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STUDIES ON α_2-MACROGLOBULIN

I. A METHOD FOR THE ISOLATION OF RABBIT α_2-MACROGLOBULIN

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SUMMARY

A procedure for the fractionation of rabbit serum is described, which allows the almost quantitative isolation of a pure α_2-macroglobulin. Briefly the method is as follows: A protein fraction is salted out with ammonium sulfate, at pH 6.8, between the molarities 1.9 and 2.4. The α_2-globulins of this fraction are isolated by preparative electrophoresis in agar-gel at pH 8.6, and the eluted proteins are submitted to preparative ultracentrifugation in a density-gradient of sucrose. The bottom fraction consists of pure α_2-macroglobulin, as shown by electrophoresis on paper, agar-gel and starch-gel, as well as by analytical ultracentrifugation and immunological tests.

INTRODUCTION

The occurrence, in human serum, of protein molecules sedimenting in the ultracentrifuge with a sedimentation rate close to 19 S was first mentioned by PEDERSEN[1]. BRATTSTEN[2] was able to localize at least part of such heavy constituents in the electrophoretic α_2-fraction of serum proteins.

Along entirely different lines, COHN *et al.*[3] described the occurrence in the Fraction III-O, prepared according to their Method 6, of a "lipid-poor β-euglobulin". ONCLEY *et al.*[4] attributed to this euglobulin a sedimentation rate of about 20 S, corresponding to a molecular weight ranging between 500000 and 1000000. They estimated this protein to account for about 20% of the material of Fraction III-O. The same component was identified in Fraction III-O prepared according to Method 10 of COHN *et al.*[5]. By further fractionation of COHN's Fraction III-O, BROWN *et al.*[6] were able to isolate an apparently pure component, to which they gave the name "heat-labile α-glycoprotein", and which they considered to be identical with COHN's lipid-poor β-euglobulin.

A third line of approach, involving a scheme of fractionation with the aid of ammonium sulphate, was followed by SCHULTZE *et al.*[7]. These authors were able to isolate a pure high-molecular-weight α_2-globulin from normal human serum as well as from COHN's Fraction III-O. This component, which they called "α_2-macroglobulin", could be shown to correspond to the heavy protein isolated by BROWN *et al.*[6].

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It soon became clear that the ultracentrifugal "19-S" peak observed in the diagram of normal serum was composed of α_2-macroglobin (two thirds) and a $\gamma_1(=\beta_2)$-macroglobulin (one third)[9].

Recently, several new methods involving preparative ultracentrifugation have been described for the isolation of human α_2-macroglobulin[10-12].

The occurrence of α_2-globulins which antigenically cross-react with human α_2-macroglobulin has been conclusively demonstrated in several species of primates[13-17]. In starch-gel electrophoresis practically all mammalian sera display a characteristic $S\alpha_2$ band[18-22] which presumably corresponds to a homologue for human α_2-macroglobulin. Precipitin-lines suggestive for such a protein can be observed in immuno-electrophoretic patterns obtained from a great variety of mammalian species. However, we have no knowledge of any work reporting the isolation of such a substance, or its properties, from any species other than man. In view of the striking elevation of serum levels of α_2-macroglobulin in human nephrosis[23-25], and the reported increase of "α_2-globulins" in the sera of animals with experimental nephrosis[26-28], the isolation and characterization of such α_2-macroglobulins in laboratory animals was attempted. The present report is concerned with the isolation from rabbit serum of what appears to be the true homologue for human α-macroglobulin.

MATERIALS AND METHODS

Analytical methods

Electrophoresis on filter paper was performed under a potential gradient of 3 V/cm, using a conventional Michaelis barbiturate–acetate buffer (pH 8.6; I 0.1). Strips were stained for proteins with amido-black-10 B and, for carbohydrates, by the periodic acid-Schiff technique.

Electrophoresis on agar-gel according to WIEME[29] was performed at room temperature under a potential gradient of 20 V/cm, using a barbiturate buffer of pH 8.4 and I 0.05.

Starch-gel electrophoresis was carried out according to the vertical method of SMITHIES[30]. The starch-gel was composed of 12 g of hydrolyzed starch (Connaught) per 100 ml of buffer (0.025 M H_3BO_3 and 0.070 M NaOH). The electrode vessels contained a buffer of 0.30 M H_3BO_3 and 0.06 M NaOH.

Immunoelectrophoreses were performed according to a modification[31] of SCHEIDEGGER's[32] micromethod.

Analytical ultracentrifugation diagrams were obtained with a Spinco (Model-E) Ultracentrifuge.

Preparative methods

Preparative electrophoresis was performed in 1.5-cm thick layers of 0.6% (w/v) agar-gel, made in Michaelis buffer (pH 8.6; I 0.033). The potential gradient was 1 V/cm, and the duration of the run was 20 h, at room temperature. Recovery of the proteins from the gel was obtained by homogenization followed by centrifugation at 20000 rev./min for 20 min, or by elution with saline. (The details of this method will be published elsewhere.)

Preparative ultracentrifugation was carried out with the aid of a Spinco (Model-L) Ultracentrifuge, using the Rotor SW-39. The fractionations were obtained in a

density-gradient of sucrose according to the procedure described by KUNKEL[33]. Usually 1 ml of the protein fraction was layered on top of the gradient.

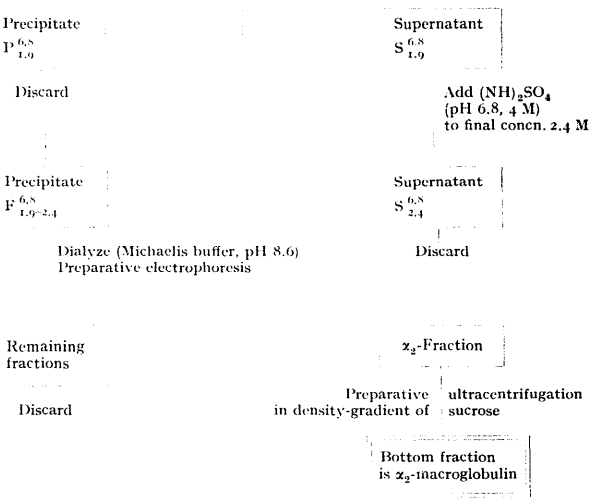
Salting out was performed with 4.0 M ammonium sulphate solution that had been previously adjusted to a pH of 6.8 by the dropwise addition of a 10% solution of Na_2CO_3. Measurements of pH were carried out at room temperature, using a Beckman Model-G pH-meter.

Procedure for the isolation of rabbit α_2-macroglobulin

After unsuccessful attempts to apply the salting-out procedure described by SCHULTZE *et al.*[7] for human α_2-macroglobulin to the isolation of α_2-macroglobulin from rabbit serum, the following scheme of fractionation was developed (Scheme I).

SCHEME I

METHOD FOR THE ISOLATION OF α_2-MACROGLOBULIN FROM RABBIT SERUM



Step 1. Salting out with 1.9 M $(NH_4)_2SO_4$

1 vol. of rabbit serum is diluted with 2 vol. of a 1/15 M phosphate buffer (Sørensen) (pH 6.8). The diluted serum is then precipitated at a final molarity of 1.9,

by addition of the calculated volume of neutralized 4 M $(NH_4)_2SO_4$, and the mixture is allowed to incubate at room temperature overnight. The precipitate (called $P^{6.8}_{1.9}$) is discarded by filtration through Whatman No. 1 filter paper.

Step 2. Salting out of the supernatant with 2.4 M $(NH_4)_2SO_4$

The ammonium sulphate concentration of the supernatant (called $S^{6.8}_{1.9}$) is raised to 2.4 M by addition of the calculated amount of the same neutralized 4.0 M $(NH_4)_2SO_4$ solution. After incubation at room temperature overnight, the supernatant (called $S^{6.8}_{2.4}$) is discarded by filtration through Whatman No. 1 filter paper. The precipitate (called $F^{6.8}_{1.9\text{-}2.4}$) is quantitatively removed from the filter paper and dissolved in 0.1 of the initial serum volume of a 1/15 M phosphate buffer (Sørensen) (pH 6.8). Sufficient 4.0 M neutralized ammonium sulphate solution is added again in order to obtain a final concentration of 2.4 M and, after filtration, the precipitated paste of protein is removed and dissolved in 0.1 of the initial volume of distilled water.

Step 3. Preparative electrophoresis

The purified fraction $F^{6.8}_{1.9\text{-}2.4}$ obtained by Step 2 is dialyzed against Michaelis barbiturate buffer (pH 8.6; I 0.03). During the dialysis a negative pressure is applied to the external dialysis fluid, so as to obtain a concentration of the contents of the membrane by passage of the fluid from the protein solutions into the external fluid. The final protein concentration to be attained is 60 mg/ml.

The yellow protein solution is now submitted to preparative electrophoresis as described in the experimental section of this paper, and after completion of the run, the electrophoretic α_2 fraction (which stands out as a dark yellow band) is removed from the agar-gel and eluted. The resultant product is called $F^{6.8}_{1.9\text{-}2.4}\text{-}\alpha_2$.

Step 4. Preparative ultracentrifugation

The fraction $F^{6.8}_{1.9\text{-}2.4}\text{-}\alpha_2$ is concentrated by dialysis under vacuum to a protein content of 30 mg/ml. The concentrated protein solution is then layered on top of the density-gradient of sucrose solution as described in the experimental section. The meniscus is destroyed by gentle stirring with a glass rod, and the tubes are spun for 22 h at 35000 rev./min in the Rotor SW-39 of the Spinco Model-L preparative ultracentrifuge. The bottom fraction of the sedimented proteins is recovered by piercing a small hole through the walls of the Lusteroid tubes and by drop-wise collecting the contents in a set of small test tubes.

It was observed in the course of these experiments that the presence of α_2-macroglobulin in preparations from rabbit origin was constantly associated with a golden-yellow color. This peculiarity can be exploited for the appropriate pooling of the fractions.

Sucrose is removed by dialysis against phosphate buffer (Sørensen) (pH 7.2, 1/15 M).

RESULTS

Analyses of the successive fractions

Filter paper electrophoretic diagrams of the successive fractions, indicating the relative areas of the various electrophoretic protein peaks are given in Fig. 1. As the fractionation proceeds, a progressive enrichment in components with α_2-mobility becomes apparent, the final preparation resulting in a practically pure α_2-globulin.

Comparative protein and carbohydrate stainings (Fig. 2) of the same fractions show a progressively rising carbohydrate content of the selected components.

In starch-gel electrophoresis (Fig. 3) the rabbit α_2-macroglobulin has been found to occupy the same position as that of human α_2-macroglobulin, *i.e.* under the form of the $S\alpha_2$ band. This circumstance proved invaluable in ascertaining the presence or absence of this protein in the various fractions.

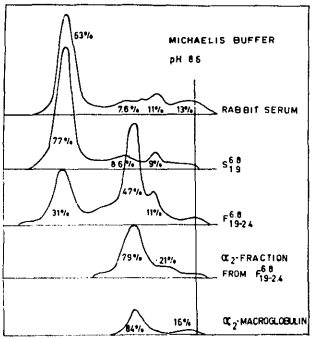


Fig. 1. Electrophoretic analyses of rabbit-serum fractions. A progressive enrichment in α_2-fractions is obtained. The final product consists of pure α_2-macroglobulin (the slow material accounting for 16% of this product is an artifact due to trailing on the filter paper).

Analytical ultracentrifugation confirmed the progressive enrichment in heavy components sedimenting with a sedimentation rate of about 19 S (Fig. 4). Quantitative data on the relative areas of the various sedimentation patterns are given in Table I.

TABLE I

ULTRACENTRIFUGAL ANALYSIS OF RABBIT-SERUM FRACTIONS

Designation of fractions	*Sedimentation rate**	*Composition of fractions (%)*
Serum	3.5	87.8
	6.0	9.7
	15.0	2.5
$S^{6.8}_{1.9}$	3.6	97.5
	15.5	2.5
$P^{6.8}_{1.9}$	3.4	24
	6.0	69
	16.0	7
$S^{6.8}_{2.4}$	3.6	100
$F^{6.8}_{1.9-2.4}$	3.7	76
	16.2	24
$F^{6.8}_{1.9-2.4-\alpha_2}$	3.3	35
	17.6	65
α_2-Macroglobulin	19.6**	100

* Uncorrected for concentration and viscosity.
** Extrapolated to zero concentration, uncorrected for viscosity.

Although the paper-electrophoretic patterns indicate the presence of substantial amounts of α_2-globulins in the fractions rejected during the fractionation process, both the starch-gel analyses and the analytical ultracentrifugations establish that these losses do not occur in the 19-S α_2-macroglobulin. Recovery of the desired component in the final material is almost quantitative, although small losses do occur especially at the preparative electrophoresis step.

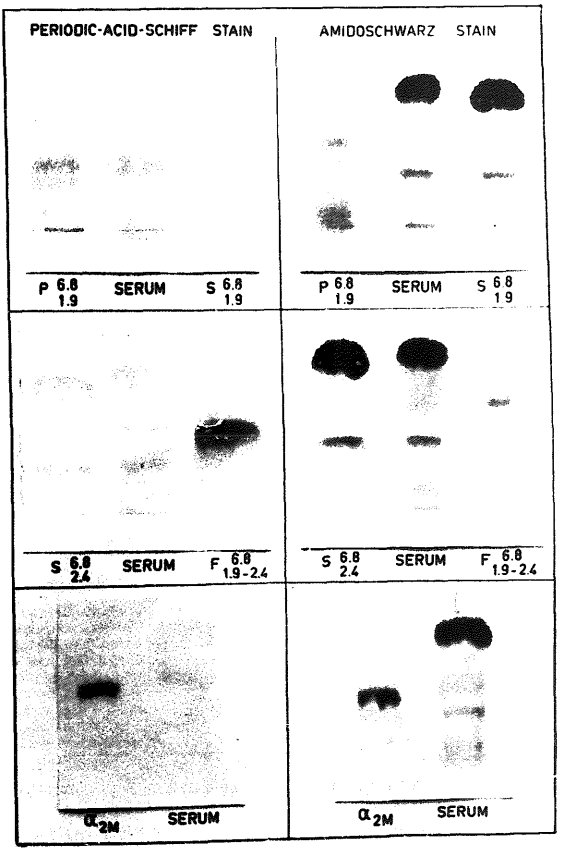


Fig. 2. Comparative stainings for protein and carbohydrate of rabbit-serum fractions. Note the progressive rise of the carbohydrate content in the selected fractions.

Criteria of purity of the final product

The α_2-macroglobulin preparations obtained by the fractionation procedure here described have constantly proven to be homogeneous by all criteria of purity that were applied. The protein showed up as a single and sharp $S\alpha_2$ band upon starch-gel electrophoresis and as a homogeneous α_2-peak in agar-gel electrophoresis. These

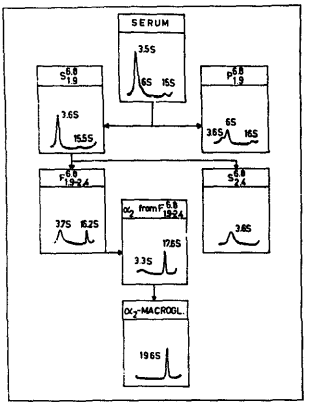


Fig. 3. Ultracentrifugal diagrams of rabbit-serum fractions. Patterns obtained in the ultracentrifuge at 59780 rev./min. The concentration of the protein solutions ranged from 0.5 to 2.4% (w/v). Sedimentation rates are uncorrected for concentration and viscosity.

Fig. 4. Starch-gel electrophoresis of normal rabbit serum and of rabbit α_2-macroglobulin. Left and centre: two different preparations of rabbit α_2-macroglobulin (1.0 and 0.5% (w/v) protein). Right: normal rabbit serum.

facts indicate that the apparent heterogeneity displayed on paper-electrophoresis (Fig. 1) was due to adsorption onto the substrate. In the ultracentrifuge a single peak, sedimenting with a rate of 19.6 S (extrapolated to zero concentration) was obtained.

Upon immuno-electrophoresis and in OUCHTERLONY plate analysis all preparations displayed only one precipitin line when tested against three different polyvalent chicken anti-rabbit antisera.

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