AEROSOL TECHNIQUE FOR THE STUDY OF MACROMOLECULES WITH THE ELECTRON MICROSCOPE*

by

E. DE ROBERTIS, C. M. FRANCHI AND M. PODOLSKY**

Instituto de Investigación de Ciencias Biológicas, Departamento de Ultraestructura Celular, Montevideo (Uruguay)

The resolving power of the electron microscope makes theoretically possible the direct observation of molecules having a molecular weight of about 4,000. However, to achieve the full use of this resolution is a difficult task and up to now only macromolecules with molecular weights of several hundred thousands have been observed with certainty. The different factors involved in the electron microscopy of small macromolecules are discussed by WYCKOFF[1]. In addition to the resolution of the microscope, the scattering power of the specimen, and the smoothness of the substrate, the main limiting factors reside in the specimen itself.

Electron microscope examination of macromolecules is hindered by their tendency to clump on the supporting film. When a drop of the solution is deposited on the film, regardless of its degree of dilution, the macromolecules tend to clump when the solution becomes concentrated through dessication, and it is then difficult to recognize the elementary units within these clumps. Aggregation of the macromolecules and colloidal particles is also affected by the electrical charge of the supporting film but this can be, to some extent, corrected by proper treatment of the membrane (RIBI AND RANBY[2]).

Besides, if the dilution is very large the impurities of the water and reagents become an important limiting factor. A relatively small concentration of a low molecular weight impurity may obscure completely the small macromolecules.

Progress in this field seems to depend a great deal on the development of satisfactory methods for the preparation of the material and particularly for spreading the macromolecules in such a way as to avoid clumping and other obscuring factors.

One attempt in this direction has been the use of spraying methods for the preparation of specimens to be observed with the electron microscope (BACKUS AND WILLIAMS[3]). This method gives a droplet pattern with drops of 5–20 μ in diameter, which is extremely useful for the study of large macromolecules such as viruses.

Recently this method has been used by MITCHELL[4] for the examination of some plasma proteins. However, judging from the published electron micrographs, the spreading of the macromolecules within the droplets is not as sharp as to assure complete individualization of the molecules, and a great deal of clumping occurs.

In view of this fact we had already in 1951 proposed a method for dispersing the

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** From Invar (Investigación Argentina) Guanacache 1634, Buenos Aires, Argentina.

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material into microdroplets of colloidal dimensions by turning it into a monodispersed aerosol and precipitating it on the film (DE ROBERTIS, FRANCHI AND PODOLSKI[5]).

The present report is intended to give a description of the technique used and to show some of the preliminary results obtained in the electron microscopy study of some protein macromolecules.

Technique for the production, precipitation and study of the aerosols with the electron microscope

In Fig. 1 the general set-up assembled for the production and precipitation of the aerosol is shown. Compressed air from a tank or a compressor is injected at a pressure of 10–20 lbs. per sq. inch. It passes through an air filter similar to the Seitz microfilter used in bacteriology, and then into the aerosol source. Several types of aerosol sources have been tried but the most commonly used, which gave best results, is an "Euratmos" apparatus designed by DAUTREBANDE (see[6]).

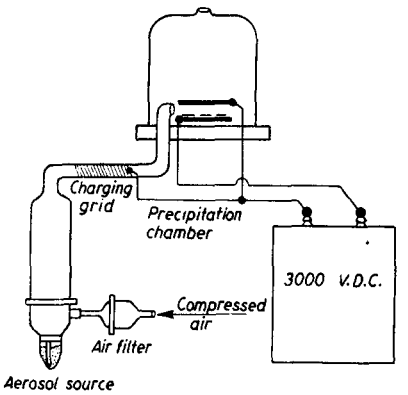

Fig. 1. Diagram of the general set-up used for the production and electrostatic precipitation of aerosols (see description in the text).

The aerosol flow then passes through a charging grid connected with one of the leads of the DC power source. From there the aerosol enters into the precipitation chamber. The diagram shows one of the designs built up in the laboratory. The electrodes are made of flat metal sheets connected with the power source. On the lower electrode the standard grids coated with parlodion films used in electron microscopy are deposited. The distance between the two electrodes can be varied but in general the separation used was 6–10 mm.

The DC power source of 2000–3000 volts is connected to the charging grid and to the two electrodes in such a way that the lower electrode (where the grids are deposited)

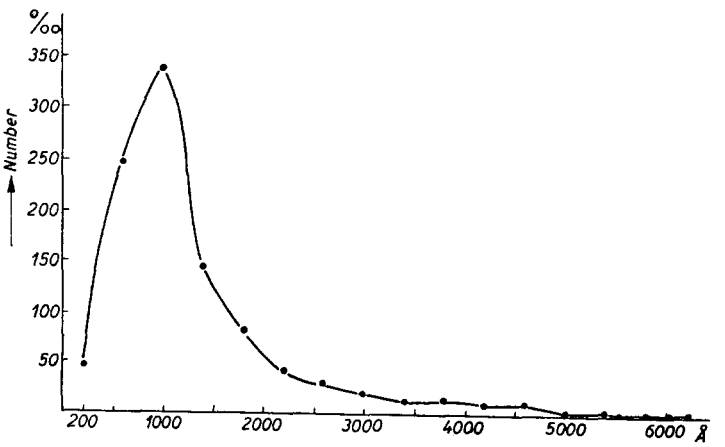

Fig. 2. Distribution curve of the diameters of 100 microdroplets of 5% dextrin (see description in the text). Air jet of 20 lbs. per sq. inch. Aerosol apparatus "Euratmos". Charged with positive DC of 3000 volts and precipitated on the negative pole for two minutes.

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Fig. 3. Electron micrograph of an aerosol of dextrin. Shadowcast with palladium at 11°. See description in the text. The same experiment as in Fig. 2. 4,700 ×.

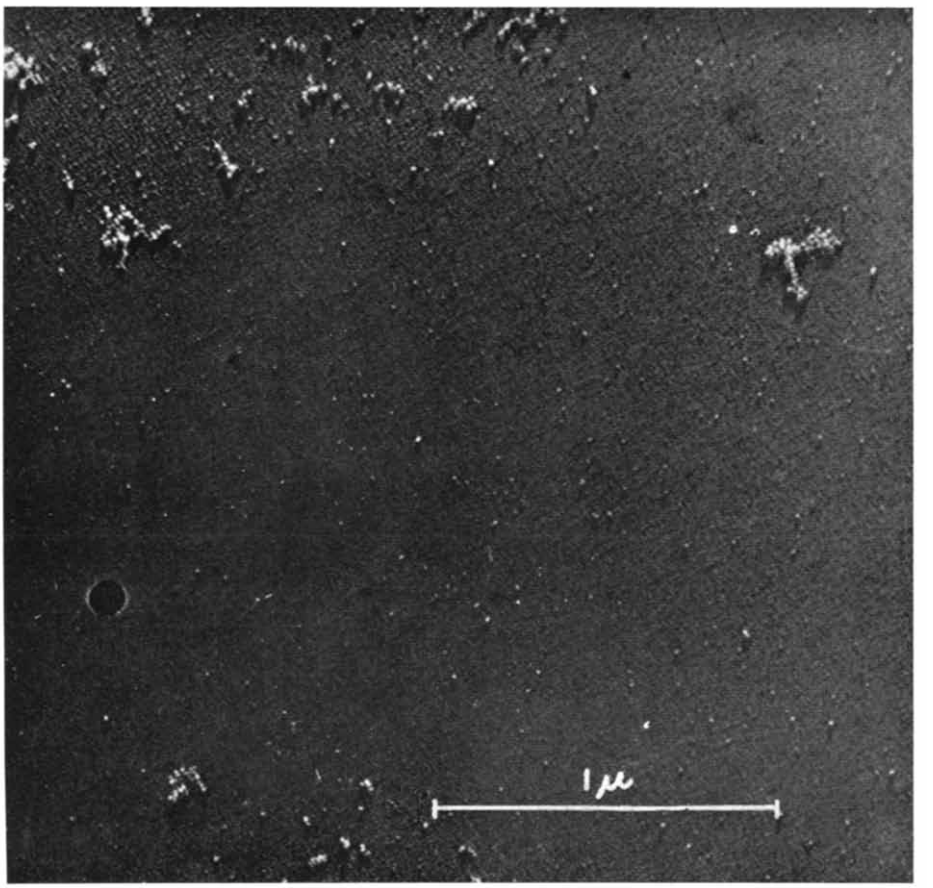

Fig. 4. Electron micrograph of an aerosol of bovine fibrinogen. See description in the text. Similar set-up as in Fig. 2 but precipitation on the negative pole for five minutes. Shadowcast with palladium at 11°. Objective lens without compensation. 43,700 ×.

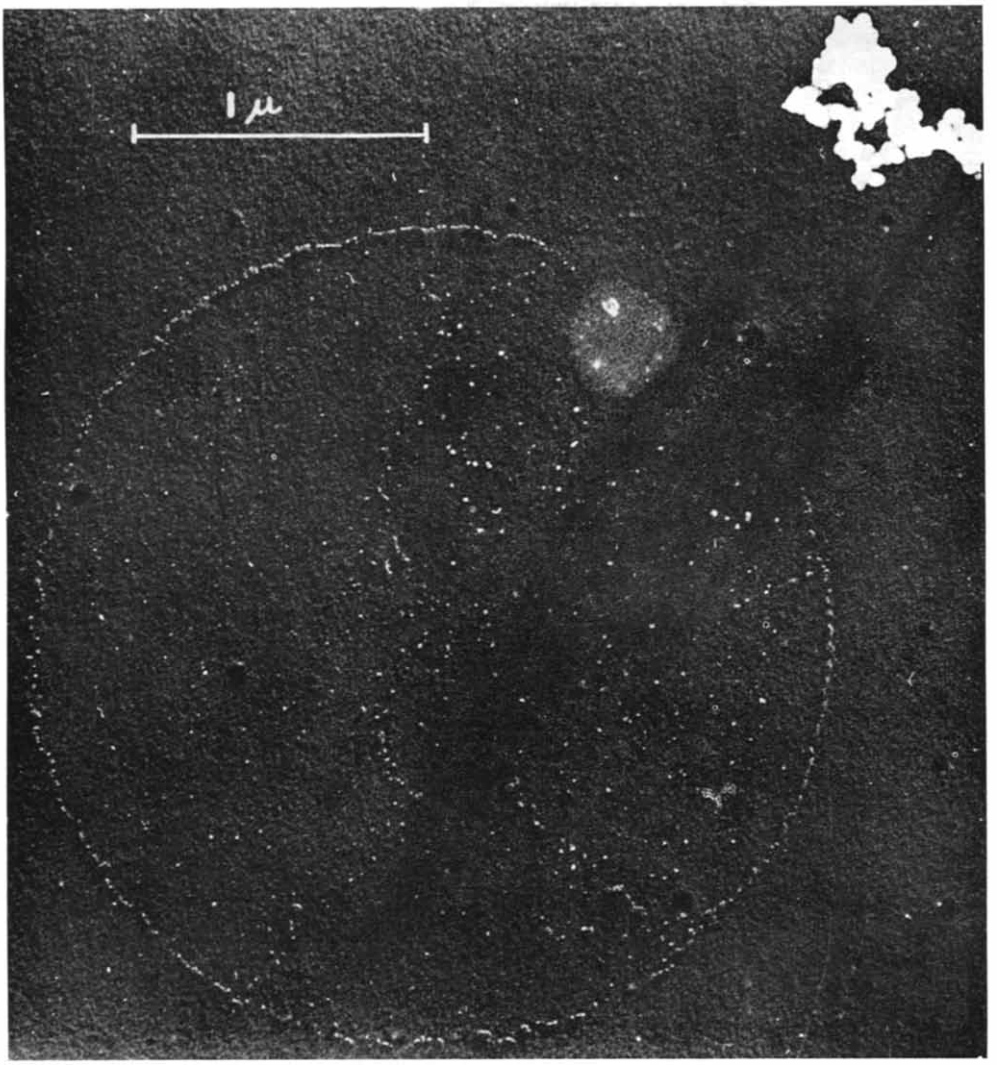

Fig. 5. Electron micrograph of an aerosol of edestin (see description in the text). Solution 10^{-6} *M* in volatile buffer of ammonium acetate and ammonium carbonate of pH 7. Precipitation on the positive pole. Shadowed with palladium at 7°. Compensated objective lens. 38,500 ×.

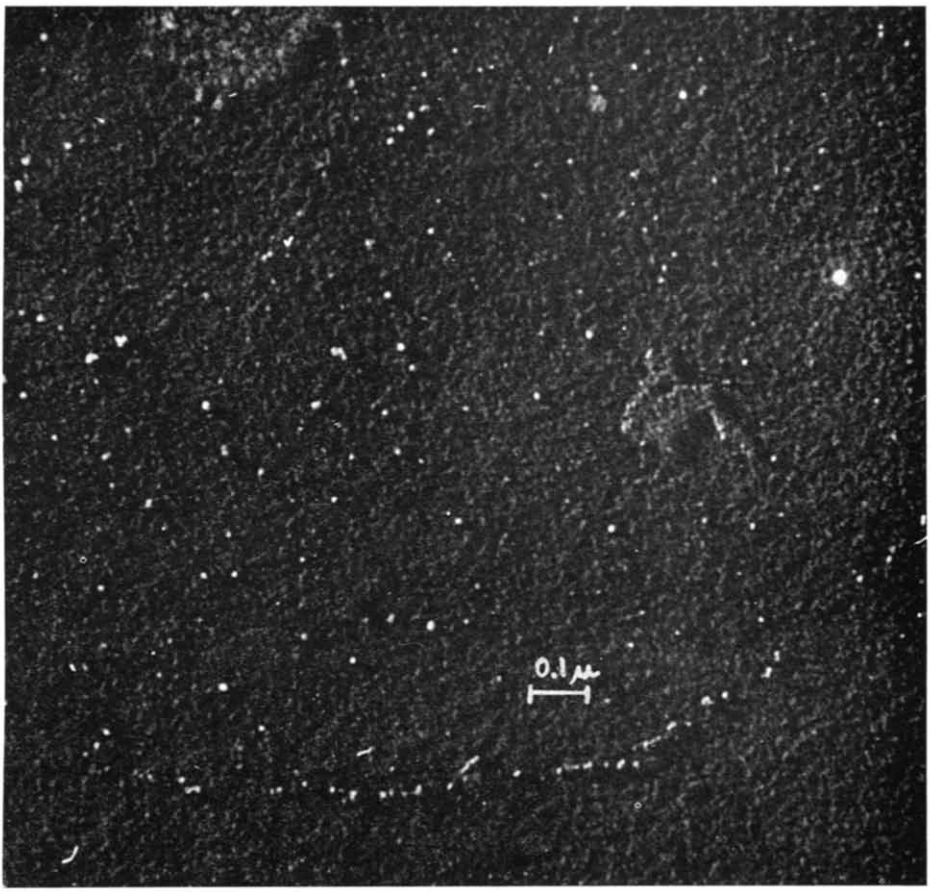

Fig. 6. Electron micrograph of an aerosol of edestin (see description in the text). Same experiment as in Fig. 5. 67,500 ×.

is connected to one pole and the higher electrode with the charging grid to the opposite one. By a simple switch mechanism the sign of the current can be inverted or the charging grid may be disconnected.

The standard results obtained with this assembly were checked by using a concentrated solution of dextrin as an aerosol source. This is a typical control experiment. A 5% solution of dextrin in distilled water was put into the aerosol source. Air was injected at a pressure of 20 lb. per sq. inch. Several factors were varied but the grids were maintained in the chamber for a constant period of two minutes. If both the charging grid and the precipitating electrode were disconnected from the DC source no precipitation of the aerosol occurred. The aerosol obtained is so stable that it would take hours to sediment by the action of gravity alone. Connecting the precipitation electrode either with the positive pole or with the negative pole and with no charge on the grid, the number of droplets sedimented was very small. Charging the grid with the negative pole and precipitating on the positive the precipitation was increased

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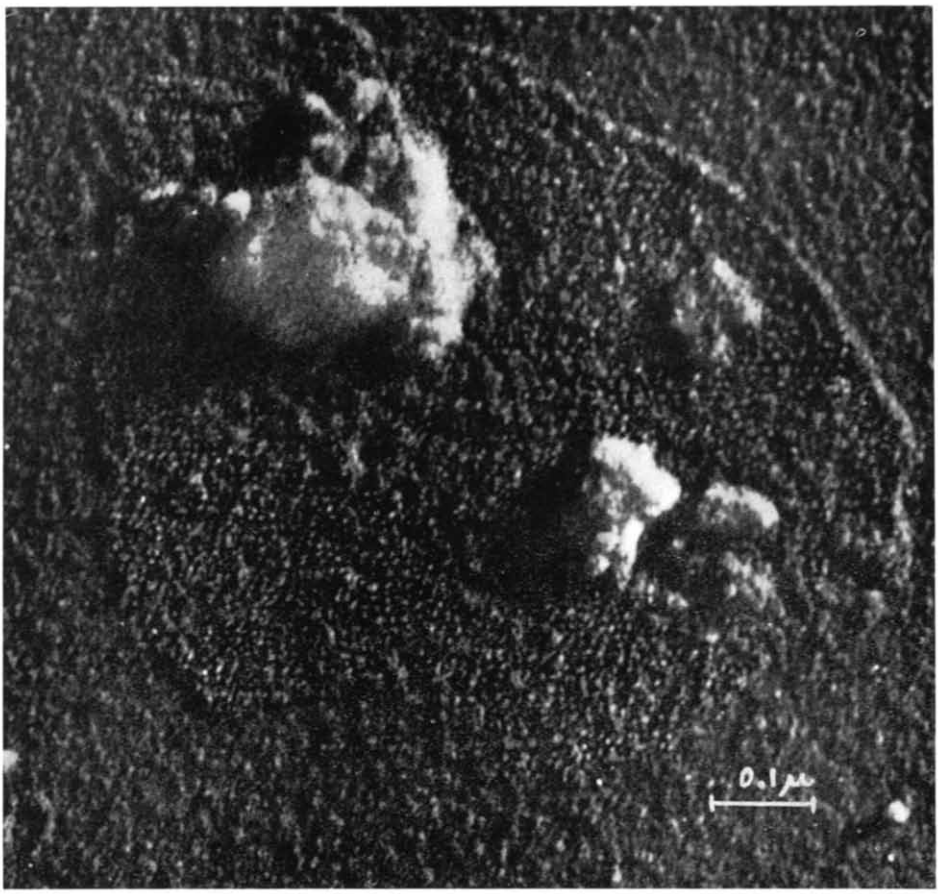

Fig. 7. Electron micrograph of an aerosol of crystalline horse carboxyhemoglobin 10^{-6} *M* solution in bidistilled water pH 7.0 charged with the negative and precipitated on the positive pole for 10 minutes. Compensated objective lens. Shadowed with uranium at 7°. 131,000 ×.

but still scarce. On the contrary by charging with the positive pole and precipitating on the negative there was a considerable deposition of microdroplets (Fig. 3).

Fig. 3 shows an electron micrograph of a typical field of an aerosol of dextrin shadowcast with palladium. There are very few droplets of about 1 μ or more but most of them are much smaller. Measurements of 1000 microdroplets gave the distribution curve of Fig. 2. This shows that 79% of the microdroplets range in diameter between 200 and 1400 A with a peak at 800 A and 94.6% range between 200 and 3000 A. Only 5.4% of the droplets have diameters larger than 3000 A.

Among other macromolecular materials aerosols of the proteins, fibrinogen, edestin and carboxyhemoglobin were prepared.

The fibrinogen (Armour) was from bovine origin (lot 128–160), contained approximately 40–50% sodium citrate and appeared as a dry powder. Solutions were dialyzed gainst a buffer solution of pH 7.6 but generally they were first treated with osmic acid

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to produce a stabilization of the protein and to avoid polymerization. The final solution used for the aerosol varied between 0.0375 g and 0.00075 g per liter.

Edestin was dissolved in the volatile buffer of ammonium acetate and ammonium carbonate of pH 7 described by BACKUS AND WILLIAMS[3] at a concentration of 10^{-6} *M*.

A highly purified sample of crystalline horse carboxyhemoglobin was dissolved in bidistilled water of pH *ca.* 7.0 and used at a concentration varying between 10^{-5} and 10^{-7} *M*.

The aerosol preparations were generally shadowed with palladium at an angle of 7°. In the case of carboxyhemoglobin uranium was evaporated.

An R.C.A. EMU 2C electron microscope was used. In this instrument the asymmetry of the lenses was corrected by the lapping technique proposed by HAMM[7] and the use of iron screws in the objective pole piece as described by HILLIER AND RAMBERG[8]. The photographs were generally taken at a magnification of 12,000 ×.

Preliminary results on the electron microscopy of protein macromolecules

In Fig. 4 a typical result obtained with an aerosol of bovine fibrinogen is shown. In addition to clumps of material, isolated particles are present on the film. These particles are spherical or slightly ovoidal in shape, casting sharp shadows. The smallest particles, probably corresponding to molecular units, are quite uniform in size having a diameter of about 100 A.

Fig. 5 belongs to an aerosol of edestin. The perfect circle made by the macromolecules probably corresponds to a large microdroplet pattern. At the periphery there is a single line of isolated spherical particles having a uniform diameter of about 85 A, probably corresponding to single molecules. Inside the circle, in addition to small particles of the same minimum size, there are larger ones representing clumps of molecules. In the upper right of the circle a large clump of material is probably a result of the dessication of the microdrop. A small clump of carbon particles casts its shadow on the circle of edestin molecules.

Fig. 6 shows at higher magnification another typical field of an aerosol of edestin. In addition to small isolated particles of 85 A there are many small clumps and one large clump of material. In this last one the individual macromolecules are clearly seen.

The aerosol of carboxyhemoglobin shows a microdrop pattern in which the spherical particles are uniform in size and have a diameter of about 55 A. As can be seen in Fig. 7 these particles, probably corresponding to single molecules, are disposed within the aerosol drop in a monomolecular layer which covers the entire surface of the film. In addition, several clumps of material are present in the microdrop in which the individual molecules disposed in a quite regular pattern can be observed.

DISCUSSION

Considerations on the technique

Aerosols have been used in electron microscopy specially for the measurement of droplet size and the number of particles of a sol (RIEDEL AND RUSKA[9], WYCKOFF[10]). The widely used methods for the examination of dusts also belong to this field. (For literature on aerosols, see DAUTREBANDE[6].)

In our work, the production of an aerosol was mainly intended as a way of dispersing a macromolecular material, considering that in diluted solutions, with a volatile

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solvent, the macromolecular units could probably be seen isolated on the film. For the same purpose the spraying technique of BACKUS AND WILLIAMS has been previously used (PORTER AND HAWN[11], MITCHELL[4]). However the dispersion produced in this case seems too coarse (5–20 μ) for the preparation of small macromolecules.

In the technique described in this paper the dispersion is of colloidal dimensions. In fact the distribution curve of the microdroplet pattern shows that almost 80% of the aerosol droplets range in diameter between 20 and 140 mμ. Considering 500 μ^3 as the mean droplet volume given by the spraying technique[3] an aerosol droplet 70 mμ in diameter corresponds to a volume of about 0.000 180 μ^3. This means a volume 27,80,000 smaller for the aerosol microdroplet than for the droplet obtained with the spraying method.

Aerosol droplets of colloidal size as those produced in our experiments are difficult to deposit only by the action of gravity. In a few preliminary experiments, after several hours of sedimentation the collection of aerosol droplets on the film was meagre. Since surface electrostatic charges are present in particles of colloidal dimensions the use of electrostatic precipitation seemed pertinent. The results are improved if the droplets are charged by previously passing them through an electrostatic field.

In addition to the problem of dispersion the smoothness and thinness of the supporting film has to be considered. The plastics used in electron microscopy have a texture with discontinuities of the order of 100 A. This makes difficult the observation of macromolecules smaller than this size, when they are scattered at random. Recently this difficulty has been overcome for macromolecules of several hundred thousand molecular weight, by obtaining replicas of crystalline configurations of the substance (HALL[12,13]). Recently replicas of lactoglobulin crystals have been obtained (DAWSON[14]). With the aerosol technique, the production of a discrete bounded microdrop pattern makes, however, the distinction between the fine structure of the specimen material and the structure of the substrate film much easier.

For example in the case of the carboxyhemoglobin aerosol (Fig. 8) the distinction of individual molecules is easily made, in spite of the fact that they are about half the size of the discontinuities in the supporting film, because they are in a monomolecular layer within the boundaries of the microdrop.

Some attempts to improve the technique by using preshadowed replicas of aerosols deposited on glass or by the use of films of SiO evaporated on glycerol did not give the expected results. In general the technique was too cumbersome and the results obtained in improving the surface structure did not compensate the work.

Protein macromolecules

Although the results obtained in the study of macromolecules with the use of the aerosol technique are considered as yet preliminary, for the case of the proteins fibrinogen, edestin and carboxyhemoglobin some general conclusions seem to be warranted. One is that the molecular weights calculated from direct measurements with the electron microscope agree, to some extent, with the molecular weight determinations made with other physicochemical methods. In this respect the main difficulty lies in the fact that we do not have any sure value for the density of the molecules in the conditions of dehydration, they are inside the electron microscope. The other conclusion is that these results in general are at variance with the predictions regarding the shapes of the macromolecules mainly deduced from dielectric dispersion and sedimentation

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experiments. These conclusions are in agreement with similar electron microscope findings of HALL[12] for the edestin molecule and MITCHELL[4] for plasma proteins. Some considerations on this problem are discussed by MITCHELL[4].

Edestin molecules have been observed with the electron microscope by STANLEY AND ANDERSON[15] and by HALL[12] in replicas of crystalline configurations. The particles were found to be spherical and of about 80 A in diameter. The molecular weight of edestin from ultracentrifugal data is of 310,000 (SVEDBERG AND PEDERSEN[16]). It is considered to be a prolate ellipsoid of revolution with an axial ratio of 9:1 as deducted from dielectric dispersion (ONCLEY[17]) or 4:1 as obtained with sedimentation experiments (COHN AND EDSALL[18]). In our results we find the isolated edestin molecules to be spherical and about 85 A in diameter. Assuming a density of 1.35 a molecular weight of 266,000 is calculated.

To our knowledge no observation of hemoglobin molecules has been previously made with the electron microscope. The molecular weight is calculated as 66,700 by chemical methods and as 63,000–69,000 by the ultracentrifuge. By dielectric constant measurements the molecule is assumed to be a prolate ellipsoid with an axial ratio of about 1.6 (see COHN AND EDSALL[18]). Our results show the hemoglobin molecule to be about spherical and of 55 A in diameter. Assuming a density of 1.33 this corresponds to a molecular weight of 69,750.

The literature regarding fibrinogen is more contradictory. While PORTER AND HAWN[11] observed the fibrinogen as particulate spherical bodies, HALL[19] concluded that the molecules were filamentous with an average length of 600 A and a width of 30–40 A. This last observation is more in agreement with physico-chemical studies which indicate an axial ratio of 20 to 1 with a molecular length of 725 A, the molecular weight being calculated at 440,000. Recently MITCHELL[4] found a globular unit of about 50 A for fibrinogen and considers that it may correspond to a product of depolymerization of the fibrinogen molecules.

Our observations are more in agreement with those of PORTER AND HAWN[11]. It shows the fibrinogen units as spheres or ovoids of approximately 100 A in diameter from which a molecular weight of 407,000 can be calculated.

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SUMMARY

A technique was developed for dispersing the macromolecular material by turning it into a monodisperse aerosol, and precipitating it electrostatically on standard grids for observation with the electron microscope.

The general set-up used is described together with control experiments indicating the size and distribution of the aerosol. About 80% of the aerosol microdroplets range between 200 A and 1400 A with a peak at 800 A.

Preliminary observations were made on aerosols of fibrinogen, edestin and carboxyhemoglobin. In all these cases the individual molecules could be recognized and some measurements made.

The possibilities of the aerosol technique are considered in connection with the study of the size and shape of macromolecules.

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RÉSUMÉ

Les auteurs ont mis au point, en vue de l'observation au microscope électronique, une technique de dispersion de matériel macromoléculaire par transformation en aérosol monodispersé et précipitation électrostatique sur des grilles standards.

L'appareillage est décrit ainsi que des expériences de contrôle qui donnent la dimension et la distribution de l'aérosol. Environ 80% des microgouttes de l'aérosol ont un diamètre compris entre 200 A et 1400 A, avec un pic à 800 A.

Des observations préliminaires, faites sur des aérosols de fibrinogène, d'édestine et de carboxyhémoglobine, permettent de reconnaître les molécules individuelles et de faire quelques mesures.

Les possibilités de la technique à l'aérosol pour l'étude de la forme et des dimensions des macromolécules sont envisagées.

ZUSAMMENFASSUNG

Es wurde eine Methode entwickelt um makromolekulare Stoffe durch Umwandlung in ein monodisperses Aerosol zu dispergieren und es elektrostatisch auf ein Gitter zur Beobachtung mit dem Elektronenmikroskop auszufällen.

Die benützte allgemeine Ausrüstung wird zusammen mit den Kontrollversuchen, die die Grösse und die Verteilung des Aerosols angeben, beschrieben. Ungefähr 80% der Aerosolmikrotröpfchen liegen in dem Bereich von 200 A und 1400 A mit einer Spitze bei 800 A.

Vorläufige Beobachtungen wurden mit Aerosolen von Fibrinogen, Edestin und Carboxyhämoglobin gemacht. In allen diesen Fällen konnten die einzelnen Moleküle erkannt werden und einige Messungen ausgeführt werden.

Die Möglichkeiten der Aerosolmethode werden im Zusammenhang mit der Untersuchung der Grösse und der Form der Makromoleküle betrachtet.

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