ULTRASTRUCTURAL STUDIES OF HUMAN AND RABBIT αM-GLOBULINS

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α_2-Macroglobulin was first isolated by Schultze et al. in 1955 (1), but its biological function has remained obscure for a long time. In 1962 Haverback et al. (2) described an α_2-globulin in human plasma which combined with trypsin or chymotrypsin. This combination did not render the enzymes inactive but made them inaccessible to certain inhibiting proteins. Mehl et al. (3) reported that the trypsin-binding protein was the macro-constituent of the α_2-region.

Recent studies have indicated that human α_2-macroglobulin plays a significant role in a number of enzymological reactions. Different authors have described its binding to plasmin (4), thrombin (5), insulin (6, 7), growth hormone (8, 9), and elastase (10). Ganrot (11) has demonstrated a correlation between the α_2M-concentration and the esterolytic activity of the trypsin-α_2M complex in human sera. Elevated α_2-macroglobulin levels, normally seen in pregnant women and in children, can also be found in patients with nephrosis. The α_2M-molecule is considered to be rather unstable, and its degradation products migrate faster than the complete molecule in starch electrophoresis (12).

The viscosity data of Schönenberger et al. (13) suggested that the α_2M-molecule had the form of a rotational ellipsoid, and Höglund and Levin (14) presented electron micrographs of prefixed α_2M-globulin molecules which had a spherical or almost spherical shape. The latter investigators used the shadow-casting technique. As the negative staining technique of Brenner and Horne (15) has proved to be a very useful tool in studies of other macromolecules, this technique was employed in the ultrastructural studies of α-macroglobulins reported here. Some of the present data have been described in a preliminary communication (16).

Materials and Methods

α-M-Globulin Preparations.—Several different sources of material were used in the present study including human and rabbit α_2M- and rabbit α_1M-globulin preparations purified as

described below. In addition, two reference preparations, representing purified human α_2M-globulins, were kindly supplied by Dr. G. Schwick, Behringwerke A G (Marburg-Lahn, West Germany) and Dr. P. O. Ganrot. The preparation obtained from Dr. Ganrot was purified as described earlier (17).

Preparatory Methods.—The αM-globulins were purified by a four step procedure starting with the removal of the low-density lipoproteins by precipitation with dextran sulphate (molecular weight, 560,000) and Ca^{2+} (5, 18). The macroglobulins were then precipitated with 7.5% (w/v) polyethylene glycol (molecular weight, 6000) (19). No lipoproteins were detectable in the macroglobulin preparation by immunodiffusion analysis employing specific antisera. The redissolved macroglobulin fraction was dialyzed against a 0.5 M NaCl solution buffered with 0.05 M phosphate (pH 7.2) containing 2% butanol and subjected to gel filtration on Sepharose 4B (Pharmacia Fine Chemicals, Uppsala, Sweden). The columns used had a diameter of 2.7 cm and a length of 150 cm and the flow rate was about 3 ml/cm² per hour. The macroglobulins were traced by immunodiffusion and, after concentration by negative pressure dialysis, they were subjected to zone electrophoresis in 0.1 M Veronal buffer, pH 8.6, μ 0.05 on polyvinyl chloride (PVC). Electrophoresis was carried out for 14–15 hr at a voltage of 10 v/cm. The individual fractions were analyzed by immunodiffusion for estimation of the relative amounts of αM- and IgM-globulins present and the absorbance at 280 mμ was determined.

Analytical Methods.—

Immunoelectrophoresis: Immunoelectrophoresis was carried out in a 1 mm layer of 1% Difco agar made up in barbital buffer, pH 8.6, μ 0.05. Electrophoresis was run for 1.5 hr at 25 v/cm and 30 ma, and the reagents in the troughs were allowed to diffuse for 48 hr.

Gel diffusion analysis: Gel diffusion was carried out in agar according to Ouchterlony (20). Specific rabbit antisera against human plasma proteins were purchased from Behringwerke AG. The sheep and goat anti-rabbit sera were prepared in this laboratory. The sheep antiserum registered both α_1- and α_2M-globulins while the goat antiserum contained precipitating antibodies only against α_2M-globulins.

Protein determination: Protein concentrations were measured by absorbance at 280 mμ in a Beckman spectrophotometer in a 1.0 cm wide quartz cuvette.

Analytical ultracentrifugation: Samples to be analyzed were dialyzed against 0.01 M phosphate buffer of pH 7.4 with 0.14 M NaCl prior to centrifugation. The runs were performed in a Spinco model E ultracentrifuge at 52,640 rpm at 20°C, using an AnD rotor with 12 mm 4° sector cells with standard or wedge windows. The sedimentation coefficient $s_{20,w}$ was calculated by conventional methods using $\bar{v}_{20} = 0.725$.

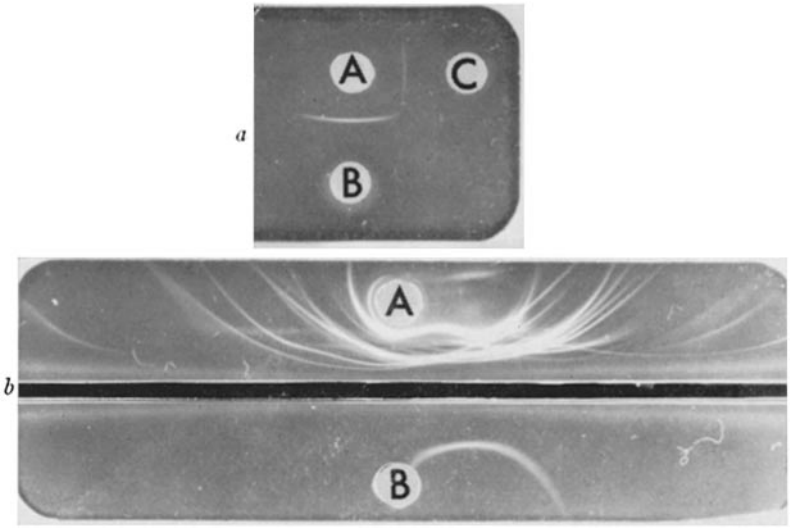
Preparation of material for electron microscopy: The purified materials, containing between 1 and 20 mg protein/ml, were dialyzed against a solution of 1% ammonium acetate, and diluted in the particular negative contrast material used. 1 drop of this mixture was placed on a 400 mesh carbon-coated grid. After a few seconds, the excess fluid was withdrawn with filter paper. The grids were allowed to dry in the air and immediately examined in a JEM-5Y electron microscope at a magnification of 50,000, using double condensed illumination. Three different negative stains were used: 2% solutions of sodium tungstosilicate (STS), potassium phosphotungstic acid (KPT), and ammoniummolybdate, all at pH 6.0. The condenser aperture was 250 μ and the objective aperture 50 or 70 μ at an operating voltage of 80 kv.

RESULTS

Ultrastructure of Human α_2M-globulin.—Electron micrographs of purified human α_2M-globulin revealed a high concentration of morphologically homogeneous particles resembling a graceful monogram of the letters H and I,

or the Russian letter ж (Fig. 1). As seen in two dimensions in the electron microscope, there appeared to be three parallel vertical bars connected at their midpoints by a horizontal bar. The average width of the molecule along the horizontal bar was 100 A and the length of the molecule was approximately 170 A.

The middle vertical bar (100–110 A) was always shorter than the two outer vertical bars. In many particles, the connection of the middle vertical bar to the horizontal crosspiece was not clearly visible, and instead of a solid central vertical bar there appeared to be two dots, one above and one below the hori-

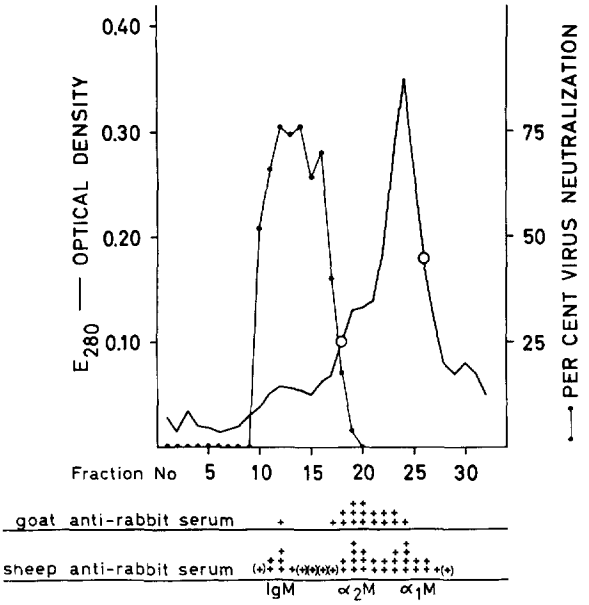


TEXT-FIG. 1. (*a*) Agar double-diffusion analysis of human α_2M-globulin. Well A, purified human α_2M-globulin; well B, rabbit anti-human α_2M serum; and well C, horse anti-human serum. (*b*) Immunoelectrophoresis of purified human α_2M-globulin compared with whole human serum. In the trough, horse anti-human serum.

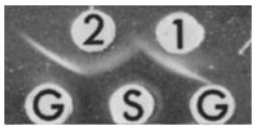
zontal crosspiece (Fig. 2). There was also some variability in the morphology of the outer two vertical bars. In certain preparations, these bars appeared capable of bending to some extent, occasionally yielding various loop formations when in contact with the central bar or with one another (Fig. 3).

In all preparations examined, there was one other type of structure constantly observed in areas of the grids containing a thick layer of the negative contrast medium. These structures resembled two small beans facing one another and had dimensions of 110 A × 125 A (Fig. 4). By inspecting particles embedded in increasing thickness of STS, a sequence was revealed illustrating the transition of the intact αM-molecule to the smaller bean-like structure. (Fig. 5). The

latter structures probably represented folded or partially collapsed αM-molecules as seen when embedded in thick areas of the negative contrast medium. The purity of the α_2M-globulin preparations examined was ascertained by gel diffusion analysis and immunoelectrophoresis (Text-fig. 1).



TEXT-FIG. 2. The final step of a representative rabbit αM-purification: zone electrophoresis on polyvinyl chloride. Below the curve are indicated the results of gel diffusion titrations of the different fractions using a goat anti-rabbit serum which precipitates α_2M- but not α_1M-globulins and a sheep anti-rabbit serum. The fractions used for electronmicroscopy and ultracentrifugal studies are encircled.



TEXT-FIG. 3. Agar double-diffusion analysis of rabbit α_2M- and α_1M-globulins. Well 2, purified rabbit α_2M-globulin; well 1, purified rabbit α_1M-globulin; wells G, goat anti-rabbit α_2M-serum; and well S, sheep anti-rabbit serum.

Ultrastructure, Antigenic Composition, and Sedimentation Constants of Rabbit α_1M- and α_2M-Globulin.—Purification of rabbit α-macroglobulins, as outlined previously, revealed two molecular populations differing in electrophoretic mobility (α_1M and α_2M). These proteins were recovered in relative amounts of about 20% α_2M and 80% α_1M (Text-fig. 2).

Examination of both rabbit α_1M and α_2M in the electron microscope (Figs. 6, 7) revealed structures indistinguishable from one another and from the human α_2M-molecules. Using a sheep anti-rabbit whole serum it was demonstrated by gel diffusion analysis that the antigenic composition of rabbit α_1M and α_2M differed. The sheep anti-rabbit serum, with precipitating antibodies against both α_1M and α_2M, showed nonidentity of the two rabbit αM-globulins (Text-fig. 3). In addition, it proved possible to produce a goat anti-rabbit α_2M-serum which demonstrated no cross-reaction with α_1M (Text-figs. 2, 3). When the purified α_1M- and α_2M-globulins were tested by immunoelectrophoresis against sheep anti-rabbit serum, characteristic lines in the respective α_1 and α_2 positions were observed.

Sedimentation constant determinations in the analytical ultracentrifuge gave $s_{20,w}$ values, extrapolated to infinite dilution, of 18.8 for α_1M and 18.2 for α_2M using $\bar{v}_{20}$ of 0.725.

DISCUSSION

The present electron microscopic data indicate that human and rabbit αM-globulins have a slender, symmetric structure with six visible appendages. In contrast, it was earlier proposed that the molecule would have an elipsoidal or spherical structure (13, 14, 21, 22). Schönenberger and coworkers (13) interpreted their data from viscosity determinations and from estimations of friction and axial ratios to suggest that the αM-molecule had the form of a rotational ellipsoid. However, since the results obtained by these methods were variable, no safe estimation of the shape of the molecule could be obtained. Höglund and Levin's electron microscopic studies (14) of prefixed human α_2M preparations suggested a spherical structure for the α_2M-molecule with a width and height of about 200 A. However, as pointed out by Rowe (23), it is probable that pretreatment of the protein solution with osmic acid or formalin caused aggregation of the molceules. In addition, Höglund and Levin used the shadow-casting technique, the resolution of which is limited by the granulation produced by the deposited metal.

The negative contrast method (15) has certain advantages in studies of macromolecules. The method is simple and gives an excellent contrast coupled with good preservation of molecular structure. However, there are factors, such as vapor pressure, osmotic gradients, and surface tension forces on a hydrophobic carbon grid, which can contribute to distortion of protein molecules even when this technique is used. Some of these effects are counteracted by the embedding effect of the contrasting material. The results of Horne and Nagington (24) and Russel and Wildy,[1] who applied the negative staining technique to poliovirus and herpes simplex virus, suggested that the viruses were at least partially infectious after the drying process on the grid. Recently, we have investigated the morphology of IgM immunoglobulins with the aid of the nega-

[1] Russel, W. C., and P. Wildy. Unpublished data cited (1963) in *Advances in Virus Research*, **10**:114.

tive staining technique (16, 25, 26). IgM antibodies, dried in 2% STS solution on a carbon-coated film, were found to retain nearly 100% of their biological activity upon subsequent redissolving in a physiological buffer. The retention of biological activity noted in these studies may be due to the tendency of the contrasting material to dry rapidly and form a rigid film around the protein molecules which tends to preserve them (27).

To reduce the interaction between the hydrophopic carbon-coated grid and the macromolecules, the "hole grids" described by Huxley and Zubay (28) were used on a few occasions in the present study. This technique revealed no difference in the shape of the αM-globulin molecules. In addition, "staining" with KPT, STS, and ammonium molybdate of the same pH revealed no difference in the average number of particles possessing the characteristic ornamental form, here considered to represent the intact αM-molecule. The STS was found to give the best contrast and the weakest granularity but the difference was slight.

Mehl et al. (3) reported that the trypsin-binding capacity of α_2M-globulins was destroyed by ammonium ions at 0.2 M or higher concentrations. In the present study, however, no alteration of the morphology was noted when an α_2M preparation, purified by ammonium sulphate precipitation (Behringwerke AG) was compared with α_2M preparations prepared using our methods.

It is interesting to note that the rabbit α_1- and α_2-macroglobulins, which were morphologically indistinguishable, also had similar sedimentation constants (18–19S). This is in contrast to the findings of Got et al. (29) who reported an S value of 16.6 for rabbit α_2M globulin. Preliminary immunodiffusion studies of the antigenic relationship between the rabbit α_1M- and α_2M-globulins have revealed the presence of strong non–cross-reacting antigenic determinants on both α_2 and α_1M.

From fluorescence depolarization measurements Jacquot Armand and Guinand (22) concluded that pig α_2M-globulin was a rigid structure. Earlier determinations by the same method (30, 31) had suggested that the IgM globulins possessed a higher degree of intramolecular flexibility. All these findings agree favorably with the electron microscopic findings made in our studies of αM- and IgM-molecules (16, 26).

SUMMARY

Electron micrographs of isolated human α_2M-molecules, obtained by the negative contrast technique, revealed morphologically homogenous structures resembling a graceful monogram of the two letters H and I. The modal values for the length and width of the α_2M particles were 170 A and 100 A, respectively. Purified rabbit α-macroglobulins contained about 80% α_1M- and 20% α_2M-globulins. The isolated rabbit α_1M- and α_2M-molecules were morphologically indistinguishable from one another and from human α_2M-molecules.

Preliminary immunoprecipitation studies demonstrated that the two rabbit

αM-globulins were antigenically different. Sedimentation constant determinations gave $s_{20,w}$ values of 18.8 and 18.2 for rabbit α_1M and α_2M, respectively.

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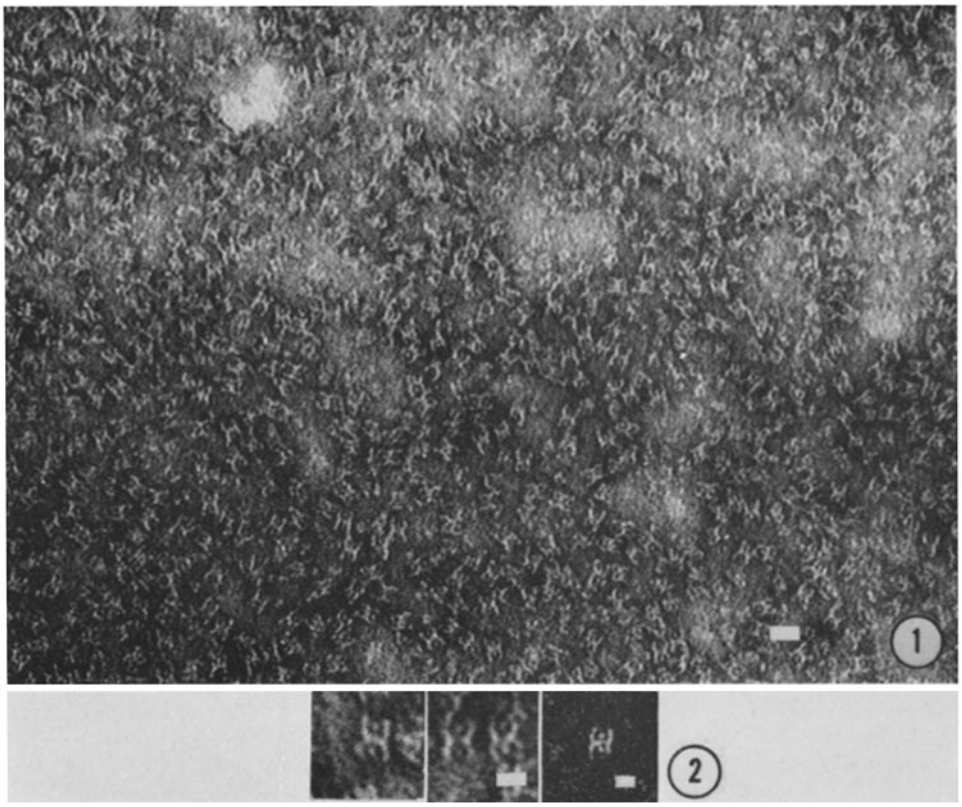
EXPLANATION OF PLATES

PLATE 82

FIG. 1. Electron micrograph of α_2M-globulin molecules purified from human serum. Scale line, 100 A. × 200,000.

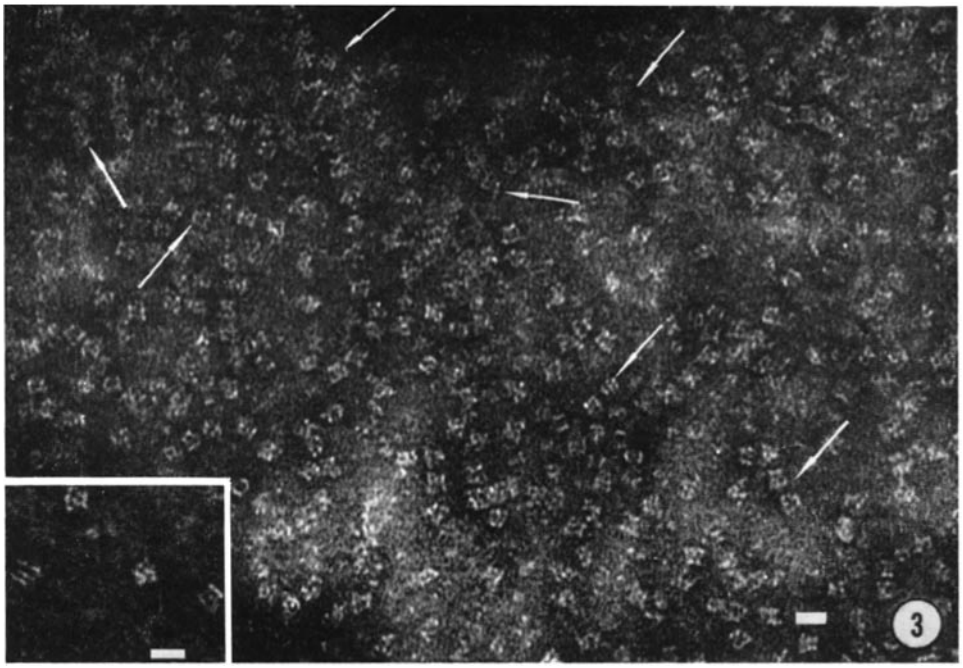
FIG. 2. Electron micrographs of human α_2M-molecules at a higher magnification. The middle vertical bar is discernible only as two dots. Magnifications: 400.000 and 250.000. Scale lines, 100 A. × 400,000 and 250,000.



(Bloth et al.: Ultrastructure of αM-globulins)

PLATE 83

FIG. 3. Electron micrographs of human α_2M-molecules with bent appendages giving rise to various kinds of loop formations (see arrows). Scale line, 200 A. × 200,000 and 250,000 (insert).

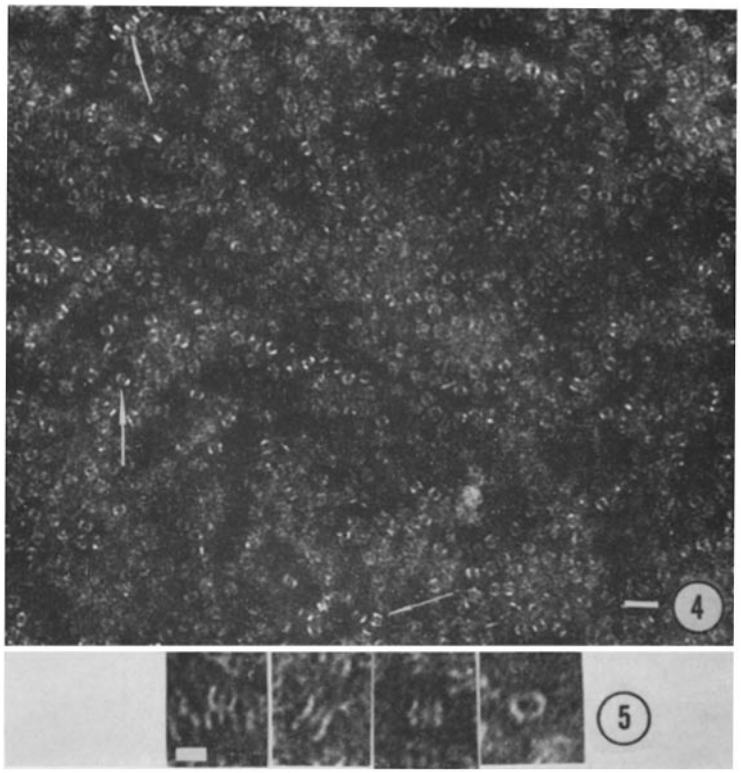


(Bloth et al.: Ultrastructure of αM-globulins)

PLATE 84

FIG. 4. Partially collapsed human α_2M-molecules embedded in a comparatively thick layer of sodium tungstosilicate. Note the presence of many particles resembling two beans facing one another. Some of these particles are indicated by arrows. Scale line, 300 A. × 150,000.

FIG. 5. Electron micrographs illustrating the transition of the apparently intact slender αM-molecule to the compressed coffee bean-like structure. This transition can be followed by examining particles embedded in increasing thickness (from left to right in the figure) of the contrasting material. Scale line, 100 A. × 400,000.

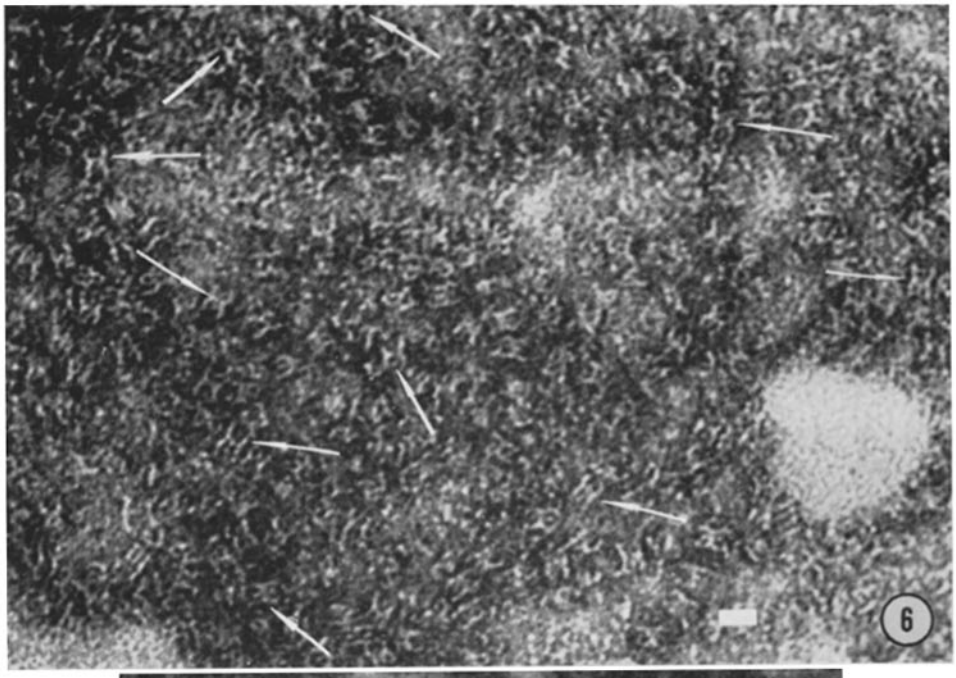


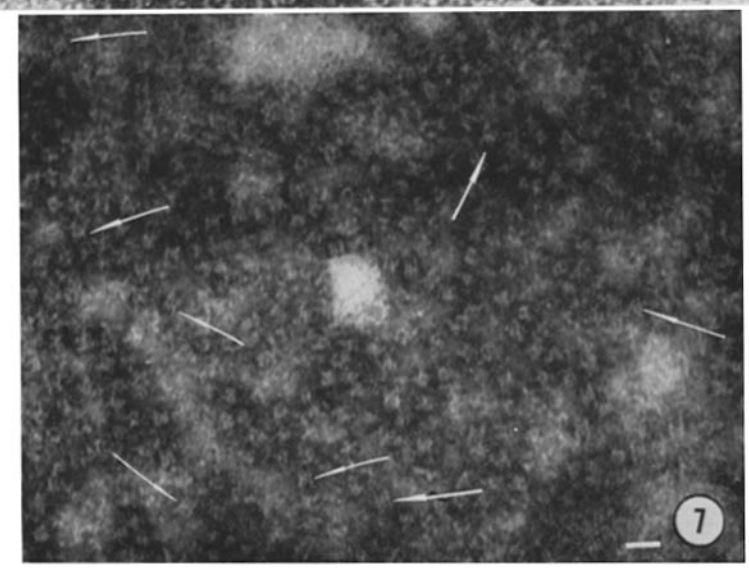
(Bloth et al.: Ultrastructure of αM-globulins)

PLATE 85

FIG. 6. Electron micrograph of rabbit α_1M-globulins in high concentration. Some α_1M-molecules are indicated by arrows. Note the great similarity to human and rabbit α_2M-molecules. Scale line, 200 A. × 250,000.

FIG. 7. Electron micrograph of purified rabbit α_2M-globulins at a magnification of 150,000. Note the presence of both apparently intact (arrows) and partly collapsed particles. Scale line, 300 A.





(Bloth et al.: Ultrastructure of αM-globulins)