A LABORATORY MANUAL OF ANALYTICAL METHODS OF PROTEIN CHEMISTRY

(INCLUDING POLYPEPTIDES)

Editors

P. ALEXANDER

R. J. BLOCK

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ANALYTICAL METHODS OF PROTEIN CHEMISTRY

(INCLUDING POLYPEPTIDES)

VOLUME 3

DETERMINATION OF THE SIZE AND SHAPE OF PROTEIN MOLECULES

EDITORS

P. ALEXANDER

CHESTER BEATTY RESEARCH INSTITUTE

R. J. BLOCK

BOYCE THOMPSON INSTITUTE FOR PLANT RESEARCH

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PREFACE

IN the last fifteen years there has been a revolution in the techniques available for the analysis and isolation of proteins. Every time a new technique has been introduced, numerous papers have appeared describing modifications to it and the research worker who wishes to employ these methods is faced with a very serious problem in deciding which particular variant to use. These volumes are intended to provide the fullest practical detail so that any scientist can follow the procedure by using this book alone and without having recourse to the original literature. No attempt has been made by the contributing authors to describe all the variants. The techniques which are described in full are ones in which all the authors have had first-hand experience and as a result the descriptions contain those small, but important, points of techniques which are often omitted from the scientific papers, but which save so much time if known. Where the techniques require a large instrument such as the ultra-centrifuge or the electron microscope, no attempt has been made to describe the working of these instruments in detail, since this is provided in the manufacturers' manuals. However, the authors have attempted to give full details of the preparation of samples before they can be used in these techniques and for the evaluation of the data. For methods which do not require large instruments or which require instruments which must, in general, be made by the investigator himself, more detailed working details are given. In each of the articles a short discussion of the background and theoretical principle is given and a more detailed description of the difficulties in interpretation. It is our hope that workers who find that they have a problem in protein chemistry will be able to turn to these volumes and, by looking through the chapters, decide which of the techniques is the most suitable for their purpose and then be able to follow this technique from the instructions provided.

In the first volume, separation and isolation procedures are discussed; the second volume concerns its analysis and reactivity, and the third volume with the measurement of the macromolecular properties of proteins.

The contents of Volumes 1 and 2 are given overleaf.

R. BLOCK
P. ALEXANDER

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By M. S. C. Birbeck

from

Chester Beatty Research Institute (Institute of Cancer Research: Royal Cancer Hospital), London, S.W.3

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TECHNIQUES FOR THE ELECTRON MICROSCOPY OF PROTEINS

By M. S. C. BIRBECK

from

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INTRODUCTION

THE resolution of modern electron microscopes lies between about 5 and 15 Å. The lower figure represents the theoretically attainable resolution which has also been obtained in practice, and the upper value is representative of the guaranteed resolution of many commercial electron microscopes. These orders of resolving power should be sufficient to visualize the smallest proteins. However, these resolving powers are only achieved with ideal objects. The methods of making protein molecules conform to such an ideal are the techniques of specimen preparation, and it is these techniques which will be described.

No attempt will be made to describe the actual manipulation of the instrument itself. This knowledge will either be possessed by a specialist operator, or alternatively may be obtained from the manual which applies to the particular instrument. Further information on the theory of the instrument will be found in textbooks by Coslett (1951), Hall (1953), and Leisegang (1956).

THE ELECTRON MICROSCOPE

The scope of this section is merely to describe the theory and practice of the electron microscope sufficiently to allow an understanding of the type of object which may be examined. From which may be inferred the type of specimen preparation which will be necessary to convert the material of interest into a suitable object.

A diagrammatic representation of an electron microscope is shown in Fig. 1. The electrons, emitted from a tungsten filament, are accelerated by a high voltage, usually in the range 40–100 kV. The electron beam then passes into the lens system. The first lens is called the condenser, by analogy with the light microscope, whose function is to focus the beam on the object, thus "illuminating" it. The electrons, after passing through the object, immediately enter the objective lens, thus producing a magnified image of the object further down the column. This image is further magnified by the projector lens and the second image falls on a fluorescent screen where the

electron energy is converted into light, thus producing a magnified image of the object. Each electron lens is a magnetic field which has a focusing effect on an electron beam and the strength of the field may be varied by altering the current in the lens winding. Hence focusing of the object is achieved by varying the current of the objective lens, and variation of magnification is obtained by varying the projector current. In most modern instruments there is also a third stage of magnification which may be switched in or out,

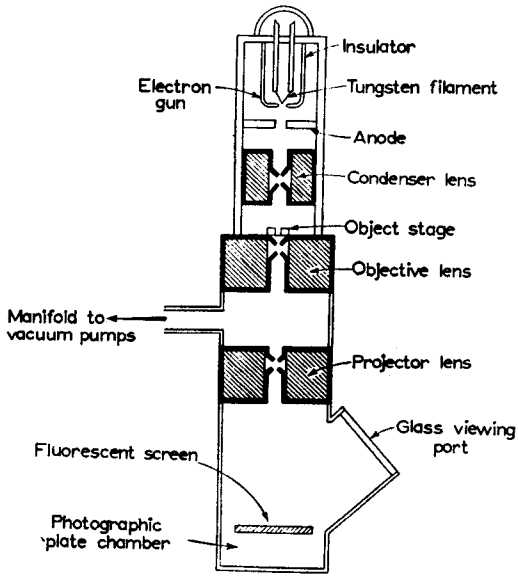

Fig. 1. Diagrammatic view of typical electron microscope.

which allows a total range of magnification from about a few hundred times up to about 100,000 times.

There are two useful measures of performance of an electron microscope. The first is resolution or resolving power. The theoretical resolution is determined by the accelerating voltage and certain parameters of the objective lens. These factors are sufficiently similar in all practical electron microscopes of more advanced design, with the result that, for these microscopes, this resolving power is about 5 Å. However, the actual practical resolution depends also on such factors as electrical stability of the circuits, mechanical stability of the object, and mechanical and magnetic symmetry of the objective lens. This latter factor gives rise to astigmatism of the image and in most modern microscopes may be corrected for by a stigmator. All these limiting factors have been sufficiently overcome in several commercial instruments to allow resolutions of better than 10 Å. However, to attain this requires very careful handling of the microscope and, under normal conditions, resolutions between 10 and 20 Å are more usual.

The second measure of performance is the contrast of the microscope.

This is equally important for biological work, for it is this factor which determines whether a protein molecule may be detected or not. The contrast depends on the scattering of electrons by the object as well as factors determined by the microscope itself, again mainly those of the objective lens. In particular the constant depends on the objective aperture, which should be sufficiently small to produce maximum contrast, without impairing the resolution. The effect of the specimen itself is more difficult to assess because the contrast in a microscope is due to two separate mechanisms. The first due to the angular scattering of the electrons is responsible for the in-focus image and may be calculated with reasonable accuracy. The second is a phase contrast effect which is obtained by very slightly defocusing the image and produces a much greater contrast for small particles. This second effect is much more difficult to calculate. It is, however, useful to consider the first mechanism in a semi-quantitative way, although it may properly only be applied to large objects (i.e. several times the resolving power). The contrast by this mechanism is proportional to the mass thickness of the object (i.e. the thickness × density). Assuming average figures for commercial microscopes, it requires about 200 Å of organic material ($\rho = 1{\cdot}3$) to produce an intensity difference on the fluorescent screen of about 10%. This means that particles of this size are the smallest that may be detected without any aids to increase their contrast (e.g. shadowing or staining). These figures may also be used to calculate that a film of platinum ($\rho = 20$) would produce the same contrast if 13 Å thick. These figures may be used to give some idea of what will be a suitable form of object for the microscope.

PREPARATION OF SUPPORTING FILMS

General Requirements

Particles to be observed in the electron microscope have to be placed on a supporting film which is transparent to electrons. These supporting films have therefore to be very thin ($\sim$ 200 Å) yet mechanically strong enough to bridge the holes in the supporting grid (q.v.). Films of various polymers have these properties and the preparation of Formvar (polyvinyl formal) films, the most commonly used plastic, will be described in detail. This method may readily be used for other polymers. Formvar films have the advantage of being extremely tough mechanically; they are also easy to prepare. One disadvantage of Formvar is that its surface is slightly etched by water, so that after shadowing (q.v.) a poor background is obtained. Nitrocellulose (Collodion) is better in this respect, as is also polystyrene, although the latter is mechanically not as strong as Formvar. Some plastics (e.g. polymethacrylates) are not suitable, as the films are disrupted by the electron radiation in the microscope. The behaviour of proteins when applied to these films depends to some extent on the surface properties of the film. Films containing polar groups (e.g. polyvinyl pyridine) which are more hydrophilic may be more suitable for certain purposes. (Birbeck and Stacey, 1959). Polystyrene, which makes very satisfactory films, is an example of an extremely hydrophobic polymer.

Supporting films may also be prepared by evaporation of certain materials in a high vacuum. Substances used for this type of film are carbon and silicon monoxide. Carbon films have exceptional mechanical stability, particularly when in the microscope, thus reducing specimen drift. They are extremely hydrophobic and this makes them in some ways unsuitable for proteins, as such films tend to cause unfolding of the molecule. They are, however, used in a somewhat modified form in the mica-replica technique which is a most useful method for proteins and therefore their preparation will be described in detail. Original descriptions of the method will be found in a paper by Bradley (1954).

Specimen Grids

The mechanical support for the film is called the specimen grid. The most usual form is a copper wire mesh. Not only do they vary in size according to the microscope, but there is a considerable variation in the pattern of holes that can be obtained. For particulate preparations, where one area of the film is identical to any other and therefore there is no necessity for the maximum clear area, the grids with round holes are the most satisfactory. These grids give the greatest stability for thin films. The Siemens microscope is supplied with grids made of platinum, containing a few round holes; these grids, although of limited use in general, may be used with advantage for this type of work.

Preparation of Formvar Films

Solution 0·1 g Formvar*

100 ml chloroform.

There are many methods of preparing polymer forms which are described in textbooks on electron microscope (Hall, 1953; Cosslett, 1951). The method described here is a simple one that has been used by the author for many years.

Take a 3 × 1 in. glass microscope slide and clean it by washing briefly with a little water and detergent, rinse with tap water and dry with a clean cloth. (It is possible to clean the slide to such an extent, e.g. by chromic acid, that the polymer films cannot be detached!) Holding the slide horizontally at one end, flood the other end (about 1½ in.) with about 1 ml of the Formvar solution, being careful to avoid any solution from reaching the fingers holding the slide. Rock the slide slightly backwards and forwards so that an even film of solution covers the flooded area, then tilt the slide vertically and place in a low humidity atmosphere to dry. This whole operation should be performed swiftly, taking perhaps about 3 sec for completion. The low humidity atmosphere is necessary because the evaporation of chloroform cools the slide, which will induce condensation of water vapour. The effect of this is to produce a film with small microscopic holes or, in severe cases, a visibly cloudy film. A simple way to attain a low

* Obtained from Shawinigen Products Corp., 350 Fifth Avenue, New York. The Formvar should be dried in a desiccator before use.

humidity is to place the slide, immediately after tilting it to the vertical position, into a warm oven (35°–70°C). In some laboratory atmospheres, placing the slide to dry over a radiator may be sufficient to prevent condensation.

This method of preparing Formvar films may be applied to other polymers, provided that a suitable solvent is used. The concentration of polymer may also require modification, as the thickness of the film will depend on the evaporation rate of the solvent and also to a certain extent on the variation of the manipulation of the slide by individual workers.

The Detachment of Formvar Films

To detach the Formvar film from the glass slide, first scratch the film around its periphery, which should leave a useful area in the centre about 1 × ½ in.

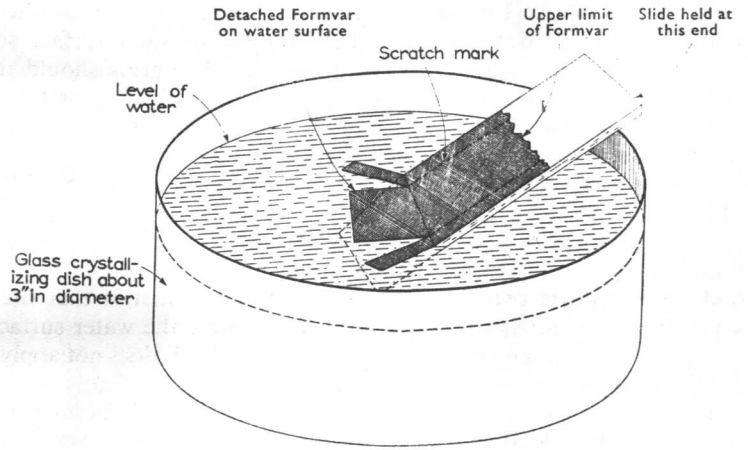

Fig. 2. The detachment of Formvar films.

Take a glass vessel, a crystallizing dish about 3 in. in diameter and 2 in. deep is convenient, and fill it with distilled water up to about ⅛ in. of the top. Lower the slide, Formvar uppermost, at an angle of about 30° into the water (Fig. 2). As the lower end penetrates the water surface, the Formvar film should detach itself, so that as the slide is progressively lowered, the Formvar film remains floating on the water surface. If the Formvar is stubborn and will not start detaching, try teasing it with a needle at the lower end. Once it starts to detach, the process usually goes smoothly. Breathing on the film also helps, but in the interests of cleanliness, this should be avoided if possible.

The film when detached should have sufficient mechanical strength to be manipulated over the water surface with a needle. It is convenient to attach it to the side of the vessel by this means. The film should also be quite colourless,* any yellow or higher interference colours indicate that it is

* *Lighting of the electron microscope laboratory.* Many of the manipulations required in electron microscopy, particularly those involving specimen grids and supporting film

much too thick, even a high light reflectance is undesirable. The ideal Formvar film, about 200 Å thick, may only be detected by a slight discontinuity of reflectance at its edge.

Transferring the Film to the Specimen Grids

The specimen grids may be covered by the Formvar film in two ways. The first method is more convenient for coating a large number ($\sim$ 20) of grids simultaneously. The grids are placed by means of fine forceps* close together but not touching on *top* of the floating formvar film. It is easy by this method to avoid any areas of the film which look uneven. A piece of platinum wire mesh about 1 in. square, cleaned by ignition, is held with forceps horizontally over the grids. The platinum mesh is then pushed downwards, still horizontal, forcing the grids and Formvar below the water interface. When about 1 in. below the water level, the mesh is tilted and brought upwards and outwards, passing through the water surface so that the mesh moves in the plane of its own surface. The grids should remain adhering to the mesh. This manipulation may require some practice, and for success it should be performed smoothly and confidently in a fairly quick sweep of the hand. The mesh should then be placed on a filter paper, grids uppermost, to blot off any surplus water and then in an oven (35°–70°C) to dry (10 min).

The second method of coating the grids is to place the grids on the platinum mesh and bring them up under the film. This may seem easier than the first method but there are two practical difficulties. The first is that the grids must be placed on the mesh while the mesh is below the water surface, for if not they float off when the mesh is submerged. (This does not apply when solid platinum Siemens grids are used.) The second difficulty is that it requires a very steady hand to prevent the grids moving about when it is lifted up under the Formvar film. This method does, however, have one advantage over the first; this is that the surface on which the protein is to be placed does not come into contact with the water or with the glass slide from which the film was cast. The surface on which the protein is to be placed is therefore potentially cleaner.

Preparation of Carbon Films

Two carbon rods about 0·5 cm in diameter with pointed ends are mounted

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on water surfaces require a good light. The ideal is daylight through a large window with an uninterrupted view of the north sky. If artificial light must be used, then one with an extensive source should be used. An X-ray viewing box fitted with a fluorescent lamp, placed at the back of the bench gives ideal illumination over the 2 ft square or so in front.

In order to make the detached films readily visible, it is convenient either to place the dish in which the films are floating on a black surface or to paint the outside of the dish black.

* Forceps for handling grids are required constantly in the electron microscope laboratory. Surgical forceps used for iridectomy, if the serrations on the tip are ground, are suitable. Watchmakers' forceps may also be used. Ideally the tips of the forceps should be spade-shaped so that they will slide under the grids.

in a vacuum chamber horizontally with the pointed ends touching, lightly pressed together with a spring.*

It is usual to employ a shadowing apparatus to provide the high vacuum which should be about 10^{-4} mm Hg. A current of about 50 A is then passed through the carbons. A current of this magnitude may not be available from the transformer of some shadowing apparatuses and the transformer may require modification. The current produces intense local heating at the point of contact of the two carbons and from this region carbon is evaporated. The glass slide to be coated is placed about 6 in. below the carbons and a film about 100 Å thick is obtained in about 10 sec. Such films are slightly brown by transmitted light. The exact time required to produce a film depends on the current and other experimental variables and is best determined by experiment.

The film is detached from the glass slide in a similar manner as that described for Formvar. It is usually necessary to smear the slide with a trace of detergent before evaporating the carbon on to it, otherwise the film will not detach. The film, when floating on the water surface, may be transferred to specimen grids in a manner exactly as described for Formvar films.

APPLICATION OF PROTEIN TO FILMS

Preparation of Protein Solution

If protein is to be dried down on to a supporting film for examination in the electron microscope, it must first be dissolved in a completely volatile

TABLE I. NECESSARY DILUTIONS FOR SPHERICAL PARTICLES

Diameter of protein particle (A°)	Approximate molecular weight	Dilution factor g/ml	
		for 1 mm diameter drops	for 10 μ drops (spray method)
30	12,000	$1{\cdot}5 \times 10^{-7}$	$1{\cdot}5 \times 10^{-5}$
100	400,000	5×10^{-7}	5×10^{-5}
300	12,000,000	$1{\cdot}5 \times 10^{-6}$	$1{\cdot}5 \times 10^{-4}$

These calculations assume that the drop is a hemisphere, that the particles are equally distributed over the supporting film and that the mean distances between particles is four times their diameter.

medium. The most generally used solvent for proteins is water, although there is no objection to using other solvents. Because the concentration of protein which is required in the water (see Table I) is small, it is imperative that the non-volatile material in the water used should be as low as possible. The water should therefore be double distilled; the second distillation

* The jig for holding the carbon rods is sold by the manufacturers of most of the shadowing apparatuses.

should be in an all-glass apparatus and the water should be stored in an all-glass vessel. Such an apparatus supplying sufficient quantities of water, usually several litres a day, is essential in a laboratory engaged in the electron microscopy of proteins. Contamination from other sources is not usually troublesome.*

For those proteins which are insoluble in distilled water it is permissible to add volatile salts. The most commonly used is ammonium acetate. As such a solution dries the concentration of salt rises and finally there may be differential evaporation of its components. In the case of ammonium acetate, it is probable that the liquid just before it dries is on the acid side of neutrality. For this reason ammonium carbonate is sometimes added. Ammonium benzoate has also been used, particularly for virus work.

Simple Method of Applying Protein Solution

The easiest way to apply a particulate suspension to the supporting film is by means of a platinum loop. A loop should be made from wire about 0·01 in. in diameter and bent into a loop about 1 mm in diameter. Such a loop, if dipped into the solution and then touched on to the grid on which the supporting film is laid, will transfer a drop about 1 mm in diameter. The concentration of protein required in the original solution to give a reasonable distribution of particles on the electron micrograph is shown in Table I. The calculation of these concentrations assumes that the particles are spherical and also that the drop is a hemisphere. Furthermore it is assumed that a distribution that gives an average particle separation five times the particle diameter is desirable.

The very high dilution required for small particles makes the spray method (q.v.) advisable. However, the dilution problem may be solved crudely by placing a drop of a more concentrated solution on the grid and then removing the drop after a few seconds with a small piece of filter paper. The effect of this is to remove the majority of the particles, but no quantitative recommendations of concentration can be given. It is a matter for experiment.

Grids prepared in this method may be dried either at room temperature or preferably in an oven with a temperature up to about 60°C, at which temperature the drops will dry in a few minutes. The grids may then be examined directly, or treated by some subsequent technique as, for example, staining or shadowing.

The Spray Method

The spray method was originally devised (Backus and Williams, 1950) to allow quantitative determinations of virus particle suspensions, but it may be applied equally well to protein solutions and it has two advantages over the simple method. The first of these is that higher concentrations of protein

* Although air filtration and air conditioning may be desirable in a laboratory in special circumstances, the problem of airborne contamination in electron microscope specimen preparation is not a major one. Most airborne particles are so large by the standards of electron microscopy that they are easily distinguished. However, smoking while actually preparing specimens should be avoided.

must be used and the second is that quantitative determinations of particles per unit volume of solution may be made. For quantitative work the unknown suspension is mixed with a calibrated suspension of marker particles and the mixed suspension sprayed on to the grid. The ratio of the counts of the two particles in the electron micrograph may be used to calculate the concentration of the unknown suspension. The necessity of using a spray of small drops follows from the fact that the whole drop must be counted. This is to avoid artifacts caused by selective deposition of particles at the edge of the drop.

The calibrated suspension of marker particles should be of the same order of size as the unknown and should give an equal distribution of particles. For virus work the Dow polystyrene 580 G. Lot 3584* is a suitable marker material; it has a diameter about 2500 Å and is remarkably monodisperse. For most proteins smaller particles would be desirable and, although latex

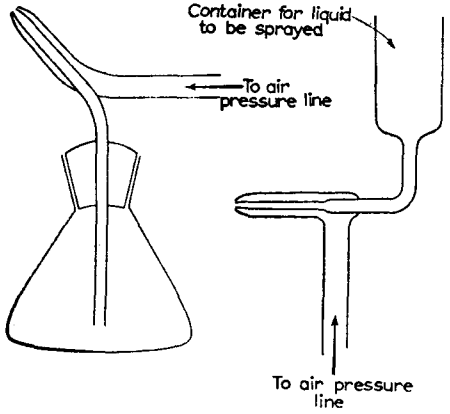

Fig. 3. Two pieces ot all-glass apparatus suitable for spraying. The diameter of the spray orifice should be about 1 mm and the air gap somewhat smaller. The other dimensions are not critical.

emulsions down to about 200 Å in diameter are available, the spread of particle size in these is greater. The ideal size of the diameter of the drop depends on the size of the particle to be examined, for proteins diameters of about 1–10 μ are suitable. Drops of 1 μ in diameter are difficult to make and elaborate devices are required (Nixon, 1958); however, 10 μ drops may be prepared with relatively simple sprays. Fig. 3 shows two pieces of all-glass apparatus which are suitable, both may be easily made in the laboratory. An air pressure of about 20 lb/in^2, most conveniently obtained from a cylinder of compressed nitrogen, will provide a dense spray of small drops. To obtain only the smallest drops it is necessary to direct the spray through a system of baffles which will catch the larger drops. The smaller ones which remain airborne are allowed to deposit on the coated specimen grids. The required amount of solution to be sprayed and the drop size are both very dependent

* Obtained from Dow Chemical Co., Midland, Michigan, U.S.A.