showed a very sharp break at the equivalence point, and the saturation enthalpy, after correction for impurity, agreed well with the value obtained from the initial slope of the titration curve. Thus the calorimetric data give no indication of heterogeneity with respect to binding enthalpy. It must be concluded either that this antibody preparation has a spread of enthalpy values much smaller than found for the free energy by fluorescence titration, or that there is no correlation between the values for ΔH_b and ΔG_b, with sites having any particular ΔH_b being uniformly distributed over all possible classes of ΔG_b. If the latter possibility is the correct one, we have here an extreme case of enthalpy–entropy compensation.

It is not at all obvious how the low value for the van't Hoff enthalpy of binding, less than half the calorimetric value, is to be accounted for. Both values clearly refer to the binding of 1 mole of ligand. The standard error of the least-squared slope of the van't Hoff plot corresponds to ± 2 kcal mole^{-1} in ΔH_b°. A possible source of the discrepancy is a systematic error in the dissociation constant. This could arise, for example, from a correlation such as suggested by McGuigan and Eisen (1968) of high binding affinity with high tryptophan content in antibody molecules. It is also possible that the Sips distribution function does not give a proper representation of the heterogeneity of binding free energies at high fractional extents of binding.

In view of the fact that the thermodynamic data for the systems studied in this work may well be atypical, it is important that accurate thermal data be obtained for a wide variety of antibody–hapten or antibody–antigen systems.

References

Barisas, B. G. (1971), PhD. Thesis, Yale University, New Haven, Conn.

Barisas, B. G., Sturtevant, J. M., and Singer, S. J. (1971), *Biochemistry 10*, 2816.

Carsten, M. E., and Eisen, H. N. (1953), *J. Amer. Chem. Soc. 75*, 4451.

Eisen, H. N. (1964), *Methods Med. Res. 10*, 94.

Farah, F. S., Kern, M., and Eisen, H. N. (1960), *J. Exp. Med. 112*, 1195.

Klotz, I. M. (1953), *Proteins 1B*, 769.

Little, J. R., and Eisen, H. N. (1968), *Biochemistry 7*, 711.

Marler, E., Nelson, C. A., and Tanford, C. (1964), *Biochemistry 3*, 279.

McGuigan, J. E., and Eisen, H. N. (1968), *Biochemistry 7*, 1919.

Nisonoff, A., and Pressman, D. (1958), *J. Immunol. 80*, 417.

Okuyama, T., and Satake, K. (1960), *J. Biochem. (Tokyo) 47*, 454.

Sips, R. (1948), *J. Chem. Phys. 16*, 490.

Human Secretory Component. Comparison of the Form Occurring in Exocrine Immunoglobulin A to the Free Form†

Michael E. Lamm* and Jacob Greenberg

ABSTRACT: The free and bound forms of secretory component have been isolated from human colostrum and characterized. Free secretory component was isolated by conventional techniques. The finding that free secretory component lacks methionine formed the basis for a method of isolating bound secretory component, which could be purified from reduced and alkylated secretory immunoglobulin A by gel filtration in 4 M guanidine hydrochloride following reaction with cyanogen bromide. Free and bound secretory components are similar with respect to antigenic determinants, molecular weight (71,000) and composition.

In recent years there has been much interest in the immune mechanisms of mucous membrane secretions, with particular attention toward their role as a first-line defense system whose properties may under certain circumstances be independent of circulating antibody. Unlike serum, where immunoglobulin G (IgG) is the predominant immunoglobulin class, in mucous membrane secretions immunoglobulin A (IgA) predominates, and in a number of situations local immunity in secretions has been shown to be mediated by IgA (Tomasi and Bienenstock, 1968; Dayton *et al.*, 1969).

In addition to quantitative differences between the IgA content of serum *vs.* secretions, IgA in secretions has distinctive structural features. Besides the H (α) and L chains of serum-type IgA, most of which occurs in molecules with a sedimentation coefficient of 7 S, the IgA in secretions occurs predominantly as an 11S molecule and has an additional component termed the secretory component (SC),[1] whose function has not been established (Tomasi *et al.*, 1965; Tomasi and Bienenstock, 1968). Recently an additional moiety, the J chain (or F component), has been described in polymeric IgA (Halpern and Koshland, 1970; Mestecky *et al.*, 1971; O'Daly and Cebra, 1971b). The J chain is not secretion specific nor is it peculiar to IgA since it also occcurs in IgM.

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[1] Abbreviations used are: SC, secretory component; BSC, bound secretory component; FSC, free secretory component; Gdn·HCl, guanidine hydrochloride; CFA, complete Freund's adjuvant.

Under normal circumstances the association of SC with secretory IgA appears to be specific, *i.e.*, SC is found in secretions either as part of the secretory IgA molecule (bound SC or BSC) or in a free form (FSC), but is not associated with any other protein (Tomasi and Bienenstock, 1968; O'Daly and Cebra, 1968). The minute amount of SC detectable in serum (Thompson and Asquith, 1970; Waldman *et al.*, 1970) is thought to result from a small proportion of the secretory IgA synthesized in mucous membranes finding its way into the circulation instead of following the usual path into the secretions.

Because of similarities in size and antigenic characteristics and because FSC can combine *in vitro* with dimeric IgA (Tomasi and Bienenstock, 1968; Mach, 1970; O'Daly and Cebra, 1971a), FSC and BSC have been assumed to be identical. Recently FSC and BSC have been isolated from rabbit colostrum and have been found to be similar in size and composition (O'Daly and Cebra, 1971b). Human FSC has been isolated (Mach, 1970; van Munster *et al.*, 1971), but progress on the bound form has been hampered by the lack of a completely satisfactory isolation procedure. The isolation of human and rabbit BSC presents different problems. Most human BSC is linked to the remainder of the molecule by disulfide bridges (Tomasi and Bienenstock, 1968; Hurlimann *et al.*, 1969; Brandtzaeg, 1970), and after these are cleaved SC must be separated from α chains. In contrast, in rabbit secretory IgA, BSC is attached by noncovalent bonds and can be liberated by denaturing agents alone from the bulk of the α and L chains (Cebra and Small, 1967; Halpern and Koshland, 1970). While the present work was being readied for publication, Kobayashi (1971) reported studies on human BSC and FSC which suggested identity. The former was isolated from reduced secretory IgA by gel filtration and immunoadsorbents. The present paper also reports isolation procedures for BSC and FSC from human colostrum together with some aspects of their structure. The method for purifying BSC is based on our finding that FSC lacks methionine.

Materials and Methods

Isolation of Secretory IgA. Human colostrum obtained within the first few days of delivery was stored frozen or lyophilized (if the latter, it was restored to the original volume when needed by the addition of water). Pools of colostrum from several women were used. Colostral IgA was prepared essentially according to Cebra and Robbins (1966). Details are given in Pincus *et al.* (1971). In brief, colostrum was centrifuged to separate lipid and cellular debris, and residual casein was precipitated at pH 4.6. The solution was then passed through DEAE-cellulose in sodium phosphate buffer (pH 7.0) at 4°, and the fraction eluting with 0.1 M NaCl–0.01 M phosphate (pH 7.0) was taken. This material was passed through Sephadex G-200 in 0.2 M NaCl at 4°. The void volume contained pure secretory IgA (yield 5–10 mg/ml of colostrum). (The second peak from the G-200 column was used as the source of FSC; see below.)

Isolation of BSC. With the knowledge that FSC lacks methionine (see below) we were able to devise a procedure for isolating BSC following the reaction of secretory IgA with cyanogen bromide. (Hitherto we had never been able to obtain BSC completely free of α chains.) Accordingly, 2% secretory IgA was extensively reduced with 0.1 M dithioerythritol in 7 M Gdn·HCl–0.5 M Tris-HCl (pH 8.2) for 1 hr at room temperature, then alkylated at 0° with a 10% excess of iodoacetamide (recrystallized from petroleum ether (bp 40–50°) and ethanol) for 30 min, and subsequently thoroughly dialyzed *vs.* water. The solution was made 70% in formic acid, and the protein was reacted with cyanogen bromide (3 mg/mg of protein) for 24 hr at room temperature in order to fragment the α chains at methionine residues (Gross and Witkop, 1962). The reaction mixture was next passed through Sephadex G-25 in 0.2 N acetic acid, and the protein eluted at the front was lyophilized. The SC chains which were unaffected by the cyanogen bromide could then be partially purified by one passage through Sephadex G-200 or Sepharose 4B in 4 M Gdn·HCl at room temperature. One of our antisera *vs.* FSC was able to precipitate specifically with SC, even after the above treatment, and was used to monitor the column effluent directly by double diffusion in agar gel (Ouchterlony analysis). The effluent could also be monitored by electrophoresis in polyacrylamide gels in sodium dodecyl sulfate after removal of the Gdn·HCl by dialysis. In order to obtain highly purified BSC appropriate fractions were concentrated by ultrafiltration (Amicon XM-50 membrane) and repassed through the same guanidine column. The final yield of BSC was about 1 mg/20 mg of starting secretory IgA.

Isolation of FSC. The starting material was the second Sephadex G-200 peak from the final step in the isolation of secretory IgA (see above). The protein solution was concentrated by ultrafiltration and dialyzed *vs.* 0.01 M sodium phosphate (pH 6.8). It was next loaded on a column of hydroxylapatite (Bio-Gel HT, Bio-Rad Laboratories; 75 ml/300 mg of protein) equilibrated with the same buffer at 4°, and then stepwise elution was performed with increasing molarities of the above buffer at pH 6.8, first 0.02 and then 0.04 M. The latter fraction contained most of the FSC and was devoid of lactoferrin. At this stage the FSC was still contaminated but could be further purified by preparative acrylamide electrophoresis. The yield was about 20 mg/l. of colostrum.

Isolation of α Chains. Extensively or partially reduced and alkylated secretory IgA was filtered through Sephadex G-200 in 4 M Gdn·HCl, and the peak tubes of the α-chain fraction were taken and passed again through the same column.

Antisera. (a) Antisera *vs.* native BSC were prepared by the procedure of Pincus *et al.* (1971) in which rabbits undergoing immunization with secretory IgA receive suppressive doses of rabbit antiserum *vs.* serum-type IgA. This scheme results in a suppression of the antibody response to α and L chains and an enhanced response to BSC. The resulting antisera were absorbed with IgA myeloma protein and normal human serum to render them specific for SC. (b) Antisera *vs.* BSC were also prepared by immunizing rabbits with 0.05 mg of purified extensively reduced and alkylated BSC in complete Freund's adjuvant (CFA) in the footpads. (c) Antisera *vs.* FSC were obtained by immunizing rabbits in the footpads with 0.1 mg of purified protein in CFA. (d) Antisera *vs.* pooled human colostrum were derived from rabbits injected intramuscularly in several sites with a total of 0.5 ml of colostrum in CFA. The rabbits were boosted 1 month later with 1 ml of colostrum subcutaneously in multiple sites. In all cases rabbits were bled at intervals 4–8 weeks after the first injection of antigen.

Amino Acid Analyses. These were performed, after 20- or 70-hr hydrolysis *in vacuo* in constant-boiling HCl at 110°, with a Spinco Model 120C amino acid analyzer. Values for labile amino acids were extrapolated to zero hydrolysis time, and for slowly released amino acids the 70-hr values were used. Half-cystine was measured either as *S*-carboxymethylcysteine after extensive reduction and alkylation or as cysteic acid after performic acid oxidation (Hirs, 1967). Homoserine was sought (Ambler, 1965) by taking up the flash-evaporated acid

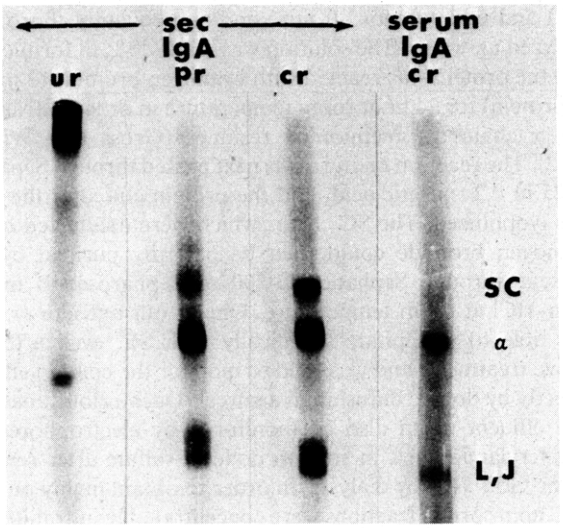

FIGURE 1: Electrophoresis in acrylamide gels in sodium dodecyl sulfate of unreduced (ur), partially reduced (pr), and completely reduced (cr) secretory IgA, and completely reduced serum IgA (a myeloma protein). Direction of migration is from top to bottom. SC, α, L, and J refer to the positions of secretory component, α chain, and L and J chains. Protein moving more slowly than SC contains aggregates, and the mobility of the fastest moving band in the unreduced secretory IgA is consistent with an L-chain dimer.

hydrolysates in 1 ml of pyridine–acetate (pH 6.5) and leaving at 110° for 1 hr to convert any homoserine lactone to homoserine. After drying, the sample was immediately analyzed on the long column of the amino acid analyzer with a starting buffer of pH 3.20. Tryptophan was measured spectrophotometrically in 6 M Gdn·HCl (Edelhoch, 1967).

Carbohydrate Analyses. Hexosamine was measured on the short column of the amino acid analyzer after 4-hr hydrolysis *in vacuo* in 3 N HCl at 110°. Corrections for losses during hydrolysis were based on the losses in standard solutions treated in the same manner. Hexose was measured by the orcinol method (Neuberger and Marshall, 1966) with galactose standards. The analyses for tryptophan, hexosamine, and hexose were done on aliquots of a solution to which a known amount of norleucine had been added; the results obtained were related to protein in terms of the amount of alanine in a 20-hr acid hydrolysate of another aliquot after correction for losses by the recovery of norleucine.

Extinction Coefficient. The OD_{280} in guanidine determined in the analysis for tryptophan together with the carbohydrate and amino acid content (corrected for handling losses by the recovery of norleucine (see above)) of the same solution were used to calculate an extinction coefficient for extensively reduced and alkylated BSC.

Analytical Ultracentrifugation. A Spinco Model E ultracentrifuge was used. (a) Sedimentation velocity of secretory IgA was performed at 0.6 mg/ml (ultraviolet optics) and a speed of 60,000 rpm. (b) Sedimentation equilibrium by the meniscus depletion method of Yphantis (1964) with interference optics was kindly done by Dr. P. A. Small, Jr. Runs were done on 0.25-mg/ml solutions (initial concentration) in 5 M Gdn·HCl at 20° as described previously (Small and Lamm, 1966). Two different speeds were used: 27,690 and 35,600 rpm. A $\bar{V} = 0.710$ ml/g was calculated from the amino acid and carbohydrate composition (Cohn and Edsall, 1943; Schachman, 1957; Gibbons, 1966). However, the $\bar{V}$ actually used for calculations was 0.70 to correct for interactions with the solvent (Hade and Tanford, 1967). Weight-average molecular weights were calculated by a computer using a least-squares fit of the experimental points.

Electrophoresis. (a) Analytical electrophoresis in polyacrylamide gels was performed in sodium dodecyl sulfate (Shapiro *et al.*, 1967; Weber and Osborn, 1969) or in urea with a discontinuous buffer system (Reisfeld and Small, 1966). After fixation gels were stained with Coomassie Brilliant Blue. For electrophoresis in detergent with 5% gels the following standards, all extensively reduced and alkylated, were used: rabbit muscle phosphorylase, mol wt 96,000 (Appleman *et al.*, 1963; Seery *et al.*, 1967); bovine serum albumin, mol wt 68,000 (Peters and Hawn, 1967; Ullmann *et al.*, 1968; Reisler and Eisenberg, 1969) and rabbit H and L chains, mol wt 53,000 and 23,000, respectively (Small and Lamm, 1966). (b) Preparative electrophoresis was kindly done by Dr. G. Goldstein in a Büchler Poly-Prep 100 apparatus in a 7% polyacrylamide gel (volume, 60 ml; no spacer gel) at 50 mA with the Davis–Ornstein (1968) buffer system. The eluent was monitored by OD_{280} and protein determinations (Lowry *et al.*, 1951).

Specific Immune Precipitation from Solution. Specific precipitation was done on 0.006 mg of purified FSC iodinated with ^{125}I by the method of McFarlane (1958) together with cold carrier FSC (up to 0.015 mg) in the antibody excess zone using 0.2 ml of rabbit antiserum specific for SC (obtained from a rabbit originally immunized with secretory IgA) in a total volume of 0.25 ml. Control tubes had normal rabbit serum. All solutions were centrifuged at 2500 rpm for 30 min before being used. The tubes were incubated at 37° for 30 min and then kept at 4° for 3 days. Tubes were counted and then centrifuged at 2500 rpm for 30 min at 4°. The precipitates were collected, washed four times with cold saline, and counted.

Results

Secretory IgA. The colostral IgA used as the source of BSC had a sedimentation coefficient, $s_{20,w}$, of 10.6 S, in agreement with previous work (Tomasi and Bienenstock, 1968). By immunoelectrophoresis at a concentration of 10 mg/ml it gave a single precipitin line with two different antisera which gave many lines with whole colostrum. When labeled with ^{125}I, it was 100% precipitable by an antiserum specific for SC (Pincus *et al.*, 1971). This result indicates not only purity but also that all molecules of secretory IgA contain SC, in agreement with O'Daly and Cebra (1968) and Mestecky *et al.* (1970).

Isolation and Characterization of BSC. Unlike rabbit secretory IgA (Cebra and Small, 1967; Halpern and Koshland, 1970), in order for human BSC to be released from secretory IgA, disulfide bonds must be split (Tomasi and Bienenstock, 1968; Hurlimann *et al.*, 1969; Brandtzaeg, 1970). We confirmed this as can be seen in the experiment shown in Figure 1 in which electrophoresis in the dodecyl sulfate was performed on alkylated and on reduced and alkylated secretory IgA. Only after reduction, either partial or complete, is there a separate band due to SC. (The two fastest bands in the reduced preparations probably are L and J chains, and the band between them and SC contains α chains.) In the unreduced protein the position of the fastest band is consistent with the L-chain dimers of the IgA_2 subclass which are not covalently linked to H chains (Grey *et al.*, 1968). Electrophoresis of completely reduced and alkylated serum-type IgA (an IgA, K myeloma protein) yields only α and L chains.

TABLE I: Composition of SC and α Chain.

	Amino Acids (Moles %)			Amino Acid and Carbohydrate Composition of BSC (Residues/71,000 g of Native Protein)
	BSC	FSC	α Chain[a]	
Lys	4.8	6.2	3.8	25
His	1.0	1.0	1.9	5
Arg	4.6	3.8	3.9	24
Asx	9.9	9.4	7.3	52
Thr	6.1	5.3	9.5	32
Ser	9.8	12.0	9.8	52
Glx	10.9	11.3	9.5	58
Pro	5.4	4.2	9.3	28
Gly	9.6	11.0	7.8	50
Ala	5.4	5.7	7.0	29
Cys/2[b]	2.5	2.4	3.1	13
Val	9.1	8.1	7.6	48
Met	0[c]	0.05[d]	0.53	0
Ile	3.1	2.9	1.6	16
Leu	9.2	8.0	9.7	49
Tyr	3.5	3.7	2.6	18
Phe	3.0	3.1	3.2	16
Trp	2.0	2.0[e]	1.9	11
Hexose				47
Glucosamine				30[f]

[a] Analyses of 22-hr hydrolysates (no extrapolations). [b] Measured as *S*-carboxymethylcysteine in BSC and α chain, and as cysteic acid in FSC. In partially reduced α chains, the *S*-carboxymethylcysteine content was halved. [c] Not detectable as either methionine or homoserine. [d] Average of two analyses as methionine sulfone in performic acid oxidized samples. Methionine was always completely absent in analyses of unoxidized samples. [e] Not determined. Assumed to be equal to that in BSC. [f] Considered as *N*-acetylglucosamine.

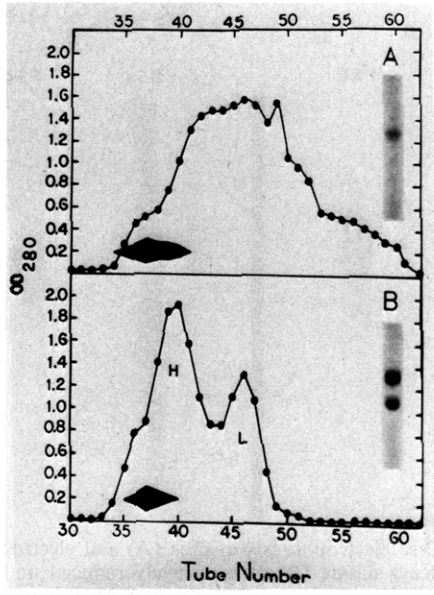

FIGURE 2: Elution patterns of extensively reduced and alkylated secretory IgA from a 2.5 × 90 cm Sepharose 4B column in 4 M Gdn·HCl (7 ml/tube). (A) The protein was reacted with cyanogen bromide and passed through Sephadex G-25 before the gel filtration shown. (B) No cyanogen bromide treatment. The black zones indicate the position and the relative amount of secretory component as determined by Ouchterlony analysis. Shown also are the electrophoretic patterns (sodium dodecyl sulfate–acrylamide gels) of the pooled contents of tubes 36–38 of each effluent, which show almost pure SC in part A and appreciable contamination with α chains (lower of the two bands) in part B.

The final step in the isolation of BSC was gel filtration in 4 M Gdn·HCl after the reaction of extensively reduced and alkylated secretory IgA with cyanogen bromide. A representative elution pattern is shown in Figure 2A. The shoulder on the ascending side of the first peak contained the bulk of the SC as monitored by Ouchterlony analysis and electrophoresis in the dodecyl sulfate. When this material was repassed through the same column, a single symmetrical peak eluted, and from this pure SC could be obtained after dialysis of the peak tubes *vs.* water (in which SC was still soluble) and lyophilization. The next fraction after SC (the tubes with the highest optical densities in Figure 2A) contained mostly material of about 43,000 molecular weight, according to mobility in electrophoresis in the dodecyl sulfate, which must contain fragments of α chain. There was also a small amount of material with a molecular weight similar to that of intact extensively reduced and alkylated α chain, which by this method is about 56,000, in agreement with Dorrington and Rockey (1970), Abel and Grey (1971), and O'Daly and Cebra (1971b). In Figure 2B is the elution pattern, from the same column as in part A, of extensively reduced and alkylated secretory IgA (no cyanogen bromide treatment). The shoulder due to SC on the ascending side of the main peak is more prominent and better separated in part A than in part B. This illustrates that SC is more readily separated from α chains if the latter have been previously cleaved by cyanogen bromide. In addition, electrophoretic patterns from some of the tubes in Figures 2A,B, are shown to bring out further the effect of cyanogen bromide.

The purified BSC (*i.e.*, after two gel filtrations in guanidine) yielded single bands on electrophoresis in the dodecyl sulfate (up to 0.04 mg) and disc electrophoresis in urea (up to 0.05 mg) (Figure 3). In addition, two rabbits were immunized with purified BSC, and each produced antiserum that precipitated strongly with purified BSC and more weakly with native FSC and secretory IgA. They did not react with other proteins in colostrum or at all with normal human serum.

The amino acid and carbohydrate composition of BSC is given in Table I. Noteworthy is the absence of methionine and/or homoserine, even when the amino acid analyzer was overloaded for most of the other amino acids. The carbohydrate content is 19.4% by weight (10.7% hexose and 8.7% hexosamine, assumed to be present in the N-acetylated form). The extinction coefficient ($E_{280}^{1\%}$) for BSC in 6 M Gdn·HCl was found to be 12.0, which is close to the value 12.7 reported by Kobayashi (1971) for FSC in water.

The molecular weight of BSC was evaluated by two different methods. Its mobility during electrophoresis in detergent compared to standard proteins, all extensively reduced and alkylated, gave a value of about 87,000. By sedimentation equilibrium in 5 M Gdn·HCl at 27,690 rpm and 35,600 rpm the weight average molecular weights of extensively reduced and alkylated BSC were 71,900 ± 797 and 71,988 ± 990 according to least-squares fits of the experimental data. Since the native protein contains half-cystine instead of *S*-carboxymethylcysteine, its molecular weight would be 71,200. The

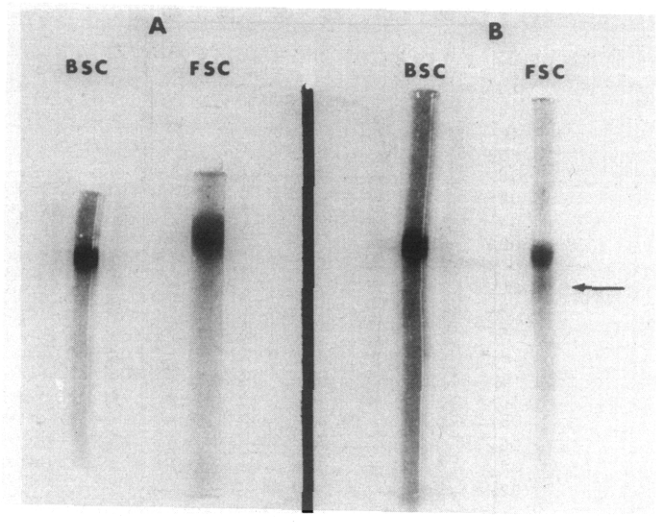

FIGURE 3: Disc electrophoresis in urea (A) and electrophoresis in sodium dodecyl sulfate (B) of extensively reduced and alkylated BSC and FSC. The arrow marks the contaminant in the FSC preparation.

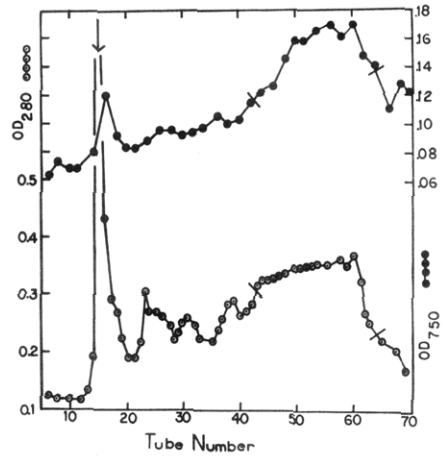

FIGURE 5: Final step in purification of FSC: preparative electrophoresis in acrylamide gel. The optical densities were read at 280 nm for native protein and at 750 nm after protein determinations by the Lowry *et al.* (1951) method. Tubes 43–64 were taken. The peak marked by the arrow is due to the Bromophenol Blue tracking dye.

ln *c vs.* r^2 plots from which the molecular weights were calculated are shown in Figure 4.

Isolation and Characterization of FSC. The final step in the isolation of FSC was preparative zone electrophoresis in polyacrylamide gel (Figure 5). The purity of the resulting FSC was evaluated in five ways. First, radiolabeled FSC was specifically precipitated in antibody excess, and was found to be 90% precipitable (control tubes 0.25%). This is considered to be a minimal estimate since the precipitates were small in size and any losses during washing would be included in the nonprecipitable radioactivity as would any radioactivity remaining in solution as soluble antigen–antibody complexes. Second, disc electrophoresis in urea-containing buffers (up to 0.1 mg) showed a single band (Figure 3A). However, on electrophoresis in the dodecyl sulfate of a 0.06-mg sample of alkylated FSC or extensively reduced and alkylated FSC there was a small amount of faster moving material (Figure 3B). Fourth, immunoelectrophoresis of FSC (10 mg/ml) with two different antisera *vs.* whole colostrum yielded only one precipitin line. Finally, three rabbits were immunized with FSC and each produced antiserum specific for SC (reaction with both BSC and FSC; precipitation in colostrum only with SC; no reaction with normal human serum).

A molecular weight of 87,000 was obtained for extensively reduced and alkylated FSC by electrophoresis in the dodecyl sulfate. By the same method the molecular weight of unreduced but alkylated FSC was 80,000. The latter value should be less accurate since conformation affects mobility, and the standard proteins were all extensively reduced and alkylated. Because the size of FSC did not change appreciably and no new bands were seen after reduction in a denaturing solvent (7 M Gdn·HCl), we can conclude, as have other investigators, that native FSC is composed of a single polypeptide chain.

The amino acid composition of FSC is listed in Table I. There was no detectable methionine in native or extensively reduced and alkylated FSC, and following performic acid oxidation there was appreciably less than 1 mole/mole of methionine sulfone, even when the amino acid analyzer was overloaded with respect to most of the other amino acids. As a check on our methodology, methionine was found in the expected amounts when proteins (ribonuclease, hemoglobin)

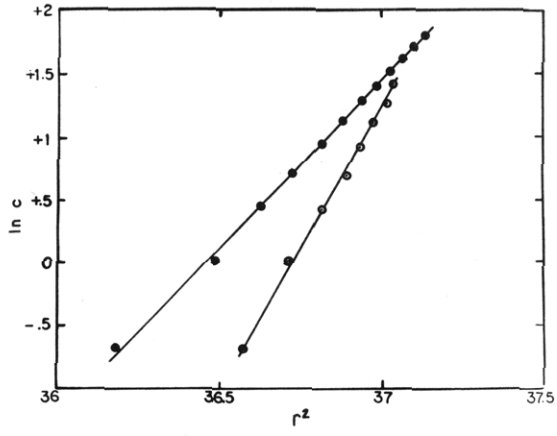

FIGURE 4: Plots of ln *c* (concentration in fringe numbers) *vs.* r^2 (cm^2) for BSC in 5 M Gdn·HCl at sedimentation equilibrium. Closed circles: 27,690 rpm; open circles: 35,600 rpm. The lines are least-squares fits of the experimental points.

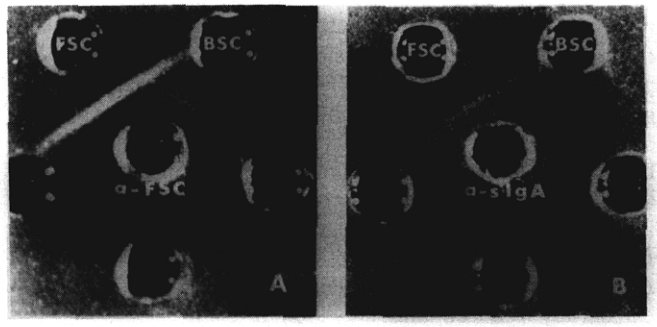

FIGURE 6: Ouchterlony analysis to show that antisera raised against (A) purified FSC and (B) SC as it occurs in secretory IgA (sIgA) react with both purified FSC and BSC. Because of conformational antigenic determinants which are present in native FSC and absent in the purified denatured BSC, there is a spur in part A, and the precipitin line *vs.* BSC in part B is weak and does not appear in the photograph.

known to contain methionine were analyzed, and homoserine (1 mole/mole) was observed in an analysis of the largest α-chain cyanogen bromide fragment.

Antigenic Determinants of BSC and FSC. In Figure 6 BSC and FSC are compared to antisera induced by each. An antiserum made against purified FSC also precipitates purified BSC (Figure 6A), and an antiserum made against secretory IgA, *i.e.*, *vs.* BSC in its native state, reacts strongly with purified FSC and weakly with purified (denatured) BSC (Figure 6B). In addition, an antiserum made against purified BSC precipitates both secretory IgA and purified FSC (not shown).

α Chain. α chain differs from SC in a number of respects including amino acid composition (Table I), antigenic characteristics (*e.g.*, antisera made *vs.* purified FSC do not react with serum-type IgA), and size (as indicated by their different mobilities in electrophoresis in the dodecyl sulfate). Of the α-chain half-cystine residues, half were reduced under mild conditions.

Discussion

Secretory component is known to occur in two forms, as an integral part of the secretory IgA molecule and in a free state. Unlike the H and L chains and probably also the J chain, which are formed in lymphoid cells, SC is found in, and is thus thought to be synthesized by, epithelial cells in various parts of the body (Tourville *et al.*, 1969; O'Daly *et al.*, 1971). In addition FSC is formed by individuals who are unable to produce IgA (South *et al.*, 1966). Because of similarities in size and antigenic determinants BSC and FSC have been considered to be different states of the same protein, and the data obtained in the present work and also by Kobayashi (1971) on human SC and by O'Daly and Cebra (1971b) on rabbit SC are consistent with this idea. In our studies BSC and FSC had similar mobilities in electrophoresis, and each reacted with monospecific antiserum prepared against the other. Also, their amino acid compositions were similar, although not identical, the main differences being in lysine, arginine, serine, proline, valine, and leucine. These differences could be real, but more likely they reflect the contaminant in the FSC noted on electrophoresis in detergent. The composition of our SC preparations is similar to that reported by Kobayashi (1971), and somewhat less so to that listed by Tomasi and Bienenstock (1968). Human and rabbit SC (O'Daly and Cebra, 1971b) have similar amino acid compositions; however, it is interesting that the rabbit protein contains 2–3 moles of methionine/mole. As discussed above, we were unable to detect methionine in human FSC, and this observation formed the basis of our procedure for isolating BSC. Of course, the lack of methionine in our BSC preparations does not in itself show that BSC is totally lacking in methionine. For example, if methionine were present in some BSC chains, perhaps as a genetic variant, such chains would have been cleaved by cyanogen bromide, and the resulting fragments would have eluted later than the main SC peak on gel filtration. It is also possible that a methionine residue could be situated near the N terminus of all BSC chains. If so, after reaction with cyanogen bromide, homoserine would be absent from the main fragment. If this were indeed the case, the putative N-terminal fragment which would be split off by cyanogen bromide would have to be small enough so as not to alter the mobility during electrophoresis in the dodecyl sulfate since there was no observable difference between the purified BSC and the BSC released by reduction alone without treatment by cyanogen bromide. In view of the lack of homoserine in our BSC and of methionine in our FSC, and also in the FSC and BSC isolated by Kobayashi (1971) without cleavage of peptide bonds, the conclusion must be that human SC is truly devoid of methionine.

The molecular weights for SC reported in the literature vary from 60,000 to 85,000. These determinations have been made by a variety of techniques including gel filtration and mobility in electrophoresis. Such methods are probably reliable only when protein is completely denatured and is migrating in a denaturing medium. These criteria were not always met. By sedimentation equilibrium Tomasi and Calvanico (1968) and O'Daly and Cebra (1971b) (rabbit) found the molecular weight to be 60,000. van Munster *et al.* (1971), using the Archibald method, and Kobayashi (1971), using sedimentation and diffusion coefficients, obtained values of 74,000 $\pm$ 6000 and 75,500, respectively, for FSC.

In the present study a molecular weight of 72,000 for reduced and alkylated BSC in 5 M Gdn·HCl was determined by sedimentation equilibrium. In no instance, including our own work, was $\bar{V}$ directly measured, and an accurate value is especially important for experiments performed in high density solvents such as 5 M Gdn·HCl. Quite satisfactory values can be calculated from composition data (Cohn and Edsall, 1943; Schachman, 1957; Gibbons, 1966), but in the case of glycoproteins the component sugars must be taken into consideration as well as the amino acids. However, one problem still remains when $\bar{V}$'s based on composition are used for calculations of experiments done in high concentrations of guanidine. In order to correct for interactions of protein with this solvent, Hade and Tanford (1967) have suggested that $\bar{V}$ be decreased by 0.01–0.02 ml/g, and we have done this in our calculations.

The difference between our molecular weight for native BSC (71,000) and that for FSC (75,500) reported by Kobayashi (1971) is due solely to the different $\bar{V}$'s used. We calculated a $\bar{V} = 0.710$ from our amino acid and carbohydrate composition. Kobayashi (1971) used a $\bar{V} = 0.730$ based on amino acid content. With carbohydrate content taken into account as well, we have calculated from Kobayashi's data a $\bar{V} = 0.716$, which would bring his molecular weight to 71,800 in close agreement with our value of 71,000.

Regardless of the problems connected with the correct choice of $\bar{V}$, there appears to be a real discrepancy between molecular weights based on ultracentrifugation and those based on molecular sieving, with molecular weights appreciably higher with the latter. Rabbit SC has a molecular weight of 64,000–70,000 (Halpern and Koshland, 1970) to 80,000 (O'Daly and Cebra, 1971b) according to migration in detergent *vs.* 60,000 by sedimentation equilibrium (O'Daly and Cebra, 1971b). O'Daly and Cebra (1971b) have suggested that molecular sieving in detergent may not be accurate for proteins containing appreciable carbohydrate when the standards used are low in carbohydrate, and Glossmann and Neville (1971) have also discussed the problems inherent in molecular weight determinations of glycoproteins by electrophoresis in detergent. In our hands electrophoresis in detergent gave molecular weights for α chains (which contain appreciable carbohydrate, though less than SC) which are in agreement with accepted values, but in the case of SC gave a much higher value (87,000) than did sedimentation equilibrium (72,000). Mach (1970) reported a value of 85,000 for human SC based on electrophoresis in detergent. Given the discrepancy between the molecular weights of SC determined by the two methods and the uncertainties connected with

molecular sieving for glycoproteins, we believe the value from sedimentation equilibrium to be the more reliable.

Human BSC, in contrast to its rabbit counterpart (Cebra and Small, 1967; Halpern and Koshland, 1970), can only be purified after cleavage of disulfide links to the remainder of the secretory IgA molecule. The reduction used to effect this cleavage is not likely to have split a symmetrical dimer of the SC moiety for two reasons. First, FSC, native or reduced, had a similar molecular weight to the BSC and gave no indication of subunits and second, the stoichiometry of the secretory IgA molecule (see below) does not accommodate more than one SC polypeptide chain.

Based on what is known of the structure of immunoglobulins in general and IgA in particular and with a knowledge of the molecular weight of SC, we can conclude in agreement with other workers that secretory IgA contains a single SC polypeptide chain. Secretory IgA has a molecular weight of close to 400,000 (Cebra and Small, 1967; Tomasi and Calvanico, 1968; Hurlimann *et al.*, 1969; Newcomb *et al.*, 1968; Kobayashi, 1971), and all molecules contain SC (O'Daly and Cebra, 1968; Mestecky *et al.*, 1970; Pincus *et al.*, 1971). The major component polypeptide chain is the α chain. A typical four-chain subunit of IgA would have a molecular weight of about 160,000. Two such units would make 320,000, leaving room for one SC chain, and also perhaps a J chain.

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References

Abel, C. A., and Grey, H. M. (1971), *Nature (London) 233*, 29.
Ambler, R. P. (1965), *Biochem. J. 96*, 32P.
Appleman, M. M., Yunis, A. A., Krebs, E. G., and Fischer, E. H. (1963), *J. Biol. Chem. 238*, 1358.
Brandtzaeg, P. (1970), *Immunochemistry 7*, 127.
Cebra, J. J., and Robbins, J. B. (1966), *J. Immunol. 97*, 12.
Cebra, J. J., and Small, P. A., Jr. (1967), *Biochemistry 6*, 503.
Cohn, E. J., and Edsall, J. T., Ed. (1943), Proteins, Amino Acids, and Peptides, New York, N. Y., Reinhold, Chapter 16.
Davis, B. J., and Ornstein, L. (1968), *in* Methods in Immunology and Immunochemistry, Vol. II, Williams, C. A., and Chase, M. W., Ed., New York, N. Y., Academic Press, p 38.
Dayton, D. H., Jr., Small, P. A., Jr., Chanock, R. M., Kaufman, H. E., and Tomasi, T. B., Jr., Ed. (1969), The Secretory Immunologic System, Washington, D. C., U. S. Government Printing Office.
Dorrington, K. J., and Rockey, J. H. (1970), *Biochim. Biophys. Acta 200*, 584.
Edelhoch, H. (1967), *Biochemistry 6*, 1948.
Gibbons, R. A. (1966), *in* Glycoproteins, Gottschalk, A., Ed., New York, N. Y., Elsevier, p 61.
Glossmann, H., and Neville, D. M., Jr. (1971), *J. Biol. Chem. 246*, 6339.
Grey, H. M., Abel, C. A., Yount, W. J., and Kunkel, H. G. (1968), *J. Exp. Med. 128*, 1223.
Gross, E., and Witkop, B. (1962), *J. Biol. Chem. 237*, 1856.
Hade, E. P. K., and Tanford, C. (1967), *J. Amer. Chem. Soc. 89*, 5034.
Halpern, M. S., and Koshland, M. E. (1970), *Nature (London) 228*, 1276.
Hirs, C. H. W. (1967), *Methods Enzymol. 11*, 197.
Hurlimann, J., Waldesbühl, M., and Zuber, C. (1969), *Biochim. Biophys. Acta 181*, 393.
Kobayashi, K. (1971), *Immunochemistry 8*, 785.
Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951), *J. Biol. Chem. 193*, 265.
Mach, J.-P. (1970), *Nature (London) 228*, 1278.
McFarlane, A. S. (1958), *Nature (London) 182*, 53.
Mestecky, J., Kraus, F. W., and Voight, S. A. (1970), *Immunology 18*, 237.
Mestecky, J., Zikan, J., and Butler, W. T. (1971), *Science 171*, 1163.
Neuberger, A., and Marshall, R. D. (1966), *in* Glycoproteins, Gottschalk, A., Ed., New York, N. Y., Elsevier, p 224.
Newcomb, R. W., Normansell, D., and Stanworth, D. R. (1968), *J. Immunol. 101*, 905.
O'Daly, J. A., and Cebra, J. J. (1968), *Protides Biol. Fluids Proc. Colloq. 16*, 205.
O'Daly, J. A., and Cebra, J. J. (1971a), *J. Immunol. 107*, 449.
O'Daly, J. A., and Cebra, J. J. (1971b), *Biochemistry 10*, 3843.
O'Daly, J. A., Craig, S. W., and Cebra, J. J. (1971), *J. Immunol. 106*, 286.
Peters, T., Jr., and Hawn, C. (1967), *J. Biol. Chem. 242*, 1566.
Pincus, C. S., Lamm, M. E., and Nussenzweig, V. (1971), *J. Exp. Med. 133*, 987.
Reisfeld, R. A., and Small, P. A., Jr. (1966), *Science 152*, 1253.
Reisler, E., and Eisenberg, H. (1969), *Biochemistry 8*, 4572.
Schachman, H. K. (1957), *Methods Enzymol. 4*, 32.
Seery, V. L., Fischer, E. H., and Teller, D. C. (1967), *Biochemistry 6*, 3315.
Shapiro, A. L., Viñuela, E., and Maizel, J. V., Jr. (1967), *Biochem. Biophys. Res. Commun. 28*, 815.
Small, P. A., Jr., and Lamm, M. E. (1966), *Biochemistry 5*, 259.
South, M. A., Cooper, M. D., Wollheim, F. A., Hong, R., and Good, R. A. (1966), *J. Exp. Med. 123*, 615.
Thompson, R. A., and Asquith, P. (1970), *Clin. Exp. Immunol. 7*, 419.
Tomasi, T. B., Jr., and Bienenstock, J. (1968), *Advan. Immunol. 9*, 1.
Tomasi, T., and Calvanico, N. (1968), *Fed. Proc., Fed. Amer. Soc. Exp. Biol. 27*, 617.
Tomasi, T. B., Jr., Tan, E. M., Solomon, A., and Prendergast, R. A. (1965), *J. Exp. Med. 121*, 101.
Tourville, D. R., Adler, R. H., Bienenstock, J., and Tomasi, T. B., Jr. (1969), *J. Exp. Med. 129*, 411.
Ullmann, A., Goldberg, M. E., Perrin, D., and Monod, J. (1968), *Biochemistry 7*, 261.
van Munster, P. J. J., Stoelinga, G. B. A., and Poels-Zanders, S. (1971), *Immunochemistry 8*, 471.
Waldman, R. H., Mach, J.-P., Stella, M. M., and Rowe, D. S. (1970), *J. Immunol. 105*, 43.
Weber, K., and Osborn, M. (1969), *J. Biol. Chem. 244*, 4406.
Yphantis, D. A. (1964), *Biochemistry 3*, 297.