Equine Antihapten Antibody. The Molecular Weights of the Subunits of Equine Immunoglobulins*

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ABSTRACT: Three independent methods have been used to determine the molecular weights of the heavy- and light-polypeptide chain subunits of equine γGab-, γGc-, and γT-immunoglobulins. Extensively reduced and alkylated proteins were filtered through standard columns of Sephadex G-100 or G-200 in 8 M urea–0.05 M propionic acid. Subunit molecular weights were obtained from the linear elution volume, V_e, *vs.* logarithm molecular weight relationship defined for each column with rabbit γG-globulin heavy and light chains and horse heart cytochrome *c*. Molecular weights also were determined by equilibrium sedimentation in 6 M guanidine hydrochloride. Partial specific volumes, $\bar{v}$, were estimated from the amino acid composition of the polypeptide chains and reduced by a factor of 0.015 ml/g to eliminate the effect of preferential binding of guanidine hydrochloride. The recovery of protein in the light fractions, obtained upon Sephadex G-200 gel filtration of extensively reduced and alkylated proteins in 0.04 M sodium decyl or dodecyl sulfate, was used as a third parameter to estimate subunit molecular weights. The three methods furnished molecular weight values which were in substantial agreement. The equine immunoglobulin heavy and light chains had molecular weights of 52,200–53,900 and 22,300–23,100, respectively. A molecular weight of 53,100–54,900 was obtained for the heavy chain of a human γA myeloma protein.

The immunoglobulins of a given species are a heterogeneous population of related but not identical classes and subclasses of proteins. A single animal may respond to antigenic stimulation with the production of a number of forms of antibody. A multiplicity of distinct molecular forms of anti-Lac (*p*-azophenyl β-lactoside) antibody have been isolated from the serum of a single horse (Rockey *et al.*, 1964; Rockey, 1967). The several equine anti-Lac antibodies have been shown to vary in electrophoretic mobility, molecular size, in the antigenic and chemical structure of their heavy chains, and in their affinity for the *p*-(*p*-dimethylaminobenzeneazo)phenyl β-lactoside hapten (Rockey, 1967). The observation that an individual animal is capable of forming a multiplicity of distinct antibodies which share specificity for a single well-defined antigenic grouping raises the question of whether or not the several molecular forms of antibody share common structural features which are relevant to that specificity.

An initial step in the structural analysis of the equine immunoglobulins is an accurate determination of the molecular weights of the constitutive polypeptide chain subunits. A molecular weight of 19,400 has been reported by Pain for the light chains of equine γG-globulins[1] (Pain, 1963). This value is not consistent with the observed recovery of protein in the light-chain fractions (29–33%) obtained upon gel filtration of reduced and alkylated equine anti-Lac antibodies in sodium decyl sulfate (Rockey, 1967). The value also is lower than the molecular weights (22,000–23,000) reported for rabbit and human light chains (Lamm and Small, 1966; Suzuki and Deutsch, 1967; Putnam *et al.*, 1967). The molecular weights of the heavy chains of different immunoglobulin classes may not be identical. The heavy chains of rabbit, human, and equine γG-globulins have molecular weights of 50,000–53,000 (Pain, 1965; Lamm and Small, 1966; Piggot and Press, 1967). Molecular weights of 65,000–70,000 have been reported for rabbit and human γM-globulin heavy chains (Miller and Metzger, 1965; Lamm and Small, 1966; Suzuki and Deutsch, 1967). The heavy chain of rabbit secretory γA-globulin has a molecular weight of approximately 64,000 (Cebra and Small, 1967). Molecular weights of approximately 77,000 and 70,000, respectively, have been determined for the heavy chains of immunoglobulins of the lemon shark (Suran and Papermaster, 1967) and of the sea lamprey (Marchalonis and

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[1] The nomenclature and abbreviations for the immunoglobulins and their subunits and proteolytic fragments have been selected to conform to the recommendations of the World Health Organization (*Bull. World Health Organ. 30*, 447 (1964)). The T component of hyperimmune equine serum and the antigenically related anti-Lac antibody and paraprotein are provisionally referred to as γT-globulins rather than γA-, IgA(T)-, or IgG(T)-globulins (Weir *et al.*, 1966; Rockey, 1967) because of considerations outlined in the Discussion.

Edelman, 1968). The heavy chain of human γE-globulin apparently has a molecular weight of approximately 75,500 (Bennich *et al.*, 1968).

In the present studies, the molecular weights of the heavy and light chains of extensively reduced and alkylated equine γGab-, γGc-, and γT-immunoglobulins have been determined by three independent methods: by gel filtration through standard columns of Sephadex G-100 or G-200 in 8 M urea–0.05 M propionic acid, by sedimentation equilibrium in 6 M guanidine hydrochloride, and from the recovery of protein in the light-chain fractions upon gel filtration of extensively reduced and alkylated proteins in detergents. The molecular weight of the heavy chain of a human γA myeloma protein also has been determined. Attention has been directed toward evaluating the stability of amino acid residues during the gel filtration in 8 M urea–0.05 M propionic acid, as the procedure offers the double advantage of isolating immunoglobulin subunits and fragments and simultaneously estimating their molecular weights.

Materials and Methods

Immunoglobulin Purification. Anti-Lac antibody was isolated from the serum of a hyperimmunized horse, fractionated into distinct antibody populations, and characterized as previously reported (Rockey *et al.*, 1964; Rockey, 1967). Equine γGab-globulins were precipitated from normal sera and from hyperimmune serum after the extraction of anti-Lac antibody, with 33% saturated ammonium sulfate, taken up in 0.01 M sodium phosphate buffer (pH 8) and passed over a column of DEAE-cellulose (Whatman DE 52, 1.0 mequiv/g) equilibrated with the same solvent at 4° (Rockey, 1967). The eluted proteins were lyophilized, taken up in distilled water, dialyzed against 0.2 M sodium chloride–0.01 M sodium phosphate buffer (pH 8), and examined by immunoelectrophoresis and in the analytical ultracentrifuge. The preparations were judged to be γGab-globulins free of other serum proteins. Refined and concentrated equine diphtheria antitoxin (Lot no. 21309, Wyeth Laboratories) was dialyzed against 0.01 M sodium phosphate buffer (pH 8) and applied to a DEAE-cellulose column equilibrated with the same solvent. γGab-Globulins were eluted at 4° with the initial solvent, and γT-globulins were recovered from the washed column by elution with a linear positive gradient of sodium chloride (final concentration 1 M) in the initial solvent. The fractions from the γT-globulin peak were pooled, concentrated either by negative pressure ultrafiltration or lyophilization, and further purified by Pevikon (Fosfatbolaget, Stockholm, Sweden) block zone electrophoresis (Müller-Eberhard, 1960) in a barbital-buffered solution of ionic strength 0.05, pH 8.6, at 4°. Human serum labled with bromophenol blue was run in parallel, and the electrophoresis was continued until the bromophenol blue labeled albumin had reached the anodal extreme of the block. Fractions from the γT-globulin peak were pooled, concentrated by negative pressure ultrafiltration, transferred to 0.2 M sodium chloride–0.01 M sodium phosphate buffer (pH 8) by dialysis, and examined in the analytical ultracentrifuge and by immunoelectrophoresis. The γT-globulin preparations contained small amounts of γG-globulins of rapid electrophoretic mobility and trace amounts of other serum proteins. Small quantities of contaminants were removed by filtering the γT-globulins through two columns of Sephadex G-150 (Pharmacia) (each column 4 × 60 cm) and one column of Sephadex G-100 (4 × 60 cm) in series, at 4°. A γT-globulin paraprotein (Montgomery *et al.*, 1968; Dorrington and Rockey, 1968) was isolated from the serum of a horse with a malignant lymphoma by Pevikon block zone electrophoresis. The extinction coefficients previously reported for equine γG- and γT-globulins (Rockey, 1967) were used to determine protein concentrations.

Human γM-macroglobulins were precipitated from sera of patients with Waldenström's macroglobulinemia by dilution with 15 volumes of distilled water. The proteins were resolubilized in saline and reprecipitated with water two additional times. The proteins, resolubilized again in saline, were passed through sterile bacterial filters (Swinney filter, Becton, Dickinson) and subjected to gel filtration at room temperature through three sterile columns of Sephadex G-200 (each 4 × 60 cm) in series in 0.2 M sodium chloride, 0.002 M EDTA, and 0.01 M sodium phosphate buffer (pH 8) (Rockey and Schwartzman, 1967). In some instances, the isolated γM-globulins also were subjected to Pevikon block zone electrophoresis. Monomeric (7 S) and polymeric (9–15 S) human γA-myeloma proteins were isolated by zone electrophoresis and Sephadex G-150 or G-200 gel filtration as described elsewhere (Dorrington and Rockey, 1968). The γM and γA proteins were examined by immunoelectrophoresis and in the analytical ultracentrifuge and were judged to be free of significant contaminating serum proteins. Rabbit γG-globulin (fraction II, Pentex) was used as purchased. Analytical ultracentrifugation and immunoelectrophoresis failed to demonstrate significant contaminating proteins.

Agar Diffusion Studies. Proteins were examined by immunoelectrophoresis (Heremans, 1960; Rockey, 1967) and in agar double-diffusion studies with the following antisera: rabbit antisera prepared against equine serum proteins, purified anti-Lac antibodies, normal γGab-globulins, diphtheria antitoxin γT-globulins, and against the heavy chains, light chains, pepsin 5S $F(ab')_2$ fragments, papain 3.5S Fab fragments, and papain Fc fragments isolated from equine γGab-globulins and γT-globulins; goat antisera prepared against equine γGab-globulin and γT-globulin heavy and light chains; rabbit antisera specific for either human κ light chains, λ light chains, γG-globulins, γM-globulins, γA-globulins, or the proteins of human serum (Rockey *et al.*, 1964); and sheep antisera specific for either rabbit γG-globulins or rabbit serum proteins. The antisera were induced by repeatedly immunizing animals with antigens incorporated in complete Freund's adjuvant (Difco Laboratories).

Subunit Preparation. Immunoglobulins were reduced with 0.05–1.25 M 2-mercaptoethanol (Mann Research Laboratories) in 0.002 M EDTA–0.2–0.5 M Tris-HCl (pH 8) containing either 0–12 M urea or 0–8 M guanidine

hydrochloride. Urea (Baker Chemical) was recrystallized three times from boiling 95% ethanol (initial solution adsorbed with charcoal) and dried on a Büchner funnel. Solutions were prepared in glass-distilled water and passed through a mixed-bed resin (Bio-Rad AG501 × 8D, analytical grade, 20–50 mesh) column immediately prior to use. Guanidine hydrochloride (Eastman Organic Chemicals) was twice recrystallized from the filtrate of a boiling 95 % ethanol–charcoal mixture, then twice recrystallized from distilled water, and dried *in vacuo* over phosphorus pentoxide. Proteins were reduced in a closed, jacketed reaction vessel at either 25 or 37° (temperature controlled by a Lauda thermostated circulator) under a stream of nitrogen for 4–24 hr. A 10–20% molar excess of solid iodoacetamide over mercaptoethanol was added and the apparent pH of the reaction mixture was maintained at 8 by the addition of 2 M Tris with a pH-Stat (Radiometer) and, when necessary, of solid Tris. The iodoacetamide (Mann Research Laboratories) was twice recrystallized from filtrates of boiling 95% ethanol–charcoal mixtures, then twice recrystallized from water, and dried *in vacuo* over phosphorus pentoxide in an opaque container. Proteins were alkylated for 30 min either at 0, 25, or 37° and then dialyzed at 4° against large volumes of distilled water.

Rabbit and equine immunoglobulin subunits were labeled with [1-^{14}C]iodoacetamide and used as internal standards for molecular weight determinations by gel filtration. Proteins were reduced with 0.05–0.15 M 2-mercaptoethanol in 0.002 M EDTA–0.2 M Tris-HCl (pH 8) and alkylated with a 20% molar excess of iodoacetamide containing 4–6% [1-^{14}C]iodoacetamide (100–120 μCi, 2.73 mCi/mmole, Tracer Lab). Radioactively labeled proteins were dialyzed exhaustively against distilled water and concentrated by negative pressure ultrafiltration. An aliquot (20–30 mg) of the ^{14}C-labeled protein was added to an unlabeled equine or human immunoglobulin (70–80 mg) and the mixture was extensively reduced in concentrated urea or guanidine hydrochloride solution and alkylated with unlabeled iodoacetamide. Proteins were dialyzed exhaustively against distilled water prior to application to the molecular weight columns. Radioactivity was measured in a Packard Tri-Carb series 3000 liquid scintillation spectrometer. Samples (50–100 μl) were dried on filter paper disks and counted in a toluene scintillation solution[2] in glass vials at a counting efficiency of 52.3%.

The heavy- and light-polypeptide chains of mildly reduced and alkylated immunoglobulins were dissociated at 4° on columns (4 × 60 cm columns, three in series) of Sephadex G-100 or G-150 in 1 M propionic acid (Fleischman *et al.*, 1962; Rockey, 1967). Mildly and extensively reduced and alkylated subunits were separated at room temperature by filtration through columns (4 × 60 cm columns, three in series) of Sephadex G-200 in 0.002 M EDTA–0.01 M sodium phosphate buffer (pH 7.5) containing either 0.04 M sodium decyl sulfate (Utsumi and Karush, 1964; Rockey, 1967) or 0.04 M sodium dodecyl sulfate (Jaquet *et al.*, 1964; Koshland *et al.*, 1966). Sodium dodecyl sulfate (sodium lauryl sulfate, U.S.P., Fisher Scientific) and sodium decyl sulfate were recrystallized three times from the filtrates of boiling absolute ethanol–charcoal mixtures. Extensively reduced and alkylated subunits also were prepared by gel filtration in 8 M urea–0.05 M propionic acid and in 5 M guanidine hydrochloride (*vide infra*). The equine immunoglobulin subunit extinction coefficients previously reported (Rockey, 1967) were used to determine the recovery of protein in the heavy- and light-chain fractions. Extinction coefficients, $E^{1cm}_{\lambda 280 m\mu}$, of 10-mg/ml solutions of extensively reduced and alkylated equine heavy and light chains in 8 M urea–0.05 M propionic acid were determined to be 14.3 and 12.7, respectively, by use of the subunit extinction coefficients in 0.04 M sodium decyl sulfate previously reported (Rockey, 1967). Prior to amino acid analysis, the isolated polypeptide chains were dialyzed exhaustively at 4° against distilled water, lyophilized, and dried *in vacuo* over phosphorus pentoxide.

Amino Acid Analyses. Lyophilized proteins were dissolved or suspended in glass-redistilled, constant-boiling (approximately 5.7 N) HCl (more than 1 ml for every 5 mg of protein) in heavy-walled Pyrex combustion tubes and frozen in a bath of solid carbon dioxide and methyl Cellosolve. The tubes were evacuated and the samples were thawed and degassed. The tubes then were sealed under vacuum (less than 50 μ of residual pressure, measured with a Stokes McLeod gauge) and the samples were hydrolyzed in an oven equipped with a circulating fan, at 110 ± 1° (Moore and Stein, 1963). Proteins routinely were hydrolyzed for 24 hr. The rates at which theronine, serine, and tyrosine were destroyed during hydrolysis, and the period necessary for maximum release of valine and isoleucine were determined from analyses of 24-, 48-, 72-, and 96-hr hydrolysates of light and heavy chains from equine γGab- and γT-globulins. Amino acid analyses were preformed with a Technicon single-column (0.6 × 128 cm, Technicon chromo-bead Type A resin) automatic amino acid analyzer equipped with an Infotronics Model CRS-10ABT digital readout system. The cysteine content of subunits was determined as *S*-carboxymethylcysteine after extensive reduction and alkylation. An improved resolution of *S*-carboxymethylcysteine and of aspartic acid was obtained by adding 5 and 3 ml of methanol to chambers 1 and 2, respectively, of the nine-chamber Autograd gradient elution device (Thomson and Miles, 1964), and developing the chromatogram continuously at 60°. Norleucine (0.20 μmole, A Grade, Calbiochem) was incorporated into each analysis as an internal standard. Conversion factors were determined from analyses of a standard amino acid mixture (0.25 μmole each of 18 amino acids, Technicon; 0.20 μmole of DL-norleucine, and 0.20 μmole of *S*-carboxymethylcysteine, Nutrional Biochemical) performed at intervals between analyses of immunoglobulin subunit hydrolysates. Amino acid chromatography peaks were integrated in the usual manner (Spackman *et al.*,

[2] The scintillation solution was prepared by adding 6 g of 2,5-diphenyloxazole (scintillation grade, Packard) and 0.5 g of 1,4-bis[2-(4-methyl-5-phenyloxazolyl)]benzene (scintillation grade, Packard) to 1 l. of toluene (Baker Chemical).

1958) and by the Infotronics digital readout system (less proline), and the two values in general agreed closely. Values obtained from the chromatography tracings were used in preference to integrator values whenever the two did not correspond closely. The isoleucine, methionine, and tyrosine values were not corrected for the formation of *allo*-isoleucine, methionine sulfoxides, and chlorotyrosine, respectively (Moore and Stein, 1963), but the formation of these components was judged to be small. The ratio of moles of tryptophan to mole of tyrosine was determined optically (Goodwin and Morton, 1946) for each subunit and used, together with the tyrosine content determined by amino acid analysis, to calculate tryptophan content. Optical densities were determined with a Zeiss PMQII spectrophotometer.

Molecular Weight Columns. Gel filtration through standard columns of Sephadex G-100 and G-200 in dissociating solvents (Whitaker, 1963; Cebra and Small, 1967) was used to determine the molecular weights of extensively reduced and alkylated polypeptide chains from equine and human immunoglobulins. Recrystallized urea (in excess of 8 M) was dissolved in distilled water and passed through a mixed-bed resin (Bio-Rad AG501 × 8D) column and the column effluent was used to prepare a solution of 8 M urea–0.05 M propionic acid. Sephadex G-100 and G-200 were allowed to swell in 8 M urea–0.05 M propionic acid for 3–5 days and then layered over glass beads and a 1-cm layer of Sephadex G-25 in columns with sintered-glass bases and minimal effluent dead spaces. The downward flow rate (1.0–2.5 ml/hr) through each column was controlled by an effluent pump (Sigmamotor). The urea solvent was passed through the column for 5 days prior to the initial experiment. Extensively reduced and alkylated subunits (total protein 100–130 mg) were applied in the urea solvent and filtered at room temperature. Column bed heights were determined during the first experiment. The dimensions of the three standard urea columns employed were: Sephadex G-100 (no. 1), 2.2 × 148.5 cm; Sephadex G-100 (no. 2), 2.5 × 146.5 cm; and Sephadex G-200, 2.2 × 162.0 cm. The gels tended to shrink and the column heights to decrease by 0.55–1.30% with prolonged use. The light chain used in the initial experiment with each column was employed as a standard in subsequent filtration experiments to correct for the small changes (1.62–4.10%) in the elution volumes observed after prolonged column use. Horse heart cytochrome *c* (protein mol wt 12,398, ferrous protoporphyrin mol wt 607; Margoliash *et al.*, 1961) and extensively reduced and alkylated rabbit γG-globulin heavy and light chains (mol wt 53,000 and 23,000, respectively; Small and Lamm, 1966) were employed as column standards. Cytochrome *c* and, in some instances, radioactively labeled rabbit γG-globulin or equine γT-globulin heavy and light chains (*vide supra*) were mixed with subunits of unknown molecular weight and used as internal standards. The logarithm of the molecular weight of each standard protein was plotted as a function of its elution volume, V_e (Whitaker, 1963), and molecular weights of unknowns were estimated from the linear V_e *vs.* log molecular weight relationship defined for each standard column.

Columns of Sephadex G-200 also were prepared in 5 M guanidine hydrochloride (2.2 × 61.3 cm columns, three in series) (Cebra and Small, 1967). The flow rate through the 5 M guanidine columns was exceedingly slow after the initial run and these columns were used only for a limited number of experiments.

Equilibrium Sedimentation. A Spinco–Beckman Model E ultracentrifuge with interference optics and a RTIC temperature control unit was used for sedimentation equilibrium experiments. Standard double-sector cells with 12-mm aluminum-filled epoxy resin centerpieces and sapphire windows were employed. An AN-D rotor was used throughout and blurring of the fringe pattern, which sometimes developed at high speeds (35,600 rpm), was compensated by a polarizing filter placed directly over the light source in addition to the Kodak 77-A filter. Fringe patterns were recorded on Kodak II-G spectrographic plates. Measurements of fringe patterns against radial distance were made with a Gaertner comparator. All fringe displacements were water-blank corrected for window distortion as described by Yphantis (1964). The system was judged to be at equilibrium when the fringe displacement at the solution–fluorocarbon junction remained constant for a period of 5–10 hr as described by Kawahara and Tanford (1966). Column height and rotor speed were adjusted to enable the use of the meniscus-depletion method developed by Yphantis (1964). The quantity $M(1 - \bar{v}\rho)$ was calculated from the slopes of linear plots of the logarithm of the fringe displacement (μ) against radial distance (centimeters) squared, r^2, according to the relationship

$$M(1 - \bar{v}\rho) = \frac{\mathrm{d} \log f}{\mathrm{d}r^2} \frac{2RT}{\omega^2}$$

Molecular weights were calculated from this quantity using the appropriate density and values for the partial specific volume, $\bar{v}$.

Solvent densities for solutions of 6.0 M guanidine hydrochloride were determined pycnometrically.

The partial specific volumes, $\bar{v}$, of the extensively reduced and alkylated heavy and light chains were estimated from the amino acid compositions reported in Table II. However, for proteins in guanidine hydrochloride it is necessary to estimate a value for the "effective specific volume" of the anhydrous protein in this solvent. This quantity includes the actual partial specific volume of the protein plus a correction term which depends upon the interactions of the protein with solvent components (Hade and Tanford, 1967). Preferential binding of guanidine has been demonstrated directly for a number of proteins (Hade and Tanford, 1967) and it has been shown that the "apparent specific volume" of certain proteins in concentrated guanidine solutions, determined by the method of Casassa and Eisenberg (1961), is 0.01–0.02 ml/g less than the corresponding quantity in dilute salt solutions (Marler *et al.*, 1964; Small and Lamm, 1966; Hade and Tanford, 1967). In view of these considerations we have used a corrected value for $\bar{v}$ in 6 M guanidine hydrochloride 0.015 ml/g lower than the partial specific volume calculated directly from the amino acid composition.

TABLE I: Sedimentation Coefficients of Equine Immunoglobulins.[a]

Equine Immunoglobulin	$s^0_{20,w}$ (S)
γGab-Globulins	6.71
γGabc anti-Lac antibody	6.78
γGc anti-Lac antibody	6.69
γT-Globulins, diphtheria antitoxin	7.12
γT anti-Lac antibody	6.94
γT paraprotein	7.22

[a] Proteins were sedimented at 5–13 concentrations ranging from 0.5 to 7.0 mg per ml. The data were treated by the method of least squares to obtain sedimentation coefficients at infinite dilution ($s^0_{20,w}$).

Analytical Ultracentrifugation. Proteins were sedimented in a Model E ultracentrifuge equipped with schlieren optics, at 20°, in 0.2 M sodium chloride–0.01 M sodium phosphate buffer (pH 8) either at 52,640 or 59,780 rpm. Sedimentation coefficients were corrected for solvent viscosity and density by use of a conversion factor of 1.048. A partial specific volume of 0.739 ml/g. (Pain, 1963) was employed for all proteins. Sedimentation coefficie, $s^0_{20,w}$, were determined at protein concentrations ranging from 0.5 to 7.0 mg per ml, and the data were treated by the method of least squares to obtain values at infinite dilution ($s^0_{20,w}$).

Results

Sedimentation Coefficients. The sedimentation coefficients, $s^0_{20,w}$, of the equine γG and γT anti-Lac antibodies and immunoglobulins are recorded in Table I. The γT-globulins consistently sedimented more rapidly than did the γG-globulins.

Amino Acid Analysis. Each acid hydrolysate was subjected to amino acid analysis on four to eight separate occasions. The average deviation from the mean value for each amino acid was determined for each hydrolysate. With the exclusions of methionine and proline and other rare instances, the average deviation from the mean was less than 4% of the mean, and in a majority of instances, less than 2%. Proline values for a single hydrolysate deviated from a mean value by an average of less than 6% of the mean. The methionine values of repeated analyses of equine immunoglobulin heavy- and light-chain hydrolysates deviated from a mean value for each hydrolysate by an average of less than 8 and 25%, respectively. Linear extrapolations of the 24-, 48-, 72-, and 96-hr hydrolysis data to zero time established the following percentages for the destruction of threonine, serine, and tyrosine during 24-hr acid hydrolysis: equine immunoglobulin light chains, threonine 2–3%, serine 4–6%, and tyrosine 3–5%; equine immunoglobulin heavy chains, threonine 2–5%, serine 5–8%, and tyrosine 2–4%. Maximum values for valine and isoleucine were obtained after 72-hr acid hydrolysis.

The amino acid compositions of the extensively reduced and alkylated subunits of the equine γGab- and γT-globulins and the human γA myeloma protein used for molecular weight determinations by equilibrium sedimentation are recorded in Table II.

The following experiments were carried out to determine whether or not amino acids (*i.e.*, methionine) were selectively lost either during the extensive reduction and alkylation, or during the subunit separation by gel filtration at room temperature. Equine γG- and γT-globulins were reduced at pH 8 with 0.1 M 2-mercaptoethanol (4 hr at 25°) and alkylated at 0° with a 10% molar excess of iodoacetamide. Polypeptide chains were separated at 4° in 1 M propionic acid, or at room temperature in 0.04 M sodium decyl or dodecyl sulfate. A fraction of each isolated chain was taken for amino acid analysis. The remaining subunit was extensively reduced in 8 M guanidine hydrochloride and alkylated at 25° with a 20% molar excess of iodoacetamide. A fraction of each extensively reduced and alkylated chain was again taken for amino acid analysis and the data were compared with that obtained prior to extensive reduction and alkylation. The remainder of each heavy chain was subjected to gel filtration at room temperature in either 0.04 M detergent or 8 M urea–0.05 M propionic acid. Proteins from the heavy-chain peaks again were used for amino acid analysis and the data were compared with that obtained prior to gel filtration at room temperature. The results of these studies for the destruction of methionine are summarized in Table III. The losses of methionine during the extensive reduction and alkylation and during the gel filtration at room temperature were minimal. A comparison of the 24-, 48-, 72-, and 96-hr hydrolysate values failed to show evidence of a time dependent destruction of methionine during the acid hydrolysis. The equine immunoglobulin light-chain hydrolysates contained less than 1 mole of methionine/23,000 g of protein (Table III). These values were substantiated by adding 0.05–0.10 μmole of methionine to the analyses, and determining the additional methionine contributed by the light chains. Table III also records the *S*-carboxymethylcysteine content of mildly and extensively reduced and alkylated subunits of equine anti-Lac antibodies and immunoglobulins. The amino acid compositions of the heavy chains, light chains, pepsin 5S $F(ab')_2$ fragments, and papain Fab fragments of equine γGab, γGc, and γT anti-Lac antibodies have been determined and will be reported elsewhere. To assure that maximum recovery of *S*-carboxymethylcysteine was obtained, extensively reduced and alkylated equine γGab- and γT-globulins on some occasions were reduced and alkylated again in 8.0 M guanidine hydrochloride at 37° and the isolated subunits were subjected to amino acid analysis. These data also are incorporated in Table III. A comparison of the 24-, 48-, 72-, and 96-hr hydrolysate *S*-carboxymethylcysteine values failed to show evidence of a time-dependent destruction during acid hydrolysis.

Molecular Weight Determinations. The molecular weights of the subunits of the equine γGab- and γT-globulins and of a human γA myeloma protein (Vu) and two γM macroglobulins (Io, St), estimated by filtration of the extensively reduced and alkylated polypeptide

TABLE II: Amino Acid Composition of the Equine and Human Immunoglobulin Subunits Used for Molecular Weight Determination by Equilibrium Sedimentation.[a]

Amino Acid	Equine γGab-Globulins		Equine γT-Globulins		Human γA Myeloma Protein
	Heavy Chain	Light Chain	Heavy Chain	Light Chain	Heavy Chain[e]
S-Carboxymethylcysteine	12.8	5.1	12.7	5.2	15.2
Aspartic acid	35.5	15.0	40.2	14.8	30.1
Threonine	43.2[b]	22.3[b]	37.0[b]	21.8[b]	50.8
Serine	50.7[b]	32.6[b]	46.2[b]	32.2[b]	48.8
Glutamic acid	42.5	19.8	45.5	19.5	43.5
Proline	35.1	12.8	37.2	13.9	49.6
Glycine	33.1	20.3	31.9	20.2	34.1
Alanine	26.5	14.2	25.0	14.5	29.3
Valine	50.8[c]	17.1[c]	51.4[c]	16.6[c]	34.3
Methionine	2.8	0.13	2.9	0.18	1.9
Isoleucine	14.5[c]	7.9[c]	13.6[c]	8.0[c]	8.3
Leucine	34.4	13.9	35.5	13.5	49.2
Tyrosine	16.8[b]	7.7[b]	17.5[b]	7.7[b]	12.9
Phenylalanine	15.3	5.8	15.5	5.1	17.3
Lysine	33.4	11.4	32.2	11.4	15.6
Histidine	10.9	2.2	12.6	2.2	9.0
Arginine	11.7	5.9	12.5	6.6	20.0
Tryptophan	8.5[d]	3.8[d]	8.8[d]	3.7[d]	9.6[d]

[a] Presented as amino acid residues per 52,000 and 23,000 g of heavy and light chain, respectively. [b] Corrected for destruction occurring during acid hydrolysis. [c] Value obtained after 72-hr acid hydrolysis. [d] Determined optically. [e] Uncorrected values from analyses of 24-hr hydrolysates.

TABLE III: Methionine and *S*-Carboxymethylcysteine Content of Equine Immunoglobulin Subunits.[a]

Mildly Reduced and Alkylated[b] Subunits	Methionine	*S*-Carboxymethylcysteine	Extensively Reduced and Alkylated[c] Subunits	Methionine	*S*-Carboxymethylcysteine
γGab anti-Lac light chains	0.24	1.0	γGabc anti-Lac light chains	0.20	5.3
γGc anti-Lac light chains	0.21	0.8	γT anti-Lac light chains	0.36	5.0
γT anti-Lac light chains	0.40	1.2	γGab-Globulin light chains	0.13	5.1
γGab-Globulin light chains	0.16	1.1			
γT diphtheria antitoxin light chains	0.21	1.2	γT diphtheria antitoxin light chains[d]	0.18	5.2
γGab anti-Lac heavy chains	3.2	5.1			
γGc anti-Lac heavy chains	3.4	3.6			
γT anti-Lac heavy chains	2.9	5.0	γGab anti-Lac heavy chains	3.0	13.6
γGab-Globulin heavy chains	3.1	4.1	γGc anti-Lac heavy chains	3.3	11.8
			γT anti-Lac heavy chains	3.4	12.4
			γGab-Globulin heavy chains[d]	2.8	12.6
γT diphtheria antitoxin heavy chains	3.2	4.3		2.6[e]	12.8[e]
			γT dithpheria antitoxin heavy chains[d]	2.7	12.6
				2.9[e]	12.9[e]

[a] Presented as amino acid residues per 52,000 and 23,000 g of heavy and light chains, respectively. [b] Reduced with 0.10 M 2-mercaptoethanol. [c] Reduced and alkylated in either 11.5–12.0 M urea or 7.5–8.0 M guanidine hydrochloride. [d] Extensively reduced and alkylated two times in 8.0 M guanidine hydrochloride. [e] Isolated subunits again subjected to gel filtration in 8 M urea–0.05 M propionic acid.

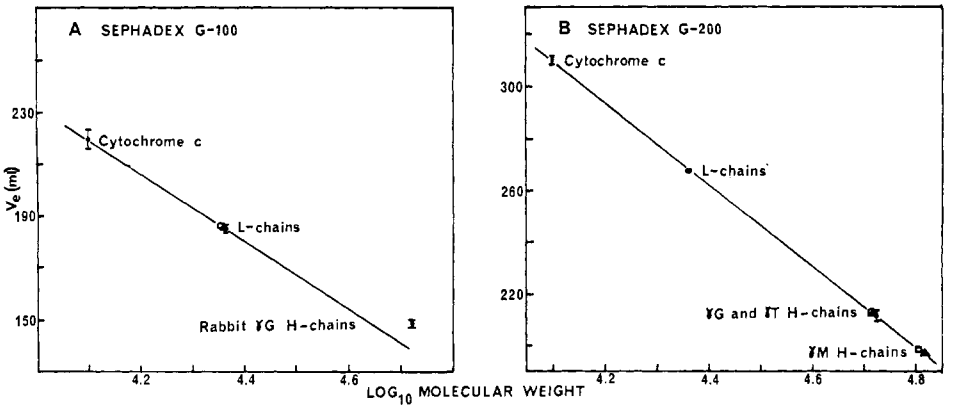

FIGURE 1: V_e *vs.* logarithm molecular weight relationships defined for standard columns of Sephadex G-100 (A) and Sephadex G-200 (B) from the mean elution volumes of rabbit γG-globulin light chains (mol wt 23,000) and heavy chains (mol wt 53,000) and horse heart cytochrome *c* (mol wt 13,000). The vertical bars indicate the average deviation from the mean for the elution volumes of the standard peptides. Column solvent: 8 M urea–0.05 M propionic acid. Column dimensions: (A) Sephadex G-100, 2.2 × 148.5 cm and (B) Sephadex G-200, 2.2 × 162.0 cm. The standard curves were used to obtain molecular weights of subunits of equine γG- (△) and γT- (○) and human γA- and γM- (□, ▲) immunoglobulins. The γA figure is not included for reasons of clarity.

chains through standard columns of Sephadex G-100 or G-200 in 8 M urea–0.05 M propionic acid, are recorded in Figure 1 and Table IV. Light-chain molecular weights were determined by Sephadex G-100 gel filtration. Equine γGab and γT subunits were run alternately with the standard rabbit γG-globulin subunits. Each protein was run six or more times and a mean elution volume was determined for each chain. Standard curves were constructed from the mean values for rabbit γG-globulin light chains and cytochrome *c* (Figure 1). The means of the elution volumes of the equine γGab and γT light chains differed from the mean elution volume of the rabbit light chain (mol wt 23,000) by 0.45 and 0.90 ml, respectively, corresponding to molecular weight differences of 600 and 800, respectively (Table IV). Heavy-chain molecular weights were determined by Sephadex G-200 gel filtration. The data were normalized by correcting the elution volumes to obtain a standard value for the light-chain elution volume. This was justified by the results of the Sephadex G-100 light-chain molecular weight determinations and by the profiles of the mixing experiments incorporating standard ^{14}C-labeled subunits (Figure 2). Each protein was run four to eight times. Standard curves were constructed from the mean values of the elution volumes for rabbit γG-globulin subunits and cytochrome *c* (Figure 1). The molecular weights of the equine γGab and γT and the human γA and γM heavy chains determined from their mean elution volumes are recorded in Table IV. The maximum deviation from the mean elution volume for each heavy chain was less than 2% of the mean. Gel filtration mixing experiments in which the subunits of one of the immunoglobulins were labeled with [1-^{14}C]iodoacetamide were carried out in the following combinations: ^{14}C-labeled rabbit γG-globulins mixed with either equine γGab-globulins, γT-globulins, or human γM-globulins; ^{14}C-labeled equine γT-globulins mixed with either human γM-globulins or γA-globulins. Representative experiments are presented in Figure 2. The ratio [1-^{14}C]-iodoacetamide in the γT heavy chains/[1-^{14}C]iodoacetamide in the γT light chains (labeled after mild reduction with 0.05 M 2-mercaptoethanol) varied from 3.7 to 4.0. The unlabeled and the ^{14}C-labeled heavy and light chains were recovered at the same respective volumes in all experiments except in those instances where γM heavy chains were included (Figure 2). Human γM macroglobulin heavy chains consistently were recovered before the ^{14}C-labeled rabbit γG and equine γT heavy

TABLE IV: Molecular Weights of Equine γGab- and γT- and Human γA- and γM-Immunoglobulin Subunits Determined by Gel Filtration in 8 M Urea–0.05 M Propionic Acid.[a]

Subunit	M × 10^{-3}
γGab light chain	22.9[c]
γT light chain	22.7[c]
γGab heavy chain	52.4[d]
γT heavy chain	52.2[d]
γA (Vu)[b] heavy chain	53.1[d]
γM (Io)[b] heavy chain	64.4[d]
γM (St)[b] heavy chain	62.5[d]

[a] Extensively reduced and alkylated proteins were filtered through standard Sephadex columns in 8 M urea–0.05 M propionic acid. Molecular weights were determined from the linear elution volume, V_e, *vs.* log molecular weight relationship defined for each column. [b] Light-chain type of human proteins: Vu and Io, κ; St, λ. [c] Light-chain molecular weight is the average of 6–8 determinations by Sephadex G-100 gel filtration (equine subunits run alternately with the standard, rabbit light chain, mol wt 23,000). [d] Heavy-chain molecular weight is the average of four to eight determinations by Sephadex G-200 gel filtration (Figure 1) (see text).

chains (Figure 2). A significant amount of aggregated material frequently was observed upon gel filtration of extensively reduced and alkylated rabbit γG-globulins in both the 8 M urea and the 5 M guanidine hydrochloride solvents.

The molecular weights of extensively reduced and alkylated equine γGab and γT and human γA polypeptide chains determined by equilibrium sedimentation in 6 M guanidine hydrochloride are presented in Table V. The plots of the logarithm of the fringe displacement ($\log f$) *vs.* the radial distance squared (r^2) for the equine heavy and light chains are presented in Figure 3. There was no substantial curvature in the $\log f$ *vs.* r^2 plots.

An additional estimate of the molecular weights of the immunoglobulin subunits was obtained from the recovery of protein in the light-chain fractions upon Sephadex G-200 gel filtration of extensively reduced and alkylated proteins in 0.04 M sodium decyl or dodecyl sulfate. The light-chain fractions were completely separated from the heavy-chain fractions when subunits were filtered through three (4 × 60 cm) columns in series (Rockey, 1967). Subunit molecular weights may be calculated from the percentage of the applied protein recovered in the light-chain fractions by assuming that there are two light and two heavy chains per molecule and that the molecular weight of the intact protein is 150,000. The molecular weight of equine γG-globulin has been reported by Pain (1965) to be 148,000–151,000. A molecular weight of approximately 150,000 for the equine γT-globulin is supported by the observation that bivalent γT anti-Lac antibody bound a maximum of 2 moles of hapten/150,000 g of protein (Rockey, 1967). The results of the detergent gel filtration experiments are summarized in Table VI. The volumes at which the subunits were recovered from a standard column set also are recorded.

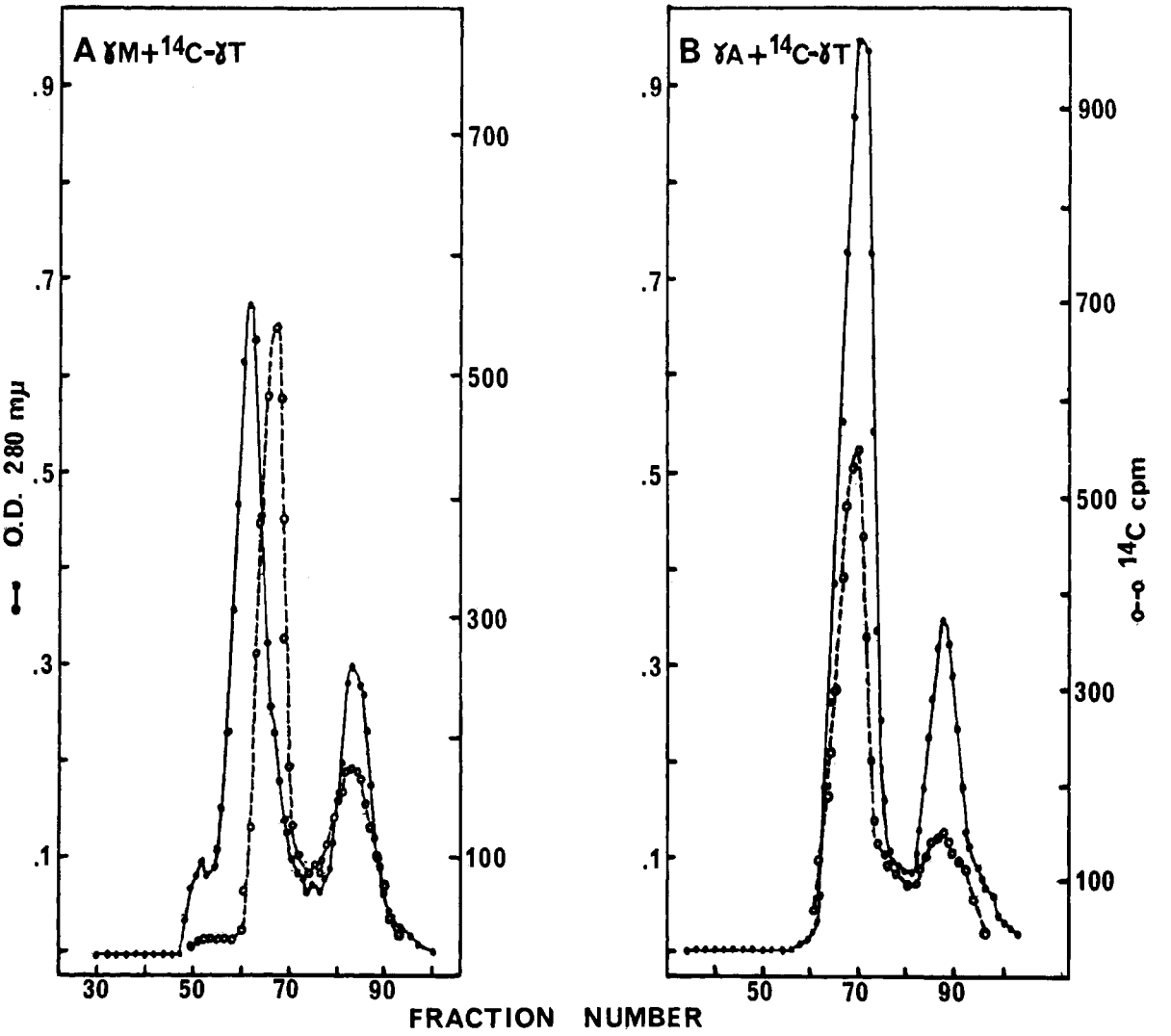

FIGURE 2: Fractionation of extensively reduced and alkylated immunoglobulin subunits by Sephadex G-200 gel filtration in 8 M urea–0.05 M propionic acid. Equine γT-globulin subunits labeled with [1-^{14}C]iodoacetamide. (A) Mixture of equine γT-globulin and human γM-macroglobulin (Io). (B) Mixture of equine γT-globulin and human γA myeloma protein (Vu). The equine γT heavy chains were recovered after the human γM heavy chains but at essentially the same volume as the human γA heavy chains. Light chains of the mixed proteins were recovered at the same volume in all instances.

Discussion

The T component of equine diphtheria antitoxin (previously designated IgA(T), Weir and Porter, 1966) and the antigenically related anti-Lac antibody (previously designated γA-globulin; Rockey, 1967) and equine paraprotein (Montgomery *et al.*, 1968; Dorrington and Rockey, 1968) have provisionally been designated γT-globulins because their relationship to the reference classes and subclasses of human immunoglobulins (Fahey *et al.*, 1967) is unclear. The nomenclature is preferred to the more restrictive revised nomenclature of Weir *et al.* (1966) (IgG(T)) because of the following considerations: The γT-globulins possess prominent distinct heavy-chain antigenic determinants, a high carbohydrate content, and a rapid electrophoretic mobility (Schultze *et al.*, 1965; Weir and Porter, 1966; Rockey, 1967). The optical rotatory dispersion curves of the equine γT-globulins lack the characteristic 240-mμ Cotton effect of equine, rabbit, and human γG-globulins (Dorrington and Rockey, 1968). An analysis of the amino acid replacements in the C-terminal nonadecapeptides of the heavy chains of the equine T component and of equine γG-, rabbit γG-, bovine γG-, and human γG_1-, γG_2-, γG_3-, and γG_4-globulins (Givol and Porter, 1965; Weir *et al.*, 1966; Press *et al.*, 1966; Prahl, 1966, 1967; Inouve and Givol, 1967) and an analysis of the required RNA codon (Nirenberg *et al.*, 1966) alterations, indicated that the equine γG-globulins were more closely related to rabbit γG-, bovine γG, and all four human γG subclass proteins than they were to the equine γT-globulin. Table VII presents this analysis. The equine γG C-terminal nonadecapeptide sequence differed from the corresponding rabbit γG, bovine γG, and human $\gamma G_1(\gamma G_2)$, γG_3, and γG_4 sequences by two, two, two, four, and three amino acid replacements, respectively. Each of the amino acid interchanges between the several pairings of the equine γG, rabbit γG, bovine γG, and human γG_1, γG_2, γG_3, and γG_4 nonadecapeptides could be accounted for by single nucleotide replacements in the RNA codons, with the one excep-

TABLE V: Molecular Weights of Equine γGab and γT Heavy and Light Chains and Human γA Heavy Chain Obtained by Sedimentation Equilibrium.

Sample	Concn[a] (mg/ml)	Speed (rpm)	$\bar{v}$[b]	M × 10^{-3}
γGab heavy chain	0.32	29,500	0.719	53.8
	0.32	24,630		52.6
	0.84	24,630		53.9
γGab light chain	0.4	35,600	0.710	23.1
γT heavy chain	0.3	29,500	0.718	52.9
	0.3	24,630		53.4
γT light	0.4	35,600	0.710	22.3
γA heavy chain	0.35	29,500	0.716	54.6
	0.35	24,630		54.2
	0.8	24,630		54.9

[a] Solvent 6.0 M guanidine hydrochloride, ρ = 1.140 g/ml. [b] Partial specific volumes estimated from the amino acid compositions reported in Table II and reduced by 0.015 ml/g (Hade and Tanford, 1967) to eliminate the effect of preferential binding of guanidine.

TABLE VI: Molecular Weights of Equine Immunoglobulin Light Chains, Estimated from the Percentage of Applied Protein Recovered in the Light-Chain Fractions.[a]

Immunoglobulin	Elution Volume[b] (ml) of Heavy Chain	Elution Volume[b] (ml) of Light Chain	% Protein in Light-Chain Fractions	Light Chain (M × 10^{-3})[c]
γGabc anti-Lac	869	1172	32.0 ± 1.7	24.2 ± 1.3
γGab anti-Lac	874	1193	29.8 ± 1.4	22.4 ± 1.0
γGc anti-Lac	868	1178	29.4 ± 1.3	22.0 ± 1.0
γT anti-Lac	883	1186	31.4 ± 0.3	23.6 ± 0.2
γGab-Globulins	873	1198	31.0 ± 0.6	23.2 ± 0.4
γT diphtheria antitoxin	869	1183	30.8 ± 1.6	23.1 ± 1.2
γA myeloma protein (Vu)	861	1181	27.2 ± 1.2[d]	
γM-Macroglobulin (Io)	809	1184	23.0 ± 0.9[d]	

[a] Extensively reduced and alkylated subunits separated by Sephadex G-200 gel filtration in 0.04 M sodium decyl or dodecyl sulfate. Equine light-chain protein determined from the optical density at 280 mμ, employing $E^{1\,cm}_{\lambda\,280\,m\mu}$ 14.0 for a 10-mg/ml solution (Rockey, 1967). Presented as the mean of two to six determinations, together with the maximum deviation from the mean. [b] Volume at which subunits were recovered from a standard set of three 4 × 60 cm columns of Sephadex G-200 in 0.04 M sodium dodecyl sulfate. [c] Calculated by assuming a molecular weight of 150,000 for the intact protein, and two light chains per molecule. [d] Extensively reduced and alkylated human γA (Vu) and γM (Io) proteins also were filtered through the standard column set. The percentage of applied material absorbing at 280 mμ recovered in light-chain fractions is recorded.

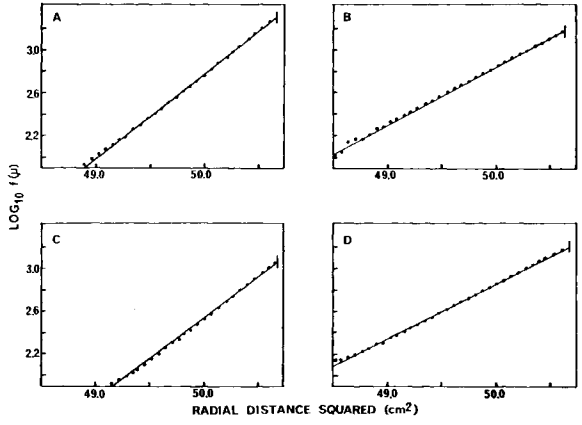

FIGURE 3: Plots of the logarithm of the fringe displacement (log $f(\mu)$) *vs.* the radial distance squared (cm^2) for sedimentation equilibrium experiments with equine immunoglobulin subunits. Solvent, 6 M guanidine hydrochloride. (A) Equine γGab-globulin heavy chains, (B) equine γGab-globulin light chains, (C) equine γT-globulin heavy chains, and (D) equine γT-globulin light chains. Vertical bars indicate the position of the solution–fluorocarbon junction at the bottom of each cell.

TABLE VII: Amino Acid Interchanges between the C-Terminal Nonadecapeptides of Equine, Rabbit, Bovine, and Human Immunoglobulin Heavy Chains, and the Required RNA Codon Alterations.

Residue[a]	Equine γT[b]	Equine γG[b]	Rabbit Gγ[c]	Bovine γG[d]	Human γG_1,γG_2[e]	Human γG_3[f]	Human γG_4[g]
2	Pro[CC(UCAG)][h]	Pro	Pro	Ala [GC(UCAG)]	Pro	Pro	Leu[CU(UCAG)] [UU(AG)]
4	His[CA(UC)]	Lys[AA(AG)]	Arg[CG(UCAG)] [AG(AG)]	Lys	Leu[CU(UCAG)] [UU(AG)]	Leu	Leu
6	Val[GU(UCAG)]	Val	Ile[AU(UCA)]	Thr[AC(UCAG)]	Leu	Leu	Leu
7	Asn[AA(UC)]	Ser[AG(UC)] [UC(UCAG)]	Ser	Ser	Ser	Ser	Ser
11	Tyr[UA(UC)]	Tyr	Tyr	Tyr	Tyr	Phe[UU(UC)][i]	Tyr
12	His[CA(UC)]	His	His	His	His	Arg[CG(UCAG)] [AG(AG)]	His
14	Glu[GA(AG)]	His[CA(UC)]	His	His	His	His	His
15	Val[GU(UCAG)]	Leu[CU(UCAG)] [UU(AG)]	Leu	Leu	Leu	Leu	Leu

[a] Residues numbered from the C terminus. [b] Weir *et al.* (1966). [c] Givol and Porter (1965). [d] Inouve and Givol (1967). [e] Press *et al.* (1966). [f] Prahl (1966). [g] Prahl (1967). [h] RNA codons from Nirenberg *et al.* (1966). [i] γG_3 proteins of Gm specificity 5+, 13+, 14+, 21−. γG_3 proteins of Gm specificity 5−, 13−, 14−, 21+ have tyrosine as residue 11 (Prahl, 1967).

tion that interchanges between lysine (equine), arginine (rabbit), and leucine (human) at one position required at replacement of two nucleotides in one of the altered RNA codons (Table VII). In contrast, two of the four amino acid alterations between the equine γG and γT nonadecapeptides required double-nucleotide replacements (histidine to glutamic acid, lysine to histidine; Table VII). These analyses of the amino acid interchanges between small segments (approximately 5%) of the heavy chains of the four species suggested that the equine γT-globulin heavy-chain gene may have arisen by gene reduplication prior to speciation. More extensive amino acid sequence data will be required to clearly establish the relationship of the equine γT-globulin to the reference human immunoglobulin classes and subclasses. An additional equine immunoglobulin candidate for the equivalent of human γA-globulin is the 10S γ_1 anti-Lac antibody and the related serum protein (Rockey, 1967).

Light chains from different species may vary in their content of cysteinyl residues. The majority of human and mouse Bence-Jones proteins examined have had five residues of cysteine per light chain molecule (Putnam *et al.*, 1967; Appella and Perham, 1967; Milstein *et al.*, 1967). However, Milstein *et al.* (1967) have described a human λ Bence-Jones protein which contained six residues of cysteine. Rabbit γG-globulin light chains contained 7 moles of cysteine/23,000 g of protein (Koshland *et al.*, 1966). The equine immunoglobulin light chains congained 5.0–5.3 moles of cysteine, determined as *S*-carboxymethylcysteine, per 23,000 g of protein. The equine γG and γT heavy chains contained 11.8–13.6 moles of cysteine/52,000 g of protein.

Cyanogen bromide cleavage fragments of equine immunoglobulin subunits are now being isolated and their molecular weights determined by gel filtration in 8 M urea–0.05 M propionic acid. Cyanogen bromide cleaves specifically at methionyl residues (Gross and Witkop, 1962) and the methionine content of the subunits therefore assumes added interest. The recoveries of methionine from the equine immunoglobulin subunits were 2.6–3.4 moles/52,000 g of heavy chain and 0.2–0.4 moles/23,000 g of light chain. A destruction of methionine may result from the formation of *S*-carboxamidomethylmethioninesulfonium salts during the alkylation of the proteins (Stark and Stein, 1964) or from the oxidation of methionine to sulfoxides (Floyd *et al.*, 1963). If the equine light chains contained 1 mole of methionine/23,000 g of protein, a loss of 60–80% would have had to have been sustained to account for the observed values. A comparison of the amino acid data before and after extensive reduction and alkylation failed to show evidence of a loss of this magnitude. Analyses of 24-, 48-, 72-, and 96-hr hydrolysates also failed to show evidence of a time-dependent destruction of methionine during the acid hydrolysis. The light-chain data, therefore, may indicate that the equine light chains were composed of two or more chemically distinct populations, one or more of which lacked methionine.

The precision of the molecular weight values determined by sedimentation equilibrium in 6 M guanidine hydrochloride is largely dependent upon the correctness of the assumptions made in estimating effective specific volumes in this solvent. A small error in $\bar{v}$ would produce a relatively large error in the term $1 - \bar{v}\rho$. The subunit molecular weights recorded in Table V are presented with this reservation. Previous studies with other proteins, where an assumed effective specific volume in 6 M guanidine hydrochloride, 0.01–0.02 ml/g less than the partial specific volume in dilute salt solutions was employed, have given molecular weights which are in agreement with information obtained by other methods (Hade and Tanford, 1967). It may be noted that the corrected $\bar{v}$ values for equine heavy and light chains presented in Table V vary only moderately from the corresponding values determined in 5 M guanidine hydrochloride for rabbit γG-globulin heavy chain (0.720 ± 0.004) and light chain (0.703 ± 0.007) by Small and Lamm (1966). The three independent methods of molecular weight determination employed in the present studies furnished subunit molecular weights which were in substantial agreement.

The molecular weights of the equine γT and the human γA heavy chains were similar to the molecular weights of the rabbit and equine γG heavy chains. The molecular weight of the equine immunoglobulin light chains (22,300–23,300) was significantly higher that the value reported by Pain (19,400; Pain, 1963) but closely approximated the values reported for rabbit and human light chains (Lamm and Small, 1966; Suzuki and Deutsch, 1967; Putnam *et al.*, 1967). The complete amino acid sequence of a λ and the tentative sequence of a κ human Bence-Jones protein have been determined and furnish the respective molecular weights of 22,607 and 23,500 (Putnam *et al.*, 1967). Human and mouse Bence-Jones proteins vary in their molecular weights as they vary in amino acid composition and in total number of amino acid residues (Gray *et al.*, 1967; Putnam *et al.*, 1967). A similar heterogeneity in the molecular weights of the equine immunoglobulin light chains is to be anticipated as the observed fractional molar content of methionine in the light chains indicated that the chains were chemically heterogeneous (Rockey, 1967). The present methods employed for molecular weight determinations are not capable of detecting such limited polydispersity.

The data support a four-chain model of a protein with a molecular weight of approximately 150,000 for each of the equine γG- and γT-immunoglobulins.

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