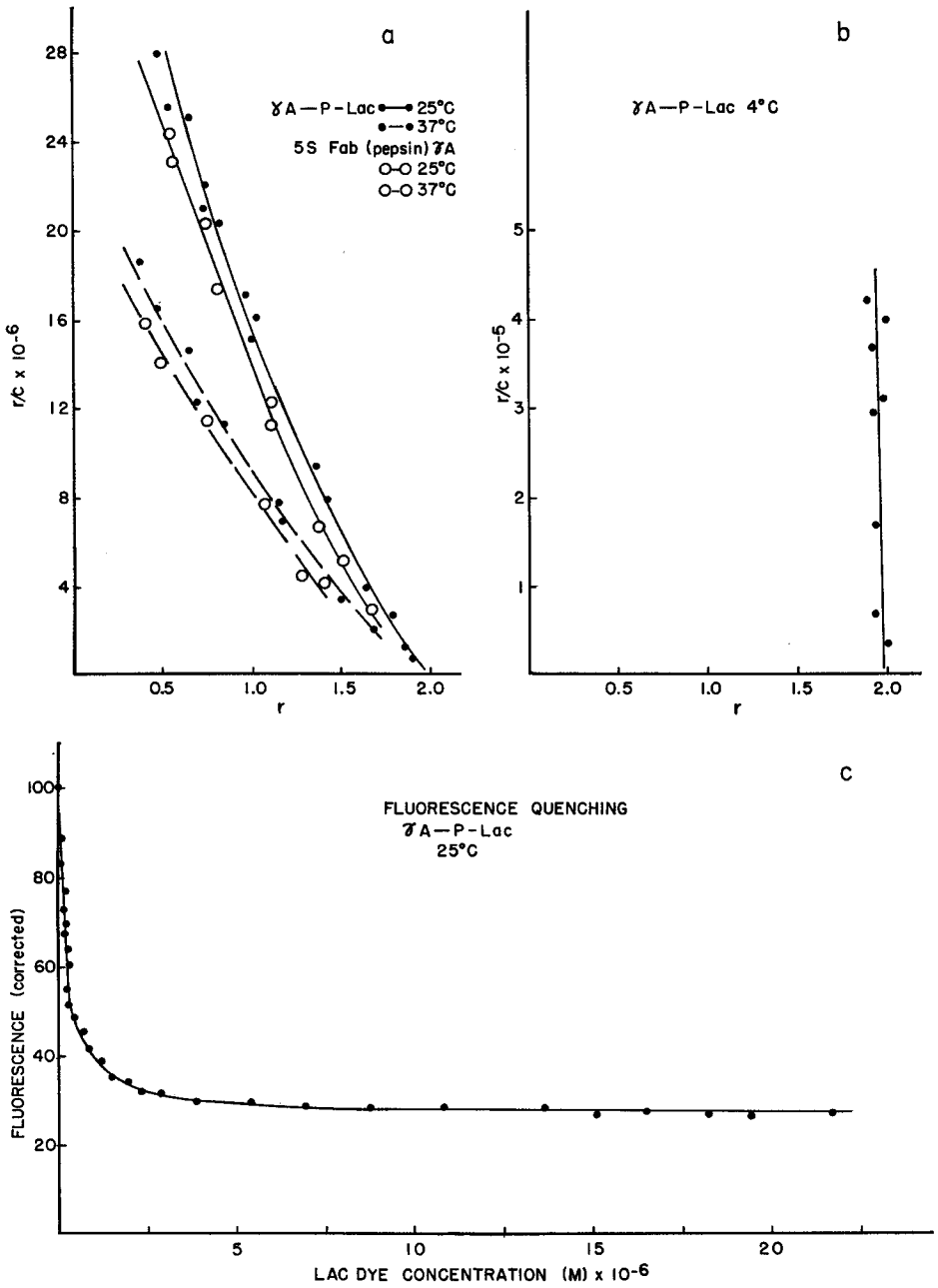

FIG. 3 *a*. The interaction of γA-anti-Lac antibody and its pepsin 5S Fab′$_2$ fragment (Fig. 1) with hapten, measured by equilibrium dialysis with tritiated Lac dye. Data presented in terms of r and c, where r is the moles of Lac dye bound per 150,000 g of γA-antibody or 100,000 g of pepsin 5S Fab′$_2$ fragment, and c is the concentration of free hapten. Equilibrium dialysis performed at 25° and 37°C.

FIG. 3 *b*. Binding of Lac dye by γA-antibody, at 4°C, at high concentrations of free Lac dye.

FIG. 3 *c*. Interaction of γA-anti-Lac antibody with Lac dye, measured by the quenching of protein fluorescence resulting upon the association of hapten with specific antibody. The antibody fluorescence data have been corrected with data obtained in an identical manner with γG-globulins prepared from nonimmune equine serum. Corrected antibody fluorescence plotted as a function of the total Lac dye concentration.

preparations. The 10Sγ_1-globulin and γM-globulin anti-Lac antibodies were isolated from preparations rich in these components either by Sephadex G-200 gel filtration (34), or by density gradient ultracentrifugation.

The optical density ratio OD (280 mμ)/OD (365 mμ) (4) was determined in neutral solvents for all antibody preparations and established that all preparations contained less than 3% antigen. The optical density ratio OD (280 mμ)/OD (251 mμ), in neutral solvents, was 2.4 or greater for all preparations. Agar diffusion studies with antisera directed against human serum albumin or hemocyanin failed to detect Lac-HSA or Lac-Hy antigens in the antibody preparations under conditions which would have demonstrated a contamination of 2–3% or greater. Diffusion of anti-Lac antibody preparations against hemocyanin failed to demonstrate the presence of precipitating antiprotein antibody. A measure of the purity of the antibody preparations was also furnished by the equilibrium dialysis data. γA-globulin antibody bound 2 moles of hapten per 150,000 g of protein at high free Lac dye concentrations (Fig. 3 *b*). The binding data of γGab-, γGabc-, and γGc-globulin antibodies at high free Lac dye concentrations extrapolated reasonably to 2 moles of sites per 150,000 g of protein (Figs. 4 *a* and 4 *b*). Protein concentrations were measured optically by employing experimentally determined extinction coefficients. The value of $E^{1\,cm}_{\lambda 280 m\mu}$ of a 1% solution of either γGabc- or γA-antibody in neutral solvents was 14.7.

All of the chromatographically and electrophoretically prepared antibody fractions were antigenically heterogeneous. All preparations formed more than a single precipitin line with anti–light (B, L) chain antisera (6, 7) or fused with the precipitin lines formed with other antibody preparations showing a clear multiplicity of lines. Fig. 5 demonstrates the two precipitin lines formed between the 10Sγ_1–anti-Lac antibody and anti–light (B, L) chain antiserum. Anti–heavy (A, H) chain antiserum, prepared against the isolated polypeptide chains of nonimmune equine γG-globulins, reacted strongly with unique antigenic determinants of the γGa-, γGb-, and γGc-globulins which were absent from the γA-globulins (Fig. 2). The antiserum also reacted with the γA-globulins, but the γA-precipitin line failed to penetrate the γG-globulin precipitin lines, and absorption of the antiserum with γG-globulins of low electrophoretic mobility removed the antibodies reacting with the γA-globulins. The anti–heavy (A, H) chain antiserum did not react with the 10Sγ_1- or γM-globulins (Fig. 5). Unique antigenic determinants of the γA-, 10Sγ_1-, and γM-globulins, which were absent from the γGa-, γGb-, and γGc-globulins, were demonstrated with other antisera. Antiserum specific for γGc-globulin was obtained by absorbing the anti-γG heavy (A, H) chain antiserum with γGab-globulins. A measure of the content of minor components in the preparations of γGab-, γGc-, and γA-globulins was furnished by the modified immunoelectrophoretic technique (33), and indicated that such immunoglobulin contamination was generally of the order of 5–25%.

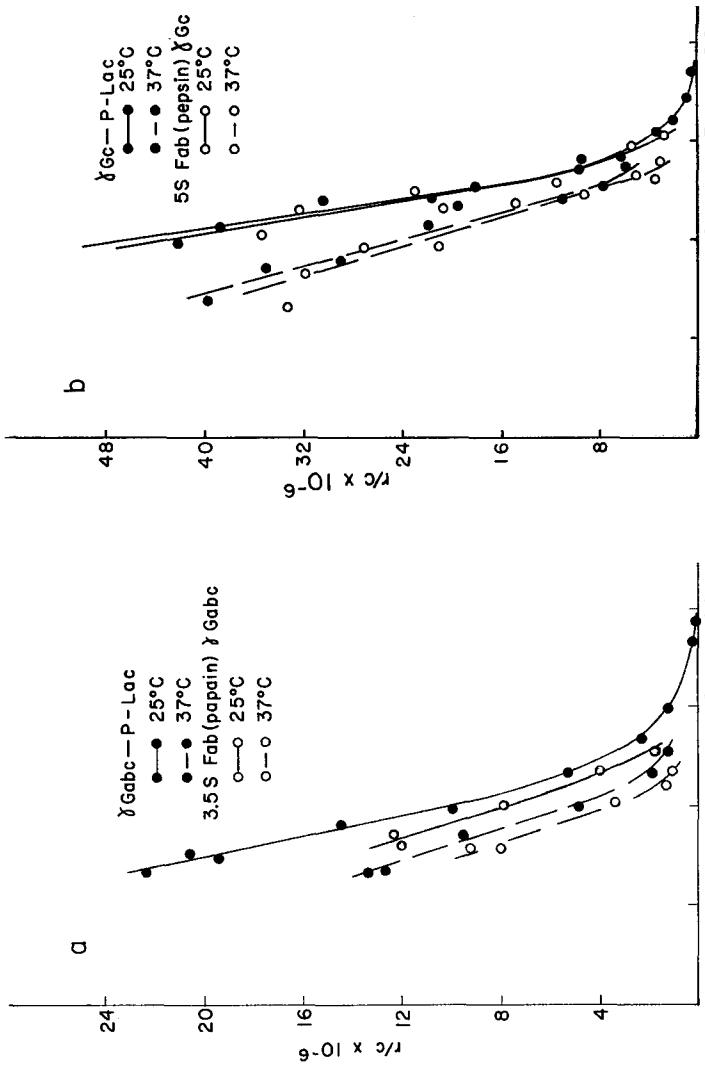

FIG. 4 *a*. Interaction of γGabc–anti-Lac antibody and its papain 3.5S Fab fragment (Fig. 2) with hapten, measured at 25° and 37°C by equilibrium dialysis with tritiated Lac dye.

FIG. 4 *b*. Interaction of γGc–anti-Lac antibody and its pepsin 5S Fab′$_2$ fragment (Fig. 1) with hapten, measured by equilibrium dialysis with tritiated Lac dye.

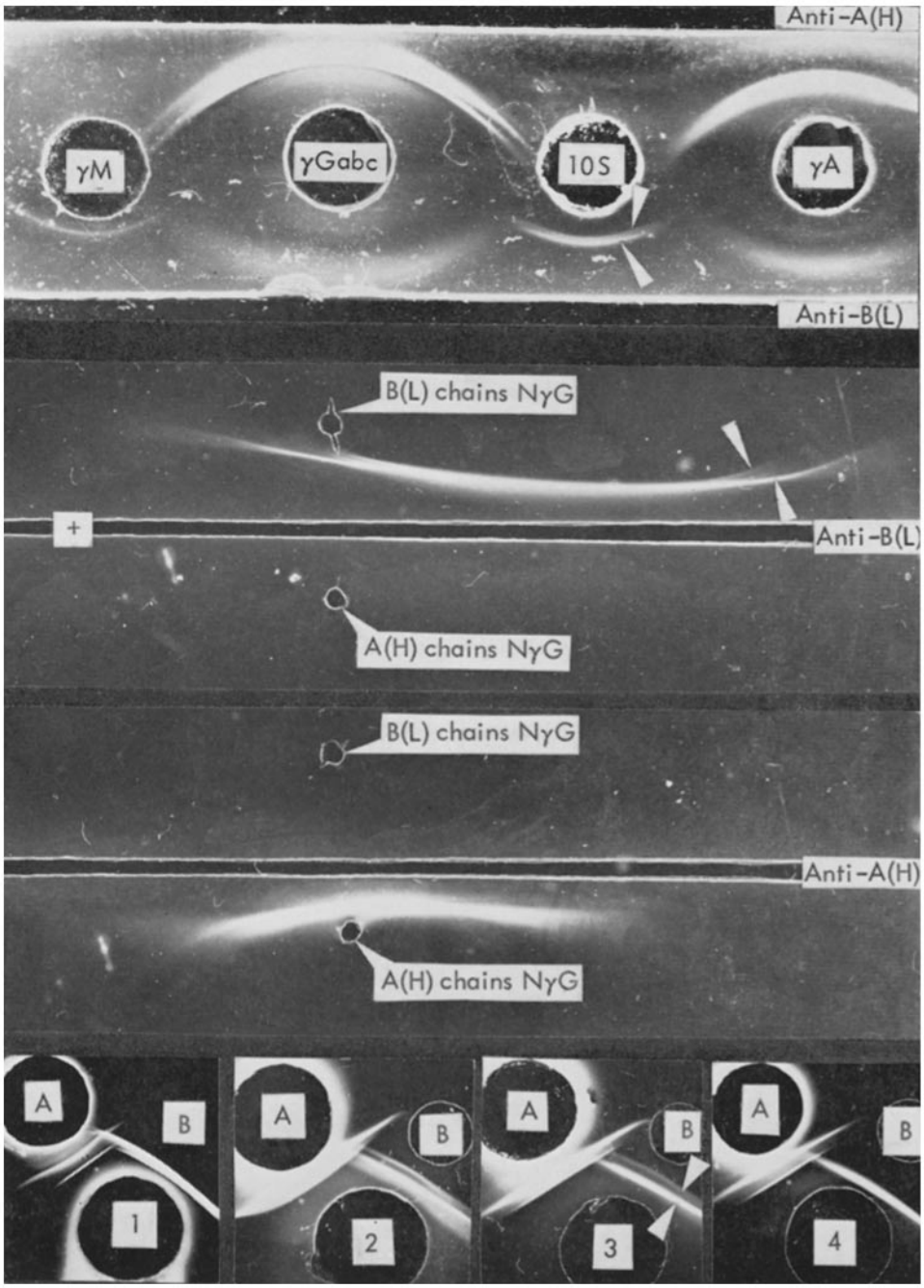

FIG. 5. Agar double diffusion analysis and immunoelectrophoresis of purified preparations of anti-Lac antibody and the heavy (A, H) and light (B, L) polypeptide chains from equine γG-globulins, with anti-heavy (A, H) chain (anti-A(H)) and anti-light (B, L) chain (anti-B(L)) antisera. γM, macroglobulin component prepared from purified anti-Lac antibody; γGabc, γGabc–anti-Lac antibody; 10S, 10Sγ_1–anti-Lac antibody; γA, γA–anti-Lac antibody; and B (L) chains NγG (B), and A (H) chains NγG (A), light (B, L) and heavy (A, H) polypeptides chains, respectively, isolated from γG-globulins prepared from nonimmune equine serum. 1, mixture of anti-A(H) and anti-B(L) antisera. 2, 3, 4, antisera prepared against equine serum. Anti-B(L) demonstrated two antigenically distinct components in the 10S γ_1-globulin antibody and in the isolated light (B, L) chains (B). Anti-A(H) did not react with the γM- or 10Sγ_1-components of anti-Lac antibody.

Enzymatic Digestion.—The course of digestion of equine immunoglobulins with pepsin was followed by immunoelectrophoresis, and by analytical ultracentrifugation. A reduction in concentration of the components with sedimentation coefficients ($s_{20,w}$) in neutral solvents of approximately 7S, and the appearance of a major component with a sedimentation coefficient ($s_{20,w}$) of approximately 5S (pepsin 5S Fab_2' fragment), was paralleled by the disappearance of prominent unique antigenic determinants of the distinct immunoglobulins. Prolonged dialysis of the protein digests against neutral solvents resulted in the removal of 28–36% of materials with absorbancy at 280 mμ. Small amounts of materials sedimenting more slowly than the 5S component remained. Sephadex G-100 gel filtration or prolonged sucrose density gradient ultracentrifugation produced preparations of more uniform size. The results obtained upon peptic digestion of γA– and γGc–anti-Lac antibodies are summarized in Fig. 1. The undigested γA-antibody was antigenically heterogeneous and contained two prominent components of γA-mobility (Fig. 1). Peptic digestion produced antigenically deficient components with lower electrophoretic mobilities (pepsin 5S Fab_2' fragments). Anti–light (B, L) chain antisera (6) precipitated the pepsin 5S Fab_2' fragments (Fig. 1). Anti-γG heavy (A, H) chain antiserum (6) reacted with antigenic determinants which were present on the intact molecules but were absent from the pepsin 5S Fab_2' fragments (Fig. 1). The γA-pepsin 5S Fab_2' fragment was antigenically heterogeneous (Fig. 1). The observed heterogeneity was not a function of the time of incubation with pepsin in that it was demonstrable in the slowly migrating pepsin 5S Fab_2' fragments early in the course of digestion and persisted even after 96 hr of incubation with enzyme. The sedimentation coefficient ($s_{20,w}$) of the γGc-antibody was 6.4S (4.8 mg/ml). The sedimentation coefficients ($s_{20,w}$) of the γA- and γGc-pepsin Fab_2' fragments were 5.3S (4.2 mg/ml) and 5.2S (2.8 mg/ml), respectively. The extinction coefficient, $E^{1cm}_{\lambda 280 m\mu}$, of a 1% solution of the γA-antibody pepsin 5S Fab_2' fragment was 14.6 in neutral solvents. The difference in electrophoretic mobility between the γGc-globulin and γGc-pepsin 5S Fab_2' fragment was less striking than that observed between the γA-globulin and the γA-pepsin 5S Fab_2' fragment (Fig. 1). Peptic digestion of mixtures of γGa–, γGb–, and γGc–anti-Lac antibodies and of preparations of γGab-antibodies resulted in a similar production of antigenically deficient pepsin 5S Fab_2' fragments. Prominent unique antigenic determinants of the γGa- and γGb-globulins, detected with anti–heavy (A, H) chain antisera, also were removed by peptic digestion. When mixtures of γGab- and γGc-globulins were digested, the unique γGc-antigenic determinants disappeared more rapidly than those of γGa- and γGb-globulins.

The course of digestion of equine anti-Lac antibodies and immunoglobulins with papain was followed by the same methods used with peptic digestion. The results obtained upon papain digestion of a mixture of γGa-, γGb-, and γGc-

globulin anti-Lac antibodies are summarized in Fig. 2. An antigenically heterogeneous component of rapid electrophoretic mobility (Fc fragments), which was readily detected with the anti-heavy (A, H) chain antiserum, was released. A portion of the Fc fragments crystallized from the digestion mixture at pH 5.6 upon standing at 4°C. The papain 3.5S Fab fragment was easily separated from the remaining soluble Fc fragments by zone electrophoresis. The sedimentation coefficient ($s_{20,w}$) of the γGabc-papain Fab fragment, in neutral solvents, was 3.5S (3.6 mg/ml). The results of papain digestion of γGc-anti-Lac antibody are illustrated in Fig. 1.

Hapten-Binding Activity.—The hapten-binding activities of preparations of γGabc-, γGab-, γGc-, and γA-anti-Lac antibodies were determined by equilibrium dialysis. The values of r/c, where r is the average number of moles

TABLE I

Anti-Lac antibody preparation	$K_0 \times 10^{-7}$ 25.0°C M^{-1}	$K_0 \times 10^{-7}$ 37.2°C M^{-1}	ΔH^0 kcal/m	ΔFu kcal/m	ΔSu eu/m
γGabc-P-Lac	1.03	0.52	−10.4	−12.0	+5.4
3.5S Fab γGabc	0.80	0.39	−10.8	−11.8	+3.3
γGc-P-Lac	4.9	2.4	−10.8	−12.9	+7.1
5S Fab_2' γGc	4.4	2.2	−10.4	−12.8	+8.1
γA-P-Lac	1.58	0.92	−8.2	−12.2	+6.7
5S Fab_2' γA	1.40	0.83	−7.9	−12.2	+7.7

The average intrinsic association constants (K_0) and the thermodynamic parameters of equine anti-Lac antibody and antibody fragment interaction with the hapten *p*-(*p*-dimethylaminobenzeneazo)-phenyl-β-lactoside. ΔH^0, standard enthalpy change; ΔFu, unitary free energy change (46); ΔSu, unitary entropy change (46); and eu, entropy units.

of hapten bound per 150,000 g of antibody protein and c is the free hapten concentration, were plotted as a function of r (14). Concentrations of free Lac dye greater than 1×10^{-6} M were readily measured optically. Concentrations of free hapten greater than 3×10^{-8} M were determined readily with tritiated Lac dye. The percentage of the moles of free Lac dye which was bound to the dialysis cell components ranged from 10% at a free Lac dye concentration of 5×10^{-5} M to 200% at a free tritiated Lac dye concentration of 1×10^{-8} M. The pepsin 5S Fab_2' fragments of γA- and γGc-antibodies and the papain 3.5S Fab fragments of γGabc-antibodies were also used for equilibrium dialysis. The data were treated in the same manner as that obtained with the intact antibodies excepting that r values were plotted as a function of the moles of Lac dye bound per 100,000 g of protein (8). Average intrinsic association constants (K_0) were obtained from the value of r/c at $r = 1$.

The average intrinsic association constant (K_0) of antibody preparations varied from a low of 3.5×10^5 M^{-1} for γGabc-globulins isolated from early

antiserum (6, 7) to a high of 4.9×10^7 M^{-1} for γGc-antibody isolated from serum of the 14th month (Fig. 4 *b*, Table I). The K_0 values obtained with γGabc–, γGc–, and γA–anti-Lac antibodies and their enzymatically produced fragments, determined at 25° and 37°C (Figs. 3 *a*–3 *c* and Figs. 4 *a* and 4 *b*) are present in Table I together with the thermodynamic parameters of antibody-hapten interaction calculated from these data. Enzymatic removal of the Fc fragments by digestion with pepsin, and dissociation of the two binding sites of digested antibody on the papain 3.5S Fab fragments did not alter the energetics of antibody-hapten interaction. The hapten-binding activity of equine anti-Lac antibodies and enzymatically produced fragments also was demonstrated by analytical ultracentrifugation employing a combination of schlieren and absorption optics.

Intrinsic association constants (K_0) were also determined for γGabc–, γGc–, and γA–anti-Lac antibodies by fluoresence quenching (25). Equine immunoglobulins in 0.2 M sodium chloride, 0.01 M sodium phosphate buffer, pH 8, had an absorption maximum at 279 mμ, and a fluorescence maximum at 345 mμ. The Lac dye absorption spectrum overlapped the protein fluorescence spectrum. Equine immunoglobulins at a concentration of 2.5×10^{-7} M gave an initial fluorescence reading in the Turner 110 fluorometer of 80–100 units. Antibody fluorescence values were corrected by adding to each value that amount of reduction of fluorescence which had occurred upon the addition of the same quantity of Lac dye to nonimmune equine γG-globulins. The corrected curve of fluorescence of γA–anti-Lac antibody, plotted as a function of the total Lac dye concentration, is presented in Fig. 3 *c*. An association constant (K_0) of 1.5×10^7 M^{-1} was calculated from an expanded plot incorporating additional data near the point at which one-half of the total reduction of corrected γA-antibody fluorescence had been obtained. The association constants obtained by the fluorescence quenching technique were generally of the same order of magnitude as those obtained by equilibrium dialysis.

Preparation of Heavy and Light Polypeptide Chains.—The heavy (A, H) and light (B, L) polypeptide chains of equine immunoglobulins were readily separated by gel filtration in either propionic acid (21) or in sodium decyl sulfate (22). The Sephadex G-200 gel filtration of mildly reduced and alkylated anti-Lac antibodies illustrated in Fig. 6 had been performed in 0.04 M sodium decyl sulfate with a single set of 3 in-series 4 × 60 cm columns. The void volume (V_0) for the column set was 618 ml. Equine γG-globulins eluted at 720 ml. Two heavy (A, H) chain components (A_1, A_2) were recovered from the γGab-, γGc-, and γA-antibodies at elution volumes of 769, 766, and 759 ml (A_1), and 864, 879, and 884 ml (A_2), respectively. The relative proportions of the two successively eluted components of the heavy (A, H) chains (A_1, A_2) varied for different molecular forms of anti-Lac antibody reduced and alkylated under the

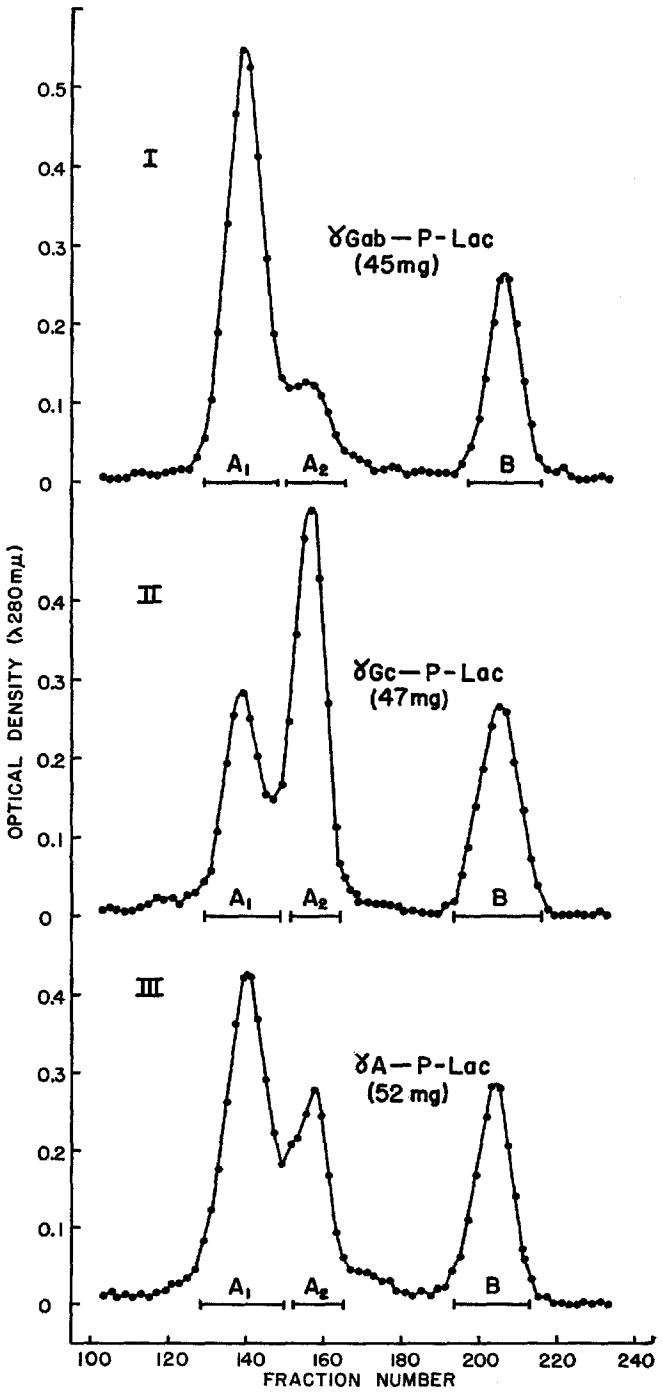

FIG. 6. Separation of the heavy (A, H) and light (B, L) polypeptides chains from γGab (γGab–P-Lac), γGc (γGc–P-Lac) and γA (γA–P-Lac) anti-Lac antibodies, reduced with 0.1 M 2-mercaptoethanol and alkylated in solvents lacking urea, by Sephadex G-200 gel filtration in 0.04 M sodium decyl sulfate. Separation was accomplished with a single set of 3 columns (4 × 60 cm) in series. Two heavy (A, H) chain components (A_1, A_2) were obtained in varying relative proportions from the different forms of anti-Lac antibody, reduced, and alkylated under the same conditions.

same conditions (Fig. 6). The volumes at which the light (B, L) chain peaks (B) eluted were 1181, 1175, and 1176 ml for the γGab-, γGc-, and γA-globulin light (B, L) chains, respectively. The recoveries of heavy (A, H) and light (B, L) chains were obtained by employing extinction coefficients which had been determined in the appropriate solvent with polypeptide chains prepared from nonimmune equine γG-globulins. The $E^{1\,cm}_{\lambda 280 m\mu}$ values of 1% solutions of heavy (A, H) and light (B, L) chains were 15.4 and 14.0, respectively, in 0.04 M sodium decyl sulfate, and 15.2 and 13.6, respectively, in 1 N propionic acid. The recovery of light (B, L) chains from the mildly reduced and alkylated γGab–, γGc–, and γA–anti-Lac antibodies of Fig. 6 were 29.6, 29.5, and 31.7%,

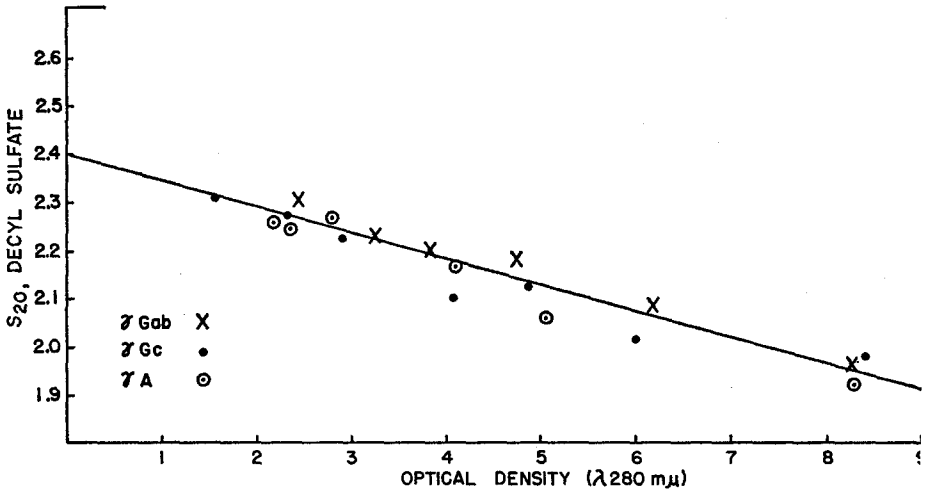

FIG. 7. Analytical ultracentrifugation of the light (B, L) chains from γGab–, γGc–, and γA–anti-Lac antibody (P-Lac) (Fig. 6), sedimented in 0.0125 M sodium decyl sulfate, 0.2 M sodium chloride, 0.002 M EDTA, 0.01 M Tris-HCl, pH 8. The s vs. c data for the three preparations have been incorporated into a single plot.

respectively, of the applied proteins. When extensively reduced and alkylated anti-Lac antibody was subjected to gel filtration in 0.04 M sodium decyl sulfate, 31–33% of the applied protein was recovered in the light (B, L) chain fractions. The recovery of light (B, L) chains upon gel filtration in propionic acid was 28–32% of the applied protein.

The light (B, L) and heavy (A, H) chain fractions of γGab-, γGc-, and γA-globulin antibodies of Fig. 6 were each pooled, concentrated by negative pressure ultrafiltration, transferred to 0.0125 M sodium decyl sulfate, 0.2 M sodium chloride, 0.002 M EDTA, 0.01 M Tris-HCl, pH 8, by Sephadex G-100 gel filtration, and examined in the analytical ultracentrifuge. The light (B, L) chains sedimented as single symmetrical peaks. Sedimentation coefficients (s) were determined at protein concentrations (c) of 1–7 mg/ml (Fig. 7). The s vs. c

TABLE II

Amino acid	γGabc-P-Lac A_1 chain			γGabc-P Lac A_2 chain			γA-P-Lac A_2 chain		
	24 hr hydrolysate	72 hr hydrolysate	Amino acid residues	24 hr hydrolysate	72 hr hydrolysate	Amino acid residues	24 hr hydrolysate	72 hr hydrolysate	Amino acid residues
S-carboxymethyl cysteine	7.2 ± 0.0	7.7 ± 0.2	7.4	7.1 ± 0.2	7.4 ± 0.0	7.2	11.4 ± 0.2	11.7 ± 0.2	11.6
Aspartic acid	35.5 ± 0.2	35.9 ± 0.2	35.7	36.0 ± 0.1	36.3 ± 0.1	36.2	38.0 ± 0.5	38.1 ± 0.7	38.0
Threonine	38.8 ± 1.2	36.4 ± 1.5	40.1*	37.9 ± 0.3	36.6 ± 0.3	39.4*	34.9 ± 0.5	31.4 ± 0.4	36.7*
Serine	43.6 ± 0.9	37.1 ± 0.9	46.8*	45.2 ± 0.3	37.7 ± 0.1	48.9*	42.7 ± 0.4	38.1 ± 0.2	45.0*
Glutamic acid	44.6 ± 0.0	43.6 ± 0.5	44.1	46.1 ± 0.4	45.4 ± 0.2	45.8	42.7 ± 0.1	42.2 ± 0.3	42.4
Proline	31.7 ± 0.2	32.1 ± 0.5	31.9	33.2 ± 0.5	35.2 ± 0.1	34.2	42.0 ± 0.1	39.3 ± 0.5	40.6
Glycine	31.9 ± 0.7	32.6 ± 0.2	32.2	30.8 ± 0.4	31.3 ± 0.1	31.0	30.2 ± 0.5	30.4 ± 0.2	30.3
Alanine	23.1 ± 0.5	24.0 ± 0.2	23.6	23.3 ± 0.3	24.0 ± 0.1	23.6	21.7 ± 0.2	22.2 ± 0.3	22.0
Valine	46.1 ± 0.2	47.4 ± 0.1	47.4‡	49.0 ± 0.4	50.4 ± 0.0	50.4‡	46.4 ± 0.8	48.9 ± 0.1	48.9‡
Methionine	3.8 ± 0.1	3.8 ± 0.0	3.8	2.7 ± 0.1	2.8 ± 0.1	2.8	3.4 ± 0.2	3.6 ± 0.0	3.5
Isoleucine	13.9 ± 0.2	14.1 ± 0.4	14.1‡	15.6 ± 0.1	15.7 ± 0.0	15.7‡	11.6 ± 0.2	12.4 ± 0.3	12.4‡
Leucine	33.1 ± 0.2	33.1 ± 0.6	33.1	34.1 ± 0.2	34.1 ± 0.3	34.1	33.7 ± 0.1	33.8 ± 0.3	33.8
Tyrosine	15.8 ± 0.1	15.6 ± 0.7	15.9*	12.6 ± 0.1	11.5 ± 0.1	13.2*	16.0 ± 0.2	16.0 ± 0.0	16.0*
Phenylalanine	14.1 ± 0.2	14.6 ± 0.5	14.4	13.7 ± 0.4	13.6 ± 0.0	13.6	13.6 ± 0.1	13.3 ± 0.2	13.4
Ammonia	76.4 ± 1.6	89.3 ± 2.9		70.7 ± 0.3	87.4 ± 2.1		55.6 ± 2.4	67.3 ± 1.4	
Lysine	30.7 ± 0.7	29.0 ± 0.5	29.8	27.6 ± 0.1	26.9 ± 0.1	27.2	30.1 ± 0.3	29.4 ± 0.3	29.8
Histidine	10.8 ± 0.0	10.1 ± 0.2	10.4	9.9 ± 0.1	9.8 ± 0.1	9.8	12.2 ± 0.1	12.8 ± 0.3	12.5
Arginine	13.4 ± 0.2	12.2 ± 0.0	12.8	12.8 ± 0.0	12.3 ± 0.1	12.6	11.6 ± 0.4	11.4 ± 0.2	11.5
Tryptophan			8.4§			7.0§			8.7§

Amino acid	γGabc-P-Lac B chain			γA-P-Lac B chain		
	24 hr hydrolysate	72 hr hydrolysate	Amino acid residues	24 hr hydrolysate	72 hr hydrolysate	Amino acid residues
S-carboxymethyl cysteine	3.0 ± 0.0	3.3 ± 0.1	3.2	4.6 ± 0.0	4.7 ± 0.0	4.6
Aspartic Acid	15.3 ± 0.3	15.6 ± 0.1	15.4	15.2 ± 0.0	15.5 ± 0.2	15.4
Threonine	21.4 ± 0.9	20.4 ± 0.0	21.9*	20.5 ± 0.3	20.2 ± 0.0	20.6*
Serine	28.9 ± 0.0	25.6 ± 0.1	30.5*	32.6 ± 0.5	30.0 ± 0.2	33.9*
Glutamic acid	19.2 ± 0.2	19.8 ± 0.0	19.5	19.3 ± 0.1	19.2 ± 0.2	19.2
Proline	11.8 ± 0.3	11.6 ± 0.2	11.7	12.0 ± 0.3	10.9 ± 0.2	11.4
Glycine	21.4 ± 0.4	21.6 ± 0.0	21.5	21.0 ± 0.1	21.2 ± 0.4	21.1
Alanine	14.6 ± 0.2	14.9 ± 0.1	14.8	14.4 ± 0.1	14.3 ± 0.1	14.4
Valine	16.7 ± 0.2	17.4 ± 0.2	17.4‡	15.5 ± 0.2	16.7 ± 0.2	16.7‡
Methionine	0.20 ± 0.0	0.20 ± 0.1	0.2	0.39 ± 0.0	0.34 ± 0.0	0.4
Isoleucine	7.9 ± 0.1	8.4 ± 0.0	8.4‡	7.0 ± 0.1	7.9 ± 0.0	7.9‡
Leucine	13.0 ± 0.2	13.3 ± 0.1	13.2	12.1 ± 0.0	12.3 ± 0.4	12.2
Tyrosine	5.6 ± 0.0	5.1 ± 0.0	5.9*	7.4 ± 0.0	7.2 ± 0.1	7.5*
Phenylalanine	5.5 ± 0.0	5.7 ± 0.1	5.6	5.4 ± 0.1	5.5 ± 0.1	5.4
Ammonia	37.3 ± 0.3	45.4 ± 1.2		33.1 ± 0.5	37.5 ± 0.8	
Lysine	12.0 ± 0.0	12.2 ± 0.3	12.1	11.8 ± 0.1	12.0 ± 0.4	11.9
Histidine	2.7 ± 0.0	2.6 ± 0.0	2.6	2.6 ± 0.0	2.6 ± 0.0	2.6
Arginine	5.3 ± 0.1	5.4 ± 0.2	5.4	5.0 ± 0.2	5.0 ± 0.4	5.0
Tryptophan			3.3§			3.9§

Amino acid composition of the heavy (A, H) and light (B, L) polypeptide chains of equine anti-Lac antibodies. Values at each hydrolysis period are the averages of two or more determinations, recorded together with the maximum deviation from average for individual determinations. Presented as amino acid residues per 50,000 and 23,000 g of γGabc-heavy (A, H) and γGabc-light (B, L) chains, respectively. Data of corresponding chains of γA-antibody normalized to give an approximately equal number (less S-carboxymethyl cysteine) of residues.

* 24 and 72 hr values extrapolated linearly to zero time.

‡ 72 hr value.

§ Determined by the method of Goodwin and Morton (35) from the optical densities in 0.1 NaOH at λ280 mμ and λ294.6 mμ.

data for the three light (B, L) chain preparations were incorporated into a single plot and treated by the method of least squares. The corrected, extrapolated sedimentation coefficient ($s^0_{20,w}$) obtained was 2.56S. More than a single component was present on analytical ultracentrifugation of heavy (A, H) chains in 0.0125 M sodium decyl sulfate. The sedimentation coefficients ($s_{20,w}$) of the principal, more slowly sedimenting components were 1.68S–3.03S.

The isolated heavy (A, H) and light (B, L) polypeptide chains of the different molecular forms of equine anti-Lac antibodies and immunoglobulins were analyzed by two dimensional agar diffusion analysis and by immunoelectrophoresis (Fig. 5). All isolated light (B, L) chain preparations were antigenically heterogeneous and contained at least two distinct components when examined with the proper antiserum (Fig. 5). The antigenic heterogeneity observed in the heavy (A, H) chain preparations was complex. A more detailed analysis of the antigenic interrelations between the different molecular forms of anti-Lac antibody, their heavy (A, H) and light (B, L) polypeptide chains and their enzymatically produced fragments will be presented elsewhere.

The hapten-binding activity of the heavy (A, H) chains from mildly reduced and alkylated γA–anti-Lac antibody was demonstrated by equilibrium dialysis (22), and by analytical ultracentrifugation employing the combined schlieren and absorption optics (4). The polypeptide chains had been separated in 0.04 M sodium decyl sulfate (22), and 31.4% of the applied protein had been recovered in the light (B, L) chain fractions. Detergent had been removed by exhaustive dialysis against the anion exchange resin (Rexyn RG 1) (22). Less than 5% of the heavy (A, H) chain protein had been lost as insoluble aggregates during concentration and dialysis. The heavy (A, H) chains bound 0.25 and 0.67 moles of Lac dye per 100,000 g of protein at free Lac dye concentrations of 1.35×10^{-5} M and 5.21×10^{-5} M, respectively. Ultracentrifugation of the heavy (A, H) chains equilibrated with Lac dye, employing the schlieren and absorption optics, demonstrated that the dye sedimented with the protein components. The principal, more slowly sedimenting component had a sedimentation coefficient ($s_{20,w}$) of 4.7S. Significant amounts of more rapidly sedimenting, aggregated materials were present. The light (B, L) chains did not bind significant amounts of Lac dye.

Amino Acid Analysis.—The amino acid composition of the heavy (A, H) and light (B, L) polypeptide chains of γGabc- and γA-globulin anti-Lac antibodies is present in Table II. The antibodies had been reduced with 0.2 M 2-mercaptoethanol in 8 M urea, and the subunits had been separated in 0.04 M sodium decyl sulfate. The heavy (A, H) chains of the γGabc-globulins were partially resolved into two components (60% A_1, 40% A_2) and the amino acid composition of each fraction was determined. The heavy (A, H) chains of the γA-globulins were recovered almost entirely in the second (A_2) component. The recovery of light (B, L) chains from the γGabc- and γA-globulins were

33.1 and 31.4%, respectively, of the applied proteins. The composition of the heavy (A, H) and light (B, L) chains of γGabc-globulins are presented as moles of amino acid residue per 50,000 and 23,000 g, respectively, of protein. The amino acid data of the γA–heavy (A, H) and γA–light (B, L) chains have been normalized to give a total number of amino acid residues, omitting the S-carboxymethyl cysteine values, which was approximately equal to that of the corresponding γGabc-antibody polypeptide chain. The absorption spectra of the heavy (A, H) and light (B, L) polypeptide chains of γGab-, γGc-, and γA-antibodies were determined in 0.1 N NaOH. A measure of the relative concentration of tyrosine and tryptophan was obtained from the absorbancy at 294.6 and 280 mμ by the method of Goodwin and Morton (35). The molar ratios for tyrosine/tryptophan were 1.99–2.12 for the light (B, L) chains and 1.84–2.04 for the heavy (A, H) chains.

Carbohydrate Analysis.—The results of analysis of anti-Lac antibodies, equine immunoglobulins, and immunoglobulin subunits for their hexose and hexosamine content are presented in Table III.

TABLE III

Sugar	γGabc (7)	γGab	γGc	γA(7)	NγG	A(H) chain	B(L) chain
Hexose	6.9	7.1	9.1	20.5	7.2	3.7	0.1
Hexosamine	7.7	7.2	10.6	14.7	8.3	4.3	0.0

Hexose and hexosamine content of equine anti-Lac antibodies, and of nonimmune equine γG-globulin (NγG) and its heavy (A, H) and light (B, L) polypeptide chains. Recorded as moles of sugar per mole of protein (immunoglobulins, 150,000 mol wt; heavy (A, H) chain, 50,000 mol wt; light (B, L) chain, 23,000 mol wt).

DISCUSSION

Eight antigenically distinct immunoglobulins have been identified in purified anti-*p*-azophenyl-β-lactoside (Lac) antibody isolated from the serum of a single horse: γGa-, γGb-, γGc-, γA (T component)-, 10Sγ$_1$-, and γM-globulins, an additional component of γA-mobility, and an additional component migrating on immunoelectrophoresis less rapidly than the γGc-, but more rapidly than the γGa- and γGb-globulins. The principal antigenic determinants which distinguish the several molecular forms of equine antibody and the related immunoglobulins have been found to be a function of that portion of the heavy (A, H) chain, termed the Fc fragment, which may be removed by enzymatic digestion without loss of antibody specificity. Unique antigenic determinants of the γGa-, γGb-, and γGc-globulins have been located on the intact papain Fc fragments with anti–heavy (A, H) chain antisera. Peptic digestion of γA-antibody removed unique γA–antigenic determinants from the active

Fab_2' fragment. Anti-γG heavy (A, H) chain antiserum demonstrated antigenic determinants of the γA-globulins (T component) which were shared with the γG-globulins. These common γG– and γA–heavy (A, H) chain determinants were absent from the pepsin 5S Fab_2' and the papain 3.5S Fab fragments. These findings indicated that the γG- and γA-globulin Fc fragments possessed both common and unique structural regions. Weir and Porter recently have reported similar observations (61). The common γG– and γA–Fc fragment antigens were absent from the $10S\gamma_1$- and γM-globulins.

Analysis of anti-Lac antibody, isolated light (B, L) chains, pepsin 5S Fab_2' fragments and papain 3.5S Fab fragments, with antisera specific for the light (B, L) chains of normal equine immunoglobulins, demonstrated antigenic heterogeneity in all preparations examined. Two principal equine light (B, L) chain variants have been defined (6, 7). These may correspond to the κ (K, type I) and λ (L, type II) light chains of other species (36, 37). The anti–light (B, L) chain antisera failed to distinguish between the different subfractions of equine anti-Lac antibodies, indicating that all classes shared common light (B, L) chain determinants.

The analytical ultracentrifugation and Sephadex G-200 gel filtration data indicated that the molecular weights of the light (B, L) chains from the γGab–, γGc–, and γA–anti-Lac antibodies were closely similar, if not identical. The s vs. c ultracentrifugation data for the light (B, L) chains from the three antibody preparations (sedimented in 0.0125 M sodium decyl sulfate) fitted within experimental error on a single linear plot, and gave a corrected sedimentation coefficient ($s^0_{20,w}$) of 2.56S. This is comparable to the sedimentation coefficient of a monomeric human light (B, L) chain (38). The light (B, L) chains from the three antibody preparations eluted at the same volume upon filtration through a standard set of Sephadex G-200 columns (in 0.04 M sodium decyl sulfate) (cf. references 39 and 40). The recoveries of protein in the light (B, L) chain fractions from the several forms of anti-Lac antibody were closely similar. Pain has determined the molecular weight of the light (B, L) chains of equine γG-globulins to be 19,400 (41). The molecular weights of equine γG-globulins and their constituent heavy (A, H) chains were reported to be 150,000 and 50,000, respectively (41, 42). Equine γA–anti-Lac antibody bound 2 moles of hapten per 150,000 g of protein at high free Lac dye concentrations, indicating that the molecular weight of the bivalent antibody was approximately 150,000 (Fig. 3 *b*) (4). Molecular weight values of 150,000–160,000 have been reported for equine T component (γA-globulins) (8, 43). The heavy (A, H) chain components of the γGab–, γGc–, and γA–anti-Lac antibodies also eluted from the Sephadex G-200 column set at closely comparable volumes. The data support a four chain model (44, 45) for equine γGab-, γGc-, and γA-globulins.

An additional parameter of the heterogeneity of the immune response is the observed variation in affinities of antibodies with specificity for a single haptenic

grouping (46). The intrinsic association constants (K_0) of antihapten antibody isolated from the serum of a single animal at different intervals in the immunization schedule may vary widely (47). In general, antihapten antibody isolated from a single serum has shown a heterogeneity of association constants (47). A heterogeneity of association constants and a progressive increase in affinities has also been demonstrated in equine anti-Lac antibody having specificity for the hapten *p*-(*p*-dimethylaminobenzeneazo)-phenyl-β-lactoside (Lac dye) (4, 6, 7, 23). The intrinsic association constants (K_0) of unfractionated, purified anti-Lac antibody, measured at 25°C, increased progressively from an initial low of 10^5 M^{-1} to greater than 10^7 M^{-1} (6, 7). The K_0 values of isolated γG–anti-Lac antibodies showed a parallel progressive rise (7). In contrast, γA–anti-Lac antibody had a high initial K_0 value of approximately 10^7 M^{-1} (4) and failed to show the progressive increase in affinity for Lac dye characteristic of γG–anti-Lac antibodies (7). A maximum K_0 value of 4.9×10^7 M^{-1} has been found with a preparation of γGc-antibody isolated from serum collected during the 14th month of immunization (Fig. 4 *b*, Table I). It is evident from the shape of the r/c vs. r plot that this preparation contained antibodies with association constants significantly higher than, and significantly lower than 4.9×10^7 M^{-1}.

Determination of the intrinsic association constants (K_0) of equine anti-Lac antibodies and of their enzymatically produced fragments at 25° and 37°C, and calculation of the thermodynamic parameters of antibody-hapten interaction, has established that removal of the inactive Fc fragments and dissociation of the two antibody-combining sites on the papain 3.5S Fab fragments did not alter the energetics of antibody-hapten interaction. These data would indicate that the variations of affinities for Lac dye present in the different populations of equine anti-Lac antibody were not a direct function of the variations in structure of the Fc fragments. Studies with rabbit γG-globulin antibodies have furnished similar data and led to similar conclusions (48, 49). If the premise that antibody specificity is totally a function of the primary amino acid sequences of the constituent subunits (50, 51) is accepted, one must anticipate variations of the sequences of either the Fd fragments (A piece) of the heavy (A, H) chains or of the light (B, L) chains which comprise the papain 3.5S Fab or pepsin 5S Fab_2' fragments, which are relevant to the observed heterogeneity of affinities. Differences in the amino acid composition of high and low affinity rabbit γG–anti-2,4-dinitrophenyl antibodies have been reported (52). It has been established that the heavy (A, H) chains of antibody contribute to the formation of the antibody-combining site (21, 22, 53). The role of the light (B, L) chains in determining antibody specificity is as yet unclear (22, 54–56) and a possible role of light (B, L) chain variation in affinity heterogeneity has yet to be defined.

The amino acid composition of the equine γGabc–anti-Lac antibody light

(B, L) chains was closely similar to that of the γA–anti-Lac antibody light (B, L) chains (Table II). The data also compared closely to the amino acid data of Weir and Porter for light (B, L) chains of equine γG- and γA-globulins (9, 61). The light (B, L) chains of the equine γGabc– and γA–anti-Lac antibodies contained less than 1 mole of methionine per 23,000 g of protein. These data indicated that the antibody light (B, L) chains were composed of two or more chemically distinct populations, one or more of which lacked methionine. Human and mouse Bence Jones proteins have been shown to vary in the methionine content of their NH_2-terminal half (57–59). Rabbit antibody light (B, L) chains have also been found to contain less than 1 mole of methionine per 23,000 g of protein (60). The amino acid composition of the heavy (A, H) chains of the equine γGabc– and γA–anti-Lac antibodies differed from that of the light (B, L) chains (Table II). The most striking variations were found in the relative contents of serine, proline, glycine, alanine, valine, leucine, and histidine. The composition of the γGabc–anti-Lac antibody heavy (A, H) chains (A_2) also differed from that of the γA-antibody heavy (A, H) chains (A_2) (Table II). Koshland, Englberger, and Shapanka have determined the amino acid composition of the heavy (A, H) and light (B, L) polypeptide chains of three rabbit γG-antihapten antibodies with high precision, and have established that the subunits of antibodies of differing specificity may differ in their primary structure (60). The data for the equine anti-Lac γGabc- and γA-antibodies indicated that the heavy (A, H) chains of two antibodies with affinity for the same haptenic grouping isolated from a single animal may also differ in their primary structure.

The results of equilibrium dialysis of the γA–anti-Lac antibody heavy (A, H) chains with Lac dye established that the isolated heavy (A, H) chains had affinity for the hapten. A maximum light (B, L) chain contamination of 0.06 moles (25,000 mol wt) per mole of heavy (A, H) chain (50,000 mol wt) was calculated from the recovery of protein in the light (B, L) chain fractions and from the maximum loss of heavy (A, H) chains during concentration and dialysis, and was not adequate to account for the molar recovery of sites obtained. Extrapolation of the r/c vs. r data to an r value of 1 per 100,000 g of heavy (A, H) chain protein gave an association constant of 0.8×10^4 M^{-1}, which was significantly lower than that of the intact γA-antibody.

The observation that a single animal is capable of incorporating as many as eight distinct heavy (A, H) and at least two distinct light (B, L) polypeptide chains into antibody with specificity for a single haptenic grouping places added demands upon the thesis which would explain the acquisition of immunological specificity by the selection of the proper subunits from available immunoglobulin polypeptide chain sequences. The genome would be required to code for eight heavy (A, H) chains for each antigenic determinant. A common

mechanism for translating antibody specificity into the sequence of the variable NH_2-terminal halves of the several polypeptide chains would be attractive. One is therefore encouraged to search for common structural features, shared by the several molecular forms of anti-Lac antibody, which are relevant to antibody specificity.

SUMMARY

Eight antigenically unique immunoglobulins have been identified in purified equine anti-*p*-azophenyl-β-lactoside (Lac) antibody isolated from a single horse. The Fc fragments of the γGa-, γGb-, γGc-, and γA-globulins have been shown to possess unique antigenic determinants. Common γG- and γA-Fc fragment antigenic determinants, which were absent from the $10S\gamma_1$- and γM-globulins, have also been observed. All antibody populations share two antigenically distinct light (B, L) chain variants.

The association of anti-Lac antibody with the hapten *p*-(*p*-dimethylaminobenzeneazo)-phenyl-β-lactoside has been measured by equilibrium dialysis and by fluorescence quenching. A variation in the affinity of anti-Lac antibody for hapten has been observed. The affinity of antibody was unaltered by enzymatic removal of the Fc fragments by peptic digestion or dissociation of the two combining sites on the papain 3.5S Fab fragments, indicating that the observed heterogeneity of affinities was not a direct function of the heterogeneity in structure of the Fc fragments. Isolated heavy (A, H) chains of γA-anti-Lac antibody have been shown to have retained affinity for Lac dye by equilibrium dialysis and by analytical ultracentrifugation, employing a combination of schlieren and absorption optics.

The heavy (A, H) chains from two physically separable, antigenically distinct antibody populations, isolated from the same animal and having affinity for the same haptenic determinant, have been found to differ in their amino acid composition. Anti-Lac antibody light (B, L) chains have also been shown to be chemically heterogeneous, and contained populations of polypeptide chains possessing, and populations lacking methionine.

The relevance of the observed structural heterogeneity of equine anti-Lac antibody to the problem of defining the mechanism of acquisition of immunological specificity is briefly discussed.

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