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Australia Antigen: Large-Scale Purification from Human Serum and Biochemical Studies of Its Proteins

J. L. GERIN, P. V. HOLLAND, AND R. H. PURCELL

Rockville Laboratory[1] of the Molecular Anatomy Program, Oak Ridge National Laboratory,[2] Rockville, Maryland 20852; Laboratory of Infectious Diseases, National Institute of Allergy and Infectious Diseases; and the Blood Bank Department, Clinical Center, National Institutes of Health, Bethesda, Maryland 20014

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Biophysical techniques are described for the large-scale isolation of Australia antigen (Au) from unit quantities of human serum by using the batch-type zonal centrifuge rotors. A three-step procedure involving isopycnic banding of the particle in CsCl density gradients and rate-zonal centrifugation on sucrose gradients resulted in a highly purified Au preparation which was used for biochemical studies of Au proteins and as immunizing antigen for the production of reagent antiserum in animals. The spherical form of Au, which was devoid of detectable nucleic acid, was composed of two major proteins (AuP1 and AuP2) and a minor protein (AuP3) of 26,000, 32,000, and 40,000 molecular weight, respectively, as determined by acrylamide gel electrophoresis. The significance of these findings to the possibility of Au subtypes is discussed.

Australia (hepatitis-associated) antigen is located on a particle 20 to 25 nm in diameter and is closely associated with serum (long-incubation or type B) hepatitis. Although it has not yet been established that Australia antigen (Au) is an infectious agent, its presence in blood known to transmit the disease and in the acute-phase serum specimens from patients with long-incubation hepatitis has been repeatedly demonstrated (2, 4, 8, 9, 12, 15). This high correlation between Au and type B hepatitis has definite value in clinical diagnosis and for epidemiological studies of the disease. Recently described sensitive serological techniques (14, 16, 17, 20-22) for the detection of Au have important application in the screening of human blood and blood products intended for human parenteral administration.

Large quantities of well characterized and highly purified antigen are needed for biochemical studies of the antigen particle and for the production of high-titered and specific animal antiserum to Au for the standardization of the various serological techniques.

This report describes in detail procedures for the preparation of purified Australia antigen by using the large capacity batch-type zonal centrifuge rotors. Au prepared by these procedures was used as immunizing antigen for the production of reference reagents for Australia antigen (Research Resources Branch, National Institute of Allergy and Infectious Diseases, National Institutes of Health) and for biochemical characterization of the Au particle. Studies of Australia antigen proteins by acrylamide gel electrophoresis are contained in this report.

MATERIALS AND METHODS

Source of Australia antigen. The sera from three units of Au-positive blood obtained by plasmapheresis of a young adult female (J. M) with chronic anicteric hepatitis were stored frozen at −70 C until used for purification of Au.

Zonal centrifugation: B-XXIX rotor. The Oak Ridge B-XXIX, an edge-unloading titanium rotor with a capacity of 1,430 ml, was used for the concentration and purification of Au from serum. The rotor and basic operating procedures were previously described (1). All centrifugation steps were done with the Beckman model L equipped with an $\int \omega^2 dt$ integrator.

Reagents. Cesium chloride (American Potash and Chemical Corp.) was purified as previously described (23). CsCl solutions in 0.01 M phosphate buffer (pH 7.4) were made by dilution of a stock saturated

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solution of CsCl in water. Sucrose solutions were prepared by dissolving crystals (Mallinckrodt, analytical reagent grade) in 0.01 M phosphate buffer (*p*H 7.4) with gentle mixing without warming. All solutions were filtered through 0.22-μm diameter membrane filters (Millipore Corp., Bedford, Mass.) and tested for sterility before use.

First-step isopycnic banding. The first step of purification was achieved by the isopycnic banding of Au in discontinuous CsCl gradients in two parts: the first centrifugation with serum from one unit (250 ml) of J. M. as starting sample and a second with two units (500 ml). In the first experiment, the empty rotor, while rotating at 2,500 rev/min, was loaded from the rotor periphery with one unit of serum diluted to 400 ml with phosphate-buffered saline (PBS; 0.15 M NaCl, 0.01 M phosphate buffer, *p*H 7.4) followed sequentially by 400 ml of 1.10 g/cm³ CsCl, 250 ml of 1.20 g/cm³ CsCl, 200 ml of 1.30 g/cm³ CsCl, and 180 ml of 1.40 g/cm³ CsCl. A 100-ml amount of PBS was then pushed into the rotor through the center line with a syringe, thereby displacing a like volume of 1.40 g/cm³ CsCl cushion from the periphery. The rotor was accelerated to 25,000 rev/min run for 11 hr at 5 C, and allowed to coast without braking to 2,500 rev/min (58.5×10^{10} $\omega^2 t$). The gradient was displaced from the rotor edge by pumping sterile distilled water to the rotor center at about 20 ml/min and 36 40-ml fractions were collected by hand.

For the second experiment, 500 ml of serum diluted to 700 ml with PBS, followed sequentially by 200 ml of 1.10 g/cm³ CsCl, 200 ml of 1.30 g/cm³ CsCl, and 330 ml of 1.40 g/cm³ CsCl, was pumped into the empty rotor from the periphery with the rotor rotating at 2,500 rev/min. An overlay of 150 ml of PBS was then introduced into the rotor through the center line; the rotor was accelerated to 30,000 rev/min and run for $87.5 \times 10^{10} \omega^2 t$ at 5 C. The gradient was displaced and collected as described above.

Second-step isopycnic banding. Fractions containing Au from both of the first-step banding experiments described above were pooled and used as the starting sample for the second-step purification. A 250-ml amount of 1.10 g/cm³ CsCl followed in order by 330 ml of starting sample at a density of 1.20 g/cm³ CsCl, 300 ml of 1.30 g/cm³ CsCl, and 550 ml of 1.40 g/cm³ CsCl was pumped into the empty rotor rotating at 2,500 rev/min. Two hundred milliliters of PBS was introduced through the center line as an overlay solution. The rotor was accelerated to 28,000 rev/min and run at 5 C for 22 hr (146×10^{10} $\omega^2 t$). The gradient was displaced and collected as described above except that the effluent stream was monitored in the Gilford model 240 spectrophotometer at 280 nm with an Oak Ridge flow cell with a 0.2-cm light path.

Rate zonal separation. The B-XXIX rotor was loaded at 2,500 rev/min with a linear 1,000-ml 7 to 25% (w/w) sucrose gradient generated from a Beckman 142 gradient pump followed by 430 ml of 50% (w/w) sucrose cushion. The starting sample as described in the text was injected into the center of the rotor with a 50-ml syringe and was followed by a PBS overlay. The rotor was accelerated to 28,000 rev/min, run for the period of time described in the text at 5 C, and decelerated to 2,500 rev/min to unload the gradient. The gradient was displaced from the rotor periphery by pumping water to the center, and the effluent was monitored for absorbancy at 280 nm by using an Oak Ridge flow cell with a 1-cm light path in a Gilford model 240 spectrophotometer. Thirty-six 40-ml fractions were collected and stored at 4 C until assayed for sucrose concentration and Au antigen activity.

Sedimentation coefficients. Equivalent sedimentation coefficients (S^*) were determined with the aid of the computer program developed at the Oak Ridge National Laboratory (3). Values of S^* were determined for every fraction from the recovered gradient for particles with densities of 1.14, 1.16, 1.18, and 1.20 g/cm³ in sucrose.

Density determination. The density of individual fractions was determined either by direct weighing in tared 100 μliter micropipettes or from curves relating refractive index to density. Refractive indexes were read at 20 C with an Abbe 3L-type refractometer.

Optical density measurements. Those fractions of the gradient from the second-step banding experiment which had optical density values beyond the range of the spectrophotometer were diluted and read manually against a PBS blank in a Gilford 2400 spectrophotometer. These values were multiplied by the dilution factor to give the values shown in the appropriate figure. The ultraviolet absorption curve of the final purified Au was determined manually at 5- to 10-nm intervals in a 0.3-ml microcuvette with a light path of 1 cm against a PBS blank.

Acrylamide gel electrophoresis. Acrylamide, 2-mercaptoethanol (2-ME), and sodium dodecyl sulfate (SDS) were purchased from Eastman Organic Chemicals, Rochester, N.Y.; bisacrylamide for electrophoresis was from Bio-Rad Laboratories, Richmond, Calif.; urea (ultra-pure) and protein markers were from Mann Research Laboratories, Orangeburg, N.Y.; Coomassie Brilliant Blue was from Sigma Chemical Co., St. Louis, Mo.; and sulfosalicylic acid was purchased from Fisher Scientific Co., Fair Lawn, N.J. Acrylamide was crystallized from chloroform and SDS from alcohol before use. Disc electrophoresis was performed by procedures described by Ornstein (13) and Davis (6) by using 7.5% acrylamide gels in a buffer containing 36 mM tris(hydroxymethyl)-aminomethane, 30 mM ethylenediaminetetraacetic acid at *p*H 7.6 to 7.7 in an apparatus supplied by Canalco, Rockville, Md. Twenty-five-microliter samples in 10% (w/w) sucrose and containing bromophenol blue as a tracking dye were applied by underlayering, and the gels were electrophoresed for 2.5 hr at 8 ma/gel. Gels were fixed in 12.5% trichloroacetic acid for 1 hr and stained with a fresh 1:20 dilution of aqueous 1% Coomassie Brilliant Blue in 12.5% trichloroacetic acid for 1 hr (5). Destaining was carried out in 10% trichloroacetic acid for 4 hr, and the gels were scanned at 550 nm by using the linear transport accessory of the Gilford 2400 spectrophotometer. SDS-urea-*p*H 7.6 gels were a modification of those used by Maizel (11), had an acrylamide concentration of 10% with a bis:acrylamide ratio of 1:50, and contained 0.1% SDS, 0.1 M phosphate

buffer (pH 7.6), and 4 M urea. The electrode buffers contained 0.1% SDS and 0.1 M phosphate buffer, pH 7.6; the upper buffer also contained 1% 2-ME. Twenty-five- or 50-μliter samples were applied by underlayering, and the gels were electrophoresed for 4 hr at 5 ma/gel. Gels were fixed as described above except that the 12.5% trichloroacetic acid was replaced by a 5% trichloroacetic acid plus 5% sulfosalicylic acid mixture as suggested by Chrambach et al. (5).

CF. Au was monitored during purification by using a Microtiter complement fixation (CF) test for Au which was described in a previous communication (16). Serum containing antibody to Au was obtained from a multiply transfused patient with hepatitis and was heated to 56 C for 30 min to destroy endogenous complement; purified Au was tested without prior heat inactivation. Four to 8 units of antibody and 1.7 exact units of complement were used in the test; antigen-antibody-complement mixtures were incubated overnight at 4 C before the addition of sensitized sheep erythrocytes.

Electron microscopy. Preparations of purified Au were examined in a Hitachi HU-11E electron microscope at an instrumental magnification of 50,000 and 90,000 $\times$ after negative staining with 2% uranyl acetate.

RESULTS

Isolation of Australia antigen from serum. Purification of Au achieved by the first-step isopycnic banding procedure resulted from the concentration of the complement-fixing antigen in a single zone of activity at a density of 1.20 g/cm^3 in CsCl (Fig. 1). When 500 ml of J.M. (CF titer = 1:4,096) was used as the starting sample, the peak 40-ml fraction recovered from the gradient (Fig. 1) titered 1:32,768 by CF assay representing full recovery of Au activity. The Au-positive fractions from both first-step banding procedures were pooled and used as the starting sample for the second-step isopycnic banding experiment; this pool was designated JMCsl.

Second-step banding of Au in CsCl resulted in further purification as shown in Fig. 2; the antigen was fully recovered in a very sharp zone at a density of 1.20 g/cm^3 well removed from the bulk of the contaminating serum proteins. The peak fraction and one fraction on either side of the peak were pooled; this 120-ml pool was designated JMCs2.

The third-step purification procedure consisted of a rate zonal centrifugation of the Au particles. The removal of CsCl from JMCs2 pool was accomplished by either dialysis at 4 C against PBS or exchange of CsCl by PBS by using the Amicon Diaflo apparatus with an XM-50 membrane at 50 psig of nitrogen pressure; both techniques worked equally well and there was no loss of Au CF antigen activity. In a preliminary experiment, 10 ml of JMCs2 was diluted to 30 ml with PBS and was used as the starting sample for the rate zonal centrifugation. This sample was introduced into the rotor, followed by a 150-ml PBS overlay; the rotor was accelerated to 28,000 rev/min and centrifuged for 3 hr at 5 C (10.3 $\times$ 10^{10} ω^2t). Assuming a particle density of 1.16 g/cm^3 in sucrose (7), Au sedimented as a discrete zone with an equivalent S^* value of 52 (Fig. 3). However, Au was not clearly resolved from the contaminat-

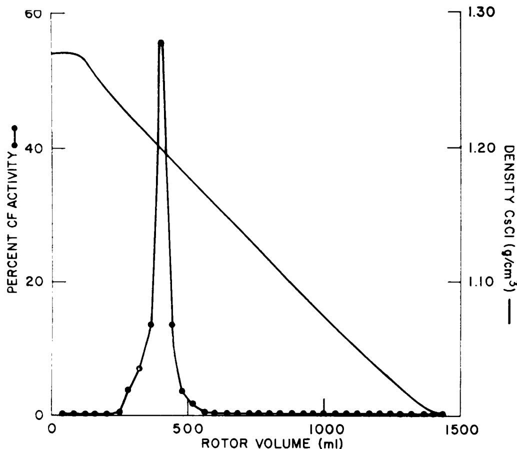

FIG. 1. *First-step isopycnic banding of Au in CsCl from 500 ml of human serum by using the B-XXIX zonal rotor. Sample was centrifuged at 30,000 rev/min for 58.5 $\times$ 10^{10} ω^2t at 5 C.*

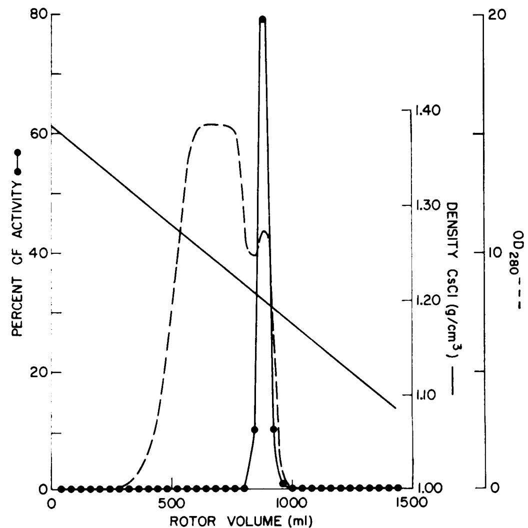

FIG. 2. *Second-step isopycnic banding of Au in CsCl by using the B-XXIX zonal rotor. Pool from first-step banding (JMCsl) was centrifuged at 28,000 rev/min for 22 hr at 5 C (14.6 $\times$ 10^{11} ω^2t).*

ing serum proteins remaining at the origin, and this procedure was repeated by using 50 ml of the dialyzed JMCs2 pool as the starting sample with a 150-ml PBS overlay. Centrifugation for 6 hr at 28,000 rev/min at 5 C (18.2×10^{10} ω^2t) resulted in good separation of Au from serum protein (Fig. 4), and the S^* of 54 was consistent with the value obtained in the preliminary experiment. Fractions corresponding to rotor volume values (Fig. 4) between 320 and 560 ml were pooled and

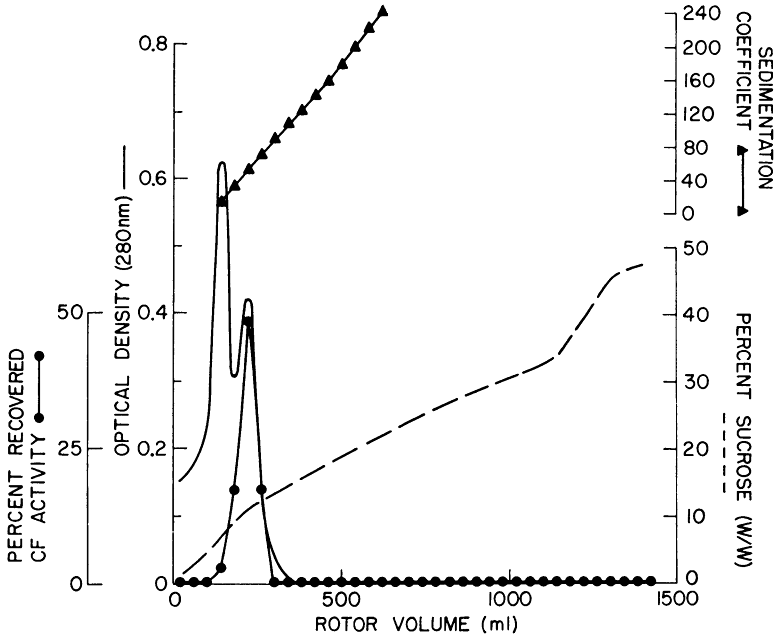

FIG. 3. *Rate zonal centrifugation of JMCs2 pool on sucrose gradient for 3 hr at 28,000 rev/min (10.3×10^{10} $\omega^2 t$).*

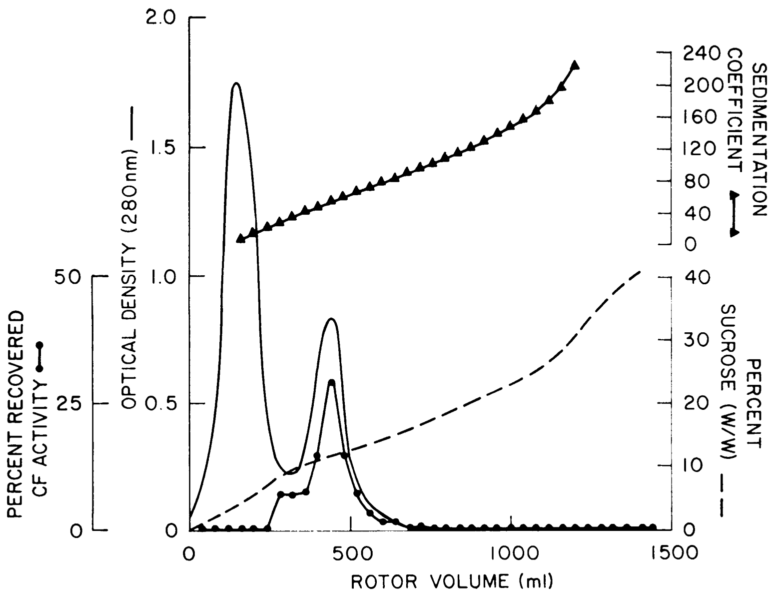

FIG. 4. *Rate zonal centrifugation of JMCs2 pool on sucrose gradient for 6 hr at 28,000 rev/min (18.2×10^{10} $\omega^2 t$).*

diluted with PBS to a volume of 410 ml. Assuming complete recovery of Au throughout the multistep procedure, the concentration of antigen in the pool, designated JMCs2R1, was about three-fourths that of the initial J.M. serum. The actual CF titer of JMCs2R1 was an unreliable estimate of recovery, since fractions rapidly lost titer with time after final purification of the Au in the phosphate-buffered sucrose gradient. The addition of equal portions of the peak Au fraction and peak serum protein fractions did not result in an increase in titer and did not suggest the removal of some small potentiating "cofactor." This loss of titer has been interpreted as due to aggregation of the purified particles (7) and does not diminish its value as an immunogen (16).

Estimation of purity. The purification of Au during the various steps of the above described procedure was evaluated by disc electrophoresis on acrylamide gels, a sensitive technique for the detection of serum proteins. Each of the pools representing different stages in the purification (i.e., JMCs1, JMCs2, and JMCs2R1) were adjusted to the same concentration of Au present in the original J.M. serum. The Au particle itself will not enter the 7.5% gel; however, except for some macroglobulins, unaggregated serum proteins will migrate in a characteristic manner. Absorption scans of stained gels (Fig. 5A-5D) demonstrated the extensive purification achieved by the ρ-s procedures. After the second-step isopycnic banding in CsCl (Fig. 5C), the major remaining serum contaminant was albumin, albeit at a much lower concentration than in the original J.M. serum (Fig. 5A). The final rate zonal step removed all traces of serum proteins detectable by this technique (Fig. 5D). Attempts to detect human serum proteins in the JMCs2R1 preparation by immunoelectrophoresis were also unsuccessful (H. Alter, *personal communication*).

Aseptic techniques were employed throughout the purification procedure, and the final pool of antigen was sterile by routine assay. The large quantity of highly purified Au was used for further biochemical studies and as immunizing antigen for the preparation of a large pool of high-titered animal antiserum. A guinea pig antiserum (V801-501-058) was prepared for the Research Resources Branch of the National Institute of Allergy and Infectious Diseases, NIH; its preparation and

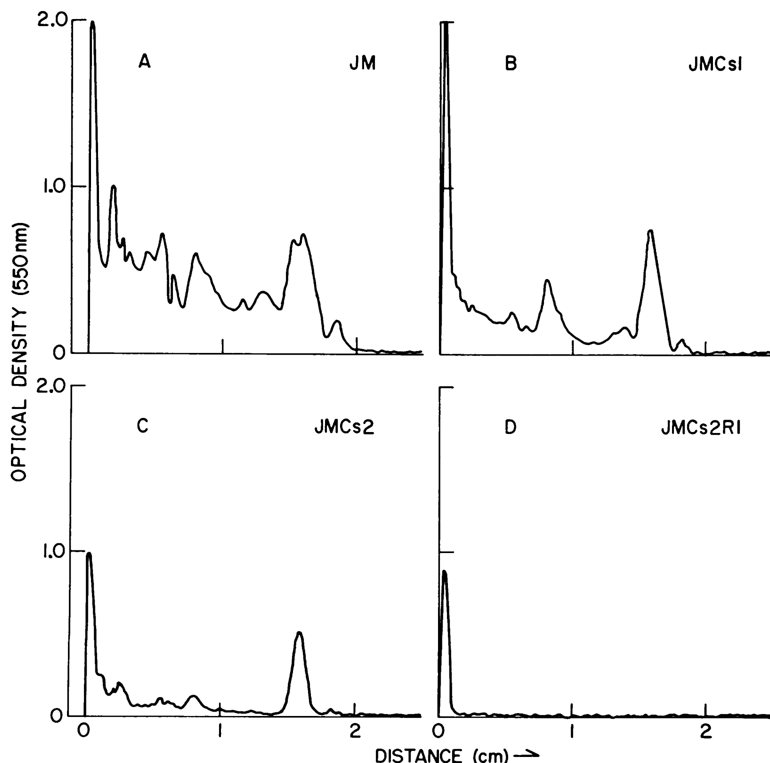

FIG. 5. *Purity of Au preparations as determined by acrylamide gel electrophoresis of normal serum proteins at each step of the purification procedure (A–D). Samples were adjusted to the same concentration of Au and electrophoresed on 7.5% gels at 8 ma/gel. Gels were stained with Coomassie Brilliant Blue and scanned at 550 nm.*

characterization will be described in a following paper (J. Gerin et al., *manuscript in preparation*).

Ultraviolet absorption spectrum. An ultraviolet absorption spectrum of the material in the peak Au fraction from the rate zonal step shown in Fig. 4 was characteristic of protein with a maximum and minimum of 280 and 250 nm, respectively (Fig. 6). The OD 260/280 ratio of 0.67 argues against the presence of nucleic acid in the spherical form of the Au, certainly below the level of one genome per particle.

Electron microscopy of JMCs2R1 revealed only spherical forms of Au, and careful measurements of 100 particles yielded an average diameter of 22 nm (range 21 to 23 nm).

Proteins of Australia antigen. Samples of the JMCs2 and JMCs2R1 pools adjusted to the same concentrations of Au were diluted fivefold with distilled water and pelleted by overnight centrifugation at 40,000 rev/min in the Spinco 50 Ti rotor. Samples of Au for electrophoresis on SDS-urea (*p*H 7.6) gels were prepared by resuspending the pellets in a solution of 0.1 M phosphate buffer (*p*H 7.6), 1% SDS, 6 M urea, and 1% 2-ME to contain 2 mg/ml and were solubilized by heating to 60 C for 10 min.

Two major polypeptides (AuP1 and AuP2) of approximately equal concentration were evident in both of the Au-containing pools (Fig. 7), and a third minor protein, tentatively designated AuP3, was consistently observed. Protein markers (bovine serum albumin, bovine serum albumin dimer, chymotrypsinogen A, and cytochrome *c*) were run on separate gels in the same experiment, and, from the plot of log molecular weight versus relative migration (19) with regard to the bromophenol blue tracking dye (Fig. 8), molecular weights of 26,000, 32,000 and 40,000 were obtained for AuP1, AuP2, and AuP3, respectively.

DISCUSSION

The serum (J.M.) used in these studies contained a high concentration of Au with predominantly "spherical" forms which were homogeneous with respect to their buoyant density

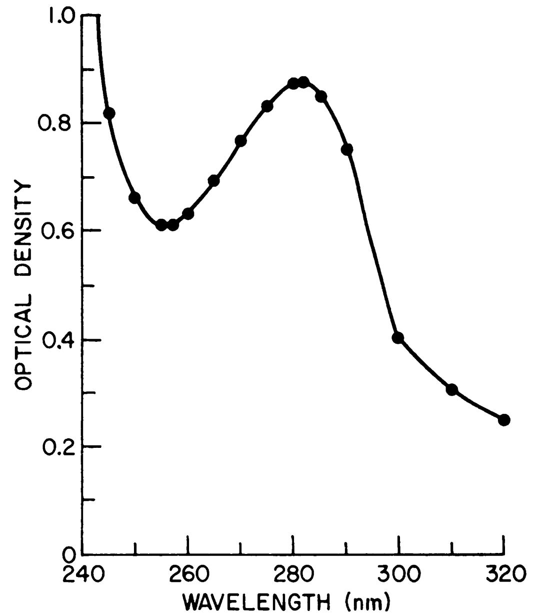

FIG. 6. *Ultraviolet absorption spectrum of purified Australia antigen.*

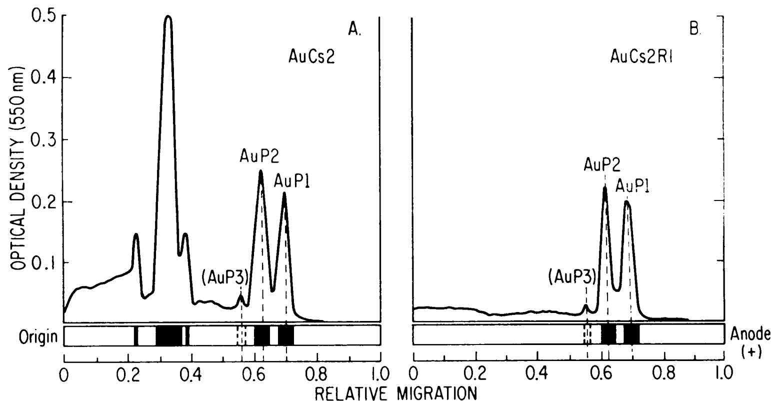

FIG. 7. *Proteins of Australia antigen. Preparations of Au after second-step isopycnic banding (A) and final rate separation (B) were solubilized by heating for 10 min at 60 C in 6 M urea, 1% SDS, and 1% 2-ME. Samples were electrophoresed on 10% SDS-urea-pH 7.6 gels at 5 ma/gel. Gels were fixed, stained with Coomassie Brilliant Blue, and scanned at 550 nm. Migration is expressed relative to the bromophenol blue tracking dye. Two major proteins, AuP1 and AuP2, were observed as was a minor component, tentatively labeled AuP3.*

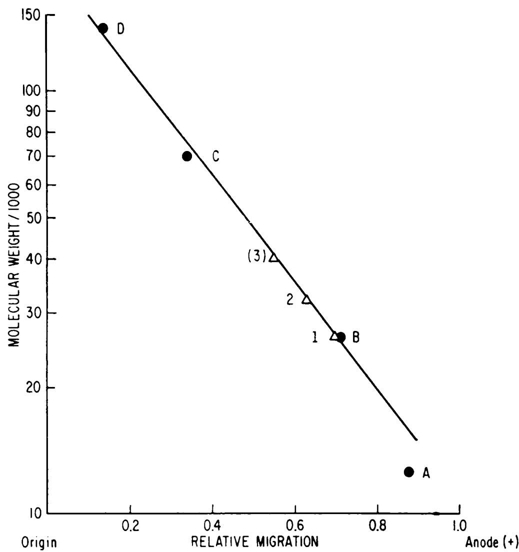

FIG. 8. *Molecular weights of Australia antigen proteins as determined from a standard plot of log molecular weight versus relative migration. The standard plot was made by using marker proteins [(A) cytochrome c; (B) chymotrypsinogen A; (C) bovine serum albumin (BSA); (D) BSA dimer] treated just as the Au samples and electrophoresed in the same experiment but on separate gels. Au proteins are numbered 1, 2 and (3) in order of decreasing mobility.*

in CsCl and sedimentation behavior. The large capacity batch-type B-XXIX zonal centrifuge rotor is well suited for the isolation and purification of Au from unit quantities of serum by the ρ-s procedures. Excellent recovery of Au CF activity was observed through both of the isopycnic banding steps (Cs1 and Cs2) and the dialysis of the Cs2 pool while appreciable amounts of serum proteins were still present (Fig. 5C); however, after rate zonal centrifugation of JMCs2 on phosphate-buffered sucrose gradients and the removal of stabilizing serum proteins, extensive aggregation occurred resulting in a loss of CF titer without diminution of immunogenicity (7, 16).

Consistent values were obtained for the sedimentation coefficient of Au over an approximately fivefold concentration range (52 and 54*S*) by using the computer program analysis (3); these values are in conflict with earlier reported determinations of 110*S* (7; M. Nicolson, *personal communication*), one of which (7) was obtained with Au from a different lot of J.M. serum. This earlier determination was made by using internal markers whereas the computer programs assumed a nonosmotic rigid particle with a particle density of 1.16 g/cm^3 and does not account for different solvation properties. Schober (*personal communication*) reported an average sedimentation coefficient ($S_{20,w}$) of 39 for Au from a number of sera. Careful measurements of 100 particles made from a negatively stained preparation of Au with an *S* value of 54 yielded an average value of 22 nm (range 21 to 23 nm), whereas the preparation with a 110*S* had a mean diameter of 25 nm. Although the sedimentation rate of Au can be used to advantage for the purification of the antigen, its value may well vary from preparation to preparation. This fact and the apparent lack of nucleic acid strongly suggest that the spherical form represents viral coat antigen assembled as an incomplete particle rather than an infectious virion itself.

An earlier report (7) of a CF-reactive antigen with a buoyant density of 1.39 g/cm^3 in CsCl has been repeatedly observed on the first step banding of some serum specimens, and this high density antigen has an OD 260/280 ratio consistent with that of a nucleic acid-containing particle (Gerin, *unpublished data*). Attempts to extract nucleic acid from the 20-nm spherical form of the particle have been unsuccessful.

Au from J.M. serum consists of two major proteins, designated AuP1 and AuP2, of 26,000 and 32,000 molecular weight, respectively. On the basis of stain intensity, AuP1 and AuP2 are present in equal concentration in the Au particle; however, different proteins vary in affinity for the Coomassie Blue stain and the actual relative concentrations of the Au proteins may differ from that indicated by the absorbancy scan of Fig. 7. A third minor polypeptide (AuP3) with a molecular weight of 40,000 may be a structural component of the Au particle, since the final purification step did not change its concentration (5 to 10%) with respect to AuP1 and AuP2; however, the possibility that this component might represent a serum protein somehow complexed to the Au particle cannot be disregarded. The finding that Au consists of at least two proteins is an important one in view of recent evidence for the existence of various Au specificities (10, 18), one of which appears to be a common or group-specific antigen. LeBouvier (*personal communication*) identified by immunodiffusion two specificities to Au in antiserum prepared against JMCs2R1, one being a common "a" antigen; the possibility that AuP1 and AuP2 correspond to the two specificities and that AuP3 represents a third specificity not detectable by immunodiffusion because of its low concentration merits further study. The presence of two or more distinct antigens associated with the particle known as Australia antigen certainly raises questions concerning nomenclature.

Animal and human antiserum intended for the large scale screening of donor blood units and prepared against mixtures of Australia antigens with undefined antigenic determinants would be difficult to standardize for use as a reagent. The two or more antigens associated with the particle may differ in their potency as immunogens and antisera could be expected to vary widely in their ability to detect a given specificity by any given assay system. Such variations between antisera have been repeatedly noted in a number of laboratories. The preparation of an antiserum pool from adjusted subpools of monospecific antiserum of known potency would seem the more satisfactory approach to the problem of standard working reagents.

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