Acknowledgment

We are especially pleased to acknowledge the excellent technical assistance of Mrs. Birgitta Kiefer.

References

Barisas, B. G. (1971), Ph.D. Thesis, Yale University, New Haven, Conn.
Carsten, M. E., and Eisen, H. N. (1953), *J. Amer. Chem. Soc. 75*, 4451.
Day, L. A. (1963), Ph.D. Thesis, Yale University, New Haven, Conn.
Day, L. A., Sturtevant, J. M., and Singer, S. J. (1963), *Ann. N. Y. Acad. Sci. 103*, 611.
Eisen, H. N. (1964), *Methods Med. Res. 10*, 94.
Eisen, H. N., and Siskind, G. W. (1964), *Biochemistry 3*, 996.
Farah, F. S., Kern, M., and Eisen, H. N. (1960), *J. Exp. Med. 112*, 1195.
Hinz, H. J., Shiao, D. D. F., and Sturtevant, J. M. (1971), *Biochemistry 10*, 1347.
Klotz, I. M. (1953), *Proteins 1B*, 769.
Little, J. R., and Eisen, H. N. (1968), *Biochemistry 7*, 711.
Lumry, R., and Rajender, S. (1970), *Biopolymers 9*, 1125.
McGuigan, J. E., and Eisen, H. N. (1968), *Biochemistry 7*, 1919.
Nisonoff, A. (1964), *Methods Med. Res. 10*, 134.
Nisonoff, A., and Pressman, D. (1958), *J. Immunol. 80*, 417.
Nisonoff, A., Wissler, F. C., and Woernley, D. L. (1960), *Arch. Biochem. Biophys. 88*, 241.
Porter, R. R. (1959), *Biochem. J. 73*, 119.
Singer, S. J. (1965), *Proteins 3*, 269.
Sips, R. (1948), *J. Chem. Phys. 16*, 490.
Steiner, R. F., and Kitzinger, C. (1956), *J. Biol. Chem. 222*, 271.
Sturtevant, J. M., and Lyons, P. A. (1969), *J. Chem. Thermodyn. 1*, 201.
Velick, S. F., Baggott, J. P., and Sturtevant, J. M. (1971), *Biochemistry 10*, 779.
Velick, S. F., Parker, C. W., and Eisen, H. N. (1960), *Proc. Nat. Acad. Sci. U. S. 46*, 1470.

Structural Units of Canine Serum and Secretory Immunoglobulin A*

Herbert Y. Reynolds† and John S. Johnson

ABSTRACT: Immunoglobulin A was isolated from canine serum and compared to canine secretory IgA (colostral) to clarify molecular weight and antigenic differences. Serum and secretory IgA differ in molecular weight by approximately 75,000 and have sedimentation coefficients of 10 and 11.7 S, respectively; yet disulfide reduction decreased the sedimentation coefficient of both molecules to approximately 7 S. Gel filtration of unreduced secretory IgA in 6 M guanidine-HCl dissociated 13% of the molecule in a manner analogous to the dissociation of rabbit colostral IgA. A portion of this dissociated protein, which was approximately the size of γ chains, possessed antigenic determinants specific for secretory IgA which were localized to the Fc_α fragment produced by tryptic digestion. The presence of a secretory piece-like protein on canine secretory IgA seems certain, although it was not identified specifically in polyacrylamide gels. It is proposed that both serum and secretory IgA molecules consist of two covalently linked subunits, each composed of two α and two light chains. While the greater mass of canine secretory IgA may be due in small part to a slightly larger α chain, most of the extra mass can be accounted for by the attachment of an additional but as yet incompletely characterized component which is similar to the secretory piece found in humans and rabbits.

Johnson and Vaughan (1967) described an immunoglobulin in canine serum, salivary, and bronchial secretions, called intermediate Sγ1, which they thought was analogous to IgA. Immunoprecipitation reactions discussed in that study suggested that antigenic differences existed between the serum and secretory IgA molecules and that these differences were lost after reduction and alkylation of the salivary protein. Subsequently, Vaerman and Heremans (1968) demonstrated immunologic homology between canine intermediate Sγ1 present in serum and external fluids and IgA from similar human fluids through shared antigenic determinants which were recognized by a group of rabbit anti-human IgA antisera. Additional work by Vaerman and Heremans (1969, 1970) revealed that disulfide reduction and alkylation of canine serum and milk whey immunoglobulins changed the sedimentation coefficient of the IgA proteins from 11 to 7 S, suggesting that the parent molecules consisted of two covalently linked subunits.

Canine serum IgA exists principally as a large molecule which is distinctly different in its sedimentation behavior from the 7S serum IgA of humans and rabbits, although polymeric forms of serum IgA have been found in small amounts in these species (Tomasi and Bienenstock, 1968). Because antigenic differences have been difficult to detect consistently between canine serum and secretory IgA, the possibility existed that secretory IgA might not have the extra protein component, designated secretory or transport piece, which is attached to secretory IgA in humans and rabbits.

In this report an isolation method for canine serum IgA is

* From the Laboratory of Clinical Investigation, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland 20014. *Received August 19, 1970.* Presented in part at the Federation of American Societies for Experimental Biology, Atlantic City, N. J., April 12–17, 1970. Referred to as Abstract 2227, *Fed. Proc. 29*, 1970.

† To whom to address correspondence.

described. Using purified serum IgA and secretory IgA, obtained from colostrum, the various polypeptide components of the IgA molecules are examined to define the antigenic and molecular weight differences which exist.

Materials and Methods

Whole canine blood, collected in an acid–citrate–dextrose solution, was obtained from pure bred American and English foxhounds quartered at the National Institutes of Health farm, Poolesville, Md. The plasma was separated and stored at 4°. Canine colostrum was collected within the first 24-hr parturition and the whey was separated as previously described (Reynolds and Johnson, 1970a).

Reagents. Chemicals used were of reagent or analytical grade and their sources have been cited (Reynolds and Johnson, 1970a). Catalase, L-(tosylamido-2-phenyl)ethyl chloromethyl ketone trypsin, and soybean trypsin inhibitor were obtained from Worthington Biochemical Corp. Proteins of known molecular weight used to calibrate gel filtration columns were either purchased in a Pharmacia calibration kit (9 KA) or had been well characterized before their use. Crystallized serum albumin (Armour lot W69102) was further purified by zone electrophoresis on Pevikon. The μ chains (mol wt 70,000) were isolated from an extensively reduced and alkylated human macroglobulin; γ chains (mol wt 50,000) and light chains (mol wt 22,500) were similarly isolated from a human IgG_1 myeloma by gel filtration in 6 M guanidine-HCl.

Sephadex G-100 and 200 and Sepharose 4B and 6B were obtained from Pharmacia; Whatman preswollen microgranular diethylaminoethylcellulose (DE-52) was obtained from Reeve Angel.

Antisera. Antisera against purified canine immunoglobulins were raised in New Zealand white rabbits. Details of immunization and antisera purification have been described previously (Reynolds and Johnson, 1970a,b). Additional antisera were prepared in strain 2 guinea pigs immunized with intact secretory IgA isolated from clarified colostrum and with IgA fragments to be described. A goat (N.I.H. 369) was immunized intramuscularly with colostral IgA. The guinea pig and goat antisera were used unabsorbed. An unabsorbed rabbit anti-IgG Fab antiserum was used to identify fragments containing light chains produced by trypsin digestion of colostral IgA.

Chromatography. Gel filtration and anion-exchange chromatography followed previously described methods (Reynolds and Johnson, 1970a,b). Borate–saline (Helmkamp *et al.*, 1960) or Tris(2-aminohydroxymethyl-1,3-propanediol)-HCl buffers were used. Two guanidine gel filtration methods were used: (1) Sephadex G-200 in 5 M guanidine-HCl as described by Cebra and Small (1967); and (2) a modification of the procedure of Fish *et al.* (1969), employing Sepharose 6B in a 6 M guanidine-HCl solvent. Distribution coefficients were calculated (Flodin, 1961; Andrews, 1964) and used to determine calibration curves. A marker, ribonuclease, was included with each protein mixture separated on the guanidine columns. Proteins chromatographed on the guanidine columns were reduced with 0.1 M dithiothreitol for 2 hr at 37° in the presence of 7 M guanidine-HCl and alkylated with 0.13 M iodoacetamide. Polypeptide chain separation was also done by Sephadex G-100 gel filtration in 1 N propionic acid at 4° (Fleischman *et al.*, 1962).

Polyacrylamide Gels. An alkaline analytical polyacrylamide gel disc electrophoresis method (Reynolds and Johnson, 1970a–c) was used for estimation of molecular weights as described by Hedrick and Smith (1968). Urea polyacrylamide gels were prepared by the method of Reisfeld and Small (1966). All gel polymerizations and electrophoreses were controlled at 25–26°. Calibration proteins used include: pepsin (34,000); ovalbumin (45,000); human hemoglobin (68,000); apoferritin (460,000)—all obtained from Mann; ferritin (460,000), obtained from Pentex (lot 16); ceruloplasmin (160,000), a gift from Dr. A Chrambach; canine IgGd ($7S_{\gamma 1}$), (150,000); rabbit colostral IgA (390,000), a gift from Dr. A. Lawton; a human IgM (900,000), prepared from the serum of a patient with Waldenström's macroglobulinemia.

Ultracentrifugation. Sedimentation velocity experiments were performed in the Spinco Model E ultracentrifuge. In calculating $s_{20,w}$ values the equations of Svedberg and Pederson (1940) were used and all values were extrapolated to infinite dilution.

Molecular weights and the simultaneous measurement of partial specific volumes ($\bar{V}$) were determined by the differential sedimentation equilibrium technique in H_2O and D_2O solvents using an ultraviolet scanner and multiplexer as described by Edelstein and Schachman (1967). The H_2O solvent consisted of 0.02 M Tris–0.1 M NaCl (pH 8.6) and had a density of 1.0032; the D_2O (International Chemical and Nuclear Corp.) solvent consisted of 0.02 M Tris–0.1 M NaCl (pH 8.6) and had a density of 1.1102. Densities of the solvents were determined by pycnometry in 25-ml Gay-Lussac bottles at 20°.

Alternatively, $\bar{V}$ was determined with bromobenzene–kerosene gradients (Linderstrom-Lang and Lanz, 1938). Density standards of analytical grade recrystallized potassium chloride (KCl) were used to calibrate the gradient. All proteins were dialyzed extensively against a KCl solution with a density of 0.99975. Measurements were made at 30°.

Extinction coefficients were calculated in a synthetic boundary cell with interferometric optics at 6000 rpm. Crystallized bovine serum albumin was dissolved in 0.14 M NaCl, buffered at pH 7.4 with sodium phosphate, and used as the reference protein. The refractive index increment of all proteins was assumed to be the same. Proteins were reduced and alkylated before extensive dialysis in the 6 M guanidine-HCl solvent. Optical density units were read at 280 nm. Fringe shifts of serum and colostral IgA and the α- and L-chain preparations were compared to the bovine serum albumin standard and the $E^{1\%}_{1\text{ cm, }280}$ foundf rom the OD/mg ratio. These data are listed in Table I.

Sucrose Density Ultracentrifugation. Samples were layered on 5-ml sucrose density gradients (10–40%) and centrifuged in a SW-39 rotor at 35,000 rpm for 18 hr at 4°. Markers included in each gradient were a 7S rabbit IgG anti-dinitrophenyl antibody labeled with ^{125}I (Fauci *et al.*, 1970) and 11S crystalline beef liver catalase (Worthington Biochemical Corp.). Fractions of approximately 175 μl were collected and counted in a gamma counter (Nuclear-Chicago) to identify the position of the 7S marker. Catalase activity was determined by the method of Allen *et al.* (1965) and expressed as the change in absorbancy per minute at 230 nm.

Results

Isolation of Canine Serum IgA. Fresh plasma was recalcified with $CaCl_2$ to give a final concentration of 0.025 M, and incubated at 37° for 30 min. Serum was separated from the fibrin clot by centrifugation. Lipoproteins were removed at 0°, according to the method of Linquist and Bauer (1966) by the addition of 0.25 M $MnCl_2$ to a final molarity of 0.1 M and with heparin (200 units/ml of plasma). After separating the lipo-

TABLE I: Extinction Coefficients.

Preparation	Solvent	$E^{1\%}_{280,\ 1\ cm}$
Colostral IgA	PBS[a] (pH 7.4)	11.80
Serum IgA	PBS	14.08
Colostral α chains	6 M Guanidine-HCl	10.07
Colostral IgA L chains	6 M Guanidine-HCl	7.32
Serum IgA α chains	6 M Guanidine-HCl	10.63
Serum IgA L chains	6 M Guanidine-HCl	8.37
BSA	PBS	6.67
BSA	6 M Guanidine-HCl	6.49

[a] PBS, phosphate-buffered saline; BSA, bovine serum albumin.

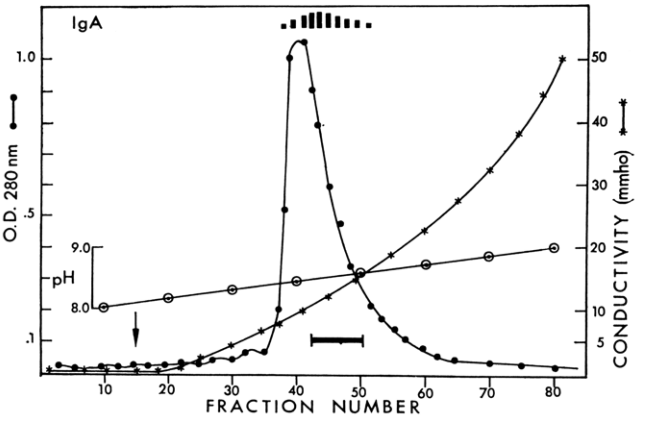

FIGURE 2: Fractions rich in IgA (shown between 50 and 60% GBV in Figure 1) were chromatographed on DEAE-cellulose in 0.02 M Tris-HCl (pH 8.0). The chromatograph was developed with a continuous salt gradient. Elution fractions (4 ml) were collected. The horizontal bar indicates IgA fractions which were pooled.

protein precipitate by centrifugation, the serum was precipitated by 30% saturation with ammonium sulfate (dry $(NH_4)_2SO_4$) at 25°. The precipitate was separated by centrifugation at 2500 rpm for 10 min, and the supernatant was adjusted to 50% $(NH_4)_2SO_4$ saturation with additional dry reagent. After stirring the mixture for 3 hr, the copious precipitate was removed with two 10-min centrifugation runs at 3500 rpm. This precipitate which dissolved readily in borate–saline buffer was dialyzed extensively in that buffer to remove residual $(NH_4)_2SO_4$. Then the dialysis fluid was changed to 0.025 M sodium diethyl barbiturate buffer (pH 6.9) as described by Zschocke *et al.* (1969), and dialysis was continued at 4° for 72 hr to promote euglobulin precipitation. This precipitate was separated by centrifugation at 15,000 rpm for 15 min at 4°. To this point in the separation procedure, IgA was almost quantitatively recovered. The 50% $(NH_4)_2SO_4$ step precipitated all of the serum IgA, whereas no more than 10% was lost during the euglobulin precipitation in Veronal buffer.

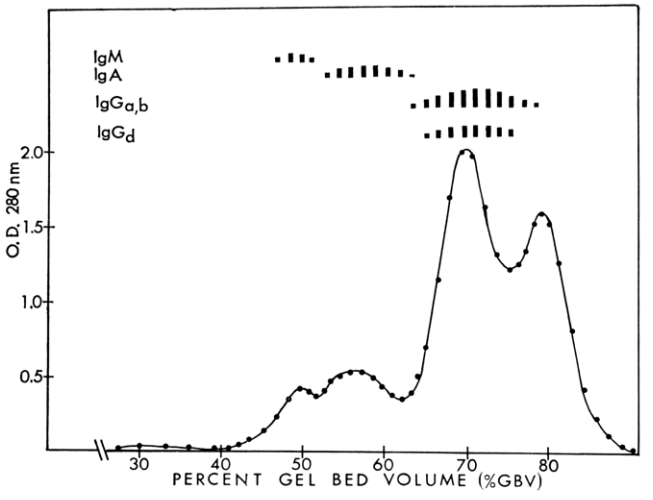

FIGURE 1: Elution diagram of a 15-ml aliquot of concentrated euglobulin depleted supernatant on a Sephadex G-200 column (2.5 (inside diameter) × 90 cm) linked in tandem to a Sepharose 4B column (2.5 cm (inside diameter) × 100 cm). The solvent was 0.5 M Tris-HCl (pH 8.0). Relative concentrations of immunoglobulins are shown by vertical bars.

The supernatant from the euglobulin precipitation step was concentrated by negative pressure to one-third volume, and aliquots were chromatographed (Figure 1). IgA-rich fractions which eluted at 55–60% of combined gel bed volume (GBV) were concentrated and dialyzed in 0.02 M Tris-HCl buffer (pH 8.0) As an alternative to the tandem column separation, 10-ml aliquots were chromatographed on Sephadex G-200 (4.1 cm (inside diameter) × 168 cm) in borate–saline buffer (pH 8.0), with acceptable separation of the IgM and IgA protein. With the Sephadex G-200 column IgA eluted at 33–37% GBV. Fractions rich in IgA protein were applied to a DEAE-cellulose column (Figure 2); IgA eluted at pH 8.6 and conductivity between 8 and 15 mmhos. These IgA fractions were subjected to a final gel filtration on Sephadex G-200 (2.18 cm (inside diameter) × 114 cm) in borate–saline (pH 8.0) to remove aggregates. The isolated IgA protein was dialyzed against 0.2 M Tris-HCl buffer (pH 8.6) to improve its stability for storage at 4°. The yield of purified serum IgA was 15–20%.

Comparative Studies of Serum and Secretory IgA. Ultracentrifugation of serum IgA gave a homogeneous sedimenting boundary in 0.2 M Tris-HCl buffer (pH 8.6) at 56,000 rpm. The sedimentation coefficient at infinite dilution was 10 S, which was determined from the linear regression plot (slope −0.136) of corrected sedimentation coefficients for 12 concentrations of serum IgA. Secretory IgA, isolated from colostrum and intestinal fluids, has a sedimentation coefficient of 11.7 S (Reynolds and Johnson, 1970c). Serum and colostral IgA preparations (Figure 3), upon zone electrophoresis in agar gel, had similar electrophoretic mobilities and were free of other contaminating proteins.

Molecular weight determinations of IgA were performed in a series of analytical polyacrylamide gels, each with a dif-

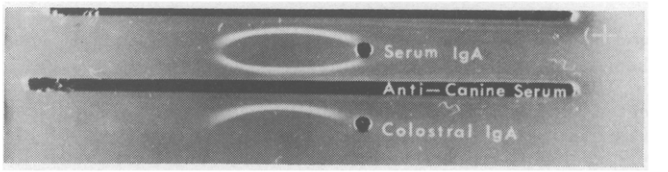

FIGURE 3: Immunoelectrophoresis patterns of serum IgA (4.0 mg/ml) and secretory IgA (3.8 mg/ml) in 1.2% agar were developed with rabbit anti-canine whole serum.

TABLE II: Molecular Weights of Colostral and Serum IgA.

Source of IgA Prepn	Mean Retardation Mobility Coef	Mean Mol Wt and Range[a]	$\bar{V}^b$	$\bar{V}^c$	H_2O Slope d ln c/dx^2	D_2O Slope	Speed rpm ($\times 10^{-3}$)	Mol Wt[d] ($\times 10^{-3}$)
Colostrum	0.401	372.0 (340.0-400.0)	0.728		0.4184		4.4	355.586
					0.4319		4.4	367.059
					0.5936		5.2	361.173
				0.735	0.5481	0.4161	5.2	343.218
							Mean	356.759
Serum	0.341	300.0 (275.0-310.0)		0.731	0.4507	0.3448	5.2	277.778
				0.743	0.4377	0.3276	5.2	282.663
							Mean	280.221

[a] Data from analytical polyacrylamide gels. [b] $\bar{V}$ from density gradient column data. [c] $\bar{V}$ from differential sedimentation equilibrium. [d] Sedimentation equilibrium ultracentrifugation.

ferent acrylamide monomer concentration in the separating gel. The acrylamide concentration ranged from 3.3 to 7% in each series of five or six gels. The IgA preparations showed single protein bands in each gel. The mean retardation of mobility coefficient (Ferguson, 1964) was calculated from seven electrophoresis runs of serum and colostral IgA and molecular weights were found from a calibration curve. In addition, molecular weights of the IgA preparations were determined by sedimentation equilibrium ultracentrifugation at 20°. Plots of the log concentration *vs.* distance from center of rotation for IgA in aqueous solvent (0.02 M Tris–0.1 M NaCl, pH 8.6) were made from ultraviolet scans obtained at 72 hr and showed linear relationships. Aggregated material was not detectable. Molecular weight data are listed in Table II.

Ultracentrifugation of fresh canine serum in a 10–40% linear sucrose gradient (Figure 4) showed that most of the IgA sedimented slightly less rapidly than the 11S catalase marker and that no appreciable 7S IgA was present. After reduction with 0.05 M dithiothreitol and alkylation with 0.1 M iodoacetamide the sedimentation of serum IgA coincided with the 7S marker. Similarly, the sedimentation coefficient of the bulk of colostral IgA was changed to approximately 7 S.

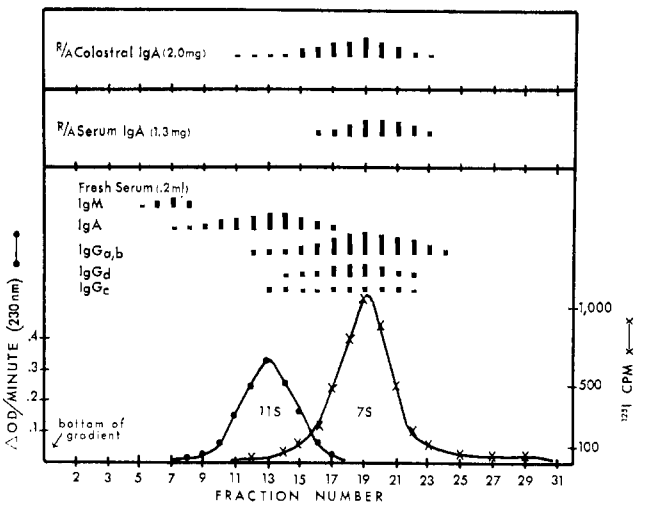

FIGURE 4: Three simultaneously centrifuged sucrose density gradients contain fresh canine serum and reduced and alkylated (R/A) preparations of serum and colostral IgA. Fractions from each graient were comparable and the positions of the 7S and 11S markers were the same in each gradient. The relative concentrations of immunoglobulins are denoted by vertical bars. The radiodinated 7S marker position (×) is shown as counts per minute (cpm). The position of the 11S catalase marker (●) was determined enzymatically and is plotted as ΔOD/min.

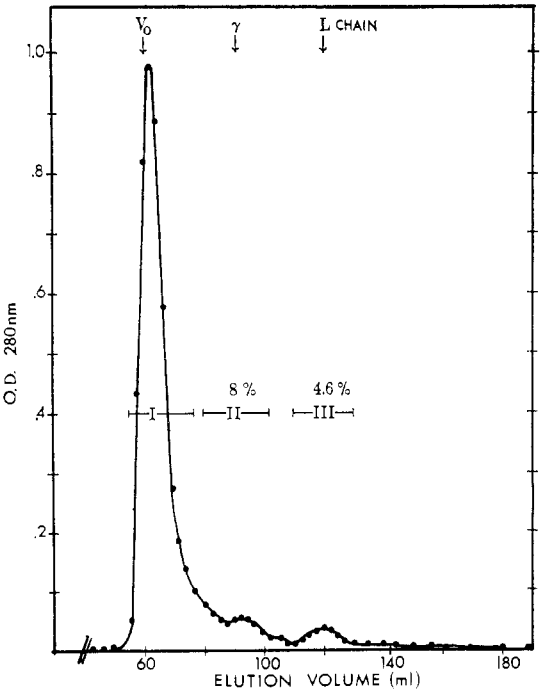

FIGURE 5: Alkylated colostral IgA (12.3 mg) was applied to a calibrated Sepharose 6B column (2.5 cm (inside diameter) × 41.5 cm) and eluted with 6 M guanidine-HCl (pH 4.8). Effluent fractions of 1.9 ml were collected. A small peak containing iodoacetamide which eluted at 216-ml volume is not shown. The void volume (V_0) was determined with intact human IgM.

TABLE III: Summary of Gel Filtration Experiments in Guanidine-HCl.

Colostral IgA	Protein Applied (mg)	mg of Protein Recovd (H + L)	% H (α) / H + L as OD	% L / H + L as OD	Mass Ratio	K_d of α Chain	Mol Wt of α Chain ($\times 10^{-3}$)	K_d of L Chain	Mol Wt of L Chain
1	14.5	13.0 (90)	67.3	32.7	0.60/0.40	0.109	60.0	0.227	26.0
2	13.5	10.3 (77)	67.5	32.5	0.60/0.40	0.111	58.8	0.242	24.0
3	28.8	16.7 (60)	70	30	0.65/0.36	0.202	62.5	0.380	31.3
4	26.0	19.2 (74)	67	33	0.60/0.40	0.220	58.6	0.426	25.3
Mean		75%	68	32	0.61/0.39				
Colostral IgA-Pool I									
5	7.8	5.2 (67)	78	22	0.72/0.28	0.095	65.0	0.252	23.5
6	10.7	6.6 (61)	72	28	0.64/0.36	0.219	59.5	0.432	24.3
7	8.85	2.1 (24)	71	29	0.68/0.32	0.220	58.6	0.438	23.7
Mean			74	26	0.68/0.32		60.43[a]		25.44[b]
Serum IgA									
1	15.0	11.1 (74)	64	36	0.61/0.39	0.120	55.0	0.251	23.5
2	15.2	11.0 (72)	76	24	0.73/0.27	0.126	53.5	0.256	23.0
3	6.9	5.7 (83)	68	32	0.64/0.36	0.102	60.5	0.240	24.5
4	7.0	5.0 (71)	67	33	0.63/0.37	0.110	58.0	0.240	24.5
Mean		75%	69	31	0.65/0.35		56.75[a]		23.88[b]

[a] Difference in molecular weight of α chain of colostral/serum $t = 2.19$, $p = 0.056$. [b] Difference in molecular weight of L chain of colostral/serum $p > 0.20$.

Colostral IgA was equilibrated with 7 M guanidine-HCl, alkylated with 0.02 M iodoacetamide, and chromatographed on a calibrated Sepharose 6B column in 6 M guanidine-HCl (pH 4.8) (Figure 5). Approximately 7–13% of the measured optical density units, determined from five experiments, were dissociated from the IgA molecule and are designated pool II and III in Figure 5. The material which eluted in the peak of pool II corresponded to the elution position of γ chains chromatographed on the same column and the peak of pool III coincided with the L-chain elution position. In 4% polyacrylamide urea gels pool II material showed banding similar to pool I, whereas pool III showed a six- to eight-band pattern typical of light chains (Reisfeld and Small, 1966). A distinct band analogous to a secretory piece-like protein (Cebra and Small, 1967) was not evident. Fractions from pools II and III were concentrated by negative pressure in $^{8}/_{32}$-in. dialysis tubing and dialyzed against distilled water to remove residual guanidine. These concentrates were used for animal immunizations. Pool I material, designated as "stripped" colostral IgA, was concentrated and used in further reduction and alkylation experiments described below.

Serum and colostral IgA, reduced with 0.1 M dithiothreitol in 7 M guanidine-HCl for 2 hr at 37° and alkylated with 0.13 M iodoacetamide, were chromatographed on a Sepharose 6B column in 6 M guanidine-HCl (pH 4.8) (Figure 6). Distribution coefficients, K_d, for the α- and L-chain components were compared to a calibration curve of reference proteins (plotted as K_d *vs.* logarithm of molecular weights) and estimates of the molecular weight for serum and secretory α and L chains were made. Table III summarizes data obtained from guanidine-HCl chromatography. Stripped colostral IgA, designated as pool I in Figure 5, was concentrated, and dialyzed against 7 M guanidine-HCl before it was subsequently reduced and alkylated as above. These experiments are listed as numbers 5, 6, and 7 in Table III. Colostral IgA expt 3, 4, 6, and 7 were performed on a Sephadex G-200 column (1.45 cm (inside diameter) × 92 cm) in 5 M guanidine-HCl; therefore, the distribution coefficients are somewhat different from those found with Sepharose 6B. A chromatogram and the calibration curve from this Sephadex G-200 column have been illustrated previously (Reynolds and Johnson, 1970c). The remaining

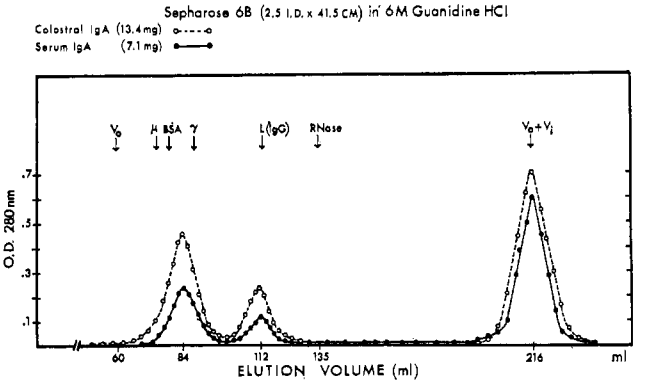

FIGURE 6: Elution diagram of two reduced and alkylated IgA preparations. The column, solvent conditions, and fraction sizes are the same as in Figure 5. The elution positions of additional calibration proteins are shown.

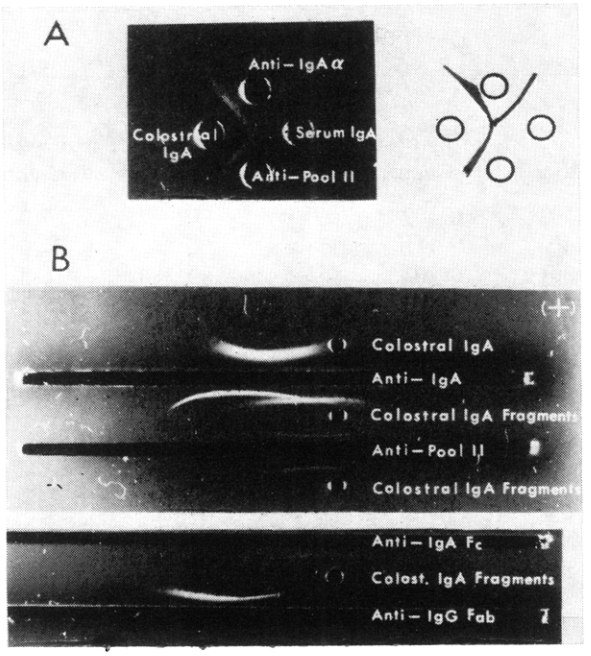

FIGURE 7: (A) Canine colostral IgA (3.8 mg/ml) and serum IgA (4 mg/ml) are reacted with an unabsorbed guinea pig anti-canine colostral IgA pool II (see Figure 5) and with rabbit anti-canine IgA, absorbed with IgG Fab. Anti-IgA α identified common determinants on α chains of colostral and serum IgA, whereas anti-pool II reacted only with colostral IgA. (B) Immunoelectrophoresis of colostral IgA (3.8 mg/ml) and colostral Fc and Fab fragments (5 mg/ml) precipitated with an unabsorbed anti-IgA antiserum. Anti-IgA Fc is the anti-IgA α shown in part A. Anti-pool II reacted only with the anodal or Fc α fragment of IgA. A rabbit anti-canine IgG Fab identified the cathodal Fab α fragment.

gel filtrations in guanidine were done on the Sepharose 6B column.

The mean protein recovery from the chromatograms was 75% for colostral and serum IgA which was somewhat low compared with yields from rabbit colostral IgA (Cebra and Small, 1967). The recovery of heavy- and light-chain protein after reduction and alkylation of the stripped colostral IgA (expt 5, 6, and 7) was less than that from other IgA preparations, suggesting that the stripped colostral IgA was more susceptible to degradation under the conditions imposed. When the yields of α and L chains were compared (calculated as the total optical density units in heavy- or light-chain peaks divided by the sum from both peaks), approximately a 2:1 ratio of α to L chains was found for both colostral and serum IgA. The L-chain yield after reduction and alkylation of the stripped colostral IgA was slightly less than that obtained from the parent molecule, which was consistent with the loss of L chains in the preliminary dissociation of the intact molecules. The mass ratio of α- to L-chain yields showed that slightly greater mass was associated with the α-chain fractions isolated from stripped colostral IgA and serum IgA; whereas relatively more mass was found in the L-chain pool from intact colostral IgA.

Immunochemical Characterization of IgA. Antisera to various purified intact secretory IgA preparations isolated from colostrum and from intestinal secretions raised in 14 rabbits, 10 guinea pigs, and 1 goat gave homogeneous precipitin lines against secretory and serum IgA proteins. IgA in its native form in a variety of external canine fluids (serum, saliva, colostrum, milk, small bowel, and colonic secretions) likewise did not give immunologic evidence of unique antigenic determinants on the secretory molecules with the numerous antisera tested. Antisera made to reduced and alkylated colostral IgA did not recognize any antigenic differences in the serum and secretory IgA molecules. However, immunization with protein dissociated from intact colostral IgA, shown as pool II in Figure 5, resulted in antisera which gave a precipitin line against native colostral or intestinal secretory IgA but not against serum IgA. Such antisera were obtained from 2 of 12 guinea pigs which had been immunized with various batches of pool II dissociated material. The unique reactivity was present in bleedings taken about 2 weeks after immunization; later bleedings from these animals showed loss of the specific reactivity. Figure 7A illustrates the specificity of an unabsorbed antiserum. The guinea pig antiserum, designated anti-pool II, recognized colostral IgA but not serum IgA; whereas, rabbit anti-α-chain antiserum identified α chains in both IgA preparations.

In order to localize the reactivity of anti-pool II antiserum, Fc_α and Fab_α fragments were produced from colostral IgA by a modification of the method of Plaut and Tomasi (1970). Colostral IgA (15 mg) in 1 ml of 0.2 M Tris-HCl buffer (pH 8.6) was reduced with 0.003 M dithiothreitol for 30 min at 25°, and alkylated with 0.007 M iodoacetamide. L-(Tosylamido-2-phenyl)ethyl chloromethyl ketone trypsin and the reduced and alkylated IgA, equilibrated with 0.1 M Tris-HCl (pH 8.0) and 0.1 M $CaCl_2$, were independently heated to 56° before mixing in a 1:25 w/w ratio of enzyme to substrate. Digestion was stopped after 30 min at 56° by the addition of soybean trypsin inhibitor. Undigested IgA was removed by gel filtration and approximately 5 mg (a 33% yield) of IgA fragments was recovered. Two fragments were identified by immunoelectrophoresis and are illustrated in Figure 7B. Only the electrophoretically slower or cathodal fragment contained L-chain determinants, which is analogous to the Fab fragment produced from IgG. The anodal fragment contained α-chain determinants and was precipitated by the guinea pig anti-pool II antiserum. These colostral IgA fragments were designated as Fab_α and Fc_α, respectively, because of their electrophoretic mobilities in zonal electrophoresis and their reactivity with specific antisera. Further molecular weight or size characterization was not done; however, the IgA fragments appeared analogous with the well-characterized fragments of IgG (Edelman *et al.*, 1968). The unique secretory IgA determinants recognized by anti-pool II were associated with Fc_α.

Discussion

The mass of canine secretory IgA is greater than that of serum IgA, and molecular weight determinations document a difference between serum and colostral IgA of approximately 75,000. Although the molecular weight of secretory α chain is slightly greater than that of serum α chain using comparable measurement techniques, the implication that there may be differences in composition of the serum and secretory α chains is considered preliminary. Even if substantiated by further experiments, this finding contributes minimally to the difference in mass of the two IgA molecules. The extra mass associated with the secretory molecule is presumed to be the effect of additional protein attached to it.

Intact colostral IgA can be stripped of approximately 7–13% of its optical density units (Figure 5) with a strong dissociating solvent, subsequent alkylation, and gel filtration. Two elution peaks coincide with the elution positions of γ-

and L-chain calibration proteins. By analogy from data obtained with rabbit IgA (Cebra and Small, 1967), pool II protein should contain secretory piece (T chain) and pool III should consist of light chains. Urea polyacrylamide gels of the concentrated material in pool II did not identify a discrete fast-migrating protein band interpretable as a T chain; pool III gels showed the multiple banding pattern characteristic of light chains. Guinea pig anti-pool II antisera did recognize, however, antigenic determinants on the canine secretory IgA molecules which were not present on serum IgA (Figure 7A). Furthermore, these determinants were associated with the electrophoretically rapid or Fc_α fragment produced from colostral IgA. Using rabbit colostral IgA, Lawton (1970) has shown that the Fc fragment is the site of secretory piece attachment.

Canine serum IgA has a corrected sedimentation constant of 10 S. After disulfide reduction and alkylation, its sedimentation rate in linear sucrose gradients decreased to approximately 7 S which is similar to the value established for human serum IgA. This is consistent with the view that canine serum IgA is a dimeric structure composed of 7S monomers. Similarly, colostral IgA was reduced to 7S sedimenting subunits. Mass ratios calculated for serum and colostral α and L chains (Table III) indicate that equal numbers of α and L chains were dissociated from extensively reduced and alkylated IgA molecules. Therefore, the most probable structural model for canine serum IgA, based on our molecular weight data, would consist of two covalently linked subunits each of which is composed of two α and two L chains linked by disulfide bonds. The canine secretory IgA model would differ from its serum counterpart in that an extra polypeptide component of approximately 50,000 molecular weight possessing unique antigenic determinants is noncovalently bound to the Fc region of the secretory α chain. It is proposed that this component is analogous to human and rabbit secretory piece and is responsible for the greater mass and sedimentation coefficient found for secretory IgA.

Acknowledgments

The authors thank Miss Lois Renfer for excellent technical assistance. Experiments employing the Model E ultracentrifuge equipped with ultraviolet scanner and multiplexer were performed in the laboratory of Dr. Antonio Gotto of the National Heart and Lung Institute and are gratefully acknowledged.

References

Allen, P. Z., Sirisinha, S., and Vaughan, J. H. (1965), *J. Immunol. 95*, 918.

Andrews, P. (1964), *Biochem. J. 91*, 222.

Cebra, J. J., and Small, P. A., Jr. (1967), *Biochemistry 6*, 503.

Edelman, G. M., Gall, W. E., Wyadal, M. J., and Konigsberg, W. H. (1968), *Biochemistry 7*, 1950.

Edelstein, S. J., and Schachman, H. K. (1967), *J. Biol. Chem. 242*, 306.

Fauci, A. S., Frank, M. M., and Johnson, J. S. (1970), *J. Immunol. 105*, 215.

Ferguson, K. A. (1964), *Metabolism 13*, 985.

Fish, W. W., Mann, K. G., and Tanford, C. (1969), *J. Biol. Chem. 244*, 4989.

Fleischman, J. B., Pain, R. H., and Porter, R. R. (1962), *Arch. Biochem. Biophys., Suppl. 1*, 174.

Flodin, P. (1961), *J. Chromatog. 5*, 103.

Hedrick, J. L., and Smith, A. J. (1968), *Arch. Biochem. 126*, 155.

Helmkamp, L. W., Goodland, R. L., Bale, W. F., Spark, I. L., and Mutschler, L. E. (1960), *Cancer Res. 20*, 1495.

Johnson, J. S., and Vaughan, J. H. (1967), *J. Immunol. 98*, 923.

Lawton, A. R. (1970), *Proc. Conf. Secretory Immune System Vero Beach, Fla., Dec. 10–13, 1969.*

Linderstrøm-Lang, K., and Lanz, H. (1938), *C. R. Trav. Lab. Carlsberg 21*, 315.

Lindqvist, K., and Bauer, D. C. (1966), *Immunochemistry 3*, 373.

Plaut, A. G., and Tomasi, T. B., Jr. (1970), *Proc. Nat. Acad. Sci. U. S. 65*, 318.

Reisfeld, R. A., and Small, P. A., Jr. (1966), *Science 152*, 1253.

Reynolds, H. Y., and Johnson, J. S. (1970a), *J. Immunol. 104*, 1000.

Reynolds, H. Y., and Johnson, J. S. (1970b), *J. Immunol. 104*, 888.

Reynolds, H. Y., and Johnson, J. S. (1970c), *J. Infect. Dis. 121, Suppl.*, 118.

Svedberg, T., and Pederson, K. O. (1940), The Ultracentrifuge, Oxford, Clarendon Press.

Tomasi, T. B., Jr., and Bienenstock, J. (1968), *Advan. Immunol. 9*, 1.

Vaerman, J., and Heremans, J. F. (1968), *Immunochemistry 5*, 425.

Vaerman, J., and Heremans, J. F. (1969), *Immunochemistry 6*, 779.

Vaerman, J. P., and Heremans, J. F. (1970), *Immunology 18*, 27.

Zschocke, R. H., Grieble, H. G., Bach, G. L., and Anderston, T. O. (1969), *J. Immunol. 102*, 625.