ION ETCHING OF TOBACCO MOSAIC VIRUS AND T4 BACTERIOPHAGE

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ABSTRACT A technique for the etching of biological materials by accelerated nitrogen ions is described. Upon exposure to such a beam, tobacco mosaic virus becomes thinner in diameter and the head of T4 becomes smaller. Experiments on radioactively labeled T4 suggest that the particle's protein is more susceptible to removal by the incident ions, and that the residual material is predominantly nucleic acid.

INTRODUCTION

Electron microscopists have long been aware that prolonged exposure of a specimen to the electron beam results in deterioration of the image. Partly, this is due to direct radiation destruction of the biological material by the incident electrons, which is known to be a major factor in limiting the amount of retrievable information by electron microscopy. Deterioration of electron diffraction patterns has been used for evaluating the extent of such damage (Glaeser and Thomas, 1969; Glaeser, 1971). To circumvent the deleterious effect of impinging electrons Williams and Fisher (1970) developed a technique whereby the beam is manipulated so that the area of the specimen finally photographed has received only minimal irradiation. Alternatively, one can employ high voltage electron microscopy to reduce damage to the specimen (Kobayashi and Sakaoku, 1965).

Thach and Thach (1971) used autoradiography of labeled specimens to show that there was an actual loss in the mass of a sample exposed to the electron microscope beam. Diminution in contrast of the image as well as direct weighing have also been described for measuring the loss of substance caused by electron irradiation (Reimer, 1965). Recently, the limitation imposed upon attaining high resolution information on biological material using the electron microscope due to specimen damage by the beam has been reviewed (Beer et al., 1975).

It has been shown that the bombardment of tobacco mosaic virus (TMV) with nitrogen ions removes portions of the virus as a function of accelerating voltage and exposure time to the beam (Rizk and Bendet, 1972). The diameter of the rod-shaped virus thus treated was reported to become thinner, and it was suggested that the protein subunits might be more susceptible to the ion etching, and that the residual material being observed might represent the nucleic acid. To test the possibility that ion etching is selective in its removal of material, and to obtain a quantitative approxima-

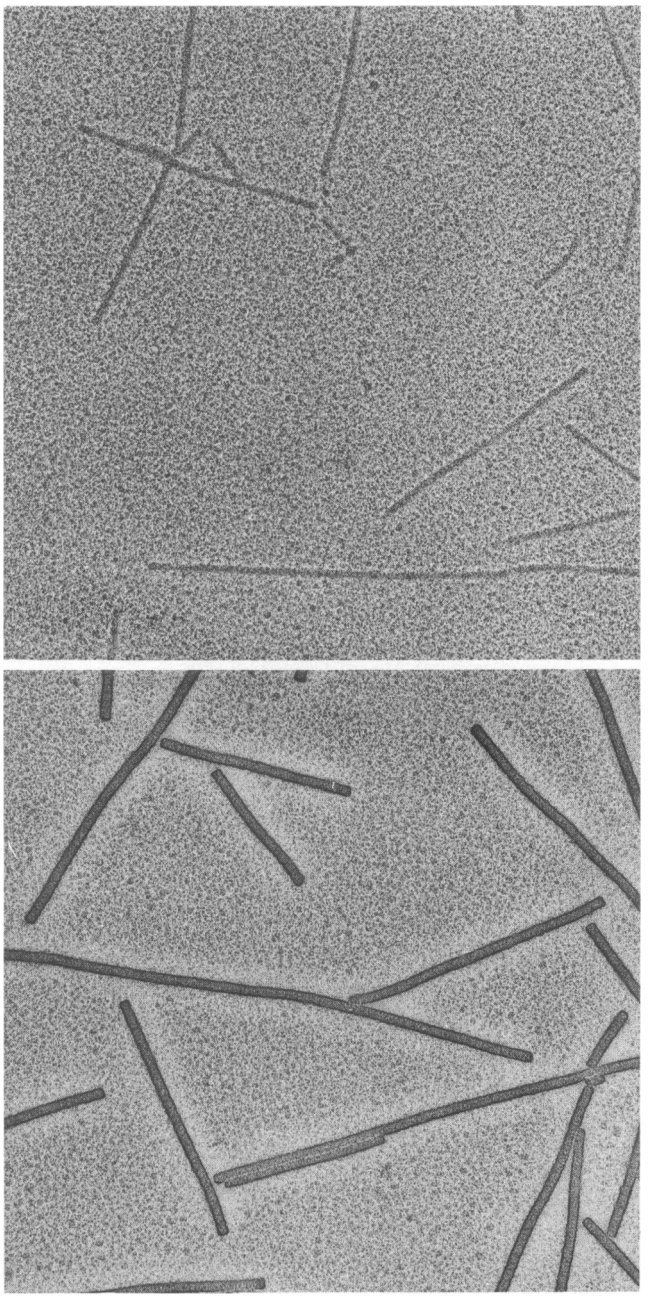

FIGURE 1 Control (*left*) and ion-etched (*right*) TMV. Magnification 56,000 ×.

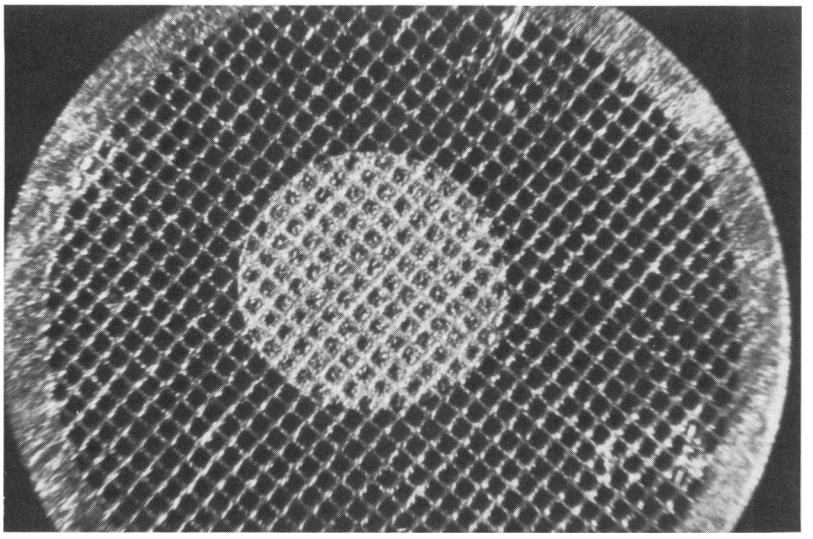

FIGURE 2 Photomicrograph of carbon-coated electron microscope grid showing centrally etched area.

tion of the rate and amount of biological material lost by exposure to accelerated nitrogen ions, the additional experiments described below on TMV, bacteriophage T4, and labeled T4 particles were performed.

MATERIALS AND METHODS

The ion gun used in these experiments has been described (Bendet and Rizk, 1973). Incorporated in the apparatus are monitoring devises for measuring the ion current, vacuum, and accelerating voltage. Several samples can be irradiated simultaneously in that the ion beam is approximately 1 cm in diameter. In our laboratory the ion gun rests upon, and is evacuated by, a Kinney SC-3 high vacuum evaporator (Kinney Vacuum Company, Boston, Mass.).

The protein of T4-D bacteriophage was labeled by adding 2.5 mCi of $H^{35}SO_4$ to a 10 ml culture of *Escherichia coli* type B 10 min after having been multiply infected (M.O.I. = 4) and reinfected at t = 8 min. Growth took place on RG glucose medium (Georgopoulos et al., 1973). Labeling the nucleic acid of the virus involved growing the bacteria in 10 ml of RG medium supplemented with 200 μg/ml of deoxyadenosine. The infection procedure was as described above, with 10 μCi of [^{3}H]thymidine being added at t = 10 min for labeling.

The T4 bacteriophage was purified by exposure to two cycles of differential centrifugation, two sedimentation runs in CsCl step density gradients and sedimentation equilibrium in CsCl for 24 h. The banded virus was dialyzed against and stored in 0.1 M Tris buffer. Before ion etching it was dialyzed against 1% ammonium acetate.

Ion etching of the radioactive virus consisted of placing either 10 μl of ^{35}S-labeled phage or 25 μl of ^{3}H-labeled phage on a microscope cover glass which acted as the mechanical support. After exposure to the beam, each sample-bearing cover glass was placed in a glass scintillation counting vial to which 0.5 ml of 10% SDS was added, and heated in boiling water for 2 min. Radioactivity was measured in a Packard Tri-Carb model 3380 scintillation counter (Packard Instrument Co., Inc., Downers Grove, Ill.).

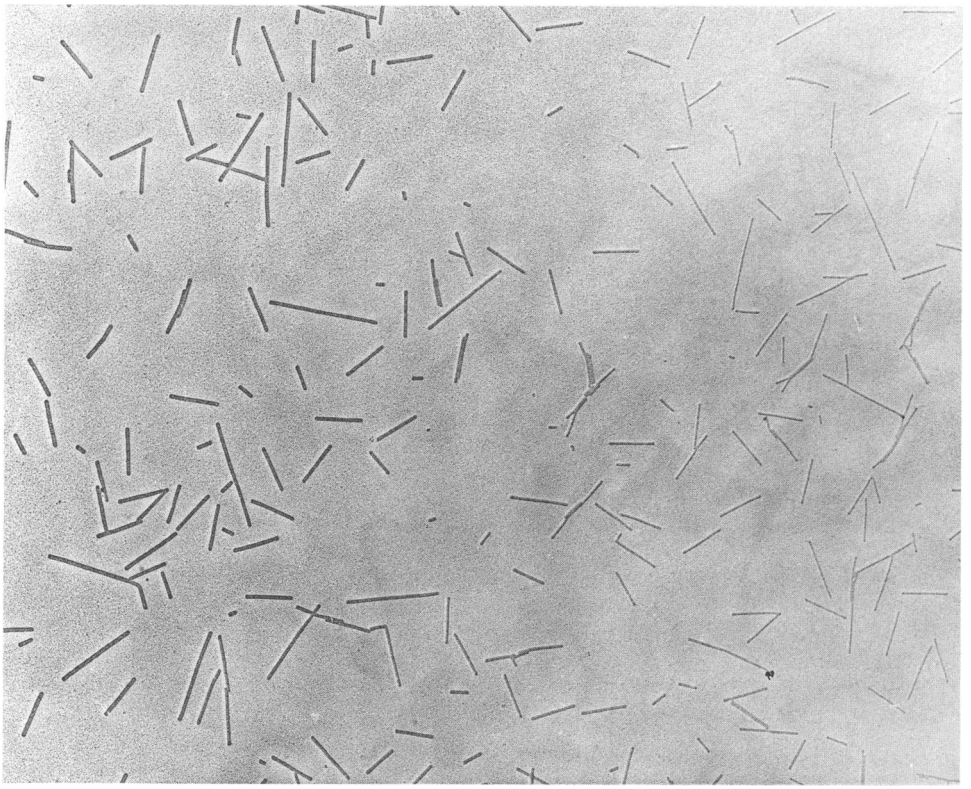

FIGURE 3 Transition zone between etched and unetched TMV.

RESULTS

An example of the effect of ion etching TMV can be seen in Fig. 1. The procedure consisted of placing a drop of the virus on a carbon-coated collodion-covered grid and removing the excess liquid with filter paper. The dried grid was then placed in the target area of the ion etching apparatus, and when an optimum vacuum developed nitrogen was bled into the system until an approximate final pressure of 5 μm of Hg was achieved. Etching was carried out at 7.5 kV for 40 s, after which the sample was rotary shadowed with uranium.

To provide an internal control each grid can be covered with a special specimen support containing a 1 mm diam central hole. This allows etching to occur only at the exposed central area of each specimen grid, so that comparisons can be made between the virus particles located in the center of the grid and exposed to the beam with those particles situated at the grid's periphery and protected from the beam. A photograph of an electron microscope grid irradiated in this manner is shown in Fig. 2 (Bendet and Rizk, 1973). This procedure allows one to observe in the microscope that portion of the grid containing the transition zone between etched and unetched areas, as illus-

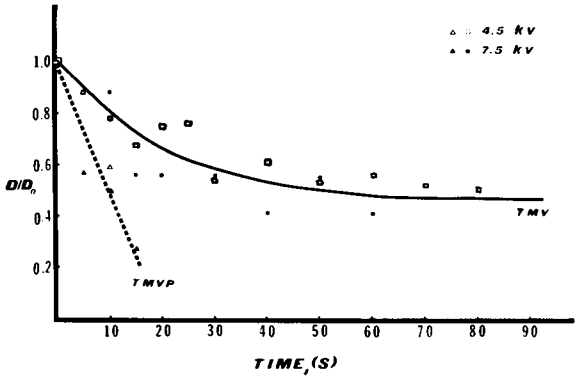

FIGURE 4 Relative diameters of TMV and repolymerized TMV protein ion etched for given periods of time.

trated in Fig. 3. It is readily apparent that this provides a rigorous control in that all of the TMV seen situated over one opening of a 300 mesh screen, has been shadowed equally and photographically enlarged identically.

Initial observations of the N_2 ion etching of TMV at 7.5 kV as a function of time indicated that the diameter of the virus particles rapidly decreases and then remains relatively constant (Bendet and Rizk, 1973). The effect of accelerated nitrogen ions on repolymerized TMV protein has been found to be much greater than on the intact virus. Fig. 4 depicts the relative rates of etching of TMV and its nucleic acid-free repolymerized protein, as arrived at by measuring particle diameter, D, relative to the control, D_o. This series of measurements would suggest that the repolymerized protein is more susceptible to the ion etching, and that the nucleic acid, either free or as nucleoprotein, reflects that material which is more resistant to the beam.

To test the hypothesis that ion etching can preferentially remove particular constituents of a virus, experiments similar to those described above were carried out on T4 bacteriophage, in which either the protein or nucleic acid was radioactively labeled. The appearance of T4 bacteriophage in the electron microscope after exposure to a 5 kV nitrogen beam for 40 and 100 s, respectively, can be seen in Fig. 5. Included in the figure for comparative purposes is a section of transition area between ion-etched and nonirradiated virus. Besides the obvious decrease in the bacteriophage's head size and tail diameter, it can be seen that the essential morphology of the particle is retained, as manifested by the compactness and angular profile of the head.

In a set of ion-etching experiments on T4 bacteriophage performed at 2,3,4, and 5 kV as a function of time, relative head volumes were calculated from measured head dimensions assuming prolate ellipsoids of revolution. All of the experiments indicated an initial rapid decrease, followed by a more gradual decrease, in head volume (Fig. 6). It can be seen that the effect of 5 kV ions is greater than 2 kV ions, while the results at 3 and 4 kV (for clarity only the points for 3 kV are indicated in Fig. 6) are intermediate in nature.

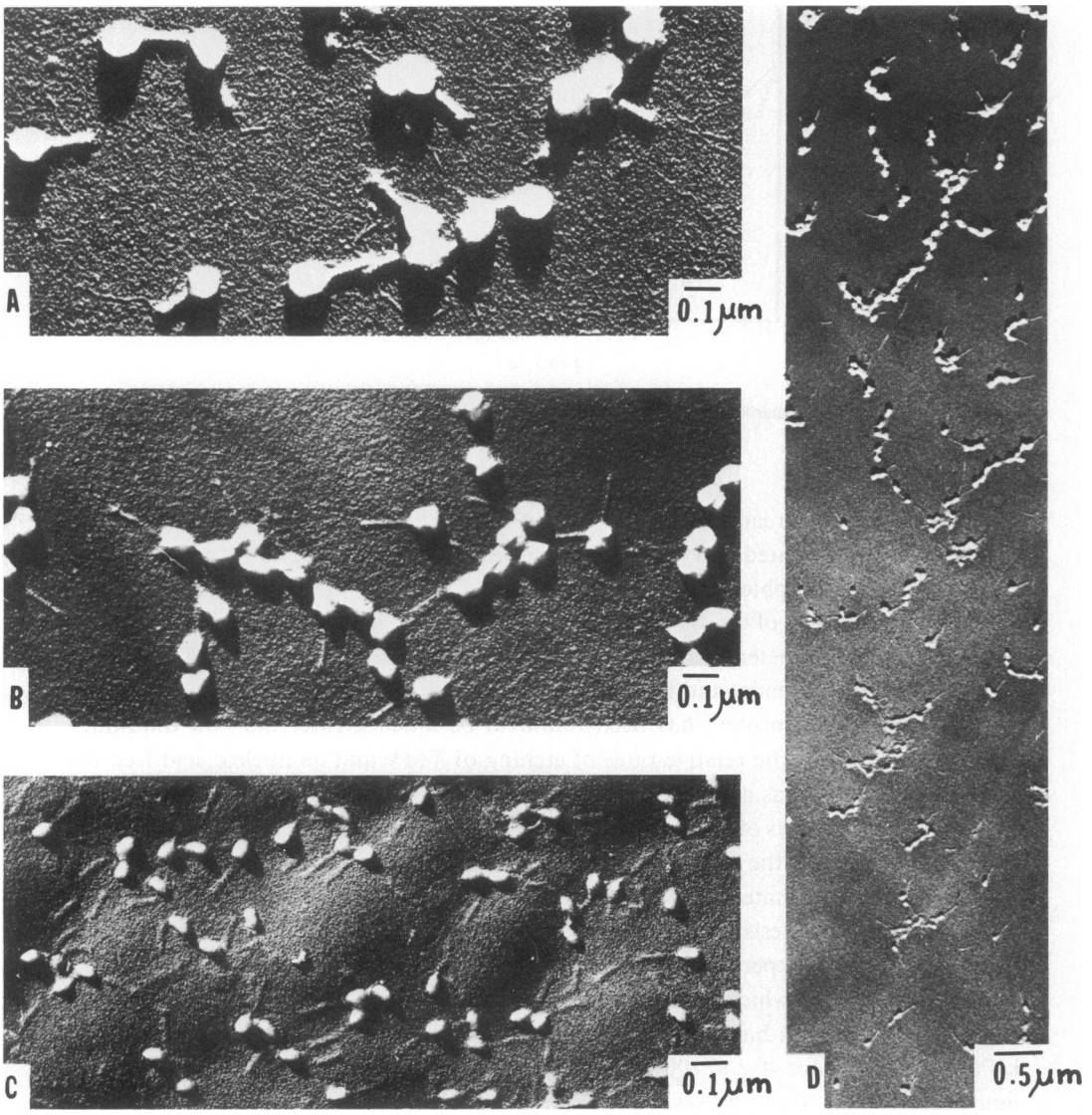

FIGURE 5 Ion-etched T4. (*A*) Control; (*B*) 40 s exposure; (*C*) 100 s exposure; and (*D*) transition zone.

The results on T4 bacteriophage make it tempting to speculate that the remaining portion of the head is predominantly nucleic acid, with the protein having been removed preferentially. Support for this hypothesis derives from ion-etching experiments carried out on bacteriophage containing either ^{35}S-labeled protein or nucleic acid incorporating ^{3}H-thymidine. The amount of radioactivity remaining associated with the virus as a function of time of exposure to accelerated nitrogen ions is indicated

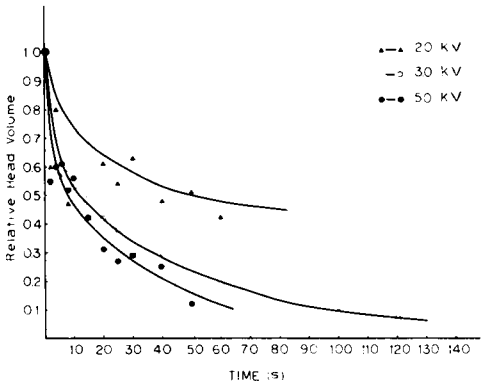

FIGURE 6

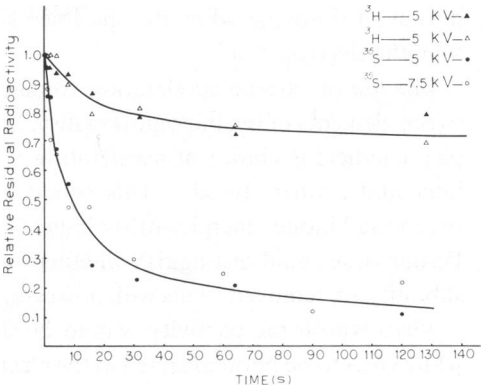

FIGURE 7

FIGURE 6 Relative head volumes of T4 bacteriophage ion etched at 2, 3, and 5 kV for given periods of time.

FIGURE 7 Relative residual radioactivity of T4 labeled with either ^{35}S containing protein or [^{3}H]thymidine containing nucleic acid ion etched for given periods of time.

for a series of experiments in Fig. 7. All counting rates were normalized, with unetched control virus aliquots producing approximately 10,000 and 70,000 cpm for ^{3}H and ^{35}S, respectively. The curves in Fig. 7 show that while approximately 80% of the ^{35}S disappears after exposure to the beam for 2 min, at least 70% of the ^{3}H remains. This would support the interpretation that the protein subunits are more susceptible to ion etching, and that the residual material may represent the nucleic acid of the virus. It is satisfying to note, too, that the relative radioactivity curve for ^{35}S closely approximates the result obtained for T4 head volume.

DISCUSSION

Although the effect of the electron beam upon biological specimens in the electron microscope is to produce both a degeneration of the electron image as well as a decrease in the mass of the objects being viewed, this investigation was undertaken with the expectation that some structural information would be forthcoming from the study of ion beam-specimen interactions. It seemed conceivable that ion etching of a biological specimen, in which some materials were removed more rapidly than others, could prove useful in selectively depicting particular components of a cell or virus particle. Thus, as an adjunct technique to the electron differential staining of cellular components, the results of ion etching might be regarded as analogous to those obtained with frozen biological materials which have been cut and then etched by sublimation of the ice from their surfaces, in the technique developed by Steere (1957).

The results of this study would indicate that the loss in mass of a specimen in the microscope (Thach and Thach, 1971) could be interpreted as being due to the heavy ions produced by the high voltage field in the incomplete vacuum of the instrument. Dubochet (1975) has indicated that besides the mass loss being related to vacuum con-

ditions, it is greater when the specimen side of the grid faces the objective lens rather than the electron gun.

The use of varying accelerating voltages was intended to aid in discriminating between elements of the biological material having different binding energies. For example, a judicious choice of accelerating voltage might lead to distinguishing between ionic and covalent bonds. This would assume that a specific relationship exists between the binding energies of biological materials in their dry form and in solutions. Perhaps one could distinguish whether individual atoms are released or intact protein subunits are removed. This will, however, require additional experimentation.

Virus whose radioactivity was to be determined was ion etched on cover glasses while virus to be photographed in the electron microscope for particle dimension measurements was supported on carbon-coated collodion-covered grids. The glass supports were used because extensive etching produced undulations and many torn areas in the carbon collodion film as well as a much more granular background. The observation that there is a decrease in the measured radioactivity suggests that the etched material has left the glass support surface and has probably been evacuated. It could, however, account for the irregular background noted on collodion-covered grids etched for extended periods.

Conceivably, the ion-etching technique should allow one to gain some insight into the orientation of the nucleic acid within virus particles. Thus, experiments have been initiated in which etched bacteriophage are suspended in solution with the expectation that after some elapsed period of exposure to the ion beam sufficient protective head protein will have been removed so that the remaining nucleic acid will be visualized when such material is observed in the electron microscope. In addition, experiments are under way to determine whether either of the two general models postulated for the structure of the flamentous bacteriophage fd—the DNA located in an axial hole, or two parallel strands of DNA surrounded by protein—are tenable, or whether a completely different model must be formulated.

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