MACROMOLECULAR FINESTRUCTURE BY DARK FIELD ELECTRON MICROSCOPY

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ABSTRACT The potential of dark field electron microscopy for examining details of unstained unshadowed macromolecules has been studied. Images produced by scattered electrons show sufficient contrast and resolution to reveal the helical nature of short sections of DNA or the cleft containing the active site in ribonuclease. Spreads of transfer RNA were observed to contain many trifoliate structures.

INTRODUCTION

In order to detect a small biological object with bright field electron microscopy it is generally necessary to enhance the contrast by staining or shadowing it with heavy atoms. Without such contrast agents a carbon protuberance with a height of about 70 A is required at an accelerating voltage of 100 kv to produce a 5% variation (assumed to be the detectable limit) in light intensity in the image plane (1). In dark field operation at the same voltage a 5 A protuberance of carbon on a 100 A carbon film will be detectible as a 5% variation in intensity (1). Since carbon films of a thickness of 100 A or less are readily produced it should be possible by dark field microscopy to detect small biological objects with thicknesses of 10 A or even less without resorting to contrast enhancing methods.

Dupouy et al. (2) have studied images produced in dark field by the "contrast-stop" method in which a fine wire placed across the objective aperture is used to stop the central beam of undeflected electrons. Unstained bacterial flagella are readily observed, although the outlines and details of these and other objects of similar size appear indistinct. This lack of sharpness of the image is thought to be caused in part by the asymmetry introduced by the wire at the position of the objective aperture in the microscope. In part, however, many of the scattered electrons have suffered energy losses to varying degrees after traversing any specimen, thereby producing chromatic aberrations in the image. To circumvent the disturbances caused by the wire I have tilted the beam to obtain dark field conditions (1). Although this results in a loss of slightly more than 50% of the electrons scattered into the aperture compared to the "contrast-stop" method, the resolving power, a func-

tion of the instrument, should now be at least equal to that in bright field operation. The problem of chromatic aberrations in the image should be reduced for a thin specimen on a thin substrate, since at a given energy of the incident beam energy losses by the electrons traversing the object, while depending on the nature of the specimen, depend also on its thickness (3). I have studied the potential of dark field electron microscopy by examining different macromolecules each less than 50 A in thickness, supported on a very thin layer of carbon. Helices were observed in DNA filaments, horse-shoe-shapes for ribonuclease, and variations of a clover-leaf form in spreads of transfer RNA (tRNA).

MATERIALS AND METHODS

The carbon support was produced by condensation of carbon on freshly cleaved mica. The layer was floated on a clean water surface and lowered on submerged microscope grids covered with a carbonized layer of plastic (Formvar or cellulose acetobutyrate) containing holes no greater than a few microns. For larger holes the carbon layer was too fragile. A drop of solution containing the specimen at 10 to 100 ng/ml in a suitable buffer was placed on the grids, and the specimen was allowed to diffuse to the carbon surface for 30 sec up to several minutes. For longer diffusion times or higher concentrations the macromolecules tended to overlap making the observation of isolated molecules in thinner regions of the support too rare an event. The grid still bearing the drop, was then washed in the same buffer several times before any buffer still adhering was withdrawn and the surface permitted to dry. The specimen was examined at high power in a Siemens Elmiskop 1 which had been placed in the dark field mode by tilting the electron gun. Care had to be taken to avoid all contamination of the specimen by the breakdown of oil vapours in the microscope and to avoid conditions under which the carbon support evaporated under the action of the beam. To accomplish this when using standard 3 mm grids a special cold trap was constructed, but a cold trap provided with the instrument for use with 2.3 mm grids proved to be adequate as well. In order to increase the light intensity at the highest electron optical magnification ($\times$ 80,000 was used generally) a pointed filament was used in the electron gun. Even at this magnification the macromolecules could not be seen directly on the microscope screen. Therefore focussing was accomplished on the occasional larger object seen in the field of view or on the thin carbon film itself, since after dark accommodation one could discern variations in thickness of this film. Plates taken at random of areas of carbon film over holes in the plastic support were then examined for focus, absence of movement of the specimen during the 15 sec exposure, and for suitable specimen concentration. Whenever the first two criteria were met, macromolecules, if present in the original drop, could be seen on the photographic plate.

RESULTS AND DISCUSSION

The technique was tested using polyoma and calf-thymus DNA and ribonuclease as examples of biological materials whose structures and dimensions are well known from previous biochemical and crystallographic studies (4–8). Structures or particles such as bacteriophage tails or ferritin which have been thoroughly studied in the electron microscope using bright field techniques proved to be unsuitable as test materials in the dark field mode since they were either too thick, producing too many chromatic aberrations in the image, or presented special problems such as

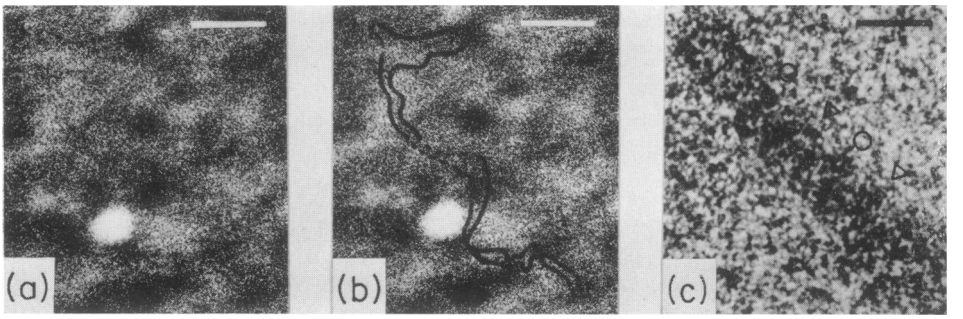

FIGURE 1 (*a*) Reversal image of thin carbon film used as support for macromolecules. The lighter areas in the photograph correspond to thinner areas in the film. The region shown contains portions of polyoma DNA circles opened from the superhelical form with 50 mg/ml ethidium bromide in 0.01 M sodium chloride 0.001 M sodium citrate. The filaments are visible only in small sections for reasons mentioned in the text. The most obvious filament in Fig. 1 *a* is sketched in Fig. 1 *b*. The bar represents 500 A. (*c*) A portion of a filament of calf-thymus DNA spread from a solution of 0.5 M ammonium acetate pH 7.0. A double-helical substructure can be seen with a diameter of 20 A and a period of about 35 A for one complete turn. The open triangles indicate one turn on one strand while the circles indicate one turn on the other strand. The bar represents 25 A.

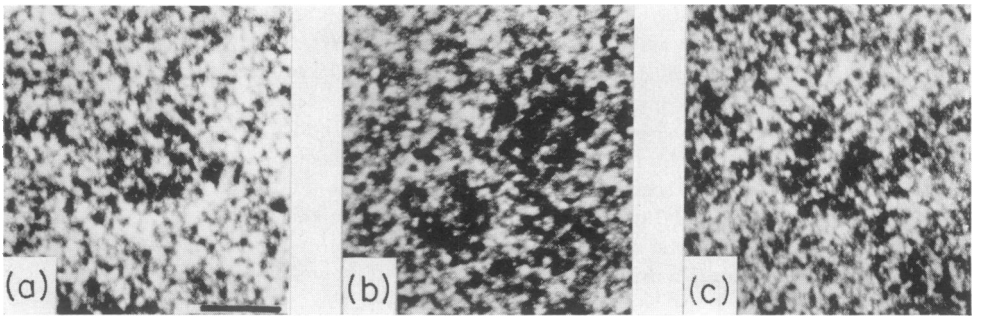

FIGURE 2 Structures seen in a spread of pancreatic ribonuclease dissolved in water. The bar represents 20 A.

Bragg reflections from the crystalline ferritin core. The potential of the dark field method is however well illustrated for the chosen test molecules, the DNA and ribonuclease in Figs. 1 *c* and 2, and also for a relatively unknown structure, tRNA, in Fig. 3.

Before discussing these structures the present major difficulty of the method must be mentioned. This arises from the combination of the great sensitivity of the technique to variations in thickness or density and the fact that the thin carbon support used at present has relatively large variations in thickness, with thinner and thicker regions interspersed at 300–500 A. The effect is shown in Fig. 1 *a*. In the photograph the lighter areas indicate thinner regions in the film, the lightest area being a hole. Photographic techniques designed to make the background more uniform, shading

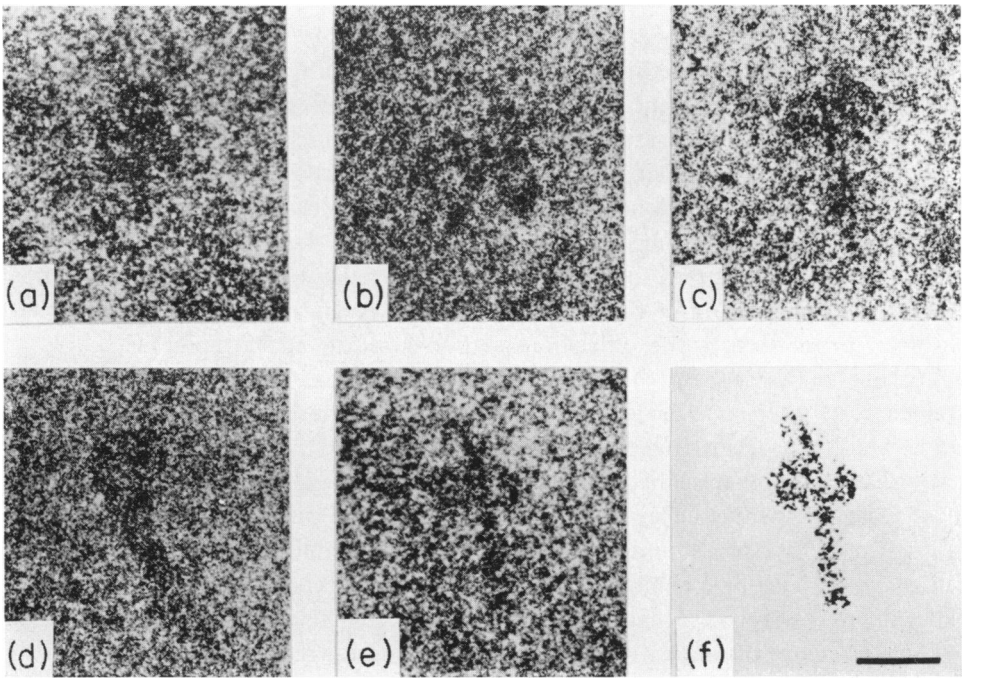

FIGURE 3 Structures seen in spreads of E. coli tRNA dissolved in water. (*f*) Darkest portions of the central structure seen in (*e*). The bar represents 50 A.

for instance, are so difficult in this case as to be impractical. Moreover, to aid visualization of a molecule of interest one has to resort to contrast-enhancing methods such as reversing the negative once or several times. These methods add to the difficulties with the background over larger areas, since they increase the contrast not only between molecule and support but also between areas of differing support thicknesses. For this reason the unstained and unshadowed polyoma DNA which had been spread on the carbon film in Fig. 1 *a* is visible only in short sections in the figure, although it could be followed as a faint line on the original negative for a length of almost 1 μ. The most obvious filament is shown sketched in Fig. 1 *b*. Over a region of 200–300 A in diameter, however, especially in thin sections, the contrast between molecule and support can be enhanced to such an extent that DNA filaments and also details of their structure can be discerned far more easily. While most often perfect helices were not observed, DNA spread from buffers of higher ionic strength did at times show almost ideal Watson-Crick double helices for a few turns (Fig. 1 *c*). In the section shown, the helix has an outer diameter close to 20 A and a pitch of about 35 A for one complete turn. This is in excellent agreement with the 20 A and 34 A obtained from crystal data (5).

The variation in thickness of the carbon support suggested that a macromolecule

of smaller extent would provide a more ideal test material, since in this case the entire molecule would at times lie in an extremely thin portion of the support. Ribonuclease, whose structure is known from the crystallographic work of Wyckoff et al. (7, 8) is a molecule satisfying this condition. Pancreatic ribonuclease (Sigma Chemical Co., St. Louis, Mo.) was in general observed to exhibit a U-shape (Fig. 2) that in form and dimensions was satisfyingly close to the structure calculated from the crystal data. While some structure of the order of 4–5 A is seen in Fig. 2, it should be pointed out that the useful resolution is limited by the fact that it is not known what proportion of the scattered electrons is caused by the molecule itself and what proportion by the amorphous carbon substrate at that point.

Mixtures of *Escherichia coli* tRNA (Calbiochem, Los Angeles, Calif.) were examined as an interesting example of macromolecules whose structures are relatively unknown. It has been suggested from maximum base pairing considerations of known sequences that such molecules exist as trifoliate structures (9). Spreads of these molecules, active in protein synthesis in vitro and purified further by passage through a methylated albumin kieselguhr column, contained structures shown in Figs. 3 *a*–3 *e*. Trifoliate configurations are quite prominent among these. Since one could infer from Fig. 1 *c* that the sugar-phosphate backbone is seen as the darkest part of the image of nucleic acids, it was thought that printing of only the darkest portion of the structures in Figs. 3 *a*–3 *e* might give a clearer indication of the substructure of these forms. In Fig. 3 *f* the densest parts of the structure in 3 *e* are shown. The three "leaves", as suggested by the single stranded loops in the models (9, 10), are quite obvious. There is, however, no clear indication of base-pairing in this region, perhaps as a result of the low ionic strength of the solution used. The "stem" of the configuration in the figure, corresponding probably to the CCA terminus of the tRNA molecule, does not seem to be a double helix. Its diameter, about 15 A, is too small for this (12) nor does it have a helical form similar to that of the DNA in Fig. 1 *c*. It may consist of two strands, perhaps not completely base-paired slightly twisted. Assuming that these structures do represent tRNA this is not an untenable conclusion, since a few known sequences indicate that bases in this part of the molecule are mismatched at several positions (9–11). The total length of the configurations seen, assuming single-stranded loops and a double-stranded stem, is in the order of 300–320 A, or still sufficiently short to fit the 75–80 nucleotides known to constitute tRNA molecules. It is tempting then to say that these are the structures of tRNA, but final proof awaits purified preparations of single species of these molecules.

While the potential of dark field electron microscopy has been shown here for only three macromolecules, the method with further improvement should be very helpful for the study of other unstained unshadowed structures about 50 A in size or smaller down to perhaps the resolution limit of the microscope. Moreover, specific techniques such as the base sequence determinations of DNA and other nucleic

acids with the help of base specific heavy metal markers (13) should be greatly facilitated by its application.

I wish to thank Professor G. Dupouy in whose laboratory I carried out the preliminary studies as a post-doctoral fellow, Dr. M. Blackstein for his gift of polyoma DNA, and Drs. H. E. Johns and L. Siminovitch for their encouragement.

The work was supported by the National Cancer Institute of Canada.

Received for publication 25 March 1969.

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