The Immunoglobulins of Sheep Colostrum*

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ABSTRACT: Sheep colostrum contains at least three antigenically distinct immunoglobulins which can be separated by Sephadex G-200 gel filtration. On the basis of analogous characteristics of human immunoglobulins, two of the three immunoglobulins were designated γA_2 and γA_1. The third immunoglobulin, being present in the largest quantity, was identical with immunoglobulin γG_1 of sheep serum. Immunoglobulin γA_2, sedimenting at 15 S, degraded in the presence of 4 M urea. Among the products of this degradation were heavy and light-chain dimers and another polypeptide resembling human and rabbit secretory piece. The 15S protein contained approximately 4% each of hexose and hexosamine. The isolated heavy-chain monomer of this immunoglobulin had a molecular weight of approximately 63,500, and the molecular weight of the secretory piece was estimated to be 26,500. Immunoglobulin γA_1, sedimenting at 10.8 S, failed to undergo extensive degradation in 4 M urea. It appeared to be composed of two 7S proteins probably linked through interchain disulfide bridges. Immunoglobulin γA_1 was present in smaller amounts than immunoglobulin γA_2. The carbohydrate content and the molecular weight of the heavy chain of immunoglobulin γA_1 were similar to that of γA_2 but the former was not associated with secretory piece. Immunoglobulin γG_1 was the main constituent of the 6.5S component of colostrum. Other subclasses of immunoglobulin G, usually present in serum, were found in ovine colostrum only in trace amounts.

Recent studies of human immunoglobulin γA have established the presence of at least two antigenically distinct subclasses, a major one, γA_1, accounting for approximately 70% and a minor one, γA_2, accounting for the balance of all γA myeloma proteins (Feinstein and Franklin, 1966; Kunkel and Prendergast, 1966; Vaerman and Heremans, 1966). The γA_2 subclass has an unusual subunit arrangement which has so far not been encountered in other immunoglobulins. In the usual arrangement, the 7S protein is a mixed tetramer composed of two light chains linked to two heavy chains by a single disulfide bond each, with disulfide-bond linkage between the two heavy chains. Immunoglobulin γA_2 appears to be a 7S protein, composed of one disulfide bond linked light-chain dimer and one similarly linked heavy-chain dimer. The two dimers form a mixed 7S tetramer held together by noncovalent forces only (Grey *et al.*, 1968). A similarly arranged immunoglobulin γA_2 was found in six of six mouse γA myeloma proteins, suggesting that it must be the predominat γA subclass in this species (Abel and Grey, 1968).

An outstanding characteristic of immunoglobulin γA is its quantitative and probably functional dominance in fluids at interfaces between the internal and external environment (Tomasi and Zigelbaum, 1963; Hanson, 1961). Immunoglobulin γA in such fluids possesses an additional polypeptide chain, the transport piece (Tomasi *et al.*, 1965) or secretory piece. The synthesis of this polypeptide in epithelial cells does not require the presence of immunoglobulin γA (South *et al.*, 1966). Combination of the secretory piece with other immunoglobulin classes has not been reported to date.

In the course of a study of sheep colostrum we found two subclasses of immunoglobulin γA. These show many similarities to the exocrine immunoglobulin γA of other species (Grey *et al.*, 1968; Abel and Grey, 1968; Cebra and Small, 1967; Sell, 1967; Newcomb *et al.*, 1968; Brandtzaeg *et al.*, 1968) and yet have characteristics which are either distinct or which have not been demonstrated in other species to date. Sephadex G-200 gel filtration of ovine colostrum allowed the separation of immunoglobulin γA_2 from γA_1 and also from immunoglobulin γG. Immunoglobulin γA_2, unlike its analogs encountered in other species, exists exclusively as an oligomer of 7S protein subunits. By contrast, immunoglobulin γA_1 was composed of two 7S protein subunits only. The secretory piece was associated only with the oligomeric immunoglobulin γA_2, binding it probably by noncovalent forces rather than disulfide bonds. The latter appear to be the predominant links between immunoglobulin γA and secretory piece in man (Tomasi *et al.*, 1965) and in the rabbit (Cebra and Small, 1967).

Materials and Methods

Ovine Colostrum. Colostrum was obtained on the first day *postpartum* from four sheep, of which one had been hyperimmunized with dinitrophenylated bovine serum albumin and another with the synthetic heteropolymer L-glutamic acid, L-lysine, and L-alanine, present in molar ratios of 42, 28, and 30. The colostra were cleared by centrifugation at 16,000*g* for 16 hr in a refrigerated centrifuge. The clear solution was diluted with equal amounts of physiological saline and filtered with 0.1 M phosphate buffer (pH 7) in columns (2.5 × 100 cm) of Sephadex G-200.

Separations of Component Chains. Immunoglobulins appearing under the first three peaks of the gel filtration were exhaustively dialyzed against 4 M urea. The immunoglobulins were also reduced by 0.1 M 2-mercaptoethanol and alkylated with 0.2 M iodoacetamide at pH 8.2 in the presence of 4 M urea. Solutions containing either 20 or 50 mg of protein were ap-

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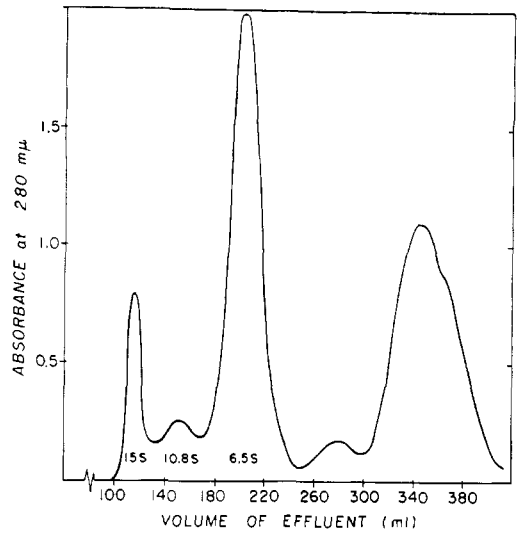

FIGURE 1: Sephadex G-200 gel filtration of 5 ml of colostral whey (approximately 170 mg of protein) diluted with an equal volume of physiological saline. Column (100 × 2.5 cm) filtered with 0.1 M phosphate buffer (pH 7.0).

plied to a Sephadex G-150 column (2.5 × 70 cm) and eluted with 0.1 M acetic acid in 4 M urea. The molecular weight of the effluent components was estimated by the method of Andrews (1964). On this column, the excluded volume, V_0, was 82 ml and the included volume, V_i, was 183 ml. Plots of the square roots of molecular weight and K_D, the latter being equal to peak elution volume minus V_0 divided by V_i, utilized markers of separated light chains and γ chains of ovine immunoglobulin G.

Starch Gel Electrophoresis. Vertical starch gel electrophoresis was carried out in 12% hydrolyzed starch in 8 M urea, buffered with 0.035 M glycine at pH 8.8 and with 0.3 M borate buffer at pH 8.2 in the electrode vessels for 18 hr at 5 V/cm at 4° (Cohen and Porter, 1964a).

Carbohydrate Analyses. Hexose was measured both by the anthrone (Kabat and Mayer, 1961) and the orcinol–sulfuric acid methods (Soerensen and Haugaard, 1933) with an equal mixture of mannose and galactose as the standard. Hexosamine was measured with Ehrlich's reagent using *N*-acetylglucosamine as the standard (Kabat and Mayer, 1961).

Physical Measurements. The extinction coefficient, $E^{1\%}_{1cm}$, at 280 mμ for the two subclasses of immunoglobulin γA was 12.

The partial specific volume, $\bar{v}$, for the heavy chains of both subclasses of immunoglobulin γA was assumed to be 0.732; $\bar{v}$ for the light chains was assumed to be 0.703. Both values were adopted from the experimental data of Cebra and Small (1967) for the analogous chains of rabbit immunoglobulin γA in 5 M guanidine-HCl.

Sedimentation velocity experiments were performed in the Spinco Model E analytical ultracentrifuge, usually at protein concentrations of 2 to 5 mg per ml. The s values were not corrected for the effects of concentration. High-speed short-column sedimentation equilibrium experiments (Yphantis, 1964) were done with isolated, reduced, and alkylated heavy and light chains. The protein was analyzed, where feasible, at different concentrations, in 4 M urea–1 M acetic acid and also in 0.1 M phosphate buffer (pH 7). M_w was calculated from plots of fringe displacement *vs.* radius. M_z was calculated as described by Yphantis (1964).

TABLE I: The Sedimentation Coefficients of the Three Immunoglobulin Fractions Isolated by Sephadex G-200 Gel Filtration.

Peak	$s_{20,w}$, HPO_4^{2-}, pH 7	$s_{20,w}$, 4 M Urea[a]	$s_{20,w}$, 4 M Urea–0.01 M MeSH[b]	Immunoglobulin Class
1	15	9, 6.5, 4.5, 1.5	4.5, 1.9, 1.5	γA_2
2	10.8	8.5, 5.8	5.8	γA_1
3	6.5	5.8	5.8	γG_1

[a] The underlined number indicates the major fraction. [b] MeSH, mercaptoethanol.

Antisera. Antisera were prepared in rabbits against immune precipitates containing sheep immunoglobulins (antiserum 911) and against whole colostral whey (antisera 929 and 967) and in guinea pigs against isolated immunoglobulin γA_2 (antiserum T). Where indicated antisera were absorbed with either slow immunoglobulin γG_2 or fast immunoglobulin γG_1 (Heimer *et al.*, 1969) to remove anti-light-chain activity.

Immunological Analyses. Immunoelectrophoresis and immunodiffusion studies were performed by the methods of Scheidegger (1955) and Ouchterlony (1949).

Results

Gel Filtration. Fractionation through Sephadex G-200 of colostrum yielded five distinct fractions. The fractions appeared at a similar elution volume in all four colostra tested. Colostra of hyperimmunized sheep, however, appeared to contain more protein in the first three fractions. As subsequent analyses revealed the absence of immunoglobulins in the last two fractions, they are not further discussed in this paper. A typical Sephadex G-200 gel filtration of colostrum is shown in Figure 1. Quantitatively the third component was the largest and the first component always exceeded the second. Fractions from the center of the three peaks were pooled separately and refiltered separately on Sephadex G-200. This procedure resulted in the preparation of solutions containing a single protein only when tested in the ultracentrifuge or by immunochemical techniques.

Analytic Ultracentrifugation. The sedimentation coefficients of the first three eluted fractions, corrected for solvent but not concentration effects and obtained on 2–5 mg of protein/ml, are shown in Table I. This table also lists the sedimentation coefficients of the same fractions exposed for 24 hr to 4 M urea in the presence and absence of 0.01 M mercaptoethanol.

The material appearing under the first peak had a sedimentation coefficient of 15 S, and will be referred to henceforth as immunoglobulin γA_2. In 4 M urea it dissociated to at least four distinct components of 9, 6.5, 4.5, and 1.5 S (Figure 2A). The 9S and most of the 4.5S component disappeared in 4 M urea containing 0.01 M mercaptoethanol (Figure 2B). On increasing the mercaptoethanol concentration to 0.1 M, only the most slowly sedimenting components remained (not shown in Figure 2).

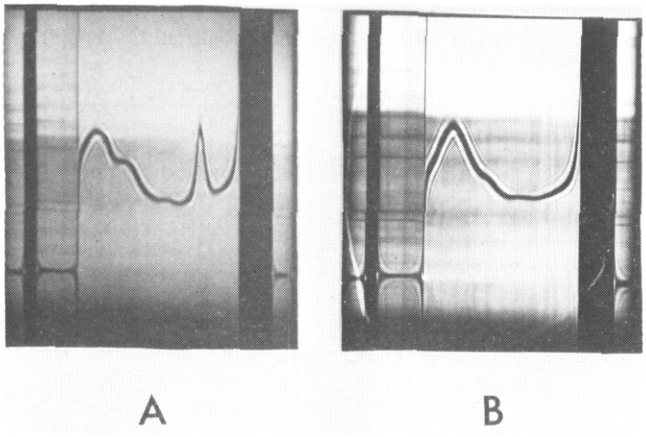

FIGURE 2: Ultracentrifuge patterns of immunoglobulin γA_2. (A) In 4 M urea, after 80 min at 56,000 rpm. (B) The same material in 4 M urea and 0.01 M mercaptoethanol, after 80 min at 56,000 rpm.

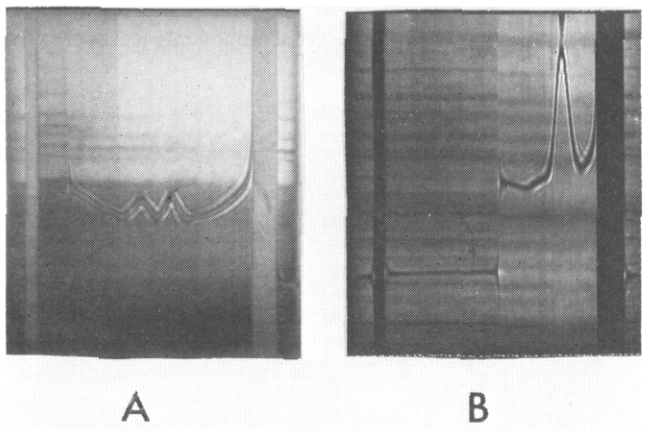

FIGURE 3: Ultracentrifuge pattern of 3.2 mg/ml of immunoglobulin γA_1. (A) In 4 M urea, after 80 min at 56,000 rpm. (B) The same material (7.6 mg/ml) in 4 M urea and 0.01 M mercaptoethanol, after 80 min at 56,000 rpm.

The material appearing under the second peak obtained by Sephadex G-200 gel filtration had a sedimentation coefficient of 10.8 S. This material was designated as immunoglobulin γA_1. After 24 hr in 4 M urea this fraction contained components sedimenting at 5.8 and 8.5 S (Figure 3A). Addition of 0.01 M mercaptoethanol to this system resulted in a nearly complete conversion to the 5.8S material (Figure 3B). On increasing the mercaptoethanol concentration to 0.1 M only a 1.5–1.2S component was observed (not shown in Figure 3).

The material appearing under the third peak in the gel filtration had a sedimentation coefficient of 6.5 S. This material was shown to be immunoglobulin γG_1. It failed to undergo dissociation in 4 M urea or 4 M urea–0.01 M mercaptoethanol. In 0.1 M mercaptoethanol it had approximately the same sedimentation coefficient as the fully reduced 15S and 10.8S material.

Electrophoresis. The three immunoglobulins obtained by Sephadex G-200 gel filtration were evaluated by a number of electrophoretic techniques. They could not be distinguished from one another by cellulose acetate (barbital buffer, pH 8.2) or by starch gel electrophoresis (discontinuous buffer, glycine and borate, pH 8.8). The three immunoglobulins were also compared in alkaline urea–starch gels (Figure 4A). Immunoglobulin γA_2 dissociated in the absence of the reducing agent to a number of components, with the most anodal one migrating more rapidly than serum albumin. By contrast immunoglobulin γA_1 and γG_1 remained intact and were seen as heterodispersed broad bands near the origin, indistinguishable from the 6.5S immunoglobulin γG_1 fraction prepared from ovine serum (Heimer *et al.*, 1969).

The alkaline urea–starch gel patterns of the three immunoglobulins reduced in 0.1 M mercaptoethanol and alkylated with iodoacetamide (Figure 4B) brought out common features as well as differences. The three immunoglobulins had three evenly spaced components migrating toward the cathode. These components were assumed to be light chains as they appeared to give patterns identical with those of light chains isolated from ovine serum immunoglobulin γG_2. The other components seen in Figure 4B, however, had distinct mobilities and are believed to be mainly heavy chains. The most rapidly migrating component was confined to immunoglobulin γA_2 and is considered by us to be the secretory piece. The reasons for this designation are set forth in the Discussion section.

Immunodiffusion and Immunoelectrophoresis. A number of antisera proved to be useful in distinguishing the three immunoglobulins. Antiserum 929 absorbed with serum immunoglobulin γG_2 distinguished between immunoglobulin γA_1 and immunoglobulin γG_1, showing no reactivity with immunoglobulin γA_2. This antiserum could be made specific for immunoglobulin γA_1 by absorption with immunoglobulin γG_1. Antiserum T, also absorbed with serum immunoglobulin γG_2, was a specific antiserum to immunoglobulin γA_2.

A mixture of the two antisera (929 + T, absorbed with immunoglobulin γG_2) was used in the immunoelectrophoresis

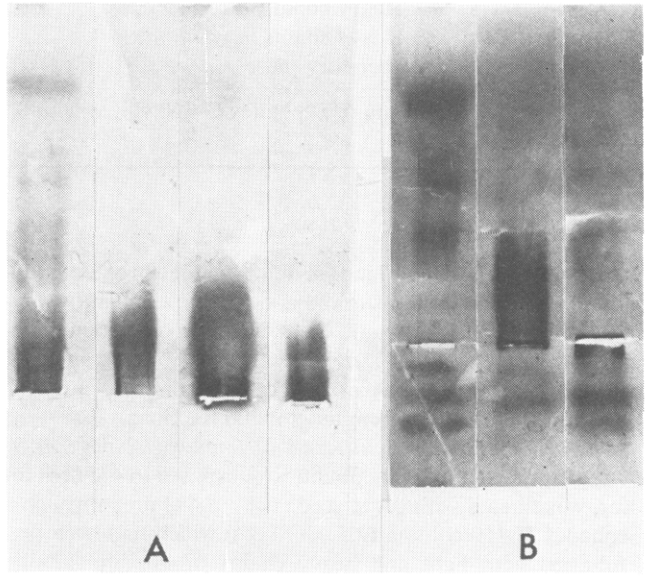

FIGURE 4: Alkaline urea starch electrophoresis. (A) Of (from left to right) immunoglobulin γA_2, γA_1, and γG_1 of colostrum and a 6.5S immunoglobulin γG_2 of sheep serum. (B) Of (from left to right) immunoglobulin γA_2, γA_1, and γG_1 of colostrum after 18-hr exposure to 0.10 M mercaptoethanol. Samples run at 5 V/cm for 18 hr at approximately 4°. Gels sliced and stained with Amido Black. Anode on top.

TABLE II: Summary of Analyses of Components Recovered from Sephadex G-150 Gel Filtration of Immunoglobulin γA_2 in 0.1 M Acetic Acid and 4 M Urea.

	Urea Starch Gel Pattern[a]						Immunodiffusion[b]	
	Direct			0.1 M Mercaptoethanol				
Component	Heavy	Light	SP[c]	Heavy	Light	SP[c]	967 Heavy	911 Light
A	+	+	+	+	+	+	+	+
B	+	+	+	+	+	+	+	+
C	+	−	−	+	−	−	+	±
D	+	−	−	+	−	−	+	−
E	−	+	−	−	+	−	−	+
F	−	−	+	−	−	+	−	−

[a] Alkaline urea-starch gel patterns were obtained for each fraction before and after reduction with 0.1 M mercaptoethanol. Characteristic bands of heavy and light chains or secretory piece seen in fractions are denoted by +. [b] Analyses were performed with two antisera; antiserum 967 was made specific for heavy chains of immunoglobulin γA_2 and γA_1 by absorption with immunoglobulin γG_2; antiserum 911 was made specific for light chains by absorption with reduced and alkylated heavy chains of immunoglobulin γG_2. [c] Secretory piece.

TABLE III: Estimation of Molecular Weights of Polypeptide Chains of the Immunoglobulins Based on Elution Volumes from Gel Filtration.[a]

Immuno-globulin	Constituents	Elution Vol	$\sqrt{K_D}$[b]	Known Mol Wt	Estimated Mol Wt
γG_1	Light chains	183	0.74	22,500	
	Heavy chains	135	0.54	53,000	
γA_1	Light chains	183	0.74	22,500	
	Heavy chains, α	126	0.49		63,500
γA_2	Light chains (dimer)	147	0.57		45,000
	Heavy chains, α (dimer)	93	0.25		127,000
	Heavy chains, α (monomer)	126	0.49		63,500
	Secretory piece	173	0.70		26,500

[a] A 70 × 2.5 cm column of Sephadex G-150 eluted with 0.1 M acetic acid in 4 M urea. [b] $K_D = (V_E - V_0)/V_i$, where $V_0 = 82$, $V_i = 183$.

experiments shown in Figure 5. Each of the three immunoglobulin fractions tested showed a single arc of slightly varying mobility (wells A, B, and C). Admixture of immunoglobulin γA_2 with γA_1 (well D), γA_2 with γG_1 (well E), and γA_1 with γG_1 (well F) gave two arcs each, confirming the antigenic distinctness of the three immunoglobulin fractions.

Characterization of Constituents of Immunoglobulin γA_2. A 50-mg sample of immunoglobulin γA_2, exposed to 4 M urea for 4 hr, was filtered with 0.1 M acetic acid in 4 M urea through a Sephadex G-150 column (2.5 × 70 cm) which had been precalibrated with light and heavy chains of immunoglobulin γG_2. Immunoglobulin γA_2 dissociated to six components, shown as A to F in Figure 6A. Effluent portions corresponding to each component, with the extent of pooling of individual fractions shown by horizontal lines on the elution diagram, were examined by urea-starch gel electrophoresis before and after reduction with 0.1 M mercaptoethanol. The individual samples were also analyzed by immunodiffusion using antiserum 967, absorbed with immunoglobulin γG_2, for the detection of heavy chains and antiserum 911, absorbed with heavy chains of serum immunoglobulin γG_2, for the detection of light chains. The results of these analyses, summarized in Table II, suggest that components A and B were oligomers containing light and heavy chains, component C was a heavy-chain dimer, component D a heavy-chain monomer, component E a light-chain dimer, and component F the secretory piece.

Characterization of Constituents of Immunoglobulin γA_1. The Sephadex G-150 column which served in the characterization of immunoglobulin γA_2 was also used to resolve the components resulting from the reduction (with 0.1 M mercaptoethanol) and alkylation of immunoglobulin γA_1. The elution profile (Figure 6B) was reminiscent of that of other reduced immunoglobulins. The major peak at 126 ml and the minor

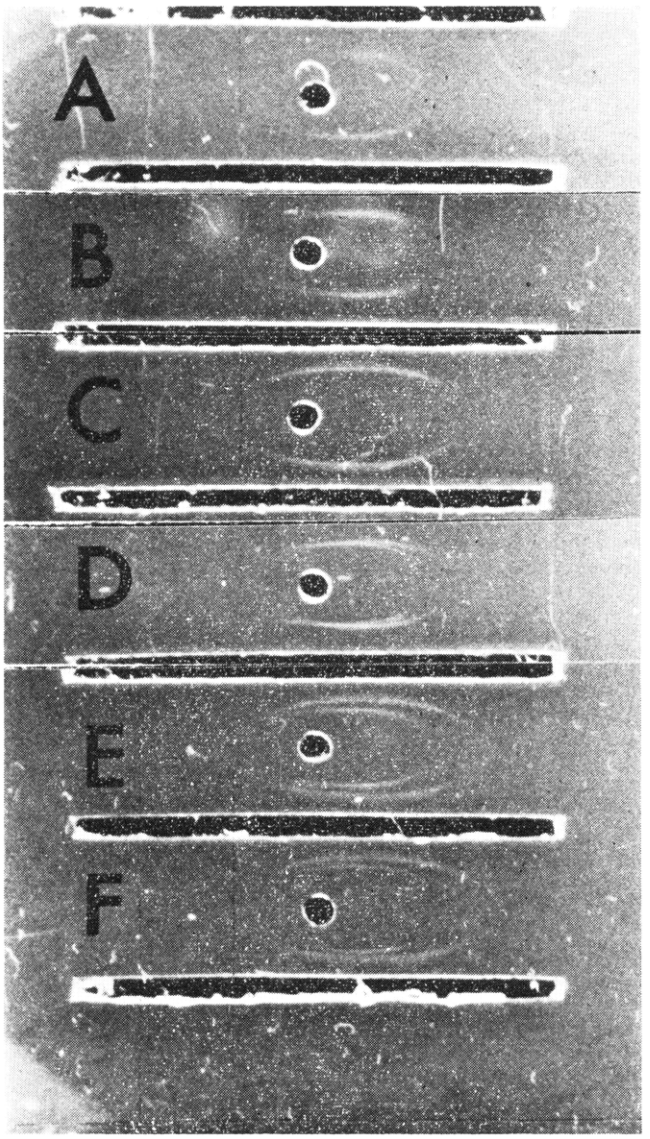

FIGURE 5: Immunoelectrophoresis with mixture of antiserum 929 and T (absorbed with immunoglobulin γG_2) in troughs: wells A, γA_2; B, γA_1; C, γG_1; D, $\gamma A_2 + \gamma A_1$; E, $\gamma A_2 + \gamma G_1$; F, $\gamma A_1 + \gamma G_1$. Anode is to the left.

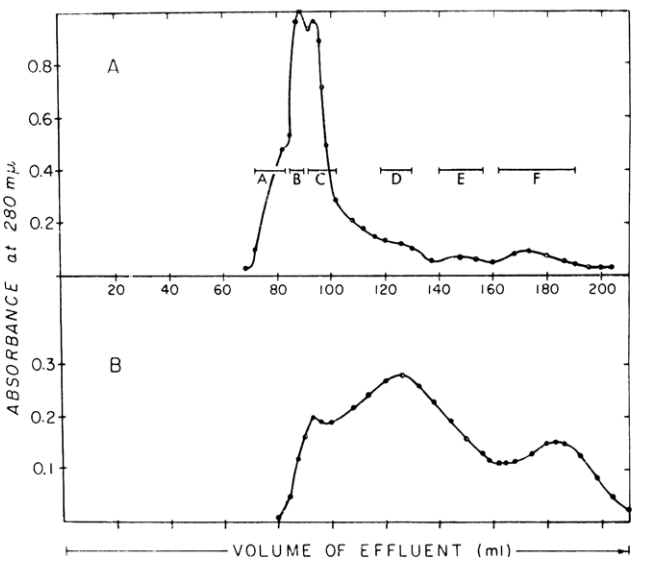

FIGURE 6: Sephadex G-150 gel filtration on column (70 × 2.5 cm) with 0.1 M acetic acid in 4 M urea. (A) 50 mg of immunoglobulin γA_2. Bars indicate the extent of pooling of each of the six components. Pooled samples were analyzed by starch gel electrophoresis and immunodiffusion. (B) 20 mg of reduced and alkylated immunoglobulin γA_1.

one at 183 ml were identified with antisera 967 and 911 (as used in the characterization of immunoglobulin γA_2) as heavy and light chains, respectively. The small amount of material at 93 ml was probably composed of heavy-chain dimers. When tested by alkaline starch gel electrophoresis, heavy-chain bands in pooled samples of the 123-ml peak could clearly be distinguished from the light-chain bands of the 183-ml peak. The light-chain pattern was indistinguishable from the one obtained with the other immunoglobulins. The ratio of recovered heavy to light chain was nearly 3:1 which is commensurate with the ratio expected from their respective molecular weights (*vide infra*).

Estimation of Molecular Weights of the Constituents of the Three Immunoglobulin Fractions. As the molecular weights of light and heavy chains of ovine serum immunoglobulin γG were known from previous studies (Heimer *et al.*, 1969; Harrison and Mage, 1967), they were used as markers in the construction of the $\sqrt{K_D}$ *vs.* $\sqrt{MW}$ plot. Table III shows the elution volumes, $\sqrt{K_D}$, and known molecular weight of the various constituents recognized from the Sephadex G-150 gel filtration experiments. Table III also includes the molecular weights of the heavy chain of immunoglobulin γA_1 and also of the heavy chain (monomer and dimer), light chain (dimer), and secretory piece of immunoglobulin γA_2 estimated from the $\sqrt{K_D}$ *vs.* $\sqrt{MW}$ plot shown in Figure 7.

Sedimentation Velocity and Equilibrium Studies of Separated Chains. The molecular weights of the reduced and alkylated heavy chains of immunoglobulin γA_2 and A_1 fractions and light chains of the latter, all in 4 M urea, were determined by sedimentation equilibrium studies. Table IV contains the M_w at different rotational speeds, calculated from linear plots of

TABLE IV: M_w Values of Heavy- and Light-Chain Immunoglobulin γA_2 and γA_1.[a]

Rotor Speeds	Heavy-Chain γA_2	Heavy-Chain γA_1	Light-Chain γA_1
22,000	M_w = 71,000[c]		
24,000	M_w = 63,500[d]	M_w = 71,500[e]	
26,000	M_w = 59,800[b]	M_w = 58,900,[f] 61,400[g]	
28,000	M_w = 60,000[d]		
32,000			M_w = 24,100[h]
36,000			M_w = 25,400[h]

[a] All runs were performed in 4 M urea. [b] At 0.60 mg/ml. [c] At 0.40 mg/ml. [d] At 0.20 mg/ml. [e] At 0.35 mg/ml. [f] At 0.50 mg/ml. [g] At 0.30 mg/ml. [h] At 0.40 mg/ml.

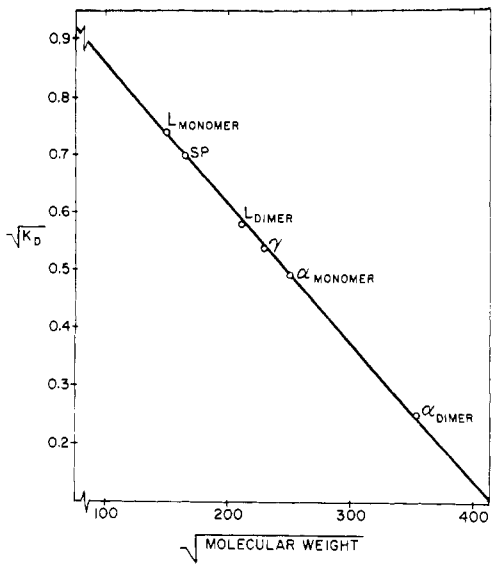

FIGURE 7: Plot of $\sqrt{K_D}$ (for definition see Table III) *vs.* $\sqrt{MW}$ for reduced and alkylated polypeptide chains filtered through Sephadex G-150 (as shown in Figure 6). The molecular weights of the markers were 22,500 for light chains, 53,000 for heavy chains of immunoglobulin γG_2 of serum. L denotes light chains; SP, secretory piece; γ, heavy chain of immunoglobulin γG_2; α, heavy chains of immunoglobulin γA_2 and γA_1.

log c *vs.* r^2. The calculated M_w checked quite well with estimates of MW made from the Sephadex G-150 elution profile. Assuming the same $\bar{v}$ value for both heavy chains, the mean value of the heavy chain of immunoglobulin γA_2 was 62,400 (±1,400) and that of immunoglobulin γA_1 was 63,900 (±2,900).

Additional studies on the heavy chain of immunoglobulin γA_2 were made in solvent systems other than 4 M urea. There was a strong tendency toward polymerization in both 0.1 M phosphate of pH 7 and in 1 M acetic acid. In the latter system a nearly linear log c *vs.* r^2 plot was obtained, but there was a slight deviation from linearity at the cell bottom. M_w (averaged on two runs in three different concentrations varying from 0.6 to 0.2 mg per ml) was 277,000 (±28,000) suggesting formation of tetramers or pentamers. A sedimentation velocity study of the heavy chain in 1 M acetic acid showed a slightly asymmetric peak with $s_{20,w} = 6$ S. This value is somewhat low considering the calculated MW in this solvent, but it may reflect a possible high axial ratio. The identical solute when run through a Sephadex G-200 column (0.9 × 50 cm) and developed in 1 M acetic acid appeared in the excluded volume of the elution profile, thus verifying the polymerized state of the heavy chain of immunoglobulin γA_2 in 1 M acetic acid.

In 0.1 M phosphate buffer at pH 7, the heavy chain of the 15S immunoglobulin sedimented as an essentially symmetric boundary at 5.9 S. The sedimentation equilibrium run, however, gave a nonlinear log c *vs.* r^2 plot, with an estimated M_z value of 118,000, suggestive of a dimer.

Carbohydrate Content. Table V contains average carbohydrate values obtained on a single determination of at least two different samples of the three immunoglobulins. The values are compared with those obtained by us for ovine serum immunoglobulin γG and γA (Heimer *et al.*, 1969) and also with known values of the three major classes of immunoglobulin of man (Cohen and Porter, 1964a). It can be seen that the hexose and hexosamine content of the two immunoglobulin γA subclasses is similar. Immunoglobulin γA of sheep and man also appears to have a similar carbohydrate content.

TABLE V: A Comparison of the Carbohydrate Content of Colostral with Serum Immunoglobulins.

Immunoglobulin	% Hexose	% Hexosamine
Colostrum		
γG_1	1.9 (±0.2)	1.2 (±0.05)
γA_2	4.2 (±0.3)	3.9 (±0.05)
γA_1	4.1 (±0.4)	3.2 (±0.7)
Ovine Serum		
γG^a	1.7	1.3
γA^a	4.0	3.5
Human Serum		
γG^b	1.2	1.1
γA^b	4.8	3.8
γM^b	6.2	3.3

[a] Heimer *et al.* (1969). [b] Cohen and Porter (1964b).

Discussion

The studies presented here show that sheep colostrum contains at least three distinct immunoglobulins separable by Sephadex G-200 gel filtration. The 15S immunoglobulin was designated γA_2 because it resembled γA_2 of man (Grey *et al.*, 1968) and the predominant γA of the mouse (Abel and Grey, 1968; Metzger and Potter, 1968) by dissociating to a number of subunits in concentrated urea in the absence of reducing agents. Our studies suggest that the subunits are heavy- and light-chain dimers, as well as secretory piece, but further studies will be required to determine whether the dissociation proceeds with or without disulfide-bond interchange, and whether complete dissociation of the 15S immunoglobulin to heavy- and light-chain dimers can be accomplished without reducing agents. It is also conceivable that the light-chain dimers recovered by us arise from a disulfide interchange, but the apparent lack of light-chain monomers in the eluents of the gel filtration experiment (Figure 6A) makes such a mechanism unlikely. The dissociation, to the extent that it proceeded in 4 M urea, was reversible, for removal of the urea resulted in formation of a major component sedimenting at 21 S. There were, however, also minor indistinct peaks with a median sedimentation coefficient of approximately 3.6 S.

When compared with the well-characterized immunoglobulin γA_2 of myelomatous mice and men (Grey *et al.*, 1968; Abel and Grey, 1968), the ovine material appears to differ in some significant respects. Whereas the murine heavy (α) chain probably has a lower molecular weight (as estimated by Sephadex gel filtration) than γ chains, ovine heavy (α) chains had a molecular weight of approximately 63,500, which is similar to the molecular weight of human α chains, calculated from recovery ratios of reduced human immunoglobulin γA_2 heavy and light chains by Sephadex gel filtration (Grey *et al.*, 1968). The molecular weight of the α chain of

immunoglobulin γA of rabbit colostrum was also approximately 63,000 (Cebra and Small, 1967). The other difference between the sheep, on the one hand, and man and mouse, on the other, is that in human and mouse serum immunoglobulin γA_2 appears to exist as a 7S or 10S protein with the 10S material predominating in human colostrum (Grey *et al.*, 1968), whereas ovine colostral immunoglobulin γA_2 sediments exclusively at 15 S.

The secretory piece, as judged from urea–starch gel electrophoresis, was associated probably only with immunoglobulin γA_2. It had a distinct anodal migration in alkaline urea–starch gels, similar to what has been found in man and rabbit. A component with this mobility was not associated with either unreduced or reduced ovine immunoglobulin γA_1 or γG_1. Lack of a specific antibody to secretory piece (we have been unable to prepare such an antibody to date) prevents us from categorically excluding its presence in trace amounts in the other four fractions obtained by the Sephadex gel filtration. We found no evidence for free secretory piece as seen in man (Tomasi *et al.*, 1965; South *et al.*, 1966; Newcomb *et al.*, 1968; Brandtzaeg *et al.*, 1968). It is possible that all secretory piece synthesized by the sheep combines readily with immunoglobulin γA_2, perhaps because the association does not require formation of disulfide bonds. Alternatively the regulation of the synthesis of the secretory piece in sheep may differ from that of man. In man and rabbit most of the secretory piece is probably attached through interchain disulfide bonds to the heavy (α) chain of immunoglobulin γA_1 (Cebra and Small, 1967; Tomasi *et al.*, 1965; South *et al.*, 1966).

Another reason for designating the polypeptide with rapid anodal mobility as secretory piece is the similarity of estimated molecular weight between it and the secretory piece of rabbit colostrum. Cebra and Small (1967) found the latter to elute slightly ahead of reduced and alkylated light chains when filtered through Sephadex G-200 in 5 M guanidine-HCl. Our results obtained on Sephadex G-150 gel filtration in 4 M urea were identical with theirs, suggesting 26,500 as the estimated molecular weight of the ovine secretory piece.

There appears to be no antigenic relationship between the ovine secretory piece and the other polypeptide chains of immunoglobulins. In this respect also our results appear to parallel those obtained in man and rabbit.

Our designation of the 10.8S fraction as immunoglobulin γA_1 is based on the following. The content of hexose and hexosamine of this immunoglobulin was found to be similar to that of immunoglobulin γA_2 and considerably greater than that of immunoglobulin γG_1. Moreover on comparing the carbohydrate content, there appear to be striking cross species similarities among all immunoglobulin classes (Table V). Furthermore, the molecular weight of the heavy (α) chains of both subclasses of ovine immunoglobulin γA were similar. This was approximately 63,500 and is significantly higher than 53,000, the molecular weight of the heavy (γ) chain of immunoglobulin γG_1. Ovine immunoglobulin γA_1 of colostrum had a sedimentation coefficient of 10.8 S, which is identical with that found by Cebra and Small (1967) but differs from 11.6 S found in human colostral material (Tomasi *et al.*, 1968). The higher sedimentation coefficient of human immunoglobulin γA_1 may be ascribed to the presence of the secretory piece. Native immunoglobulin γA_1 in exocrine solutions of man, rabbit, and the sheep is composed of two 7S proteins, believed to be linked by a labile disulfide bond.

If the two immunoglobulins described in this paper are analogous to immunoglobulin γA of other species they ought to share with them homologous heavy-chain amino acid sequences. Such work has not been undertaken by us to date, but we have looked for the possibility of the existence of cross-reacting specific antisera against human and sheep immunoglobulin γA. While Vaerman and Heremans (1968) were able to establish in 4 of 30 rabbit antisera to human immunoglobulin γA cross reactivity with canine γA, our search with 5 rabbit antihuman γA antisera has not led to cross reactivity with ovine γA. Similarly four rabbit antisheep γA antisera failed to cross react with human $\gamma\beta A$.

The 6.5S fraction obtained after Sephadex G-200 gel filtration of colostrum appeared to be free of immunoglobulin γA_1 and γA_2. It contained mainly immunoglobulin γG_1 and constituted the major immunoglobulin of colostrum. In contrast to ovine serum, colostrum appears to contain only negligible amounts of the other immunoglobulin γG subclasses recently described by us (Heimer *et al.*, 1969) and Pan *et al.* (1968). It would appear that the distribution of immunoglobulins in colostrum of various species show considerable variation. Man and rabbit and probably also the mouse have more immunoglobulin γA than γG (Tomasi and Bienenstock, 1968), whereas cattle have predominantly immunoglobulin γG, some γM, but apparently no γA (Hammer *et al.*, 1968). The dog probably occupies an intermediate position between the two groups with immunoglobulin γG predominating, but also having an "intermediate-sedimenting" immunoglobulin γG_1, possibly akin to γA (Johnson and Vaughan, 1967). Sheep colostrum, like that of cattle and dog, as demonstrated previously (Sullivan and Tomasi, 1964) and also in this paper, contain large amounts of immunoglobulin γG_1. Additionally, as shown herein, there are lesser amounts of two subclasses of immunoglobulin γA. The sheep, moreover, is only the third species in which secretory piece has been found. Its exclusive association with immunoglobulin γA_2 suggests that there may be different subtypes of secretory piece, for in man some is found covalently linked and another type is linked to the balance of the immunoglobulin γA through noncovalent bonds alone. Alternatively, the manner in which secretory piece is bound may be a characteristic of the subclass of immunoglobulin γA.

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Partial Sequences of Six Macroglobulin Light Chains. Absence of Sequence Correlates to Functional Activity*

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ABSTRACT: The amino acid sequence of portions of the variable regions of the light chains from six Waldenström macroglobulins has been determined. Four of the six proteins studied show evidence of binding activity. No distinctive sequences which correlated with the functional properties of the active proteins were observed.

The data also strengthen previous evidence that light chains associated with μ-type heavy chains are not as a group distinguishable from those associated with other heavy-chain classes.

The polypeptide chains of immunoglobulins are divisible into regions having an invariant amino acid sequence (for chains of a particular class and allotype) and regions in which the sequence is variable (Cohen and Milstein, 1967). In light chains the variable region comprises about 100 amino acids, almost exactly the amino-terminal half of the chains. Although there is much less information on the heavy polypeptide chains the variable stretch may also be about 100 amino acids long (Gottlieb *et al.*, 1968), therefore making up approximately one-fourth the length of these chains.

These amino-terminal regions are the only ones which show sufficient variability to account for the remarkable binding specificity exhibited by functional immunoglobulins. Furthermore in at least one instance it has been possible to isolate a light-chain dipeptide from a site-labeled antibody preparation and this peptide was shown to have originated from the variable region (Singer and Thorpe, 1968).

Almost all of the published sequence data on immunoglobulins was derived from the polypeptide chains of myeloma proteins or Waldenström macroglobulins. None of these proteins had defined antibody activity. In the few instances where normal serum immunoglobulins were examined these were also without known binding activity (Hood *et al.*, 1966; Cohen and Milstein, 1967; Niall and Edman, 1967). In one instance the light chains of an antibody preparation have been studied (rabbit antidinitrophenyl antibody) but the data were limited and no unique sequence could be resolved (Doolittle, 1966). Other data available indicate that the N-terminal amino acids of the κ chains from several cold agglutinins (monoclonal human γM proteins which agglutinate human red cells in the cold) were similar to those seen with other κ chains (Cohen and Cooper, 1968). In addition, the sequence of the 22 amino acids at the N-terminal end of the κ chain from a cold agglutinin was recently published (Edman and Cooper, 1968).

The discovery in our laboratory of Waldenström macroglobulins with well-defined binding properties (Metzger, 1967;

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