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Equine Antihapten Antibody. Studies on the Primary Structure and Conformation of Equine Immunoglobulins*

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ABSTRACT: Equine γGab, γGc, and γT anti-Lac (*p*-azophenyl β-lactoside) antibodies have been purified from the serum of a hyperimmunized horse. γGab- and γT-globulins have been isolated from equine diphtheria antitoxin, γGab-globulins from normal equine serum, and a γT-globulin paraprotein from the serum of a horse with a malignant lymphoma. γGab-, γGc-, and γT-globulin heavy and light chains have been prepared and their amino acid composition determined. The light-chain compositions were closely similar. Small differences were observed between the γGab, γGc, and γT heavy-chain data. Papain 3.5S Fab and Fc fragments and pepsin 5S $F(ab')_2$ fragments have been prepared from the equine immunoglobulins. Papain 5S $F(ab)_2$ fragments also have been obtained from equine γGab- and γT-globulins by hydrolysis with 2-mercaptoethanol-activated papain in the absence of free reducing agent. Equilibrium dialysis with *p*-(*p*-dimethylaminobenzeneazo)phenyl β-lactoside hapten established that the anti-Lac Fab and $F(ab')_2$ fragments retained in full the affinity for hapten of the parent γGab, γGc, and γT antibodies. Two-dimensional peptide maps have been prepared from tryptic hydrolysates of extensively reduced and alkylated equine γGab and γT heavy and light chains, and γGab and γT Fab and Fc fragments. The γGab and γT light-chain maps displayed a high degree of homology. Numerous peptide differences were evident between the γGab and γT heavy-chain maps. γGab and γT Fc fragment maps contained both common and distinct peptides. Common and distinct γGab and γT Fd fragment (Fab fragment less light chain) tryptic peptides also were identified. The amino-terminal residues of heavy chains from equine γGab- and γT-globulins have been studied. The heavy chains lacked free α-amino groups. After mild alkaline hydrolysis, glutamic acid was identified as the terminal amino acid of both γGab- and γT-globulin heavy chains by reaction with dimethylaminonaphthalenesulfonyl chloride, tentatively identifying pyrrolidonecarboxylic acid (PCA) as the unreactive terminal residue. Amino-terminal peptides lacking a free amino group have been isolated from subtilisin, α-chymotrypsin and pronase digests of γGab- and γT-globulin heavy chains. The amino acid sequences of the heavy-chain amino-terminal subtilisin tetrapeptides were equine γGab heavy chains, PCA-Val-Gln-Leu; and equine γT heavy chains, PCA-Val-Gln-Leu. No class-specific amino-terminal sequences were apparent. Optical rotatory dispersion curves of equine γGab-, γGc-, and γT-globulins have been examined. The optical rotatory dispersion spectra showed Cotton effect minima near 225 and 230 nm. The depth of the minima varied for different immunoglobulin classes, and between distinct immunoglobulins of a single class. A small Cotton effect centered near 240 nm, characteristic of the γG-globulin spectra, including equine γGab and γGc spectra, was absent from the γT anti-Lac antibody, γT diphtheria antitoxin, and γT paraprotein spectra. γGab and γT $F(ab')_2$ fragment optical rotatory dispersion spectra lacked the Cotton effect minimum near 230 nm and showed only a single minimum near 225 nm. The principal difference between the γGab and γT $F(ab')_2$ spectra was the presence of a Cotton effect near 240 nm in the γGab $F(ab')_2$ spectrum and the absence of the 240-nm effect from the γT $F(ab')_2$ spectrum. The experimental results are briefly discussed in terms of an expanded two-gene translocation hypothesis for generating immunoglobulin heavy-chain sequence variations and antibody specificity.

The immunoglobulins[1] of a given species are a heterogeneous population of related, but not identical, proteins, and in addition to diversities in antibody specificity, may be subdivided into distinct classes, subclasses, and allotypes on the basis of the antigenic and chemical structure of their heavy chains (Dubiski *et al.*, 1961; Natvig *et al.*, 1967; Rockey,

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1967). A single animal is capable of producing antibodies directed against a wide variety of natural and synthetic antigenic determinants. A single animal also is capable of synthesizing a number of different forms of antibody which share specifically for a single antigenic determinant. A multiplicity of distinct molecular forms of anti-Lac (*p*-azophenyl β-lactoside) antibody has been isolated from the horse (Rockey, 1967; Dorrington and Rockey, 1968; Montgomery *et al.*, 1969). The observation that a single animal may respond to antigenic stimulation with the production of different forms of antibody which share a common specificity raises the question of whether or not a common (conservative) mechanism exists for generating similar antibody specificity in the diverse proteins, and whether or not the diverse proteins share common structural features which are relevant to the common specificity. The study of diverse molecular forms of antibody with a common specificity produced in a single animal also affords an opportunity to define immunoglobulin structural features which are not directly relevant to the formation of the antibody combining site.

In the present communication, primary structural features of distinct equine immunoglobulins have been compared. Optical rotatory dispersion has been employed as a probe to define conformational features of different equine immunoglobulins and immunoglobulin fragments. The submolecular localization of primary structural and conformational similarities and differences between the diverse equine immunoglobulins has been further defined. The results are discussed in terms of an expanded two-gene translocation hypothesis for encoding antibody specificity into the amino-terminal sequence of the immunoglobulin heavy chain.

Materials and Methods

Preparation of Equine Immunoglobulins. Anti-Lac antibody was isolated from the serum of a hyperimmunized horse by coprecipitation with Lac-Hy (hemocyanin, *Limulus polyphemus*) (Rockey, 1967). γGabc antibodies were separated from γT antibodies by DEAE-cellulose (Whatman DE 52, 1.0 mequiv/g) column chromatography in 0.02 M sodium phosphate buffer (pH 8), at 4°. The γGab and γGc antibodies were separated from one another by Pevikon block zone electrophoresis (Rockey, 1967). γT antibodies were eluted from the DEAE-cellulose column with a positive gradient of sodium chloride and then subjected to zone electrophoresis to remove small quantities of γGabc antibodies of rapid electrophoretic mobility. In some instances the purified anti-Lac antibodies also were subjected to gel filtration at 4° through four columns (each column 4 × 60 cm) of Sephadex G-150 in neutral solvents. The optical density ratio OD(280 nm)/OD(365 nm) was determined for each antibody preparation and used, together with the ratio obtained for the Lac-Hy antigen, to calculate the maximum contamination of antibody by Lac-Hy antigen.

The γGab-globulins of equine serum were precipitated with 33% saturated ammonium sulfate, and further purified by DEAE-cellulose column chromatography in 0.01 M sodium phosphate buffer (pH 8) (Montgomery *et al.*, 1969). Equine γGab- and γT-globulins were isolated from refined and concentrated diphtheria antitoxin (lot no. 21309, Wyeth Laboratories) by DEAE-cellulose chromatography and Pevikon block zone electrophoresis (Montgomery *et al.*, 1969), and further purified by filtration through four columns of Sephadex G-150.

A γT-globulin paraprotein was isolated from the serum of a horse with a malignant lymphoma (Montgomery *et al.*, 1968; Dorrington and Rockey, 1968) by Pevikon block zone electrophoresis. The paraprotein was precipitated repeatedly (three times) with one-half-saturated ammonium sulfate, taken up in 0.2 M sodium chloride–0.01 M sodium phosphate buffer (pH 8) and subjected to Sephadex G-150 gel filtration. A human γGl myeloma protein was isolated by zone electrophoresis and Sephadex G-150 gel filtration.

Other Materials. Pyrrolid-2-one-5-carboxylic acid (PCA)[2] was synthesized by heating a solution of L-glutamic acid (A grade, Calbiochem) in water (pH 3.5) for 2 hr at 140° in a sealed ampoule (Moav and Harris, 1967). The ninhydrin-negative PCA was separated from residual glutamic acid by passage over a column of Dowex 50-X2 (200–400 mesh, H^+ form, Bio-Rad) in water.

Preparation of Immunoglobulin Subunits. Immunoglobulin heavy and light chains were prepared as previously described (Rockey, 1967; Montgomery *et al.*, 1969).

Preparation of F(ab')$_2$, Fab, and Fc Fragments. Equine and human immunoglobulins were digested with pepsin (twice recrystallized, Worthington Biochemical) in 0.2 M sodium acetate buffer (pH 4.5) at an enzyme to substrate ratio of from 1:100 to 5:100 (w/w), for 4–96 hr at 37°. The digestion mixture was filtered through four columns of Sephadex G-150 (each column 4 × 60 cm) at 4°. Immunoglobulins were digested with papain (twice recrystallized, Worthington Biochemical) in 0.2 M sodium acetate buffer (pH 5.6), 2 mM EDTA, containing 0.01 M cysteine, at an enzyme to substrate ratio of from 1:100 to 3:100 (w/w) at 37° for 4–48 hr (Rockey, 1967). In some experiments, papain was activated by exposure to 0.05 M 2-mercaptoethanol for 1 hr at room temperature and separated from residual 2-mercaptoethanol by Sephadex G-25 gel filtration under N, prior to addition to the immunoglobulin. Fab (or F(ab)$_2$) fragments and Fc fragments were isolated by Pevikon block zone electrophoresis, and further purified by Sephadex G-150 gel filtration.

Two-Dimensional Mapping of Tryptic Peptides. Extensively reduced and alkylated immunoglobulin heavy and light chains and Fab and Fc fragments were digested with TRTPCK-trypsin (L-(1-tosylamido-2-phenyl)ethyl chloromethyl ketone treated trypsin, Worthington Enzymes) at an enzyme to substrate ratio of 3:100 (w/w) at pH 8.1 in the thermostated chamber of a Radiometer pH-Stat at 37°, for 4–24 hr. A 5–8-mg sample of peptide was applied at a single point on Whatman No. 3MM paper and descending chromatography in 1-butanol–acetic acid–water (4:1:5, v/v) was followed by high-voltage electrophoresis (2000 V applied for 1.5 hr) under Varsol in a tap-water-cooled Gilson Model D electrophorator in a pyridine–acetic acid–water (1:10:289, v/v)

[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 (1964).

[2] Abbreviations used are: PCA, pyrrolid-2-one-5-carboxylic acid; DNS, dansyl(1-dimethylaminonaphthalene-5-sulfonyl) group; TRTPCK, L-(1-tosylamido-2-phenyl)ethyl chloromethyl ketone treated trypsin.

solvent of pH 3.7 (Katz *et al.*, 1959). Four identical maps were prepared for differential staining with buffered ninhydrin, acidic ninhydrin, Sakaguchi, α-nitroso-β-naphthol, platinic iodide, and Pauly and Erlich reagents (Easley, 1965).

Amino-Terminal Analysis. Equine γGab- and γT- immunoglobulin heavy chains (2–3 mg), in 120 μl of 0.1 M ammonia-free sodium bicarbonate, were mixed with 30–45 μl of DNS-Cl (Mann Research Laboratories) in acetone (1–20 mg/ml) (Gray and Hartley, 1963; Morse and Horecker, 1966). Immunoglobulin subunits also were dansylated in 8 M urea–0.5 M sodium bicarbonate (Gray, 1967). In addition, heavy chains were exposed to 1 M sodium hydroxide at 25° for 16 hr in evacuated combustion tubes, and then subjected to dansylation. The reaction products were hydrolyzed in glass-redistilled, constant-boiling (approximately 5.7 N) HCl for 12 hr at 105° *in vacuo* in sealed combustion tubes, and the hydrolysates were analyzed by thin-layer chromatography on MN Polygram SIL SHR plates (Brinkman Instruments) in 1-butanol–acetic acid–water (4:1:1, v/v).

Isolation of Heavy-Chain Amino-Terminal Peptides. Fully reduced and alkylated equine γGab- and γT-immunoglobulin heavy chains (50–100 mg) were digested in water at pH 8.1 for 4–6 hr at 37° with either pronase (Calbiochem), subtilisin (Nutritional Biochemical), or α-chymotrypsin (Worthington Biochemical) at an enzyme to substrate ratio ranging between 1:50 and 1:15 (w/w). The digest was applied to a 1 × 45 cm column of Dowex 50-X2 (200–400 mesh) in the H^+ form (Narita and Ishii, 1962; Wilkinson *et al.*, 1966; Press *et al.*, 1966) and the column was eluted at room temperature with water. Effluent fractions were subjected to ninhydrin analysis before and after alkaline hydrolysis (Hirs *et al.*, 1956). Selected fractions were pooled and further purified by high-voltage paper electrophoresis at pH 3.7. The paper was stained with ninhydrin and by the chlorine gas–potassium iodide–starch staining procedure of Rydon and Smith (1952).

In some instances at the completion of enzymatic digestion, 1-fluoro-2,4-dinitrobenzene (Calbiochem) was added to block free α-amino groups (Press *et al.*, 1966) exposed during enzymatic digestion. The dinitrophenylated-peptides were extracted five times with ether and five times with ethyl acetate, and the aqueous residue was applied to a Dowex 50-X2 (H^+ form) column. The column was eluted with water and fractions from the region of the chromatogram previously found to contain a ninhydrin-negative peptide were pooled. The pH of the eluate was adjusted to 7 with 1 M sodium hydroxide. The eluate was concentrated by flash evaporation and applied to a 1 × 30 cm column of Dowex 1-X8 (Bio-Rad) in the chloride form. The column was eluted with 0.1 M formic acid and the effluent was concentrated by flash evaporation and subjected to high-voltage paper electrophoresis.

Ninhydrin-negative peptides, isolated from subtilisin digests of equine γGab and γT heavy chains by Dowex 50-X2 (H^+) chromatography and high-voltage paper electrophoresis, on some occasions were exposed to 1 M sodium hydroxide for 16 hr at 25°. The pH was brought to neutrality by the addition of a predetermined amount of hydrochloric acid, and the alkali-treated peptides were subjected to dansylation.

Carboxypeptidase A Digestion. Peptides (0.3–0.5 mg) were digested in 0.1 M ammonium bicarbonate at 37° with diisopropylfluorophosphate-treated carboxypeptidase A (Worthington Enzymes) at an enzyme to substrate ratio of 1:50 (w/w). Aliquots of the digestion mixture were removed at 10, 60, 240, and 1440 min, acidified to pH 3 with acetic acid, and analyzed by thin-layer chromatography, in 1-butanol–acetic acid–water (4:1:1, v/v) (Hoenders *et al.*, 1968). Amino acids were identified with a ninhydrin spray and with the chlorine–potassium iodide–starch stain.

Amino acid analysis was carried out as previously reported (Rockey, 1967; Montgomery *et al.*, 1969). Cysteine or half-cystine was determined as *S*-carboxymethylcysteine after extensive reduction and alkylation. Cysteine and methionine also were determined as cysteic acid and methionine sulfone, respectively, after performic acid oxidation (Moore, 1963; Hirs, 1956).

Analytical Ultracentrifugation. Sedimentation velocity analysis was performed at 20° in a Beckman–Spinco Model E ultracentrifuge equipped with an RTIC temperature control.

Equilibrium Dialysis. Equilibrium dialysis with Lac–dye (*p*-(*p*-dimethylaminobenzeneazo)phenyl β-lactoside) hapten or tritiated Lac–dye hapten was accomplished as previously described (Rockey, 1967).

Optical rotatory dispersion measurements were performed at 25° in the thermostated cell compartment of either a Cary Model 60 spectropolarimeter or a Jasco ORD/UV5 spectropolarimeter. Cylindrical cells (Pyrocells) with 1.0-cm path lengths, and protein concentrations ranging from 0.4 to 0.8 g per 100 ml were used from 300 to 600 nm. Cells with path lengths of 2.0, 1.0, and 0.1 mm were used below 300 nm. In this spectral region a variety of protein concentrations were employed ranging from 0.02 g/100 ml between 200 and 210 nm to 0.2 g/100 ml above 250 nm. The solvent used was either 0.1 M NaCl, or 0.1 M NaCl–0.01 M sodium phosphate buffer (pH 7.0). The optical path was continuously purged with N. The results are expressed in terms of the mean residue rotation [*m'*], at each wavelength, obtained from the specific rotation, [α], by the relation

$$[m']_\lambda = \frac{3M_0}{100(n^2+2)}[\alpha]_\lambda$$

where M_0 is the mean residue weight, taken as 110 for the proteins studied, and n is the refractive index of the solvent. The data above 300 nm were analyzed according to the equation of Moffitt and Yang (1956)

$$[m']_\lambda = a_0\frac{\lambda_0^2}{(\lambda^2-\lambda_0^2)} + b_0\left(\frac{\lambda_0^2}{\lambda^2-\lambda_0^2}\right)^2$$

with λ_0 arbitrarily set equal to 212 nm. Protein concentrations were determined spectrophotometrically at 280 nm by the use of an extinction coefficient ($E^{1\%}_{1cm}$) of 14.7 for equine γG- and γT-globulins, and of 14.6 for Fab and F(ab')$_2$ fragments (Rockey, 1967).

Experimental Results

Characterization of Purified Equine Immunoglobulins. The purified equine γGab, γGc, and γT anti-Lac antibodies were examined in the analytical ultracentrifuge and by immunoelectrophoresis (Figure 1) and judged to be free of significant contaminating serum proteins. The maximum concentration of Lac-Hy antigen in the antibody preparations employed in these studies was less than 3%. The molar concentration of

TABLE I: Average Intrinsic Association Constants (K_0) of Equine Anti-Lac (*p*-Azophenyl β-Lactoside) Antibodies and Antibody Fragments.[a]

Antibody	Antibody Prepn No.	$K_0 \times 10^{-7}$ (l./mole)
γGabc anti-Lac	1	0.6
	2	1.3
	3	1.0
γGabc Fab[b]	3	0.8
γGab anti-Lac	1	1.1
	2	0.5
	3	1.2
γGab F(ab′)$_2$[c]	3	1.0
γGc anti-Lac	1	6.6
	2	4.9
γGc F(ab′)$_2$[c]	2	4.4
γT anti-Lac	1	1.8
	2	0.9
	3	1.6
	4	1.0
γT F(ab′)$_2$[c]	3	1.4
	4	0.8

[a] Determined by equilibrium dialysis at 25° with tritiated *p*-(*p*-dimethylaminobenzeneazo)phenyl β-lactoside hapten. [b] Papain 3.5S Fab fragment. [c] Pepsin 5S F(ab′)$_2$ fragment.

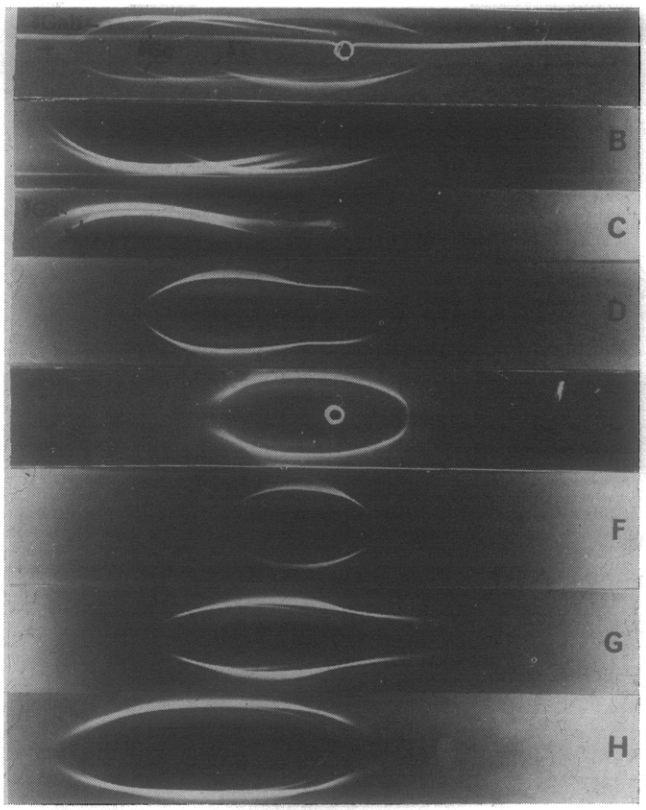

FIGURE 1: Immunoelectrophoretic characterization of purified equine immunoglobulins employed in the present studies. (A) γGabc and γT anti-Lac antibodies, specifically isolated by coprecipitation with Lac-Hy antigen. The lower trough was filled with a rabbit antiserum prepared against equine serum proteins, and the upper trough was filled with a goat antiserum prepared against γG-globulin heavy chains including rabbit γG- and equine γGabc-globulin heavy chains. The line across the middle of the slide is due to the precipitation of the rabbit γG-globulins diffusing from the lower trough, by goat antibodies diffusing from the upper trough. (B) γGabc anti-Lac antibody isolated by DEAE-cellulose chromatography. (C, D) γGab and γGc anti-Lac antibodies, respectively, separated by zone electrophoresis. (E) γT anti-Lac antibody. (F) γT-globulin paraprotein isolated from the serum of a horse with a malignant lymphoma. (G) Diphtheria antitoxin γT-globulins. (H) γGab-globulins isolated from normal equine serum. Slides B–H were developed with rabbit antisera prepared against equine serum proteins.

antibody combining sites was determined by equilibrium dialysis, at 4°, at high free Lac–dye hapten concentrations. The γT anti-Lac antibody preparations bound a maximum of 1.8–2.0 moles of hapten/150,000 g of protein. The γGabc, γGab, and γGc anti-Lac antibody binding data extrapolated reasonably to 2 moles of hapten bound per 150,000 g of protein at infinite free hapten concentration. The average intrinsic association constants (K_0) for the anti-Lac antibodies utilized in the present studies are recorded in Table I.

The γT-globulin paraprotein was present in high concentration in the equine lymphoma serum, and migrated on zone electrophoresis as a β(γ_1) component with a restricted electrophoretic mobility (Figure 2). The isolated γT paraprotein, and the γGab- and γT-globulins prepared from equine diphtheria antitoxin and normal equine serum also were judged to be free of significant contaminating proteins when examined in the analytical ultracentrifuge and by immunoelectrophoresis (Figure 1).

Amino Acid Composition of Immunoglobulin Subunits. The amino acid composition of the heavy and light chains of equine γGab, γGc and γT anti-Lac antibodies, and of normal equine serum γGab-globulins and diphtheria antitoxin γT-globulins are recorded in Table II. The light chains from the different equine immunoglobulin preparations did not vary substantially in amino acid composition. More variations were apparent within the data for the heavy chains.

Enzymatic Fragments of Equine Immunoglobulins. When equine γGabc, γGab, and γGc anti-Lac antibodies and normal serum γGab-globulins were digested with papain in the presence of 0.01 M cysteine, Fab and Fc fragments were obtained which were readily separated by zone electrophoresis. The sedimentation coefficients ($s_{20,w}$) of the Fab fragments thus obtained were approximately 3.5 S. Fragments with the electrophoretic mobilities of Fab and Fc fragments also were obtained from equine γGab- and γT-globulins after cleavage with 2-mercaptoethanol-activated papain in the absence of free reducing agent. The isolated γGab and γT fragments of low electrophoretic mobility had sedimentation coefficients ($s_{20,w}$ at 3.9 mg/ml) of 4.6 and 4.5 S, respectively, and appeared to represent disulfide-linked dimers of Fab fragments (F(ab)$_2$ fragments). The F(ab)$_2$ fragments carried light-chain antigenic determinants and lacked certain prominent heavy-chain determinants. Fc fragments which had been isolated from γGab- and γT-globulins shared certain common heavy-chain antigenic determinants, and, in addition, possessed specific γGab or γT heavy-chain determinants, respectively. The γGab and γT papain Fc fragments preparations contained small

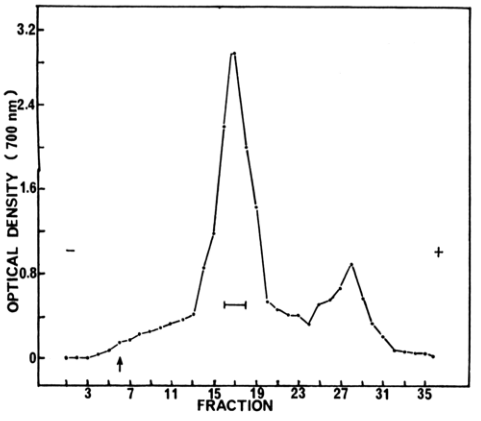

FIGURE 2: Pevikon block zone electrophoresis of the serum from a horse with a malignant lymphoma. A paraprotein of rapid and restricted electrophoretic mobility is present in high concentration. The indicated fractions were pooled and used for further study. The origin is indicated by the arrow. The anode is to the right.

amounts of Fc′ fragments which were antigenically deficient and moved with a slightly more rapid mobility toward the anode on immunoelectrophoresis, relative to the Fc fragments.

Digestion of equine γGab-, γGc-, and γT-globulins and a human γG1 myeloma protein with pepsin at pH 4.5 resulted in the production of 5S F(ab′)$_2$ fragments. The equine γGab-globulins proved to be more resistant to peptic digestion than the γGc- and γT-globulins, and a significant residue of 7S protein remained even after 96-hr hydrolysis at 37°. The γGab 5S F(ab′)$_2$ fragments were freed of residual 7S protein by Sephadex G-150 gel filtration.

Intrinsic association constants (K_0) were determined by equilibrium dialysis with tritiated Lac-dye for papain Fab and pepsin F(ab′)$_2$ fragments of γGabc, γGab, γGc, and γT anti-Lac antibodies and the parent antibodies, and are recorded in Table I. The Fab and F(ab′)$_2$ fragments retained in full the affinity for hapten of the parent antibody.

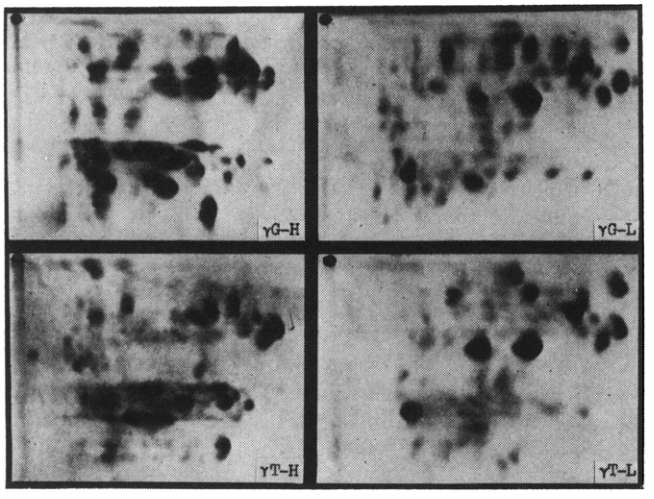

FIGURE 3: Photographs of ninhydrin-stained tryptic peptide maps prepared from extensively reduced and alkylated equine immunoglobulin subunits. γG-H, γGab-globulin heavy chains. γG-L, γGab-globulin light chain. γT-H, γT-globulin heavy chains. γT-L, γT-globulin light chains. Chromatography was performed in the vertical direction followed by electrophoresis in the horizontal direction. The origin is at upper left-hand extreme of each map.

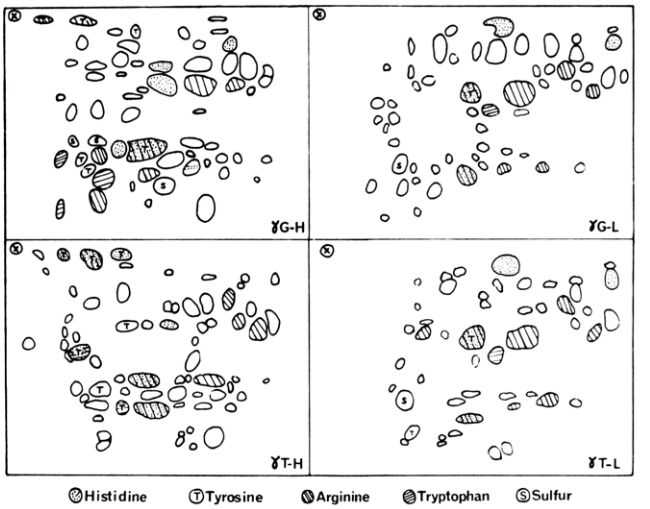

FIGURE 4: Composite tracings of tryptic peptide maps of γGab- and γT-globulin heavy and light chains stained with specific amino acid reagents: histidine (Pauly); tyrosine (α-nitroso-β-naphthol); arginine (Sakaguchi); tryptophan (Erlich); sulfur (platinic iodide). Ninhydrin-positive peptides which did not stain with specific amino acid reagents are indicated by open tracings.

The amino acid composition of anti-Lac antibody Fab and F(ab′)$_2$ fragments are recorded in Table II.

Tryptic Peptide Mapping. Extensively reduced and alkylated heavy and light chains and Fab and Fc fragments from normal and diphtheria antitoxin equine γGab-globulins and diphtheria antitoxin γT-globulins were cleaved with trypsin and subjected to two-dimensional peptide mapping. Each tryptic hydrolysate was examined repeatedly (8–12 times) and found to yield reproducible peptide maps. Tryptic digests from 2–5 preparations of each subunit or fragment were examined. Photographs of ninhydrin-stained peptide maps of equine γGab and γT heavy- and light-chain hydrolysates are presented in Figure 3. Figure 4 summarizes the results obtained with special stains for histidine, tyrosine, arginine, tryptophan, and sulfur-containing amino acids. The γGab heavy-chain maps displayed 45–58, and the γT heavy-chain maps 47–65 prominent ninhydrin-positive peptides. Fifteen peptides were identified which were common to both the γGab and γT heavy-chain maps. In addition, a large number of peptides could be identified which were distinct for either the γGab or the γT heavy chain. The γGab heavy-chain maps contained 22 peptides, and the γT heavy-chain maps 17 peptides which were absent from the maps of the alternate chain. A comparison of the heavy-chain maps from normal equine serum γGab-globulins and diphtheria antitoxin γGab-globulins did not reveal prominent differences.

A significantly greater degree of homology was evident when peptide maps of γGab and γT light-chain hydrolysates were compared (Figures 3 and 4). The γGab light-chain maps contained 39, and the γT light-chain maps 37 prominent ninhydrin-positive peptides. Thirty-one peptides were identified which were common to both light-chain maps.

The results obtained upon examination of tryptic hydrolysates of extensively reduced and alkylated γGab and γT Fab

TABLE II: Amino Acid Composition of Equine Immunoglobulin Subunits and Fragments.

	Light Chains[a]					Heavy Chains[b]					Fab Fragments[c]	$F(ab')_2$ Fragments[c]	
Amino Acid	γGab Anti-Lac	γGc Anti-Lac	γT Anti-Lac	γGab-Globulins	γT Diphtheria Antitoxin	γGab Anti-Lac	γGc Anti-Lac	γT Anti-Lac	γGab-Globulins	γT Diphtheria Antitoxin	γGabc Anti-Lac	γGc Anti-Lac	γT Anti-Lac
S-CM-cysteine[d]	5.3	5.1	5.0	5.1	5.2	13.6	11.8	12.8	12.8	12.9			
Cysteic acid[e]				5.3	5.2				12.9	13.0			
Half-cystine[f]											8.7	10.4	11.3
Aspartic acid	15.6	16.7	16.0	15.0	14.8	37.1	35.3	39.0	35.5	40.4	30.9	30.9	35.2
Threonine[g]	21.8	22.8	20.4	22.9	22.3	43.5	40.7	38.6	43.2	36.9	43.7	46.1	39.6
Serine[g]	33.4	32.0	33.6	34.2	33.8	52.4	54.8	49.0	50.7	47.1	64.9	63.4	65.9
Glutamic acid	19.6	20.2	19.8	19.8	19.5	44.5	43.8	45.4	42.5	45.8	37.2	38.0	34.9
Proline	10.8	10.3	11.2	12.8	13.9	31.4	33.4	38.5	35.1	35.5	23.7	28.3	32.0
Glycine	22.1	24.2	21.2	20.3	20.2	34.4	36.9	31.7	33.1	32.0	44.1	40.3	39.8
Alanine	14.6	15.0	14.2	14.2	14.5	25.5	24.6	23.6	26.5	25.2	31.9	29.6	27.7
Valine[h]	17.2	14.8	16.6	18.1	17.6	49.2	49.6	51.1	50.8	50.5	40.7	40.5	41.9
Methionine	0.2	0.2	0.4	0.2	0.2	3.3	3.5	3.6	3.2	3.2	1.9	1.9	2.6
Methionine sulfone[e]				0.4	0.4				4.1	4.2			0.7
Isoleucine[h]	8.2	8.3	7.7	8.0	8.1	14.9	16.4	13.5	14.5	13.1	15.9	17.8	15.0
Leucine	12.7	12.1	12.4	13.9	13.5	35.0	34.8	35.3	34.4	35.7	33.5	30.7	32.6
Tyrosine[g]	7.5	8.1	7.7	7.6	8.0	16.4	19.9	17.1	16.8	18.1	18.7	18.6	15.6
Phenylalanine	5.3	5.7	5.8	5.8	5.1	14.3	13.4	14.7	15.3	15.5	11.6	9.7	9.9
Lysine	11.9	10.6	12.0	11.4	11.4	32.6	26.7	31.6	33.4	32.2	25.0	23.3	27.5
Hisitidine	2.6	2.6	2.7	2.2	2.2	11.1	9.4	12.6	10.9	12.8	6.2	5.5	7.3
Arginine	5.5	5.4	5.2	5.9	6.6	12.2	14.8	12.7	11.7	12.6	13.0	12.5	11.6
Tryptophan[i]	3.5	4.0	3.8	3.8	3.8	8.0	10.4	9.3	8.5	9.1	10.0	10.3	8.6

[a] Amino acid residues (moles) per 23,000 g of light chain. [b] Amino acid residues (moles) per 52,000 g of heavy chain. [c] Amino acid residues (moles) per 50,000 g of papain Fab or pepsin F(ab') fragment. [d] Half-cystine determined as *S*-CM-cysteine after extensive reduction and alkylation. [e] Half-cystine and methionine determined as cysteic acid and methionine sulfone, respectively, after performic acid oxidation. [f] Uncorrected for destruction occurring during 24-hr acid hydrolysis. [g] Corrected for destruction occurring during acid hydrolysis. [h] Value obtained after 72-hr acid hydrolysis. [i] Determined optically.

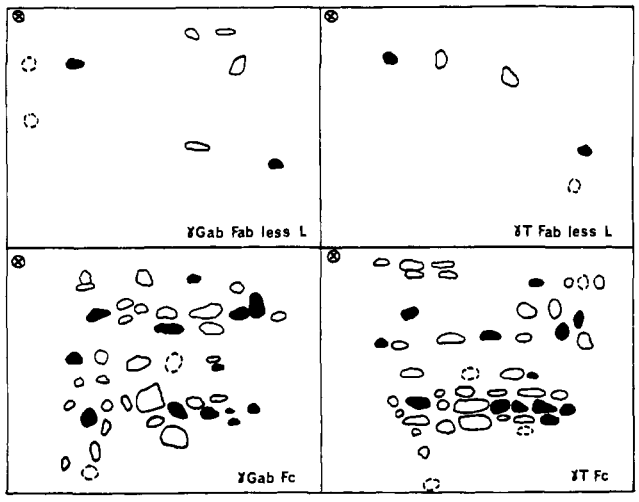

FIGURE 5: Composite tracings of tryptic peptide maps of extensively reduced and alkylated papain F(ab)$_2$ and Fc fragments of γGab- and γT-globulins. Fab maps: peptides shared by γGab and γT Fab and γGab and γT heavy-chain maps (●); distinct γGab and γT peptides present on the homologous heavy-chain map (○); distinct γGab or γT Fab peptides not identified on the homologous heavy-chain map (◌). Those peptides of the Fab maps which could be identified on light-chain maps have been eliminated from the tracings. Fc maps: peptides common to γGab and γT Fc fragments and γGab and γT heavy chains (●); unique γGab or γT Fc peptides identified on the homologous heavy-chain map (○); Fc fragment peptides which could not be identified on the heavy-chain maps (◌).

TABLE III: Molar Ratios of Amino Acids and Per Cent Recovery of Amino-Terminal Peptides from Equine Immunoglobulin Heavy Chains.[a]

Heavy Chain	Enzyme	Amino Acids in Peptide (Molar Ratio)[b]	% Recov[c]
Equine γGab	Pronase	Glu (2.3), Val (1.0)	25.5
	Subtilisin	Glu (1.8), Val (0.9), Leu (1.1)	26.5
	Chymotrypsin	Glu (2.2), Val (0.9), Leu (1.1)	21.0
Equine γT	Pronase	Glu (2.1), Val (1.0)	44.9
	Subtilisin	Glu (1.9), Val (1.0), Leu (1.3)	24.7
	Chymotrypsin	Glu (2.0), Val (1.0), Leu (1.0)	34.0

[a] Ninhydrin-negative peptides isolated from enzymatic digests of extensively reduced and alkylated equine γGab- and γT-globulin heavy chains by Dowex 50-X2 (H^+) column chromatography and high-voltage paper electrophoresis. [b] Molar ratios are mean values obtained from amino acid analyses of two to six peptides. [c] Calculated by assuming a single amino-terminal sequence. Uncorrected for losses sustained during isolation.

and Fc fragments are summarized in the composite tracings presented in Figure 5. Each Fab fragment map displayed a group of prominent peptides which were found to be shared in common by both the γGab and γT Fab fragments and the γGab and γT light chains. Two additional peptides that were shared by the γGab and γT Fab fragments were located on both the γGab and γT heavy-chain maps. Distinct γGab and γT Fab fragment peptides which were present only on the corresponding γGab or γT heavy-chain maps, and therefore appeared to be distinct γGab and γT Fd fragment peptides, also were identified. Two γGab Fab fragment peptides and one γT Fab fragment peptide were identified which could not be located on either of the corresponding heavy- or light-chain maps. These distinct Fab fragment peptides may have arisen from the carboxy-terminal regions of the Fd fragments adjacent to the site of papain cleavage. Peptide maps of the γGab and γT Fc fragments displayed 37–48 prominent ninhydrin-positive peptides (Figure 5). Thirteen peptides were identified which were common to both the γGab and γT Fc fragments, and twelve of these peptides also were located on both the γGab and γT heavy-chain maps. The γGab and γT Fc fragment maps, in addition, contained 17–31 distinct peptides which could be identified only on the corresponding γGab or γT heavy-chain map.

Amino-Terminal Residues of Equine Immunoglobulin Heavy Chains. Dansylation of equine γGab- and γT-immunoglobulin heavy chains did not reveal any residue with a free α-amino group. Pyrrolidonecarboxylic acid has been found to be the unreactive amino-terminal residue of other immunoglobulin heavy and light chains (Wilkinson *et al.*, 1966; Press *et al.*, 1966; Hood and Ein, 1968; Rüde and Givol, 1968). The cyclic PCA residue may be opened by mild alkaline hydrolysis (Dekker *et al.*, 1949), freeing an α-amino group for dansylation. PCA has been reported to be more labile to alkali treatment than are other blocking groups (Dekker *et al.*, 1949; Rüde and Givol, 1968). The heavy chains with unreactive amino-terminal residues, therefore, were again subjected to dansylation after exposure to 1 M sodium hydroxide. DNS-glutamic acid was detected in the hydrolysate from each of the heavy chains after mild alkali treatment and dansylation, furnishing evidence that PCA was the unreactive amino-terminal residue of the equine γGab- and γT-immunoglobulin heavy chains.

Isolation of Amino-Terminal Peptides. Acidic peptides from subtilisin, pronase, and α-chymotrypsin digests of extensively reduced and alkylated equine immunoglobulin heavy chains were separated from a majority of peptides possessing free amino groups by passage over a Dowex 50-X2 (H^+) column at neutral pH in water. Analysis of the column effluent before alkaline hydrolysis revealed the elution of ninhydrin-positive materials in addition to a ninhydrin-negative peptide which was identified only after alkaline hydrolysis. Selected fractions from the Dowex 50-X2 (H^+) column were pooled and further purified by high-voltage paper electrophoresis. Ninhydrin-positive materials were identified on the paper with a ninhydrin spray, and ninhydrin-negative materials were located with the chlorine–potassium iodide–starch stain. The spots were cut out, eluted, and subjected to acid hydrolysis and amino acid analysis. A cathodically migrating ninhydrin-positive component and a minor anodally migrating ninhydrin-negative component in Dowex 50-X2 (H^+) eluates from subtilisin digests were found to contain only trace amino acids. The major ninhydrin-negative γGab and γT heavy-chain sub-

tilisin peptides moved toward the anode at pH 3.7, and gave the molar ratios for amino acids listed in Table III. Ninhydrin-negative peptides of similar composition were obtained from α-chymotrypsin digests of γGab and γT heavy chains (Table III). Pronase digestion at high enzyme concentrations gave an increased number (two to three) of minor electrophoretic components which contained only trace amino acids. A ninhydrin-negative component with the electrophoretic mobility of PCA, which yielded glutamic acid on amino acid analysis after acid hydrolysis and therefore corresponded to free PCA, also was obtained in low yield from the pronase digests of both the γGab and γT heavy chains. A decrease in the number of minor electrophoretic components was observed when the concentration of pronase had been reduced. A single major ninhydrin-negative peptide was isolated from each of the pronase digests (Table III).

The possibility that the isolated ninhydrin-negative peptides with unreactive terminal PCA residues may have formed during the chromatography on Dowex 50-X2 (H^+) was considered, as PCA may form under acidic conditions by the cyclization of internal glutamine residues exposed during enzymatic cleavage (Dekker *et al.*, 1949; Hirs *et al.*, 1956). To eliminate this potential artefact, free amino groups were reacted with fluorodinitrobenzene immediately after enzymatic digestion. Dinitrophenylated peptides were extracted with ether and ethyl acetate, and ninhydrin-negative peptides were purified by Dowex 50-X2 (H^+) and Dowex 1-X8 (Cl^-) chromatography and high-voltage paper electrophoresis. Peptides, identical in amino acid composition with those initially obtained from each heavy chain, were recovered from the dinitrophenylated enzymatic digests, establishing that the ninhydrin-negative peptides were not artefacts of the isolation procedure.

Amino Acid Sequence of Amino-Terminal Peptides. Ninhydrin-negative peptides, isolated from subtilisin digests of equine γGab and γT heavy chains, were exposed to 1 M sodium hydroxide and then subjected to dansylation and acid hydrolysis. DNS-glutamic acid was identified in each hydrolysate, indicating that PCA was the terminal unreactive residue of the γGab and γT peptides. A comparison of the amino acid compositions of the subtilisin and pronase ninhydrin-negative peptides from each immunoglobulin heavy chain (Table III) tentatively identified leucine as the carboxy-terminal residue of the subtilisin tetrapeptides. The first amino acid released from each of the subtilisin tetrapeptides by carboxypeptidase A digestion was leucine, confirming its carboxy-terminal position. The second amino acid released from each peptide was glutamine. Valine was identified only after prolonged carboxypeptidase A digestion. The amino acid sequences of the subtilisin tetrapeptides therefore were γGab-globulin heavy chains, PCA-Val-Gln-Leu; γT-globulin heavy chains, PCA-Val-Gln-Leu.

Optical Rotatory Dispersion. The optical rotatory dispersion curves between 220 and 300 nm for the equine γGab, γGc, and γT anti-Lac antibodies, and for the γT paraprotein, are shown in Figure 6A,B. Table IV summarizes the Cotton effect data and the Moffitt–Yang parameters for the equine immunoglobulins. A small but consistent Cotton effect could be detected in each spectrum near 280 nm. Two major Cotton effect minima were clearly resolved for each of the equine immunoglobulins, one near 230 nm and the other near 225 nm. The 224–225-nm minimum was less pronounced than the

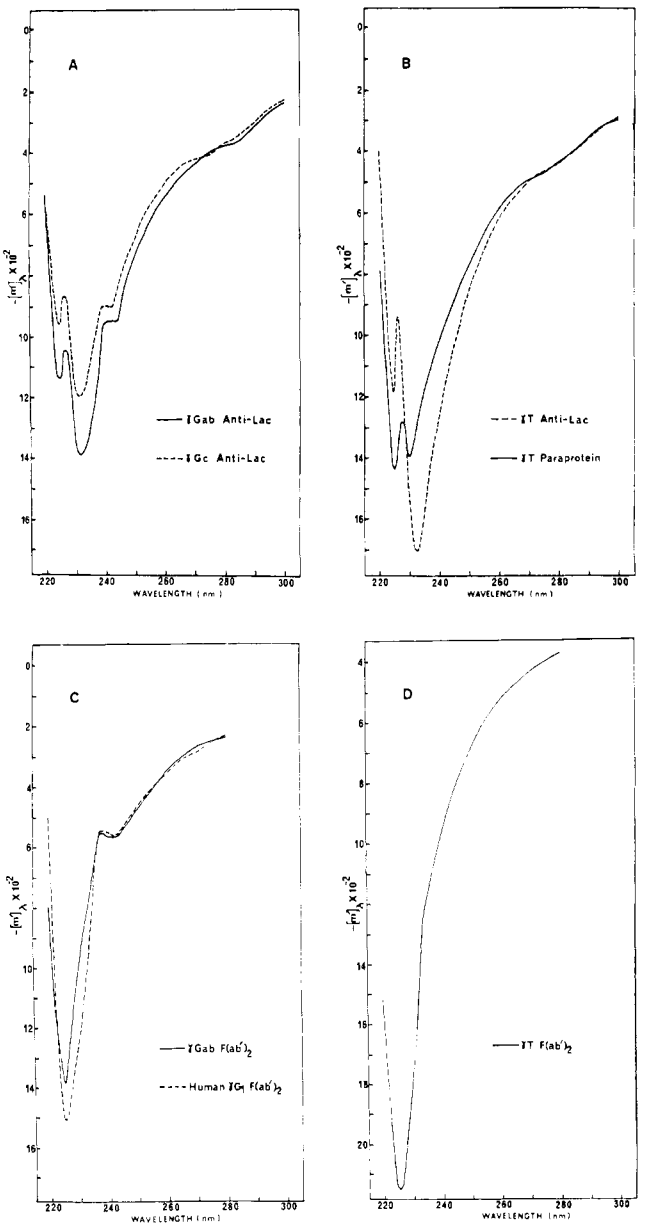

FIGURE 6: Optical rotatory dispersion spectra of equine immunoglobulins, and equine and human immunoglobulin fragments. Anti-Lac, anti-Lac antibody. $F(ab')_2$, pepsin 5S $F(ab')_2$ fragments prepared from equine γGab-globulins (γGab), γT-globulins (γT), and a human γG1 myeloma protein (Human γG1). Solvent, 0.1 M sodium chloride–10 mM sodium phosphate buffer (pH 7.0).

230–232-nm minimum for each of the γGab- and γGc-immunoglobulins examined. The depth of the minima between 220 and 235 nm and their wavelength showed only small variations within the γGab and γGc data. A much greater variation in the depth of these minima was apparent when the γT-immunoglobulin data were included in the comparison. The depth of the minimum near 230 nm was much greater for the γT anti-Lac antibody and the diphtheria antitoxin γT-globulins, while the minimum near 225 nm did not vary as greatly between the γGab, γGc, and γT antibodies. The γT paraprotein spectrum differed from the other equine immunoglobulin spectra in that the minimum at 225 nm was

TABLE IV: Cotton Effect Data, Moffitt–Yang Parameters, and Sedimentation Coefficients for Equine γGab, γGc, and γT Anti-Lac Antibodies and Immunoglobulins, and Equine γGab and γT, and Human γGl F(ab′)$_2$ Fragments.

Protein	$s^0_{20,w}$ (S)	Optical Rotatory Dispersion					
		λ_{min} (nm)	$-[m']_{min}$	λ_{max} (nm)	$[m']_{max}$	$-a_0$	b_0
γGab-Globulins	6.7	231	1230	210	3100	218	12
		224	1095				
γGab anti-Lac	6.8	232	1390	210	3300	271	3
		225	1140				
γGc anti-Lac	6.7	231	1202	210	2700	248	8
		224	959				
γT anti-Lac	7.0	232	1700	207	3400	300	10
		225	1180				
γT diphtheria antitoxin	7.1	232	2115	206	4200	378	7
		225	1324				
γT paraprotein	7.2	230	1396	206	3800	288	2
		225	1432				
γGab F(ab′)$_2$	5.2[b]	225	1380			177	2
γT F(ab′)$_2$	5.3[b]	255	2105			257	14
γGl F(ab′)$_2$[a] human	5.2[b]	225	1504			167	−4

[a] Pepsin 5S F(ab′)$_2$ fragment from a human γGl (Gm(a$^+$f$^-$)) myeloma protein. [b] $s_{20,w}$ values.

significantly deeper, and the two minima at 225 and 230 nm were of nearly equal depth.

The optical rotatory dispersion spectra of the equine γGab and γGc anti-Lac antibodies, and the γGab-globulins from normal serum, each displayed a distinct Cotton effect near 240 nm (Figure 6). In contrast, the 240-nm Cotton effect was absent from the spectra of the γT anti-Lac antibody, the diphtheria antitoxin γT-globulin and the γT paraprotein (Figure 6).

Optical rotatory dispersion curves for F(ab′)$_2$ fragments of equine γGab- and γT-globulins are shown in Figure 6C,D, and the Cotton effect data and Moffitt–Yang parameters are included in Table IV. The data for a human γG1 myeloma protein F(ab′)$_2$ fragment also are included for comparison (Figure 6 and Table IV). The F(ab′)$_2$ fragment spectra had only a single minimum between 220 and 235 nm. The depth of the minimum at 225 nm was greater for the diphtheria antitoxin γT-globulin F(ab′)$_2$ fragment than for the γGab-globulin F(ab′)$_2$ fragment. A distinct Cotton effect at 240 nm was evident in the γGab F(ab′)$_2$ spectrum and in the human myeloma protein F(ab′)$_2$ spectrum. The 240-nm Cotton effect was absent from the γT F(ab′)$_2$ spectrum.

Discussion

Octadecapeptides from the carboxy terminus of equine γG and γT (γG(T)) heavy chains have been isolated after cyanogen bromide cleavage, and their amino acid sequences determined, by Weir *et al.* (1966). The γG and γT octadecapeptides differ at four amino acid residues. Both peptides have a lysinyl residue at position 8 (numbered from the carboxy terminus). The γG peptide has an additional lysinyl residue at position 4 (Weir *et al.*, 1966). Trypsin cleaves specifically at the carbonyl group of lysinyl or argininyl residues, and the carboxyl-terminal γG and γT octadecapeptide sequence variations would have contributed three and two distinct heavy-chain (Fc fragment) tryptic peptides, respectively. The additional differences noted between the γGab and γT heavy chain, Fc fragment, and Fd fragment (Fab fragment less light chain) tryptic peptide maps indicate that variations in the γGab and γT heavy-chain amino acid sequences occur further along the carboxy-terminal half of the heavy chains, and that class-specific amino acid sequence alterations extend into the Fd fragments of the heavy chains. The distinct γGab heavy-chain peptides were identified in maps prepared from both normal equine serum γGab-globulins and diphtheria antitoxin γGab-globulins and do not reflect antibody specificity. The distinct tryptic peptides of the γT heavy-chain maps, prepared from the γT-globulins of diphtheria antitoxin, also are considered to be class specific, as the γT-globulins from this source were heterogeneous with regard to antibody specificity and contained antibodies directed against various determinants of the toxoid used for immunization. Schultze *et al.* (1965) have examined tryptic peptide maps prepared from pepsin Fab′ fragments of equine diphtheria antitoxin and tetanus antitoxin γT-globulins, and were unable to identify peptides which were associated with antibody specificity.

Identical amino-terminal tetrapeptides were isolated from the equine γGab and γT heavy chains. The uncorrected recoveries for the equine tetrapeptides were similar to the recoveries of amino-terminal subtilisin tetrapeptides obtained under the same conditions from the heavy chains of homogeneous human γG and γA myeloma proteins and a γM-macroglobulin (Montgomery *et al.*, 1970). These sequences, therefore, appear to represent the principal amino-terminal sequence of the equine γGab-globulin and γT-globulin heavy chains. Nevertheless, the results do not eliminate the possibility that other amino-terminal heavy-chain sequences also occur in the equine protein populations.

The amino-terminal half of the Fd fragment of the immunoglobulin heavy chains may display extensive sequence variations (Press and Piggot, 1967; Gottlieb *et al.*, 1968; Bennett, 1968; Wikler *et al.*, 1969; Press and Hogg, 1969; Wilkinson, 1969a; Cunningham *et al.*, 1969), and subgroups of amino-terminal heavy-chain sequences are apparent. However, in contrast to the occurrence of type-specific subgroups for the light-chain-variable regions (Milstein *et al.*, 1967; Putnam *et al.*, 1967; Hood and Ein, 1968; Langer *et al.*, 1968), the heavy-chain-variable region subgroups may be associated with more than a single heavy-chain class. The variable region of the human γG1-immunoglobulin heavy chain extends over approximately the first 114–120 amino-terminal residues (Press and Hogg, 1969; Cunningham *et al.*, 1969). A comparison of the first 105 residues of a human γM-macroglobulin heavy chain, Ou (Wikler *et al.*, 1969), and 2 human γG1 myeloma protein heavy chains, Daw (allotype Gm(a^+z^+)) (Press and Piggott, 1967; Press and Hogg, 1969), and He (allotype Gm(a^-f^+)) (Cunningham *et al.*, 1969), demonstrated that the Ou sequence shared 73 (70%) and 61 (58%) identical residues with the Daw and He sequences, respectively, when homologies were maximized. The Ou γM, Daw γG1, He γG1, and Cor γG1 (allotype Gm(a^+z^+)) (Press and Hogg, 1969) amino-terminal sequences appear to be members of the same heavy-chain-variable region subgroup. In contrast, a similar comparison between the Daw and He γG1 sequences and the first 114 residues of γG1 heavy-chain Eu (allotype Gm(a^-f^+)) (Cunningham *et al.*, 1969) demonstrated that the Eu sequence shared only 33 (29%) and 31 (27%) identical residues with the Daw and He sequences, respectively, even when homologies had been maximized by assuming deletions of several amino acid residues. γG1 heavy-chain Ste (allotype Gm(a^-f^+)) (Fisher *et al.*, 1969) also appears to be a member of the distinct Eu subgroup. The sequence PCA-Val-Gln-Leu (*cf.* Eu heavy-chain amino-terminal sequence, Gottlieb *et al.*, 1968) in addition has been identified at the amino terminus of the heavy chain of a human γA1 myeloma protein (Vu), and of an additional γG1 (allotype Gm(a^-f^+)) protein (Fi) (Montgomery *et al.*, 1970).

Rabbit Aa1, Aa2, and Aa3 allotypic determinants, located on the Fd fragments of the γG-globulin heavy chains, also have been found on rabbit γM- and γA-globulins (Todd, 1963; Kelus, 1967; Lennox and Cohn, 1967). Distinct sets of amino-terminal sequences have been associated with specific rabbit γ heavy-chain allotypes (Wilkinson, 1969a). The same sets of allotype-specific amino-terminal sequences have been identified in rabbit colostral γA-globulin heavy chains (Wilkinson, 1969b).

The amino acid sequence of the amino-terminal half of the heavy-chain Fd fragment may vary greatly between proteins of the same class, subclass and allotype. The remainder of the heavy-chain sequence (the carboxy-terminal half of the Fd fragment, and the Fc fragment) appears to be under the control of a single cistron at each subclass-specific locus (Natvig *et al.*, 1967; Herzenberg *et al.*, 1967; Kunkel *et al.*, 1969). Dreyer and Bennett (1965) have proposed a two-gene hypothesis to account for the variable amino-terminal and stable carboxy-terminal sequences of the immunoglobulin subunits (Dreyer *et al.*, 1967; Hood and Ein, 1968). An extension of this thesis for the heavy chain which would allow a given member of a set of variable genes, encoding for a single antibody specificity, to be incorporated into different allotype-, subclass-, or class-specific cistrons offers a conservative mechanism to generate a wide range of antibody specificities from the multiple alleles which may occur at a single subclass-specific heavy-chain locus (*e.g.*, multiple Gm(b) alleles at the human γG3 locus) (Muir and Steinberg, 1967; Kunkel *et al.*, 1969), and to generate a multiplicity of distinct molecular forms of antibody which share a common specificity (*e.g.*, equine anti-Lac antibodies). The linkage of the variable (amino-terminal sequence) gene and the constant (allotype- and subclass-specific sequence) gene, which may be necessary for the initiation of heavy-chain synthesis, may preclude other combinations, and offers a mechanism to account for the suppression of the remaining class-, subclass-, and allotype-specific cistrons of the diploid genome, and for the production of antibody of a single specificity, which have been observed in individual cells (Curtain and Golab, 1966; Pernis, 1967; Mäkelä, 1967). The recovery of identical amino-terminal tetrapeptide sequences from equine γGab- and γT-globulin heavy chains, and the analysis of the more extensive amino-terminal sequence data available for human heavy chains are consistent with the expanded translocation hypothesis. The occurrence of distinct equine γGab and γT Fd fragment tryptic peptides is not necessarily inconsistent with the expanded hypothesis for the equine proteins, as these distinct peptides may have arisen from the class-specific carboxy-terminal halves of the γGab and γT Fd fragments. Similar class- and subclass-specific Fd fragment peptides have been identified upon examination of tryptic hydrolysates of subunits and fragments of human γG and γA myeloma proteins and γM-macroglobulins (Grey and Kunkel, 1965; Frangione *et al.*, 1966, 1967).

The optical rotatory dispersion studies indicated that equine immunoglobins also differ in conformation. The depth of the Cotton effect minima at 230–232 nm varied between γG and γT spectra, and in general was smaller for the γG proteins. The 230-nm minimum in the γT paraprotein spectrum was more shallow than the comparable minima at 232 nm in the spectra of the two other γT proteins, indicating that this spectral feature may vary within a single class of equine immunoglobulins. Variations in the depth of the minima near 232 nm in optical rotatory dispersion spectra within a single class of immunoglobulins also have been reported for human γG-globulins (Dorrington *et al.*, 1967; Ross and Jirgenson, 1968), γM-globulins (Dorrington and Tanford, 1968), and γA-globulins (Dorrington and Rockey, 1968), and for rabbit γG antibodies which differ in affinity or specificity (Steiner and Lowey, 1966). Enzymatic removal of the Fc fragments from the equine γGab- and γT-globulins by peptic digestion under conditions shown to leave the antibody combing site undisturbed, eliminated the prominent 230–232-nm minima from the optical rotatory dispersion spectra of the residual γGab and γT $F(ab')_2$ fragments. The 230–232-nm minima of the intact proteins therefore appeared to be principally a function of the conformation of the Fc fragment of the γG- or γT-globulin heavy chains. Papain Fc fragments of rabbit γG-globulins display a minimum near 232 nm in their optical rotatory dispersion spectra (Steiner and Lowey, 1966).

The minima at 224–225 nm in equine immunoglobulin dispersion curves also displayed variations between γG and γT proteins. The spectra of the equine γGab and γT $F(ab')_2$ fragments displayed only a single prominent minimum at

225 nm. A single minimum at 224–225 nm also has been observed in the spectra of human γG, γM (Dorrington and Tanford, 1968), and γA (Dorrington and Rockey, 1968), and rabbit γG (Steiner and Lowey, 1966; Cathou and Haber, 1967) Fab, or F(ab′)$_2$ fragments. The F(ab′)$_2$ fragments of the equine anti-Lac antibodies retain in full the affinity for hapten of the parent proteins, indicating that no substantial alteration in the conformation of the F(ab′)$_2$ portion of the antibody molecule had attended peptic digestion. The optical rotatory dispersion spectra of the equine F(ab′)$_2$ fragments therefore are considered to reflect the conformations of the light chain and Fd fragment (amino-terminal half of the heavy chain) that are present in the native immunoglobulin molecules. The optical rotatory dispersion spectrum of rabbit γG-globulin has been found to be the sum of the spectra of the papain Fab and Fc fragments (Steiner and Lowey, 1966; Cathou and Haber, 1967). The 224–225-nm Cotton effect minimum is principally a function of that portion of an equine γG or γT antibody molecule, the conformation of which alone determines the structure of the antibody combining site. The optical rotatory dispersion data and the Fab and F(ab′)$_2$ fragment hapten binding data are consistent with a model for the immunoglobulin molecules in which the Fab and Fc fragments occupy separate domains of conformational interaction.

An important difference between the equine γG- and γT-immunoglobulin dispersion curves is the presence of a distinct Cotton effect centered near 240 nm in the γG spectra, and the absence of this feature from the γT spectra. Both equine γGab-globulins and γGab and γGc anti-Lac antibodies display the 240-nm Cotton effect; the dispersion curves of the three distinct equine γT-globulins lacked the 240-nm Cotton effect. The 240-nm Cotton effect was present in the γGab F(ab′)$_2$ spectrum, but again was absent from the equine γT F(ab′)$_2$ spectrum. Human and rabbit γG-globulin Fab and F(ab′)$_2$ dispersion curves display the 240-nm Cotton effect (Steiner and Lowey, 1966; Cathou and Haber, 1967; Dorrington *et al.*, 1967). This spectral feature is not apparent in human γM and γA optical rotatory dispersion curves, but, in contrast to the findings with equine γT F(ab′)$_2$ fragments, is evident in γM and γA Fab or F(ab′)$_2$ dispersion curves (Dorrington and Tanford, 1968; Dorrington and Rockey, 1968). A circular dichroism band and an optical rotatory dispersion Cotton effect centered near 240–250 nm have been associated with the disulfide bond in other peptides and proteins (*e.g.*, glycine oxytocin, vasopressin, and ribonuclease) (Beychok, 1966; Carmack and Neubert, 1967; Coleman and Blout, 1968; Kahn and Beychok, 1968; Urry *et al.*, 1968). The 240-nm Cotton effect of the γG-globulins therefore may be a function of the stereo configuration of the disulfide bonds of their F(ab′)$_2$ fragments. The disulfide bridge is inherently dissymmetric and may exist in either a right-handed or left-handed diasterio isomeric form (Carmack and Neubert, 1967; Kahn and Beychok, 1968). The asymmetry inherent in polypeptide chains may be expected to result in a preferred screw sense for each disulfide bridge of a native protein. The sign of an optical rotatory dispersion Cotton effect or circular dichroism band depends on the handedness of the disulfide bond (Carmack and Neubert, 1967; Kahn and Beychok, 1968). Dispersion curve features contributed by disulfide bridges in one configuration could be wholly or partially compensated for by the contributions of other disulfide bridges of the opposite screw sense present in the same protein. The optical activity dependent on disulfide orbital transitions also may be influenced by perturbations arising from asymmetrically placed vicinal groups (*e.g.*, electrostatic charges, dynamic coupling with other functions). Large changes in wavelength maximum or minimum and ellipticity of circular dichroism bands of disulfide functions have been observed to accompany changes in solvent polarity and pH (Carmack and Neubert, 1967; Coleman and Blout, 1968; Kahn and Beychok, 1968; Urry *et al.*, 1968). The circular dichroism band also may be reduced and displaced (*e.g.*, red shifted) by distortion of the disulfide dihedral angle from the characteristic 90° (Beychok, 1966). A 240-nm Cotton effect, which is the γG F(ab′)$_2$ fragment was dependent on conformational restrictions on disulfide bridges, could be eliminated from the γT F(ab′)$_2$ dispersion curve by any one or a combination of the above features without requiring a gross alteration in the placement of the disulfide bridges along the primary amino acid sequence. Alternately, the distribution of cysteine residues along the amino acid sequence of the Fd fragment of the heavy chain, and consequently the stereochemical configurations of the disulfide bridges may differ between γG and γT F(ab′)$_2$ fragments.

The 240-nm Cotton effect is present in diverse γG-globulin spectra and is not directly related to the unique conformation which explicitly defines the stereo configuration of an antibody combining site of a single specificity. The effect also is present in dispersion curves after mild reduction and alkylation has cleaved the interchain disulfide bonds (Dorrington *et al.*, 1967) and therefore is not a function of interchain disulfide bond stereo configurations.

The expanded two-gene hypothesis would predict that the amino-terminal half of the γG- and γT-globulin Fd fragments would not display class-specific sequences, and that the conformational features reflected in the presence or absence of the 240-nm Cotton effect would be a function of the carboxy-terminal half of the Fd fragments. This conformational probe therefore may be of special use in studying the role of the class-specific carboxy-terminal half of the Fd fragments in the specific interaction between light chain and Fd fragment required for the generation of the native antibody combining site.

The equine immunoglobulins furnish a useful system to study both the primary structural and conformational implications of the expanded translocation hypothesis for generating heavy-chain variability and antibody specificity.

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