Electron Microscopy and Physical Parameters of Human Blood Group i, A, B, and H Antigens†

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ABSTRACT: Glycoproteins with blood group i antigenic activity have been purified by affinity chromatography from human plasma and amniotic fluid. These affinity purified antigens and conventional preparations of blood group A, B, and H substances from ovarian cysts have been examined by various physical methods. Electron micrographs of shadow-cast preparations showed rod-shaped molecules with a width of 3.0 nm in all preparations. These molecules in the preparations of blood group substances, A, B, H, and amniotic i antigen, were very heterogeneous in length, ranging from 50 to over 500 nm, but the majority of molecules of the plasma i antigen had lengths which fell into a narrow distribution with a number average of 75 nm. By sedimentation equilibrium ultracentrifugation, the plasma i antigen was found to consist of two components, one with a molecular weight of 153,000, probably the predominant molecule, and one with a molecular weight of 317,000, which may represent a dimer. Gel filtration, acrylamide gel electrophoresis, and sucrose density gradient ultracentrifugation of the plasma and amniotic i antigens and the A antigen showed the plasma i to be considerably smaller and more homogeneous than the amniotic i and the A antigens, in agreement with the electron microscopy findings. Gel filtration and acrylamide gel electrophoresis gave anomalously high estimates of the molecular weights of all these antigens. These results are consistent with a model for the blood group antigens of a semirigid linear molecule having a single polypeptide backbone and frequent carbohydrate side chains radiating outward along the length of the molecule. It is possible that the amniotic i, and A, B, and H antigens and a minor population of plasma i molecules are composed of a series of linear polymers of a basic subunit which is analogous to the shorter and more homogeneous 153,000 molecular weight plasma i molecules.

There has been considerable interest in the human blood group glycoproteins. These molecules have been found in soluble form in numerous normal body fluids (Marcus, 1969), in ovarian cysts (Morgan, 1970), and are also present as components of the membranes of red cells (Poulik and Lauf, 1968; Whittemore *et al.*, 1969; Marchesi and Andrews, 1971) and possibly other types of cells. Since these molecules exist in alloantigenic forms, they have practical importance in the areas of transfusion, maternal-infant interaction, and organ transplantation. There have been correlations between blood group type disease incidence (Marcus, 1969) and alterations of blood group antigens have been found in some human neoplasms (Davidsohn *et al.*, 1971).

Although a considerable body of information has been amassed in recent years about the chemical composition of the soluble blood group glycoproteins (Morgan, 1970), the sequence of the carbohydrate chains (Watkins, 1966; Lloyd *et al.*, 1968), the structural basis of the antigenic allotypes (Kabat, 1970), and the genetic mechanism of control of biosynthesis (Grollman *et al.*, 1970; Watkins, 1970), little is known about macromolecular configuration of these antigens. Analysis of the physical parameters of blood group substances is hampered by their physical polydispersity (Marcus, 1969). Molecular weights ranging as high as $1-2 \times 10^6$ have been reported and physical studies have indicated that these antigens are highly asymmetric (Gibbons *et al.*, 1970).

In this study, we have examined by electron microscopy and other physical methods human blood group i antigen molecules, which were specifically purified from plasma and amniotic fluid using a solid-state immunoadsorbent, and conventional preparations of human A, B, and H substances.

Materials and Methods

Blood Group Antigens. The presence of glycoprotein antigen in human amniotic fluid capable of inhibiting human cold agglutinins has been previously described (Cooper, 1970). Recent studies (Cooper *et al.*, 1974) have shown that this antigen is more potent in inhibiting anti-i cold agglutinins (those that react preferentially with fetal as compared with adult red cells) than anti-I cold agglutinins (which react preferentially with adult red cells). In addition, a glycoprotein has been found in normal plasma with very strong i specificity (Cooper and Brown, 1973). The purification of these two forms of i antigen will be reported in detail elsewhere (Cooper *et al.*, 1974).

Briefly, amniotic fluid, obtained from normal deliveries, was concentrated 10× (Diaflo, Amicon Corp., Lexington, Mass.) and was dialyzed against 0.05 M Tris-HCl–0.10 M NaCl at pH 8.2 (Tris-NaCl buffer). Plasma was obtained from out-dated type O blood. A glycoprotein-rich extract of the plasma was made by precipitation of the proteins with perchloric acid (final concentration 0.75 M), and the supernatant was concentrated 10× and dialyzed against Tris-NaCl buffer. An immunoadsorbent was prepared by coupling 30 mg of a purified high titer cold agglutinin (Cooper, 1968) to 100 g (wet weight) of CNBr-activated Sepharose 2B (Porath *et al.*, 1967). The cold agglutinin employed had a greater affinity for the red cell i antigen than the I antigen, as determined by adsorption studies with adult and cord red blood cells (Cooper and Brown, 1973).

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This immunoadsorbent was used in a batch procedure for the purification of the i antigen from the concentrated glycoprotein-rich fraction of the plasma as well as that from concentrated amniotic fluid. The basic principle of the purification step was that the i antigen molecules were specifically and strongly bound to the immunoadsorbent in the cold and were eluted upon warming. Based on Lowry *et al.* (1951) protein determination and antigenic activity (see below) it was determined that the i antigen from the amniotic fluid had been purified over 10,000× and the i antigen from the plasma had been purified over 100,000× (Cooper *et al.*, 1974). Immunoelectrophoresis of these two purified antigens, at a concentration of 1 mg/ml against a goat anti-whole human serum, showed only a single weak precipitin line, in each case corresponding to the stronger precipitin line seen with an anti-i serum. Likewise, sodium dodecyl sulfate–acrylamide gel electrophoresis (see below) of 100 μg of each type of i antigen failed to show any protein bands after Coomassie Blue staining, while at the same time 10 μg of plasma protein showed nine bands and 0.5 μg of albumin was easily detected. Because of the high degree of purity most of the studies were done with these i antigens.

Blood group A, B, and H substances, prepared by extraction from human ovarian cyst fluids (Watkins, 1966), were kindly supplied by Professor Winifred Watkins. Since these antigens had not been purified by affinity chromatography, it was likely that contaminant molecules would be present. For this reason physical studies, other than electron microscopy, were performed only on the A antigen and this antigen was monitored by specific A activity rather than by nonspecific protein or carbohydrate assays. For electron microscopy of the A antigen, the A activity of sucrose density gradient fractions (see below) was used as a guide in the correlation of structure with specific A antigen.

Electron microscopy was performed on unfractionated B and H antigens and was meant only to be confirmatory of the structures seen in the purified i preparations and the fractionated A preparation.

Antigenic Activity. An automated quantitative hemagglutination-inhibition method (Technicon AutoAnalyser) was used for assaying the activity of the amniotic and plasma i antigens and the A antigen (Cooper and Brown, 1973). The soluble i antigens inhibited the agglutination of group O red cells by anti-i, and the A antigen inhibited the agglutination of group A red cells by anti-A. Since these inhibition reactions require only nanogram amounts of the antigens, they could be readily used to quantitate the i and A antigens in eluates of acrylamide slices or fractions from a sucrose density gradient or from a gel filtration column in order to locate the antigen. The aliquots of affinity purified plasma i and amniotic i and the partly purified A antigen were used as standards in the respective hemagglutination-inhibition assays, based on activity per unit weight.

Gel Filtration. Aliquots of purified amniotic and plasma i antigen and of blood group A antigen were applied sequentially to the top of a 100 × 2.5 cm column of Sephadex G-200 and were eluted with the Tris–NaCl buffer at 25°. Ten-milliliter fractions were taken (flow rate, 15 ml/hr), and an aliquot of each column fraction was assayed for the appropriate antigen activity. The appropriate active fractions were pooled and concentrated (Diaflo) and applied to a 100 × 2.5 cm column of Bio-Gel A-5. These were also eluted with Tris–NaCl buffer (flow rate, 40 ml/hr) and 10-ml fractions were taken and aliquots were assayed for antigenic activity. The column was subsequently calibrated with Blue Dextran, purified 19S IgM, and purified 7S IgG. All subsequent studies of the plasma i antigen were performed on the major peak (Figure 1b) which was retarded on the Bio-Gel A-5 gel, and the small early peak containing larger molecular weight molecules was discarded.

Acrylamide Electrophoresis. Sodium dodecyl sulfate–acrylamide gel electrophoresis was carried out essentially as described by Weber and Osborn (1969) except that pretreatment with mercaptoethanol was omitted and the gels consisted of 2.6% acrylamide and 0.13% bisacrylamide. Preelectrophoresis was carried out for 1 hr before loading with 0.1% sodium dodecyl sulfate in the tray buffer. The electrophoresis was performed for 3 hr during which time the bromophenol tracker migrated about 8 cm. The gels containing the i and A antigens were not stained but were removed from the tubes and sliced. The migration distances of the trackers were measured for each gel. Each successive slice was macerated in 1 ml of 0.01 M phosphate buffered saline (pH 7.2) and, after 12 hr of elution at 4°, the tubes were centrifuged and the supernatants dialyzed against the phosphate-buffered saline. An aliquot of each dialyzed eluate was assayed in the AutoAnalyser for antigenic activity. The relative fractional distance traveled compared to tracker position (R_F) for each blood group antigen was calculated from the position in the gel of maximum antigenic activity. Control gels were run with samples of bovine serum albumin, human IgG, 16S RNA, and 23S RNA as calibration standards.

Due to the necessity for empirical calibration in the range above 100,000 daltons, and to the lack of proteins in the size range 100,000 to 10^6 daltons, we used RNA and RNP preparations for sodium dodecyl sulfate electrophoresis and sedimentation studies, respectively, since these are well characterized physically, available in pure form in our laboratory, and have been found to deviate very little from the extrapolation of the protein calibration curve. In any case no quantitative conclusions are drawn regarding glycoproteins because of their anomalous behavior on gels relative to proteins of equal molecular weight.

Sucrose Gradient Fractionation. Fractionation of glycoprotein A and i antigens was carried out on 4-ml, 5–10% sucrose gradients, in a Beckman SW 39 head at 120,000*g* for 3 hr in 0.15 M ammonium acetate (pH 6.8). The positions of antigens in the A and i containing gradients were determined by assaying fractions for hemagglutination-inhibition activity using the AutoAnalyser. The positions in control gradients of purified 19S IgM and 5S–30S ribosomal RNA and RNP standards were determined by pumping fractions of each gradient through an ultraviolet (uv) recording system in which the position on the tracing was correlated with position in the gradient.

Electron Microscopy. Electron microscopy was carried out on a Siemens 1A electron microscope at 80 kV with a 70-μm objective aperture and a magnification of 27,000×. Calibration was based upon photographs of indanthrene olive crystals (Labaw, 1964). Micrographs were recorded sufficiently close to focus to make metal grain at the 2.0-nm level clearly resolved on the original plates. Plates were enlarged four times photographically using a contact intermediate to reverse contrast, so that the shadowing metal would appear light. Solutions of glycoproteins, dialyzed exhaustively against 0.15 M ammonium acetate, were sprayed through a high-pressure spray gun at freshly cleaved mica at a concentration of about 0.1 mg/ml. Preparations were shadow cast with platinum by rotary shadowing as described previously (Slayter and Lowey, 1967).

In making the measurements from the micrographs, a number of entire fields were measured in order to reduce selectivity, and sufficient total numbers were obtained to give smooth distribution curves. From other similarly prepared samples of

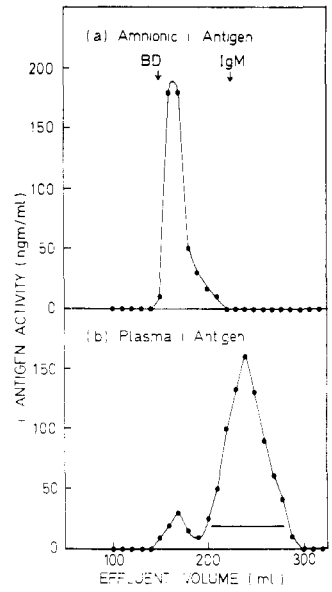

FIGURE 1: Bio-Gel A-5 filtration of (a) amniotic i antigen and (b) plasma i antigen. The affinity purified antigens were applied to the top of the column (100 × 2.5 cm) which was eluted with 0.05 M Tris–0.10 M NaCl (pH 8.2) at a flow rate of 40 ml/hr. Ten-milliliter fractions were taken and, based on purified plasma i standard, the i antigenic activity of an aliquot of each fraction was measured by the automated hemagglutination-inhibition method described in the text. Blue Dextran (BD) and IgM were used as calibration points, as was IgG (which eluted with a peak at 370 ml). The bar in Figure 1b indicates the plasma i fractions which were pooled and used for subsequent studies.

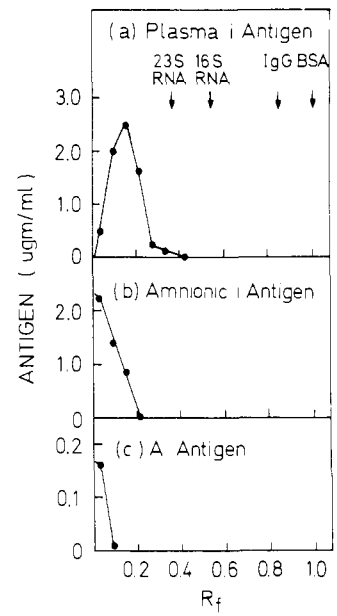

FIGURE 2: Sodium dodecyl sulfate-acrylamide gel electrophoresis of (a) plasma i antigen, (b) amniotic i antigen, and (c) A antigen. The gels contained 2.6% acrylamide, 0.13% bisacrylamide, and 0.1% sodium dodecyl sulfate and were electrophoresed for approximately 3 hr. The antigen containing gels were sliced and each slice was eluted into 0.01 M phosphate buffered saline (pH 7.2). The eluates were dialyzed against the same buffer and the appropriate i or A antigen content was assayed by hemagglutination inhibition, using as standards the purified plasma i, amniotic i, and A antigen, respectively. Control gels contained 23S RNA, 16S RNA, IgG, and bovine serum albumin whose positions were determined by uv scanning or staining. Each mobility was related to that of the tracker run with each gel.

other molecules of known size and shape, we have determined (H. S. Slayter, unpublished results) that a correction of 2.5 nm must be applied in order to compensate for the thickness of the distortion in size of the original particles by the replicating cap of metal. A discussion of limits of error in this type of measurement appears elsewhere (Slayter and Codington, 1973). Negatively contrasted specimens of crude glycoprotein were prepared as described previously (Slayter *et al.*, 1963).

Molecular Weight by Sedimentation Equilibrium. A sample of purified plasma i antigen (400 μg/ml) was dialyzed against several changes of buffer (0.05 M Tris-HCl (pH 7.5), with 6 M guanidine hydrochloride, 2.5 mM dithiothreitol, and 1 mM EDTA) for 72 hr. The specific gravity of the last dialysate was determined with a picnometer. Short column equilibrium sedimentation (Yphantis, 1964) was performed on 100 μg of the plasma i antigen using the Beckman Model E ultracentrifuge at an equilibrium speed of 13,177 rpm and a temperature of 20°. The partial specific volume ($\bar{v}$ = 0.66) of the plasma i antigen was calculated from values for the carbohydrate and amino acid composition (Cooper *et al.*, 1974).

Results

Gel Filtration. Blood group i antigen purified from amniotic fluid and from plasma and blood group A antigen all emerged at the exclusion volume of the Sephadex G-200 column in the same region as control purified 19S IgM. The active fractions for each blood group antigen were pooled, concentrated, and applied to the Bio-Gel A-5 column. The exclusion volume of the column was found to be approximately 140 ml using Blue Dextran. The amniotic i antigen emerged with peak activity at 150–180 ml of effluent, but some activity trailed through 210 ml (Figure 1a). The plasma i antigen showed a small peak at the same position as the amniotic i, but the bulk of the activity was retarded, emerging with a peak at 210–280 ml (Figure 1b). The bar indicated those fractions which were pooled and used for all other studies of the plasma i antigen. Control 7S IgG emerged with a peak at 370 ml.

This difference in elution volumes of amniotic and plasma i antigens was confirmed on repeat runs. In addition, i antigen obtained by perchloric acid extraction of concentrated amniotic fluid emerged at the same position as i antigen purified directly from concentrated amniotic fluid. Plasma i antigen which had not been perchloric acid extracted emerged at the same position as acid extracted antigen, demonstrating that the size difference between the amniotic and plasma forms of the i antigen could not be attributed to the perchloric acid step used in the purification of the latter. There was no alteration in the antigenic activity of the plasma i antigen after the perchloric acid extraction (Cooper and Brown, 1973).

In the gel filtration experiments shown here, only microgram quantities of the i antigens were applied to the columns since the elution was monitored by antigenic activity obviating the monitoring of OD_{280} material. However, during purification of the i antigens (Cooper *et al.*, 1974) several milligrams of each antigen were applied to these same columns and the OD_{280}, as well as the i activity, were monitored on each fraction. A small amount of OD_{280} was found only in those tubes containing the peak of antigenic activity.

Blood group A antigen emerged from the Bio-Gel A-5 in a pattern almost identical with that of the amniotic i antigen. Reduction and alkylation of the A antigen with 0.01 M dithiothreitol and 0.02 M iodoacetamide caused no change in the elution position of the A antigen.

Sodium Dodecyl Sulfate-Acrylamide Electrophoresis. The electrophoretic positions of the i and A antigens, determined from the serologic activity in eluates of gel slices, are shown in Figure 2. The position of control markers was determined by uv scanning at 260 nm for RNA and by Coomassie Blue stain-

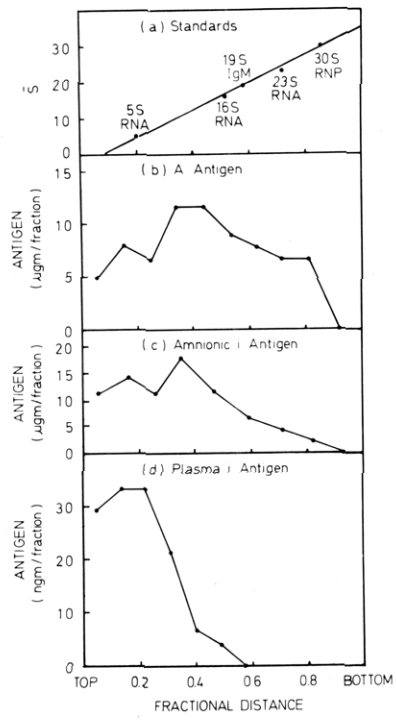

FIGURE 3: Sucrose density gradient ultracentrifugation. Four-milliliter gradients (10–20% sucrose) containing (a) RNA and protein standards and (b–d) A, amniotic i, and plasma i, respectively, were spun for 3 hr at 120,000*g*. The positions of the standards were determined by assaying the appropriate i or A antigen content in an hemagglutination-inhibition system, using as standards purified plasma i, amniotic i, and A antigen, respectively (RNP, ribonucleoprotein).

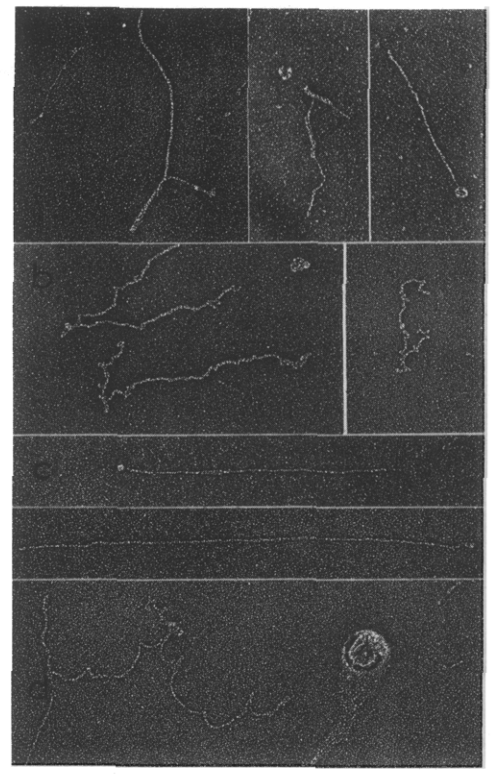

PLATE 2: Amniotic i antigen fractionated on a sucrose gradient and rotary shadow cast with platinum: (a) supernatant; (b and c) upper and lower gradient regions, respectively; (d) pellet material which showed practically no antigenic activity (see Figure 3c); magnification 37,500×.

ing for protein. Whereas the plasma i antigen (Figure 2a) entered the gel with a measured R_F of 0.14 at the peak of activity, the amniotic i (Figure 2b) and A antigens (Figure 2c) were found to enter the 2.6% gels only partially under conditions that resulted in the migration of the largest control particle, 23S rRNA (mol wt 1.1 × 10^6) with an R_F of 0.35. The total recovery of plasma i by extraction from crushed gel slices was about 40% compared with a recovery of 17% for the amniotic i and only 0.3% for the A antigen, indicating that very little of the larger glycoproteins penetrated the gel at all.

Sucrose Gradient Ultracentrifugation. Sucrose gradient fractionation was carried out on A antigen and plasma i and amniotic i antigens in order to establish an approximate sedimentation coefficient for each species (Figure 3). Fractions of each gradient were assayed for the appropriate antigenic activity with the AutoAnalyser. Based on empirical calibration with various particles of known sedimentation values (Figure 3a) the A antigen activity peaked at about 10–15 S (Figure 3b), amniotic i peaked at about 10 S (Figure 3c), and plasma i peaked at about 5 S (Figure 3d). Plasma i antigen appeared substantially more homogeneous than the other two antigens which sedimented over a broad range.

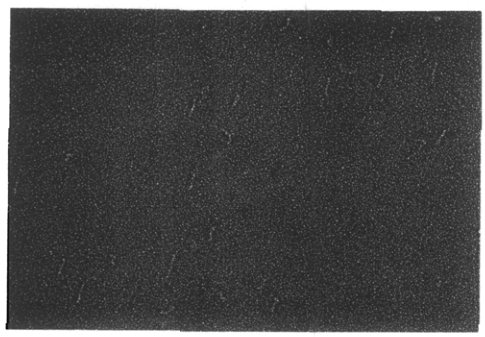

PLATE 1: plasma i antigen rotary shadow cast with platinum, 37,500×.

Electron Microscopic Results. Plasma and amniotic i antigens and blood group A, B, and H substances all consisted of rods with an average width of about 3.0 nm after correction for the thickness of the shadowing metal. A small fraction of the rods were thicker than the average. The plasma i antigen (Plate 1) was the most homogeneous and consisted of short rods about 75 nm in length. The amniotic i antigen preparation consisted of various length rods, sometimes stretched out and sometimes flexed. In addition, there were membranous blebs ranging up to 100 nm in diameter. In order to correlate structure with antigenic activity, various fractions of the sucrose gradient ultracentrifugation of the amniotic i antigen were examined by electron microscopy. The supernatant (Plate 2a) consisted largely of thick rods, suggesting adherence of lower density material, perhaps lipid. The material from the region of the gradient corresponding to the main portion of antigenic activity consisted of rods with lengths varying from 50 to over 1000 nm, the longer rods concentrating toward the bottom of the gradient (Plate 2b,c). The pellet (Plate 2d), which lacked antigenic activity, contained the membranous blebs seen in the unfractionated material.

The A antigen was also examined after sucrose gradient fractionation. The supernatant contained small particles which were shown to be human serum albumin by electrophoretic molecular weight determination on sodium dodecyl sulfate–acrylamide gels, and by immunodiffusion against anti-human albumin. Optical density at 280 nm was monitored and found significant only in the supernatant region corresponding to that shown to contain human serum albumin by the other methods. The remaining fractions consisted of various sized rods similar

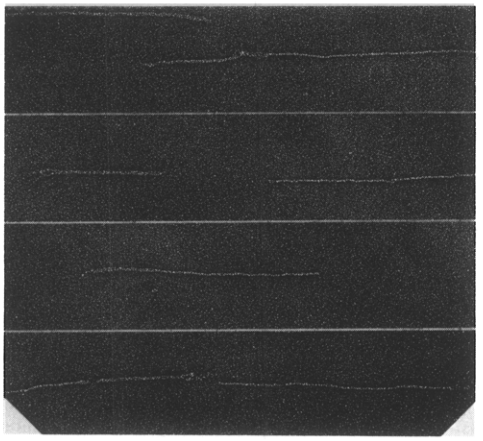

PLATE 3: A antigen purified by sucrose gradient fractionation (from near the bottom fraction of the gradient; see Figure 3b), rotary shadow cast with platinum; magnification 37,500×.

in appearance to the amniotic i antigen. The bottom of the gradient consisted of the very long rods (Plate 3). While the use of antigenic activity and optical density measurements does not rule out the possibility that extraneous glycoprotein might be included in these fractions, the fact that only rods of relatively high axial ratio were found in the region of highest activity indicates, in any case, that long rods contain the A antigenic activity. The blood group B and H preparations consisted of rods similar to those of the amniotic i and the A antigens and also contained some small particulate material. No attempt was made to fractionate the B and the H antigens further.When amniotic i antigen was examined by negative contrast, only the contaminating membranous blebs were seen since the 3.0-nm diameter rods were invisible under these conditions, as would be expected.

Figure 4 shows distributions of length for the various preparations examined. The striking finding was the uniformity of the plasma i antigen molecules. Table I summarizes the peak and average values for the dimensions of all the blood group antigens.

Sedimentation Molecular Weight. Sedimentation equilibrium analysis of plasma i antigen in 0.05 M Tris-HCl (pH 7.5), with 6 M guanidine-HCl, 2.5 mM dithiothreitol, and 1 mM EDTA, suggested sample polydispersity based on a plot of half the radius squared ($r^2/2$) *vs.* the natural logarithm of the concentration (ln *C*), as illustrated in Figure 5. This plot is slightly concave upward indicating more than one component. Plots of $1/\sigma_n$ and of $1/\sigma_w$[1] *vs.* concentration extrapolated in infinite dilution (Figure 6) intercepted the axis at almost the same point ($\sigma_{n,o} = 2.914$; $\sigma_{w,o} = 2.950$) indicating molecular weights of 153,012 and 154,937, respectively. The plot of σ_w *vs.* $1/\sigma_n$ was linear (correlation coefficient 0.996), indicating two components of molecular weights 153,039 and 317,092, respectively (Roark and Yphantis, 1969; Yphantis, 1964). This independent high degree of correlation with σ_n and σ_w allows considerable confidence in the value for the smaller component.

Discussion

Two special serological techniques were used which greatly facilitated this study. Firstly, the i antigens from amniotic fluid and plasma were specifically purified using a solid state immunoadsorbent consisting of IgM anti-i covalently bound to Se-

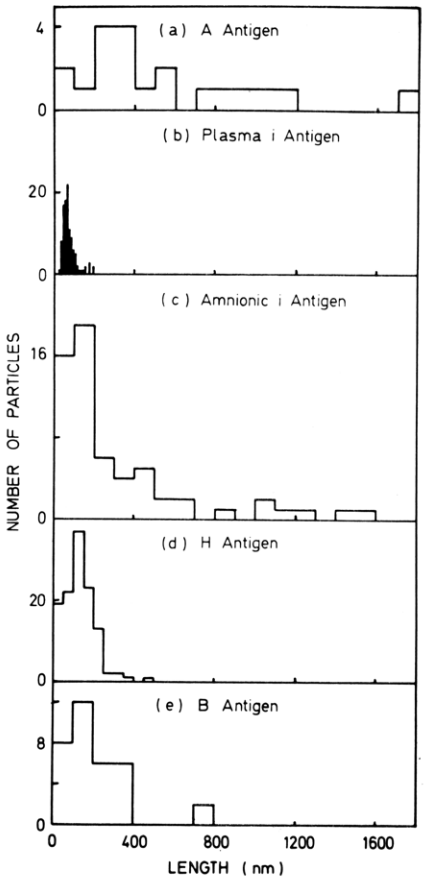

FIGURE 4: Frequency histograms of electron microscopy length measurements of (a) A antigen; (b) plasma i antigen; (c) amniotic i antigen; (d) H antigen; and (e) B antigen.

pharose. Because the binding of the i antigens to the anti-i was cold-dependent (Cooper and Brown, 1973), purification was readily achieved by mixing the crude i antigen with the immunoadsorbent at 4°, washing the immunoadsorbent at 4°, and eluting off the purified i antigen with 37° buffer. The mildness of this procedure minimized the chance for introducing artifacts in these molecules. An additional benefit of using this immunospecific method of purification is that the final preparations of i antigens contained only molecules having the antigenic groups, whereas conventional extraction methods are based on physical-chemical properties rather than the presence or absence of specific antigenic groups.

The second special technique employed throughout this study was the use of a quantitative automated hemagglutina-

TABLE I: Electron Microscopic Dimensions of Blood Group Antigens.

Material	N^a	Peak Length (nm)	No. Av Length (nm)	Wt Av Length (nm)	Peak Width (nm)
Plasma i antigen	110	65	75	90	3.0
Amniotic i antigen	64	160	370	920	3.0
A antigen	20	350	540	890	3.0
B antigen	31	150	190	300	4.0
H antigen	120	140, 390	180	280	3.0

[a] N = number of particles measured.

[1] Abbreviations used are: σ_n, number average apparent reduced molecular weight; σ_w, weight average apparent reduced molecular weight (Yphantis, 1964); RNP, ribonucleoprotein.

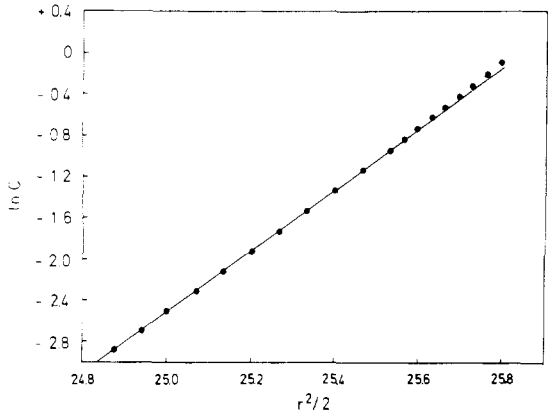

FIGURE 5: Plot of sedimentation equilibrium data for plasma i antigen. The concavity upward indicates more than one species is present; solvent, 0.05 M Tris-HCl (pH 7.5) with 6 M guanidine-HCl, 2.5 mM dithiothreitol, and 1 mM EDTA; C, concentration; r, radius.

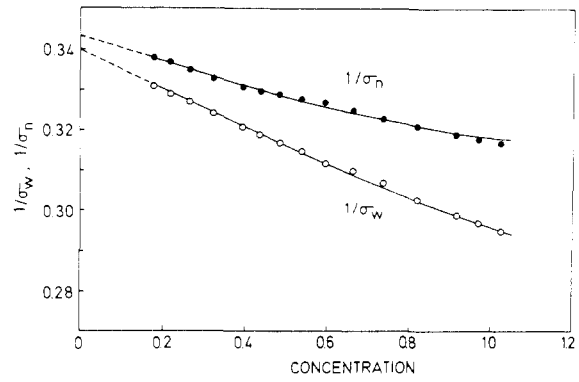

FIGURE 6: Sedimentation equilibrium for plasma i antigen. Concentration distribution indicating heterogeneity. At infinite dilution (C = 0), $\sigma_{n,o}$ = 2.914, and $\sigma_{w,o}$ = 2.950, demonstrating only one molecular species is seen with a molecular weight of about 153,000.

tion-inhibition technique (Cooper and Brown, 1973) to measure the i and A blood group antigens. This method utilized the fact that nanogram amounts of the soluble glycoprotein antigens were able to specifically inhibit the agglutination of red cells by the appropriate antibody. Thus, in this study, microgram amounts of antigen could be fractionated by gel filtration, sodium dodecyl sulfate-acrylamide electrophoresis, or sucrose density gradient centrifugation and the subsequent distribution of antigen molecules in aliquots determined by this automated technique. This was important with regard to the specifically purified i antigens, which were available only in small amounts, and was also useful for monitoring specific A antigen in the presence of possible contaminants.

A significant result of this study was the electron microscopy demonstration that the blood group antigen molecules examined were thin linear rods which appeared semirigid. These molecules, regardless of antigenic specificity, were fairly uniform in width, with an average of about 3.0 nm. The amniotic i antigen and the A, B, and H antigens were all quite heterogeneous in length consisting of molecules varying from 50 to over 500 nm long. In striking contrast, the i antigen molecules purified from the plasma, and freed of a minor fraction of larger molecules by Bio-Gel A-5 gel filtration, were far more uniform with a number-average length of 75 nm. Gel filtration, sodium dodecyl sulfate-acrylamide electrophoresis, and sucrose density ultracentrifugation experiments on the two types of i antigen and on blood group A antigen all were consistent with the electron microscopy observations on relative size of the three antigens.

Sedimentation equilibrium was performed only on the plasma i antigen because of the obvious heterogeneity of the other antigens. The major Bio-Gel A-5 fraction of plasma i antigen consisted of two components, with molecular weights of 153,000 and 317,000, respectively. The electron microscopy examination leads us to believe that the smaller component is the principal one present. From the known percentages of protein and carbohydrate and from the amino acid composition for the plasma i antigen (Cooper *et al.*, 1974), it can be calculated that a molecule of the i antigen with a molecular weight of 153,000 would contain approximately 350 amino acids. If these were aligned in a polypeptide chain in the fully extended configuration, the predicted length (0.36 nm/amino acid residue) would be 126 nm, which is somewhat longer than the number average length of 75 nm for the plasma i antigen molecules as measured from the electron micrographs. The reason for this difference is not known; it is possible that the polypeptide chain is not fully extended.

The 317,000-dalton component is likely to represent a dimer of the smaller molecule. We did not observe in the electron micrographs a second population of longer plasma i molecules. It is possible that such a minor population was present but not observed. Another possibility is that the small fraction of thicker rods seen represents side-to-side dimerization. If the assumption is made that the amniotic i and A, B, and H antigens have a molecular weight per unit length similar to the plasma i antigen, an estimate of their molecular weight can be made from their electron microscope lengths. These range from about 75,000 to 2×10^6 with variation for the different antigens.

The blood group i and A antigens showed early emergence from gel filtration columns and penetrated poorly into sodium dodecyl sulfate-acrylamide gels relative to protein controls. This anomalous behavior was best documented for the smaller plasma i molecules for which sedimentation equilibrium, sucrose density ultracentrifugation, and electron microscopy data indicated a much smaller molecule than would be estimated by the gel filtration and gel electrophoresis methods. Segrest *et al.* (1971) demonstrated that sodium dodecyl sulfate-acrylamide electrophoresis of glycoproteins gives an anomalously high molecular weight when compared with protein standards and this anomalous behavior is exaggerated when gels with low per cent acrylamide were used, as was necessitated in our study in order to have any penetration into the gels. This poor penetration could be explained by the low binding of the negatively charged sodium dodecyl sulfate to glycoproteins relative to proteins, as demonstrated in the study of Segrest *et al.* (1971) and by the linear, semirigid type of structure indicated by our electron microscopy observations. This latter factor would also explain the early emergence of the blood group i antigen from gel columns.

Our findings lend further support to a model for the blood group antigens based on a single partially extended polypeptide chain backbone with short carbohydrate side chains radiating outward at frequent intervals. It seems likely that the presence of adjacent side chains is responsible for the linear configuration and apparent rigidity of the molecule. It is possible that a molecule with dimensions similar to those for the 153,000 molecular weight plasma i antigen could be a basic biosynthetic subunit for the larger blood group molecules.

There was a distinct similarity between the electron microscope appearance of the soluble blood group antigens reported here and the cell surface glycoproteins removed by trypsin cleavage from TA-3 murine mammary carcinoma cells (Slayter and Codington, 1973). In fact, there was a striking similarity in various parameters measured between the plasma i mole-

cule and the smallest glycoprotein fragment (fraction C) found in the trypsin digest of the TA-3 cells. Parameters compared include $\bar{v}$, width, length, and protein and carbohydrate composition. However, the plasma i was about 50% larger in molecular weight by sedimentation equilibrium. It is possible that the physical properties of the soluble blood group molecules, which are probably of secretory origin, also pertain to some of the blood group glycoproteins intercalated into the plasma membrane (Marchesi and Andrews, 1971). It should be possible, using affinity chromatography methods, to purify glycoprotein molecules having i antigen groups from solubilized plasma membranes and to compare their properties with the soluble molecules described here.

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