ACTA CHEMICA SCANDINAVICA 20 (1966) 2339–2348

Isolation of Immunoglobulin A (IgA) from Human Colostrum

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The IgA in human colostrum has been isolated on a gram-scale under mild conditions, involving column electrophoresis of defatted colostrum and subsequent gel filtration of the IgA-containing fraction on Sephadex G-200. The isolated protein was pure in immunoelectrophoresis and had a sedimentation coefficient of 11.6 S. The amino acid composition resembled that of serum IgA. Aspartic acid and glutamic acid were found in the N-terminal positions of the protein.

The isolation of IgA from human blood serum was first accomplished by Heremans and Schultze.[1] Their isolation procedure was based on the observation that IgA in contrast to IgG does not precipitate in the presence of Zn^{2+} ions. The original method has been modified by Heremans and coworkers.[2–4] Purification methods avoiding the zinc precipitation and involving a combination of chromatography on cellulose or Sephadex ion exchangers and gel filtration have been described by several authors.[5–8] No matter what method is used, the isolation of IgA free from impurities is a tedious procedure, especially in view of the small amounts of IgA present in normal serum compared to the rather similar IgG. For this reason most of the investigations on the structure of IgA have been performed on M-components of the IgA-type, usually occurring in large amounts in sera from patients with IgA-myeloma and therefore much easier to isolate in large quantities.[9–12]

Since human colostrum contains large amounts of immunoglobulins antigenically related to IgA, while the amount of IgG is conspicuously low,[13–14] colostrum seemed to be a convenient starting material for the isolation of IgA. The preparation of colostrum IgA has been accomplished by several authors. Montreuil *et al.*[15] used fractionation with ammonium sulfate, while Blanc [16] obtained the protein by treatment of colostrum with Zn^{2+} ions and subsequent chromatography on DEAE-cellulose. Hanson and Johansson [17]

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isolated the milk IgA by chromatography of colostrum on DEAE-cellulose and rechromatography of a fraction containing mainly IgA. Tomasi *et al.*[18] recently described a procedure for the purification of the IgA from colostrum by starch block electrophoresis, DEAE-cellulose chromatography and gel filtration on Sephadex G-200 after acid precipitation of the casein. The present paper describes the preparation of milk IgA on a gram-scale under mild conditions involving column electrophoresis of colostrum followed by gel filtration of the IgA-containing fraction on Sephadex G-200. The isolation procedure and some main characteristics of the protein will be described in detail.

MATERIALS

Colostrum samples were collected from apparently healthy women within 72 h after delivery. Pooled material was used for the preparations.

Human serum IgA was prepared from pooled normal sera by the zinc precipitation method essentially as described by Heremans.[2] The IgA was further purified by ammonium sulfate precipitation; gel filtration on Sephadex G-200 and column electrophoresis. Only preparations immunologically free from IgG and IgM were used.

Human IgG was kindly supplied by AB Kabi (Stockholm, Sweden). The preparation did not contain IgA or IgM according to immunoelectrophoretic analyses with anti-human serum.

Antisera against human colostrum and serum IgG were obtained by weekly subcutaneous injections of rabbits with 2 ml of colostrum or 20 mg of IgG for 3—6 months. Rabbit antisera against immunoglobulins (anti-7S-γ-globulin) and human serum were obtained from Behringwerke (Marburg a/L, Germany).

METHODS

Milk fat was removed by ultracentrifugation of colostrum in a Spinco ultracentrifuge, model L, for 2 h at 40 000 rpm (146 000 *g*). After the centrifugation the clear or slightly opalescent liquid between the fatty layer at the surface and a precipitate at the bottom of the lusteroid tubes was removed through a tiny hole made near the bottom of the tubes.

Column electrophoresis was performed according to Porath.[19] In the preliminary experiments a column of the dimensions 3 × 50 cm, equipped with a device for continuous elution during the runs, was used.[20] Large scale preparations were made with an apparatus similar to the commercially available model 5800, manufactured by LKB (Stockholm, Sweden). This column had a length of 42 cm. The outer and inner diameters of the space containing the supporting medium were 11 and 6.5 cm, respectively. Both columns were cooled by circulating water at 0.5°. As supporting media were used "Pevikon" (Svenska Superfosfatbolaget, Stockholm, Sweden) passed through an 140, mesh screen, formaldehyde-treated cellulose,[21]* or Sephadex G-25 "fine". The runs were performed in tris-sulfate buffers, pH 8.2, containing 0.05 or 0.2 M tris (tris = tri(hydroxymethyl)aminomethane). Prior to the electrophoresis the defatted colostrum samples were dialysed for 2 days at + 4° against the appropriate buffer diluted 1:2 in order to obtain a sharpening of the starting zones.

The samples were layered between the bed surface and the supernatant buffer after the density of the solution was increased by adding sucrose to a final concentration of 3 % (w/v). The formation of distinct boundaries between the samples and the buffer was facilitated by use of a motor-driven syringe, introducing the sample at a flow rate of 60 ml/h. Marker substances, 2,4-dinitrophenyl (DNP) derivatives of aspartic acid, serine, and ethanolamine, were always added to the samples for observation of the electrophoretic movement and the electroendosmotic flow. The small column was run in 0.05 M tris-sulfate for 24 h at 600 V while the large column was run in 0.2 M tris-sulfate for 40 h

* A similar type of cellulose is manufactured by LKB, Stockholm, Sweden.

at 300 V. A constant flow rate during the continuous elution was maintained by a peristaltic pump. The light absorption at 254 mμ was registered by a "Uvicord" ultraviolet absorptiometer (LKB, Stockholm, Sweden). Effluent fractions were collected by a time-operating fraction collector and the optical density at 280 mμ of each fraction was determined by a Zeiss PMQ II spectrophotometer. The optical densities of the DNP-derivatives were measured at 360 mμ. Pooled effluent fractions were concentrated by means of ultrafiltration through Visking dialysis tubings (8/32), or dialyzed against distilled water and lyophilized.

Gel filtration was performed on Sephadex G-200, 140–400 mesh, in 0.02 M tris-HCl buffer pH 8.0 containing 0.2 M sodium chloride with columns of the dimensions 3 × 90 cm and 7 × 90 cm at temperatures ranging from 0.5° to 8°. The flow rates were kept at 15 and 30 ml/h, respectively. The samples were applied, collected and concentrated as described under "Column electrophoresis". *Thin-layer gel filtration* [22] *and thin-layer gel filtration-electrophoresis* [23] were performed as described earlier.

Gel electrophoresis in agar was carried out with an LKB equipment [24] using 1 % (w/v) Difco Noble agar in 0.05 M sodium barbital buffer, pH 8.4. The analyses were run for 90 min at a potential gradient of 8 V/cm. *Immunoelectrophoresis* was performed with the same equipment as described by Hirschfeld.[24] *Starch gel electrophoresis* was carried out in a horizontal apparatus, employing hydrolyzed starch from Connaught Medical Laboratory, Toronto, Canada. The analyses were performed as described by Poulik.[25]

Ultracentrifugal analyses were made in a Spinco Model E ultracentrifuge at a constant temperature of 20° ± 0.15° and 59 780 rpm. Potassium chloride-phosphate buffer, pH 7.4, ionic strength 0.2, was used as solvent. Correction of sedimentation coefficients to $s_{20,w}$ were made with an assumed value of the partial specific volume of the colostrum IgA protein of 0.72.

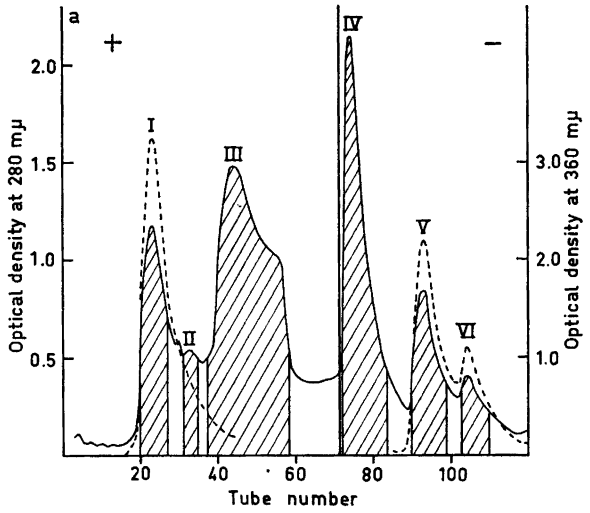
Amino acid analyses were performed by the recording method of Spackman, Stein and Moore.[26] Protein samples of about 10 mg were hydrolyzed with 2 ml of 6 M HCl in evacuated, sealed tubes for 22, 72, and 144 h in a silicon oil bath at 110°.

Amino-terminal amino acid residues were determined by Sanger's procedure.[27]

Reduction of protein. A 2 % (w/v) solution of colostrum IgA was treated with 0.1 M cysteine or penicillamine (β-mercaptovaline) for 5–16 h at pH 7.0 and room temperature. Alkylation was performed by the addition of iodoacetamide to a final concentration of 0.25 M in an ice water bath. After 1 h the solution was dialyzed for 2 days against frequently changed, ice-cold, distilled water.

RESULTS

Introductory studies. The resolution by column electrophoresis and by gel filtration on Sephadex G-200 was studied with samples of human colostrum. To avoid clogging of the columns the colostrum had been defatted by ultracentrifugation. Results of an electrophoretic run are shown in Fig. 1*a*. By measuring the ultraviolet light absorption of the effluent six peaks were obtained, peaks I–III being eluted during the electrophoretic run and peaks IV–VI after disconnection of the electric current. DNP-aspartic acid and DNP-ethanolamine were recovered in peaks I and V–VI, respectively. These fractions contained only trace amounts of proteins, *i.e.* "milk γ-globulin",[13] which was demonstrable in fractions V and VI by immunoelectrophoresis. The protein distribution after agar electrophoresis of fractions II–IV is shown in Fig. 1*b*. Fraction IV contained a major component in the γ_1-globulin region and this fraction also developed a heavy precipitate corresponding to milk IgA in immunoelectrophoresis with anti-human colostrum (Fig. 6*a*). A few additional lines were formed with anti-human colostrum. Fractions I–III formed no precipitates in immunoelectrophoresis with anti-7S-γ-globulin and were for this reason not considered to contain any appreciable amounts of IgA.



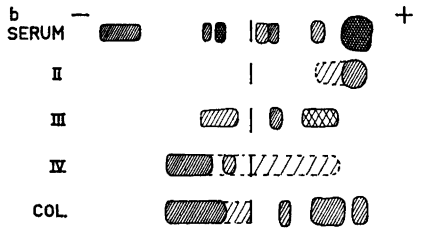


Fig. 1a. Results of column electrophoresis of 10 ml of colostrum (0.3 g protein) in 0.05 M tris-sulfate buffer, pH 8.2. The separation achieved on formaldehyde-treated cellulose during 24 h at 600 V and 22 mA in a column of the dimensions 3 × 50 cm. Continuous elution during the run at a flow rate of 15 ml/h. The vertical line before fraction IV in the figure indicates disconnection of the electrical current. ———, optical density at 280 mμ, -------, optical density at 360 mμ. Pooling of fractions indicated by hatched areas.

b. Schematic representation of agar gel electrophoresis of fractions II—IV from the column electrophoresis. SERUM and COL. represent normal serum and fresh colostrum run as references.

Gel filtration of colostrum on Sephadex G-200 gave a separation into four peaks (Fig. 2). The pooling of the effluent is indicated in the figure. Fraction I was considerably larger than the other fractions and comprised the entire asymmetrical first peak. It showed in agar electrophoresis a main component in the γ_1-globulin region and reacted in immunoelectrophoresis with anti-7S-γ-globulin. Fractions II and III contained no components in the γ-globulin region and only fraction II reacted with anti-7S-γ-globulin, forming a very faint line. Fraction IV obviously contained mainly low molecular weight constituents. The column gel filtration pattern was comparable to that obtained by thin-layer gel filtration of fresh, untreated colostrum (Fig. 3).

Isolation of colostrum IgA. By combining column electrophoresis and Sephadex gel filtration a simple procedure was achieved for the isolation of the IgA from human colostrum.

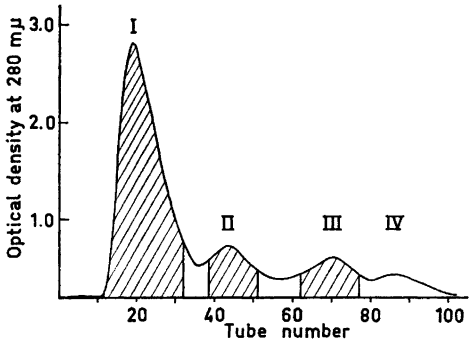


Fig. 2. Separation of colostrum (5 ml containing 0.3 g protein) on Sephadex G-200, equilibrated with 0.02 M tris-HCl pH 8.2 and 0.2 M sodium chloride. Column dimensions 3 × 90 cm. The experiment was performed with a flow rate of 15 ml/h at 8°.

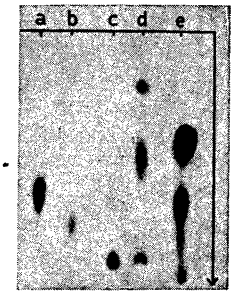


Fig. 3. Thin-layer gel filtration on Sephadex G-200 "superfine". *a.* IgG from serum. *b.* IgA from serum. *c.* IgA from colostrum. *d.* Fresh colostrum. *e.* A human serum with an increased γ-globulin fraction demonstrated with electrophoresis.

After ultracentrifugation and dialysis, the defatted colostrum 50—100 ml, containing 2—4 g of protein, was applied on the large electrophoretic column, which was packed with formaldehyde-treated cellulose or in a few experiments with Sephadex G-25 "fine". No continuous elution was used but the DNP-Asp (or DNP-Ser) and material corresponding to fraction II and part of fraction III, (Fig. 1*a*), were allowed to migrate into the anode buffer compartment,

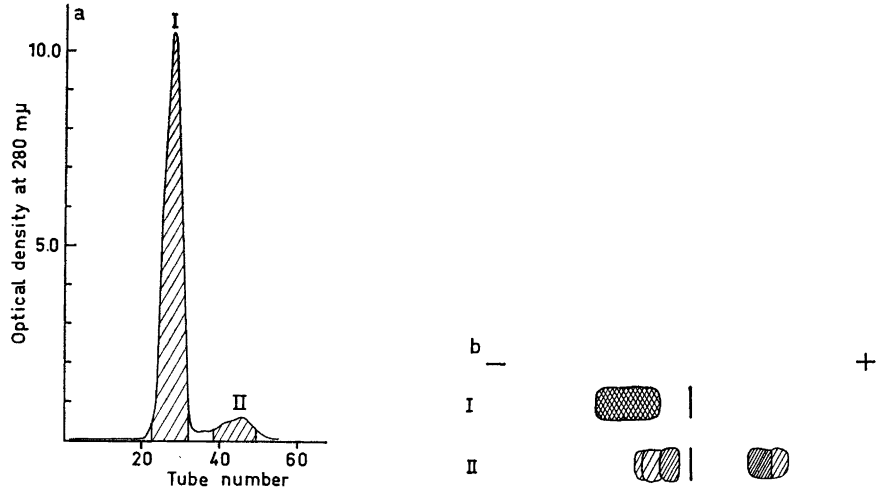


Fig. 4a. Gel filtration on Sephadex G-200 of fraction IV obtained by column electrophoresis of human colostrum (Fig. 1*a*). Column dimension 7 × 90 cm. The separation was performed with a flow rate of 30 ml/h at + 0.5°C.

b. A schematic representation of the patterns obtained by agar electrophoresis of fractions I and II (Fig. 4a).

which in these experiments had no communication with the cathode compartment. After disconnection of the electric current fraction IV was eluted, concentrated by ultrafiltration through Visking dialysis tubings and applied to the Sephadex G-200 column. In addition to the major peak (Fig. 4) consisting of IgA, a small peak was obtained, which did not contain IgA, but consisted of a variety of proteins, *i.a.* milk γ-globulin and lactoferrin (red protein).[13,17] A brief preparation schedule is shown in Fig. 5.

The yield of IgA in two preparations was 47 % and 53 % of the protein content in the defatted colostra. Immunoelectrophoretic analyses of the IgA after electrophoresis and gel filtration are shown in Fig. 6. In (*b*), (*c*) and (*d*) the isolated protein was analyzed with anti-human colostrum, anti-7S-γ-globulin and anti-human *serum* IgA, respectively. It is apparent that the colostrum IgA was immunoelectrophoretically pure when tested against the anti-human colostrum, which was the potent, multivalent anti-serum, used in earlier studies.[13,17] Starch gel electrophoresis of the isolated IgA revealed a single broad band emerging anodically from the application slit.

Ultracentrifugal studies on the isolated protein showed the presence of one main component and a small faster sedimenting component (Fig. 7). Small

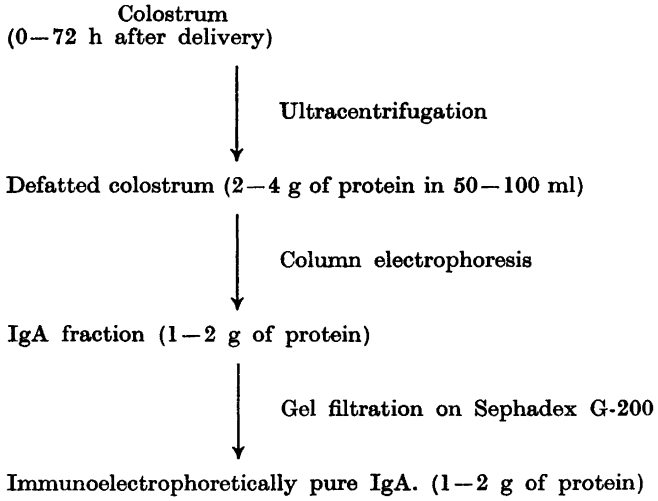


Fig. 5. Scheme for the preparation of human colostrum IgA.

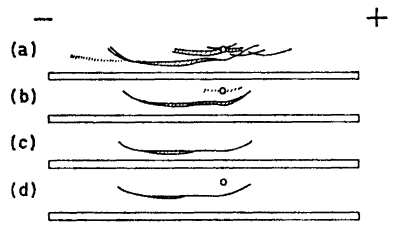


Fig. 6. Immunoelectrophoresis of IgA fractions. *a.* IgA fraction obtained by column electrophoresis tested with anti-human colostrum.
b, *c*, and *d*. Isolated colostrum IgA separated: *b*, tested with anti-human colostrum; *c*, tested with anti-7S-γ-globulin; *d*, tested with anti-serum IgA.

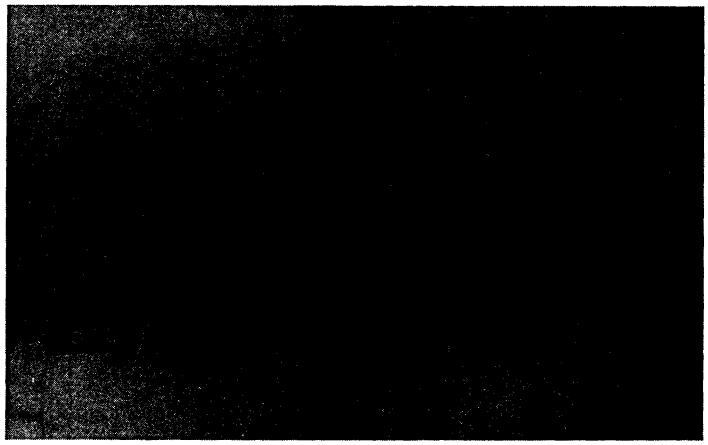


Fig. 7. Ultracentrifugal analysis of colostrum IgA at a concentration of 10 mg/ml. Rotor speed was 59 780 rpm. Sedimentation from left to right at 37 and 45 min after reaching the constant speed.

amounts of material were also observed moving through the cell during the acceleration of the rotor. Values of $s_{20,w}$ were calculated assuming a partial specific volume of 0.72 for colostrum IgA. By extrapolating the $s_{20,w}$ for the main component to infinite protein concentration, a value of 11.6 S for $s^{\circ}_{20,w}$ was obtained. The $s_{20,w}$ of the minor component was around 16.5 S. The possibility that the obtained sedimentation coefficient around 12 S might be due to a polymerization of 7 S protein during the preparation was ruled out by the results of thin-layer gel filtration of fresh colostrum, showing a large component with the same localization as the isolated IgA. (Fig. 3). Combined thin-layer gel filtration-electrophoresis revealed that this component was located in the electrophoretic γ-globulin region. It might be noted that the migration of IgA at thin-layer gel filtration was concentration-dependent with a retardation of samples with a high protein content. At lower concentrations than used in Fig. 3, the colostrum IgA had the same migration rate as the macroglobulin fraction of human serum.

Attempts to split the 12 S protein into smaller units were made by treatment with 0.1 M cysteine or penicillamine followed by alkylation with iodacetamide. The addition of the reducing agents gave an almost instantaneous disappearance of the slight opalescence present in the IgA solutions. Thin-layer gel filtration of the treated proteins on Sephadex G-200 showed, besides the main component, a faint spot with a somewhat higher migration rate than the IgG reference. IgA that was merely alkylated behaved exactly like the original IgA. The sedimentation patterns of IgA, treated with cysteine and iodoacetamide revealed the appearance of a small 6 S component, whereas the 17 S component had disappeared. Control ultracentrifugal runs on merely alkylated protein showed the same pattern as that obtained for the untreated IgA. It should be noted, however, that the sedimentation coefficient of the

main component was diminished to 10 S, possibly owing to changes of the protein conformation.

The amino acid composition of the protein determined by ion exchange chromatography is shown in Table 1. No tryptophan analyses were made. The values for serine, threonine and tyrosine were extrapolated to zero hydrolysis time, while the maximum value for valine was used. The average values of the amino acid residues account for 84 % of the protein. Assuming carbohydrate and tryptophan contents of 10 % and 3 %, respectively, amino acid recoveries of about 97 % were obtained.

Table 1. Amino acid analyses of colostrum IgA by the method of Spackman, Stein and Moore.[26] The values are expressed in per cent amino acid residues of the protein.

Amino acid	Time of hydrolysis in hours				Mean
	22	22	72	144	%
Asp	6.94	6.95	6.88	6.99	6.94
Thr	6.72	6.80	6.18	5.55	6.99[a]
Ser	6.71	6.74	5.56	4.17	7.34[a]
Glu	9.74	9.82	9.72	9.87	9.79
Pro	5.97	5.84	6.13	5.88	5.96
Gly	3.28	3.25	3.31	3.30	3.29
Ala	3.79	3.77	3.80	3.81	3.79
½ Cys	2.40	2.88	2.44	2.15	2.74[a]
Val	5.63	5.81	6.39	6.38	6.39[b]
Met	0.55	0.56	0.57	0.58	0.57
Ile	2.11	2.24	2.22	2.15	2.18
Leu	8.01	7.74	7.06	7.83	7.66
Tyr	5.18	4.01	4.12	3.54	4.77[a]
Phe	3.83	3.60	3.79	3.62	3.71
Lys	4.67	4.62	4.40	4.50	4.55
His	1.83	1.85	1.65	1.77	1.78
NH_3	2.06	1.65	4.43	1.69	0.77[a]
Arg	4.07	4.94	4.97	4.87	4.71
					83.91

[a] Extrapolated to zero hydrolysis time, assuming a first order reaction for the destruction.
[b] Mean of values at 72 and 144 h.

Aspartic acid and glutamic acid were found to occupy the N-terminal positions of the protein as determined by Sanger's method. The proportion of aspartic to glutamic acid was 1:1. Trace amounts of three other DNP-derivatives were also found.

DISCUSSION

The present method for the isolation of colostrum IgA involves a mild treatment of the material and is suitable for preparations of the protein on a gram-scale. Furthermore the procedure yields a material with a more complete representation of the IgA population than methods employing ion exchange chromatography where only a selected part of the IgA is obtained in a pure form.[17]

The amino acid composition of the protein is approximately the same as that of IgA in *serum*, reported by Heimburger *et al.*[28] The N-terminal acid residues (Asp and Glu) are also the same as found in three immunoglobulin classes (IgG, IgA, and IgM) in human serum.[29]

The sedimentation pattern indicated a heterogeneity of the protein preparation that might be the result of impurities present, which do not form immunoprecipitates with anti-human colostrum, or be due to an association of IgA molecules. No separation of the 12 S and 17 S components could be obtained by gel filtration on Sephadex G-200, due to almost complete exclusion of both components from the gel phase. A separation might be achieved by recycling on Sephadex G-200 [30] or by gel filtration in the presence of a mild reducing agent, provided that the 17 S component is split into 7 S units. The sedimentation pattern after reduction of the protein with cysteine can well be interpreted in this way. It is quite evident from the reduction experiments with cysteine and penicillamine that the 12 S component is resistant to the action of these reducing agents. This was also noted by Tomasi *et al.*[18]

The sedimentation coefficient of the colostrum differs from that of the main part of the *serum* IgA, which has a sedimentation coefficient of 7 S.[1,31] Investigations of myeloma proteins of the IgA type have shown heterogeneity both in the ultracentrifuge [32,33] ($s_{20,w}$ = 7 S, 10 S, and 14 S) and on starch gel electrophoresis.[32] By thin-layer gel filtration Johansson and Rymo demonstrated such a heterogeneity in two cases of myeloma type IgA.[22] Though very probable it cannot as yet be definitely stated that the proteins with higher sedimentation coefficients are polymer forms of the 7 S protein.

The colostrum IgA might represent a polymerized *serum* IgA or a serum IgA with a sedimentation coefficient of 12 S, or it might be synthesized in the mammary gland. A local synthesis of IgA in the *salivary gland* has been shown by Tomasi *et al.*, who found similar types of IgA common to certain external secretions, *i.e.* parotid saliva, colostrum, and lacrimal secretion.[18,34] The main part of the colostrum IgA isolated by Tomasi *et al.* had a sedimentation coefficient of 11.4 S which is in accordance with the findings of the present authors. Immunological specificity of the colostrum IgA compared to *serum* IgA has been demonstrated by Hanson [13] and more recently by Tomasi *et al.*[18] South *et al.*[35] quite recently suggested that the specific structure of colostrum and saliva IgA was due to a protein, attached to the IgA in the gland. This protein, designated "transport piece", most probably contains the specific IgA determinant in colostrum.

Acknowledgements. We are indebted to Mrs. Birgit Johansson and Miss Inger Karlsson for skilful technical assistance. This investigation was financially supported by the *Swedish Medical Research Council*, grant T277.

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Received June 23, 1966.