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Immunochemical Studies of Human Serum Fractionated by Gel Filtration with Sephadex G-200. (29054)

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The fractionation of serum proteins by gel filtration with Sephadex G-200 was described recently(1,2,3). Gel filtration with Sephadex G-200 separates serum proteins on the basis of their molecular size. Molecules greater than approximately 200,000 in molecular weight do not penetrate the gel matrix and are eluted first. Smaller molecules appear in later effluents depending on the degree of their retention by penetration and subsequent displacement from the gel. The present studies were undertaken to determine the degree of retention by Sephadex G-200 of a wide variety of the serum proteins which may be characterized immunochemically.

Materials and methods. Sera. Sera were obtained from 3 human donors. The donors were subject to various atopic allergies but were normal in other respects. Prior to gel filtration, the sera were dialyzed against 0.1 M sodium chloride buffered at pH 8.0 by addition of 10% by volume of borate buffer(3).

Antisera. Horse anti-whole human sera were purchased from Serpasteur, Pasteur Institute, Paris, France. Specific anti-γ-globulin and anti-β_2A (γ_1A) globulin sera were prepared as described elsewhere(3). Specific antisera to ρ-pre-albumin, fast (ρ) lipoprotein, slow (α_2 and β_1) lipoproteins, α_2 macroglobulins, β_2M (γ_1M) macroglobulins and (β_1) siderophilin were purchased from Behringwerke A. G., Marburg, Germany.

Gel filtration with Sephadex G-200. Sephadex G-200 was obtained from Pharmacia Fine Chemicals, Inc., Rochester, Minn. The Sephadex was washed 10 times with 0.1 M sodium

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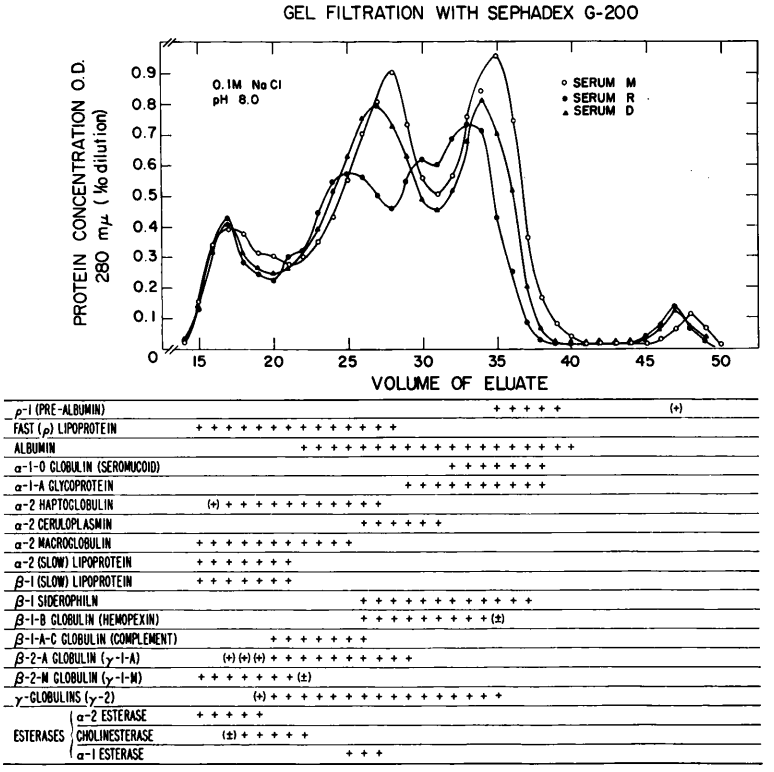

FIG. 1. Proteins are listed from top to bottom in order of decreasing electrophoretic mobility in agar with the exception of esterases. Nomenclature follows that of Grabar and Burtin (6). α-2-(slow) lipoproteins are of α-2 mobility in agar and of density <1.019 and β-1-(slow) lipoproteins are of β-1 mobility in agar and density 1.019-1.063. Volume of eluate in ml and tube number are the same.

chloride buffered with 10% by volume borate buffer (pH 8.0) and the "fines" were removed by decanting after each wash. A column 130 cm in length and 1.0 cm in diameter was packed with 2.5 g of Sephadex G-200 by gravity flow. A 2 ml volume of human serum was applied to the column and eluted, at room temperature, with the borate buffered chloride solution. The flow rate was 1 ml per hour at approximately 60 cm of water pressure and the eluates were collected in 1 ml volumes.† Protein concentrations of the eluates were measured by their absorption at 280 mμ in a Beckman DU spectrophotometer.

Immunoelectrophoresis. The micro method of Scheidegger(4) was used with minor modification.

† Subsequent to this study, flow rates of 4-6 ml per hour have been obtained through a similar column by packing and running under much lower pressure.

Double gel diffusion. A modification of the method of Ouchterlony(5) was used.

Ultracentrifugation. Analytical ultracentrifugation was carried out in a Beckman-Spinco model E ultracentrifuge.

Specific characterization reactions. After immunoelectrophoresis of the eluates, the numerous precipitin bands developed by a horse anti-whole human serum(6) were identified by the specific reactions for esterases(6, 7), lipids(6,10), ceruloplasmin(6,8), haptoglobin(6,9) and β_1B globulin (hemopexin) (9). To facilitate identification of the hemopexin and haptoglobin, an excess of hemoglobin was added to the fractions and the peroxidase activity of the bound hemoglobin determined after immunoelectrophoresis.

Results. The protein elution patterns of the 3 sera following Sephadex G-200 filtration are shown in Fig. 1. The 3 sera produced

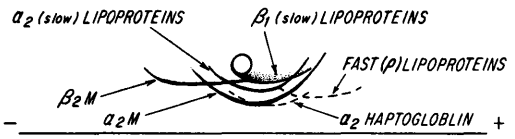

FIG. 2. Diagram of immunoelectrophoretic pattern of serum proteins obtained from the ascending portion of first peak (tube 16). Proteins represented by dotted lines were identified only by specific staining reactions.

quite similar protein elution curves, all being characterized by 3 main peaks. An immunoelectrophoretic pattern of the proteins associated with the ascending portion of the first peak (tube 16) is shown in Fig. 2. Six precipitin bands were formed by the horse anti-whole human serum. Those precipitin lines that required special staining methods to be visualized are indicated by dotted lines (Fig. 2). The fast (high density) and slow (low density lipoproteins were identified by specific lipid staining with Sudan Black(10). The precipitin line tentatively identified as α_2 (slow) lipoprotein has a slower electrophoretic mobility than the α_2 (slow) lipoprotein previously described (ref. 6, p. 146). The β_2M macroglobulins, α_2M macroglobulins and lipoproteins were identified by the use of specific antisera in double diffusion studies. That the α_2 precipitin arc closest to the antibody trough was haptoglobin was suggested by the identification of haptoglobin in tube 16 by starch gel electrophoretic analysis and by comparison with a known haptoglobin preparation. When the immunoelectrophoretic precipitin arcs of tube 16 were tested for esterase activity(7), the activity was associated with the α_2-lipoprotein precipitin band.

The ascending portion of the first peak (tube 16) from one gel filtration experiment was examined in the analytic ultracentrifuge Fig. 3A. The fraction was found to contain components with sedimentation coefficients of approximately 8 and 20S. In another experiment, a diluted serum sample was concentrated by sedimenting the proteins in the preparative ultracentrifuge (Beckman-Spinco, model L) at 39,000 rpm for 20 hours. The supernatant solution above the gel pellet was

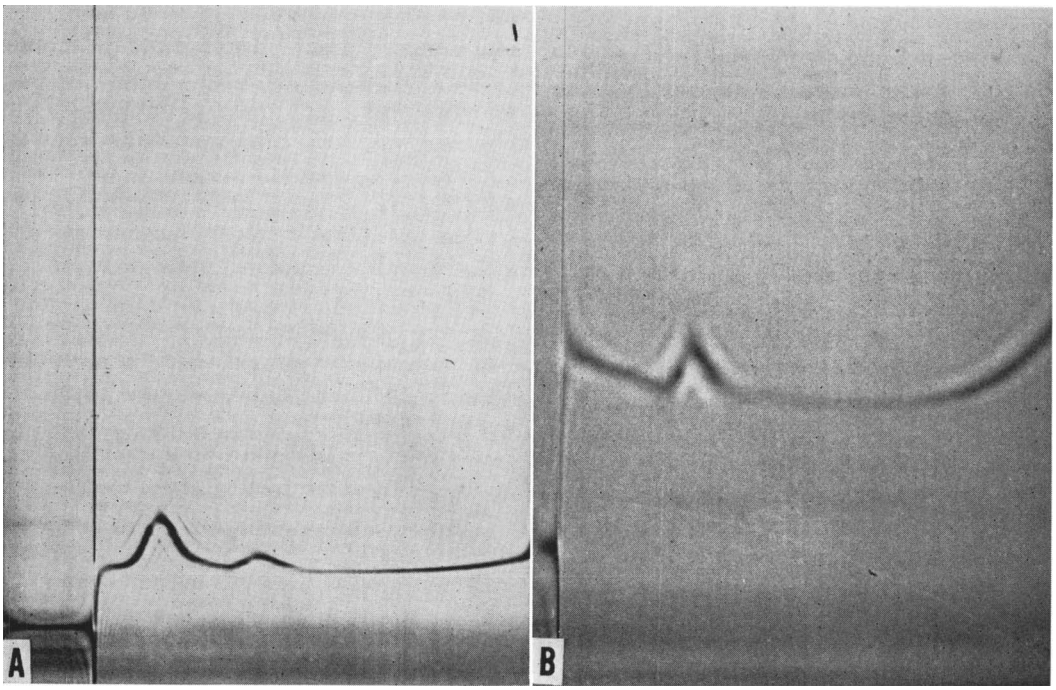

FIG. 3. Ultracentrifuge patterns obtained from ascending portion of first peak (tube 16) from a run with unconcentrated serum (A) and a serum sample that had been concentrated by preparative ultracentrifugation (B). Both pictures were taken after 24 min at 59,780 rpm. Peaks move from left to right on sedimentation.

removed, the pellet redissolved and fractionated on the Sephadex G-200 column. Material from the first peak of this run contained only a single macroglobulin component (approximately 17S) as shown (Fig. 3B) by analytical ultracentrifugation. These data and the similar experiments of Flodin and Killander(1) would suggest that the slow 7-8S peak is lipoprotein, presumably of high molecular weight.

The immunoelectrophoretic and double gel diffusion analyses of all the eluates are shown in Fig. 1. Following the elution of the 19S α_2M and β_2M globulins, lipoproteins, and haptoglobin in the first protein peak, the β_2A globulins, cholinesterase, γ-globulins and complement (β_1AC globulin) appeared in the trough between the first 2 peaks. As estimated in the double gel diffusion plates, the maximum concentration of β_2A globulins appeared in the eluates of the ascending portion of the second peak while the greatest concentration of γ-globulins appeared later in the eluates of the second major peak. Siderophilin (transferrin), ceruloplasmin and hemopexin all appeared initially in the eluates representing the second major peak and their greatest concentration appeared to be in the trough between the eluates of the second and third peaks. Even though albumin was first detected in the eluates of the ascending portion of the second peak, its maximum concentration as estimated by immunoelectrophoretic analysis was in the eluates of the third peak. Seromucoid and α_1 A-glycoprotein were also in maximum concentrations in the eluates of the third major peak. Pre-albumin was detected in the eluates of the descending portion of the third peak. No precipitin line was developed in double diffusion in gel between 2 horse antisera against human serum and the eluates of the small final protein peak. A third horse antiserum formed a single weak precipitin line with the eluates in Ouchterlony tests, but not in immunoelectrophoresis. Absorption of this antiserum with the eluates did not visibly remove antibodies to any of the serum protein components, as demonstrated by immunoelectrophoresis.

Discussion. The data presented concur with the previous reports using Sephadex G-200 gel filtration for fractionation of human sera (1,2,3). The serum proteins were separated into 3 major peaks. Those proteins of molecular weight greater than 200,000 formed the first peak. The order of elution of the proteins was well correlated with what is known of the molecular weights of these proteins(6,11,12). The increasing degree of retention on Sephadex G-200 column was associated with a decreasing order of molecular weight (Table I).

TABLE I. Protein Elution and Molecular Weight.

Proteins	Molecular wt
α-2 (slow) lipoprotein	3.2×10^6
β-1 " "	—
β-2M globulin	1×10^6(13,14)
α-2 macroglobulin	820,000
fast (ρ) lipoprotein	195,000
α-2 haptoglobin 2-2 and 2-1	400,000 and 220,000(15)
β-1-A-C globulin (complement)	—
β-2A globulin	?*
γ- "	160,000
α-2 ceruloplasmin	151,000
β-1-B globulin (hemopexin)	—
β-1 siderophilin	90,000
albumin	69,000
α-1-A glycoprotein	54,000
α-1-O globulin (seromucoid)	44,000
ρ-1 (pre-albumin)	61,000

Proteins are listed in order of position of the midpoint of the series of tubes in which protein antigen was detected. Molecular weights were taken from reference(11) except as noted.

* Ultracentrifuge data(16) would suggest that the β-2A globulins are of nearly the same size as the γ-globulins and that small amounts of higher molecular weight materials with β-2A specificity may be found.

It was necessary to perform the gel filtration with a low pressure head because increasing the hydrostatic pressure caused a marked diminution in the already slow flow rate. This was probably due to compression of the gel by the increased pressure.

The appearance of the haptoglobin among the proteins of the first peak was consistent with the types of haptoglobin found. Sera M and D were type 2-2 while R was 2-1. It is possible that some of the haptoglobin may have been combined with hemoglobin and therefore accelerated in its passage through the Sephadex column.

Gel filtration with Sephadex G-200 offers

a useful means for separation and characterization of serum proteins, particularly in conjunction with other methods of protein separation(17). Its use for the relative separation of various antibody activities in human sera has been described(1,2,3).

Summary. Three human sera were fractionated by gel filtration with Sephadex G-200. The distributions of 19 serum proteins or serum protein activities were studied by immunoelectrophoresis, double gel diffusion with specific antisera and specific characterization reactions. The serum proteins were separated into 3 major peaks and eluted in order essentially depending on their molecular size. The first peak contained the α_2M and β_2M macroglobulins, haptoglobin and lipoproteins. The second peak contained mostly γ-globulins and third peak consisted primarily of albumin.

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Hypophysectomy and the Lipolytic Action of Epinephrine *in vitro.** (29055)

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The prompt increase in plasma free fatty acid (FFA) concentration which follows epinephrine administration(1,2) is abolished by hypophysectomy(3,4). Pretreatment of hypophysectomized monkeys with triiodothyronine or thyrotropin restored responsiveness to epinephrine, but corticotropin and adrenal steroids were without effect in this regard(3). However, in the dog, this sequel of hypophysectomy was reversed by pretreatment with ACTH or adrenal steroids(4).

The present experiments were undertaken in an attempt further to elucidate the role of the pituitary in the lipid mobilization induced by epinephrine.

Materials and methods. Male rats weighing approximately 100 g were obtained from the Charles River Breeding Laboratories. Hypophysectomized rats were studied 2-6 weeks post-operatively. In all cases, hormone preparations were administered daily for the 5 days preceding sacrifice. The epididymal fat bodies were removed under nembutal anesthesia following a 24-hour fast. Pairs of adipose tissue

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