BBA 76079

HIGH-VOLTAGE ELECTRON MICROSCOPY OF WET WHOLE CANCER AND NORMAL CELLS

VISUALIZATION OF CYTOPLASMIC STRUCTURES AND SURFACE PROJECTIONS

D F PARSONS, V R. MATRICARDI, J. SUBJECK, I UYDESS AND G WRAY

Electron Optics Laboratory, Biophysics Department, Roswell Park Memorial Institute, Buffalo, N Y. 14203, and U S Steel Corporation, Applied Physics Laboratory, Research and Development Center, Monroeville, Pa 15146 (U S A)

(Received June 12th, 1972)

SUMMARY

A preliminary report is given of the observation of several types of cells (melanoma, 3T3, Ehrlich ascites tumor, bovine spermatozoa) in the wet state inside a differentially pumped (aperture limited) hydration chamber constructed for a high-voltage microscope. The hydration chamber functions efficiently and allows rapid insertion and examination of wet specimens on a routine basis. Initial work has shown that the scattering of water layers or drops is strong and has a pronounced effect on the resolution and contrast of the specimen. Methods have been developed for controlling the water layer thickness.

Cytoplasmic details (nuclei, mitochondria, melanin granules, axial filaments of spermatozoa) have been visualized in wet whole cells.

Attempts to observe cell movements in the hydration chamber have not yet been successful, but are continuing with special attention to minimizing radiation damage and optimizing medium nutrient composition

INTRODUCTION

The successful development of differentially pumped, aperture limited hydration chambers for both conventional (100 kV and 200 kV)[1–5] and high-voltage (650–1200 kV) microscopes (refs 6–8; P. R. Swann and N. J. Tighe, personal communication) has made rapid examination of wet specimens a practical technique. Previous attempts to use environmental chambers closed by thin windows[9–16] led to problems of frequent breakage of the windows, and their contamination. The scattering by windows also decreases contrast and resolution.

In preliminary reports we have demonstrated the practical use of the differentially pumped hydration chamber in making possible high resolution electron diffraction of wet unfixed protein crystals[17,18] and cell membranes[19].

The biological significance of electron microscopy of whole wet cells has only

recently been appreciated. It is hoped that the examination of whole wet cells will give more realistic morphologic detail without the artifacts associated with thin section and other electron microscope techniques. Occasionally, claims have been made that the cells examined in a hydration chamber could be maintained in a living state[10,15,16,20]. However, to date no convincing evidence has been produced that this has, in fact, been achieved. Here we will report the first observation of internal structures in whole unstained wet cells and also initial observations of the form of cells' surface projections as seen by this new technique. A preliminary report[6,7] of some of this work was made previously.

METHODS AND MATERIALS

Cells

Cultures of human melanoma cells (M7821 provided by Dr G. Moore, Roswell Park Memorial Institute), Ehrlich ascites tumor cells (provided by Dr E. Mayhew of Roswell Park Memorial Institute), and normal and transformed Balb/3T3 cells (provided by Drs Todaro and Aaronson of the National Cancer Institute and by Dr M. Burger of Princeton University) were utilized in these experiments. Cultures of the melanoma and Ehrlich ascites were grown in Roswell Park Memorial Institute medium 1640 supplemented with 10% fetal calf serum, penicillin and streptomycin. The Balb/3T3 cells were cultured in Dulbecco's modified Eagle medium (Associated Biomedical Systems, Buffalo, N.Y.) supplemented with calf serum, penicillin and streptomycin. Gold grids, covered with Formvar and evaporated carbon were utilized instead of copper due to the toxicity of the latter type. The grids were sterilized by exposure to ultraviolet light prior to their use in the cell culture experiments. These were then placed into the wells of Falcon Micro Test II Culture Plates and covered with fresh media.

The cells, having been grown to a density of approximately $2.5 \cdot 10^5$ cells per ml after trypsinization, were centrifuged and resuspended in a new medium. Several drops of this medium were then used to seed the Micro Test II wells. The culture plates were then inserted into a 37 °C incubator with a 10% CO_2–air mixture, for 24 h.

Grids chosen for the hydration experiments were removed from the Micro Test II wells within a 100% humidity box. They were then washed gently in phosphate-buffered saline, and the excess fluid removed with torn filter paper. The grids were then mounted on dry specimen rods and enclosed within a small humidified transfer chamber used to transport the hydrated specimen to the microscope. Examination of a wet grid could be begun in less than 30 s.

Over 500 photographs of wet cells were taken (mainly using Kodak No-Screen X-ray Film[21], but some on Kodak Electron Image Plates). There was considerable variation in the amount of cytoplasmic detail visible in different preparations. In some cases lack of detail was due to poor penetration of the 800-kV beam through thick spindle shaped cells. In the case of wet, spread 3T3 cells it was due to excessive thickness of the water of culture medium layer present on one or both surfaces of the specimen grid. Microscopy using a Zeiss Lebedeff-type interference microscope inside a humidity box showed that thick water or medium layers (up to 10 μm thick) were trapped in the grid squares on the reverse side of the grid. Methods for better

control of the water or medium layer thickness are being developed. Interference microscopy showed that thin wet films were stable provided the humidity box was above 90 % relative humidity and a fast stream of water-saturated air was driven into the box.

Whole cell mounts of the cultured 3T3 cells were also prepared for electron microscopy by a modification of the procedure described by Porter *et al.*[22]. Cells grown on gold grids were removed from their culture media and immersed into 2 % OsO_4–phosphate-buffered saline solution for 10–15 min, or in 2 % glutaraldehyde in phosphate-buffered saline for 10–15 min both at 4 °C. These specimens were then washed for 30 min in distilled water The grids were dried in air and examined in a Siemens Elmiskop Ia at 100 kV by bright field (20-μm aperture) and dark field (displaced 20-μm aperture) methods. Basic-lead stained thin sections of OsO_4-fixed cultured 3T3 cells embedded in Epon were also examined to cross-correlate structures observed in the wet whole cells, air-dried whole cells (Porter-type preparations) and thin sections.

Hydration chamber

A differentially pumped hydration chamber was designed and built for the U.S. Steel 1.0 MeV electron microscope at Monroeville, Pa. The differentially pumped chamber was chosen rather than a thin-film window chamber because of its ease of operation and its ability to scan most of the specimen grid. Also, contamination of the specimen is not a problem since a continuous flow of hydrocarbon-free water vapor flows over the specimen. The theory, details of construction and performance of three different types of differentially pumped chambers, will be described elsewhere (R. C. Moretz, V. R. Matracardi, G. G. Hausner, Jr and D. F. Parsons, in preparation). Only a brief outline will be given here.

The hydration chamber stage (Fig. 1) is located in the gap of the objective pole-piece so as to provide straight-line access to the specimen position. The specimen on its own translation rod, is sandwiched by two 100-μm apertures 1.2 mm apart. The apertures form the walls of the hydration chamber and allow the electron beam to pass through the top aperture, through the wet specimen and out the bottom aperture. External to these two apertures there is an intermediate chamber limited

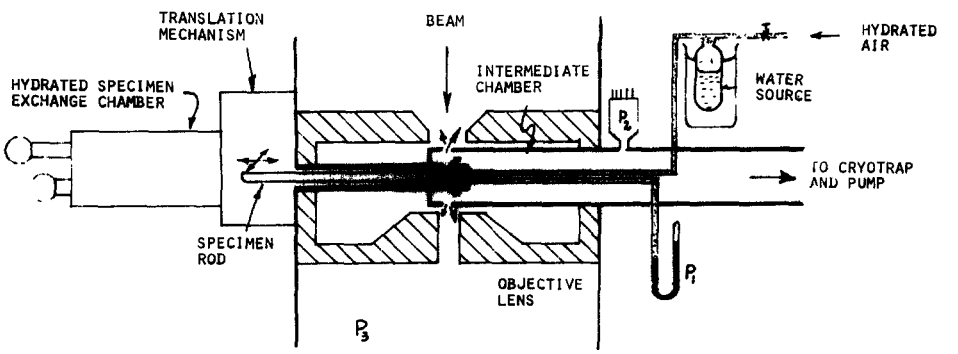


Fig 1. Diagram of the differentially pumped hydration stage used on the U S. Steel 1 0 MeV electron microscope The wet specimen is immersed in water vapor and enclosed by two small apertures The escaping water vapor is pumped out of the system

by two 200-μm apertures and colinear with the two 100-μm apertures. This outer chamber is pumped independently by a cryotrapped mechanical pump. The normal specimen translation mechanism was used to center the whole aperture assembly on the electron beam.

The water vapor pressure inside the hydration chamber was measured by a mercury manometer (0–100 torr) and a Bourdon gauge (0–760 torr). The water vapor pressure in the hydration chamber was controlled by adjusting the temperature of the external water reservoir. The observed pressure of the hydration chamber agreed with the value[23] required by the water reservoir temperature to within 0.5 torr. In all the experiments reported here only water vapor was used inside the chamber.

The 1 MeV microscope had to be fitted with a new objective upper pole-piece with a larger gap (increased from 10 mm to 22 mm) in order to accommodate the chamber. This required a larger than normal objective current which caused some heating of the objective lens. The objective current was run overnight to stabilize the temperature and the average temperature of the hydration chamber was 29 °C (the design of the pole-piece and the camber will be improved to allow operation of the chamber at room temperature, and also at 37 °C). Care was required to keep the apertures from blocking with dirt particles by not allowing room air to be drawn into the chamber. No particular problem was encountered with aligning the aperture of the hydration chamber or maintaining their alignment as a whole with respect to the beam.

For insertion of a new specimen through the air–water vapor lock, the chamber was brought to atmosphere with water-saturated air or helium so that the wet specimen at no time encountered dry air. Wet specimens appeared to remain wet indefinitely, provided the exterior water reservoir temperature matched that of the hydration chamber. A special gas mixing device allowed water vapor-saturated mixtures of helium and oxygen to be added to the 20–30 torr water vapor pressure. It was found that 200 torr total pressure of O_2·He (1:5, v/v) gave too much scattering for dark field work although, pictures were satisfactory by bright field. A later design of hydration chamber will minimize this gas scattering.

Operation of the U.S. Steel high-voltage microscope for wet specimen

Observation

The high-voltage microscope could be equipped with the hydration stage in 1–2 h and the microscope restored to normal use in 30 min. Once the aperture assembly was centered on the beam the stage could be used continuously to examine numerous wet specimens. However, occasionally the apertures were blocked by a piece of dirt, but this could be blown out of place by Freon gas. To do this it was only necessary to disconnect the intermediate chamber mechanical pump connection.

Bright field images were obtained using a 20-μm objective aperture which was manipulated in the normal way, beneath the hydration stage. Dark field pictures were taken by using strioscopic apertures consisting of 10-μm etched tungsten wire welded across a 100-μm platinum aperture manipulated in the plane of the objective aperture. 800 kV acceleration voltage was used throughout.

Image recording and viewing

Various recording and viewing methods have been explored[21] to reduce beam radiation damage to the specimen to a minimum. Kodak No-Screen Medical X-ray

Film was used for most micrographs (it is 17 times more sensitive than Kodak Electron Image Plates at 800 kV). However, the X-ray film required more careful exposure control (use of a Faraday cage or probe to measure beam current) in order to make it possible to print image details with good contrast. We found the Logetronic Mark III printer a considerable asset in obtaining prints from the X-ray films. The decreased emulsion resolution (2.5 times less than the Kodak Electron Image Plates) did not affect our results since most micrographs were taken so as to give a final magnification of 10000 × or less.

RESULTS

In this work we have had to work out a new method of handling wet cell preparations. The micrographs reported, however, should not be taken as illustrating the maximum detail and contrast that the method of high-voltage microscopy of spread unstained wet cells can produce, since the technique of consistently obtaining the optimum thickness of wet materials has yet to be optimized.

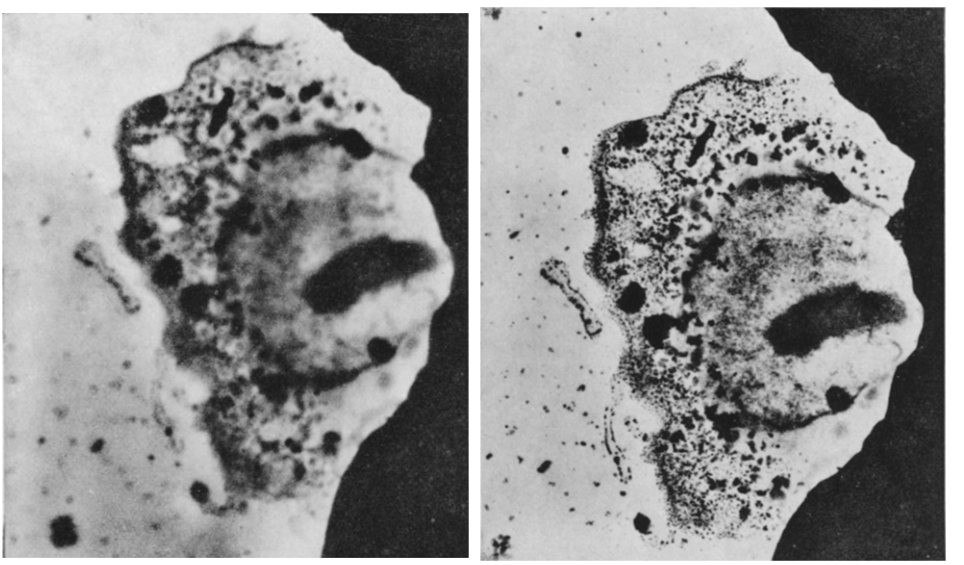


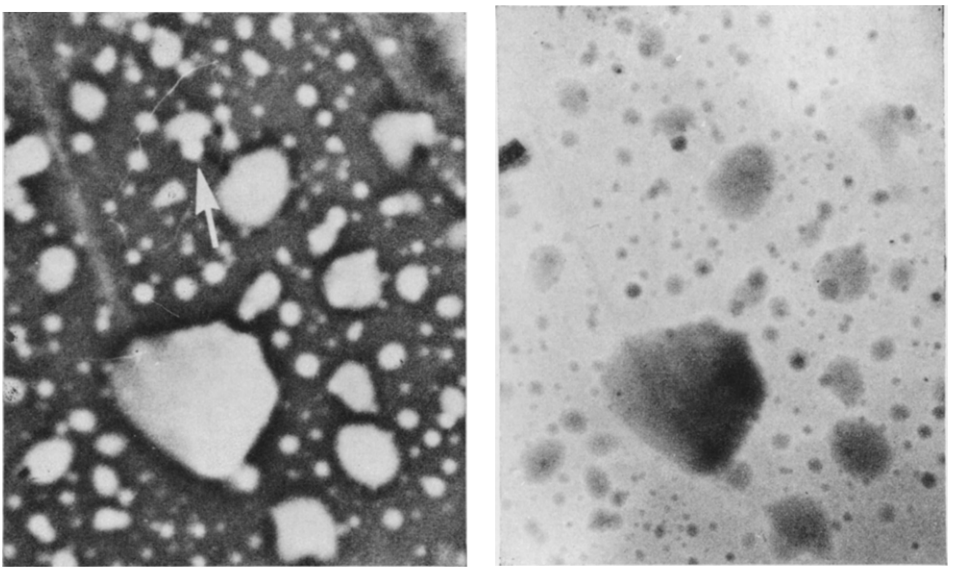
Fig 2 All micrographs taken at 800 kV unless otherwise stated 0 25 μm Epon section of 3T3 cell, OsO_4 fixed and basic lead acetate stained, covered with a thick layer of water There is considerable blurring presumably due to chromatic aberration produced by the inelastic scattering, resulting from excessive water layer thickness Photographed in the hydration chamber with 24 torr of water present Magnification, 5000 ×.

Fig 3 The same as Fig 2 after shutting off the water vapor supply to the hydration chamber and drying the specimen The detail of the specimen is now sharp. Magnification, 5000 ×

The effect of diffuse scatter by too thick a water film can be judged from Figs. 2 and 3. A water film has been placed on top of a 0.25-μm thin section and photographed both wet and dry The surprisingly high contrast of the water droplets is shown in

dark field in Fig. 4 and in bright field in Fig. 5. Water droplets condensed after raising external water source 1–2 °C higher than the temperature of the hydration chamber. In many cases the water droplets condense preferentially around small particulate deposits on the film (arrows in Figs 4 and 5). This suggests that our hydration chamber provides a convenient microsystem for study of the nucleation of super-saturated water vapor atmosphere, a problem of considerable interest to atmospheric science. A study of the contrast and nucleation of water will be reported separately.

A sensitive indication of partial drying was the appearance of cracks around the nuclear membrane. In some cases, highly electron transparent bubbles appeared on or near these cracks. In a smaller number of cases such bubbles (0.2–0.8 μm diameter) appeared in micrographs of cells showing no evidence of drying. In later experiments, where more care was taken to prevent drying, such bubbles were seen rarely. It is suggested that a crack in a thick layer of partly dried cells allowed beam penetration and gas bubble formation opposite the crack in a water layer on the back surface of the grid.



Figs 4 and 5 Water drops condensed on the specimen support film following raising the temperature of the external source by 2–3 °C The drops vary in size between 0 2 and 9 μm They appear to have condensed preferentially around denser particles (arrows) Fig 4, dark field and Fig 5, bright field Magnification, 2400 ×

If care was not taken to keep the beam intensity at a minimum several kinds of radiation and heat damage effects were observed. Prolonged exposure for half an hour at a time caused a clearing of the illuminated area of Epon sections covered with a water layer. We also observed some crystallization of dense material (possibly a lead compound) on the surface of wet, lead-stained Epon sections. These effects were eliminated by keeping the beam intensity low by monitoring it with a Faraday cage or probe and a vibrating reed electrometer and the use of the extra high electron speed X-ray film[21].

Cytoplasmic structures

Melanoma cells

A low power view of the wet, unstained melanoma cell is shown in Fig 6. The dense melanin granules are dispersed around lighter cytoplasmic structures, such as vacuoles and mitochondria. Higher magnification views of the cytoplasm are

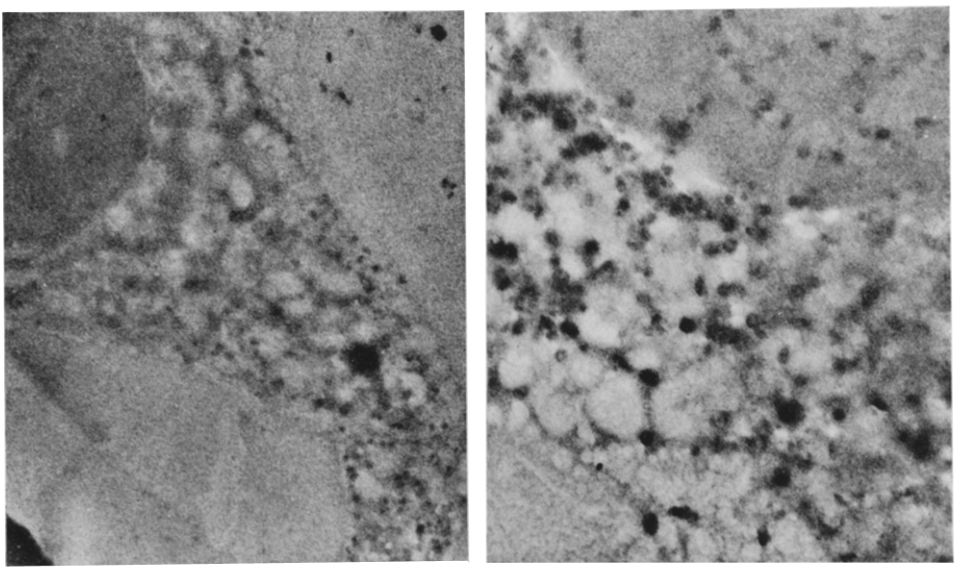


Fig 6 Portion of the nucleus and cytoplasm of a wet melanoma cell showing melanin granules and light vacuoles Magnification, 2400 ×

Fig 7 Wet spread melanoma cell after washing in distilled water The characteristic melanin granules are seen dispersed around the mitochondria and other cell vacuoles and also above or below the nucleus Magnification, 5000 ×

shown in Figs 7 and 8. The granules in the cultured melanoma cells (Fig. 7) show high contrast without staining, the high contrast may be due to a combination of high density and crystalline arrangement of the melanin[24]. Our results show that the half empty and doughnut shapes of many melanin granules seen in thin section (Fig. 9) is not an artifact of thin-section preparation since we see the same shape in the wet untreated cells (Fig. 8). Staining of our wet cells with osmium vapor produced no significant increase in the contrast of the granules

The melanin granules serve as convenient high contrast intracytoplasmic markers. To date we have not observed their movement in sucessive photographs which might have indicated preservation of cytoplasmic streaming. However, experiments are continuing at lower electron beam exposure levels.

In the melanoma cells the melanin granules serve to outline the approximate position of the mitochondria and cytoplasmic vacuoles (Figs 7, 8 and 9) In these preparations (thickness of water layer unknown), unexpectedly, the mitochondria do not appear as denser objects in the cytoplasm.

The nucleus appeared too thick in these preparations to be penetrated by the 800 kV beam It has the appearance of a uniform dense body with nucleoli and clumped

chromatin barely visible (Fig. 7). Further studies are required with flatter cell preparations having thinner enveloping water layers to establish how much of the nuclear structure can be revealed by our technique. However, it is likely that even higher acceleration voltage (preferably with filtering of the inelastic scatter) will be needed to give detail of such thick structures.

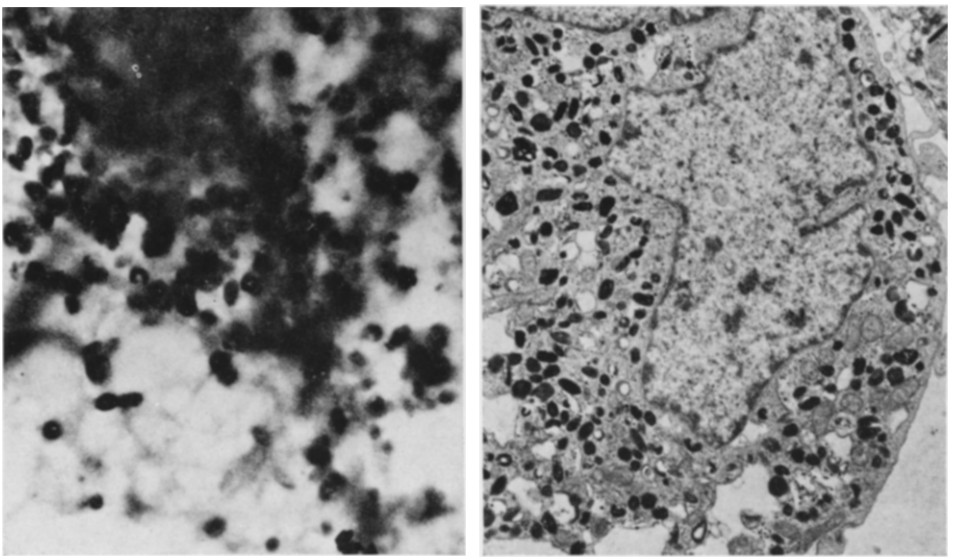


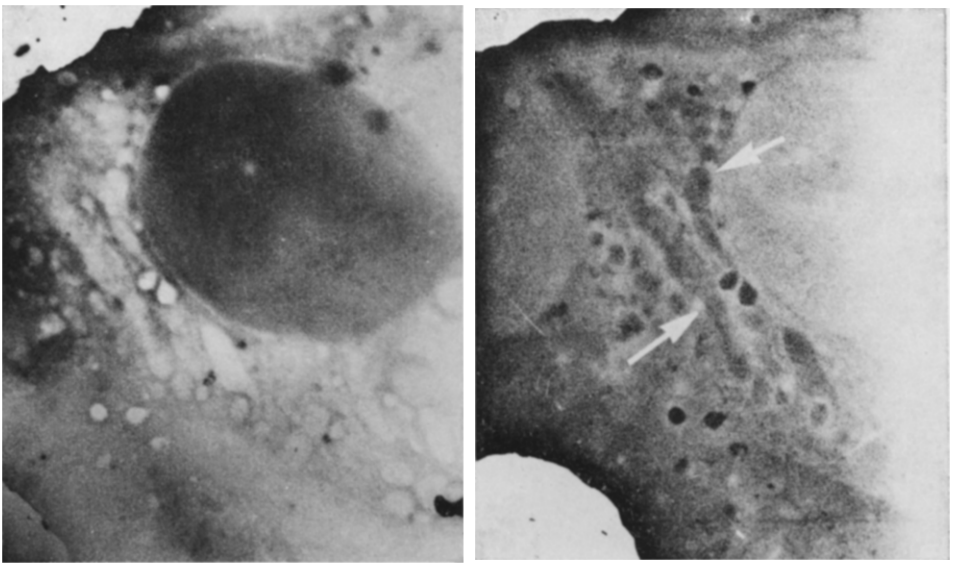
Fig 8 Portion of the cytoplasm of a wet spread melanoma cell showing the highly scattering granules dispersed around other less dense cytoplasmic structures (mitochondria and vesicles) Some of the melanin granules show the same doughnut appearance as seen in thin sections (Fig 9). Magnification, 7200 ×

Fig 9 Thin section of melanoma cell. Some of the melanin granules show the same doughnut appearance as in the unfixed preparation of Fig 8 The number and size of vesicles and of the mitochondria can be compared with the light vesicle areas in Figs 6–8 Magnification, 7000 × Micrograph contributed by Dr A Sandberg, Roswell Park Memorial Institute.

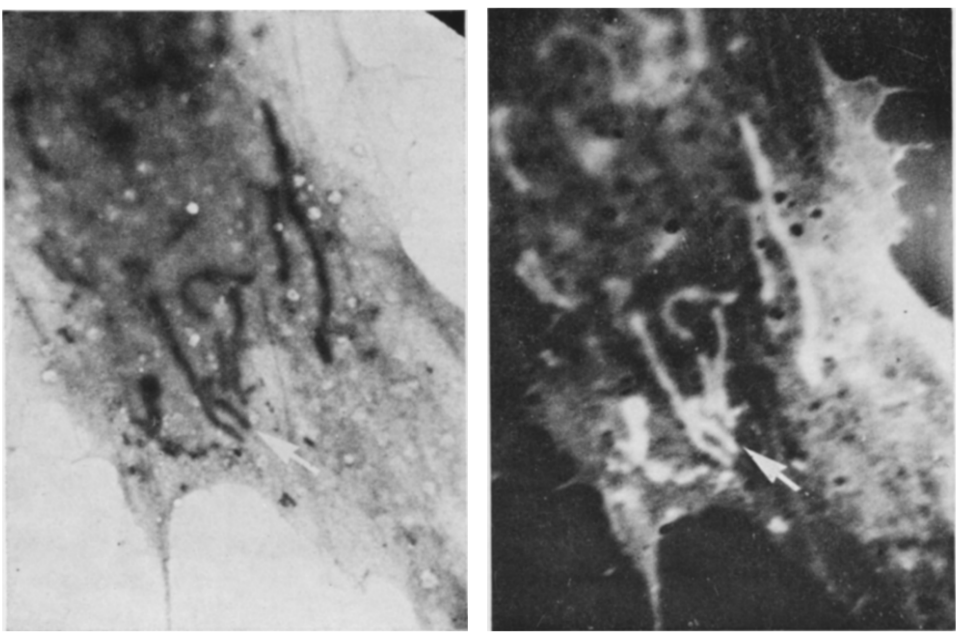
3T3 cells

3T3 cells gave good spreading on the gold grid and showed mitochondria more clearly than the melanoma cells (Figs 10 and 11). Both bright field (Fig. 10) and dark field show that the mitochondria have lower contrast (less scattering) than the surrounding cytoplasmic fluid. However, these mitochondria may be somewhat swollen or otherwise damaged. At this magnification and degree of discrimination, the contrast level of the cytoplasm is an average of the cytoplasmic fluid and the numerous small structures in it (endoplasmic reticulum, vesicles, ribosomes, microtubules, *etc.*).

The mitochondria in Porter-type spread cell preparations are shown in Figs 12 and 13. The unstained glutaraldehyde-fixed preparation (Fig. 12) gives a good idea of the shape of the mitochondria in dried preparations. These preparations were examined dry at 100 kV accelerating voltage. The glutaraldehyde-fixed preparations show that the mitochondria scatter more than the surrounding material resulting from drying down of the cytoplasm. Note that the dried mitochondria appear much thinner than the wet ones of Figs 10 and 11. However, the mitochondria around the



Figs 10 and 11 Thinly spread wet 3T3 cell showing nucleus and cytoplasm The elongated mitochondria (arrows) are plainly visible in the cytoplasm (compare with the Porter-type preparation in Fig 12) These mitochondria (which may be damaged) scatter less than the surrounding cytoplasm as seen in both bright field (Fig 10) and dark field (Fig 11) Magnification, 5000 ×

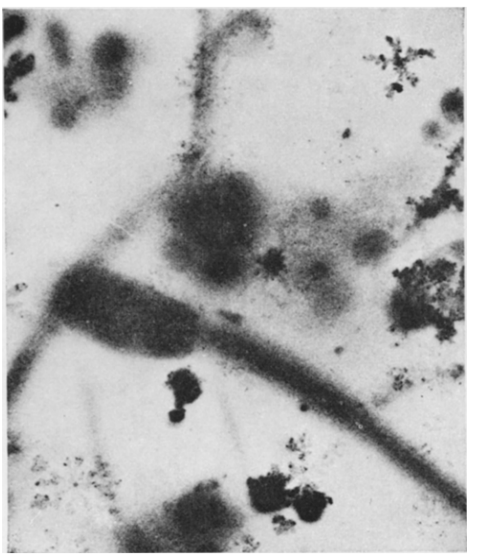


Figs 12 and 13 Porter-type preparation of 3T3 cells grown on gold grids Fixed in glutaraldehyde (no OsO_4 fixation), and washed with distilled water and air dried Photographed at 100 kV acceleration voltage (20-μm objective aperture) on a Siemens Ia electron microscope Fig 12 bright field, Fig 13 dark field The mitochondria (arrows) are thinner than those of the wet cells of Figs 10 and 11, presumably because of the shrinkage on drying and also because the mitochondria appear longer and thinner at the periphery of the cell Magnification, 6400 ×

nucleus (Fig. 10) in live cells, viewed in the light phase-contrast microscope, frequently appear wider and more ovoid than those in peripheral thin portions of the cytoplasm (Fig. 11).

Bovine spermatozoa

A low power view is shown in Fig. 14. We examined the visibility of the mitochondrial sheath in the wet preparations. In Fig. 14, the mid-piece cytoplasm has very low contrast and the wet layer thickness is probably too great for good contrast. In one preparation (probably with thinner water layer) the mitochondrial sheath has medium contrast (Fig. 15) and the individual mitochondria appear to form a spiral around the axial filaments. The high contrast of the axial filaments is encouraging for attempting to visualize the microtubular structures of cell processes.



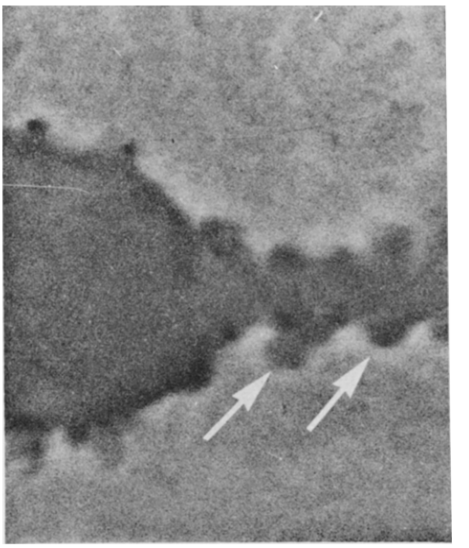


Fig 14 Low magnification view of bovine spermatozoa (supplied by Dr S H Hu) The preparation was partially dried to reduce the aqueous medium thickness layer and improve contrast This resulted in some precipitation of salts but no detectable morphological change The head and axial filament have high contrast but the mitochondria sheet of the mid-piece is only faintly visible in this preparation Magnification 2400 ×.

Fig. 15 High magnification of the head and mid-piece of a sperm from the same preparation as in Fig 7, but apparantly from an area of thinner water layer thickness The mitochondria here show densely (arrows) and could be wrapped spirally around the axial filaments Magnification, 9200 ×.

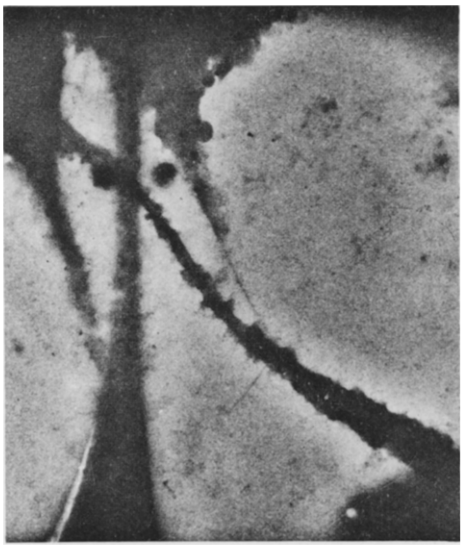
Cell surface

Contact inhibition and loss of inhibition

Both normal and transformed 3T3 cells showed overlap of their processes (Figs 16 and 17) even if the main bodies of the cells showed contact inhibition and were not overlapping. A comparison of the overlap of terminal processes of normal and transformed 3T3 cells is being made.

Fine cell processes

The elongated ends of wet cell preparations of both 3T3 cells and melanoma cells were surrounded by very fine processes (Figs 18 and 19). Their presence can



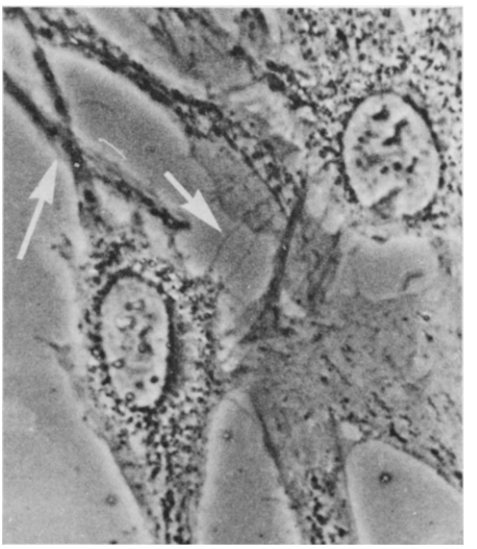
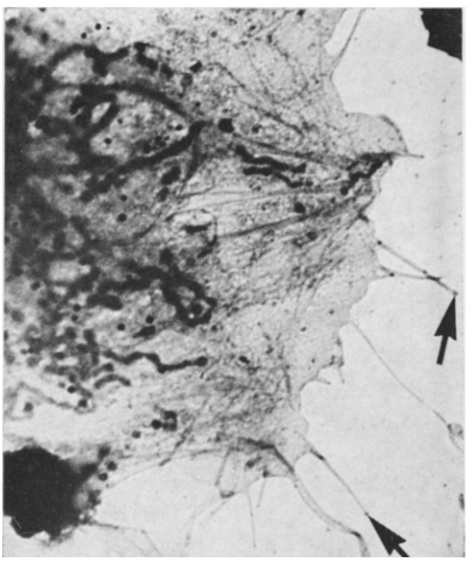


Fig 16 Overlap of the tails of processes of 3T3 cells (transformed) In some of the non-transformed 3T3 processes are also overlapped but not the cell bodies Magnification, 2400 ×

Fig 17 Light microscope, phase-contrast photograph of the overlapping processes (arrows) of non-transformed 3T3 cells The fine processes of the surface can also be seen (arrows) Porter-type preparation (OsO_4 fixed and distilled water washed) of cells Magnification, 1920 ×



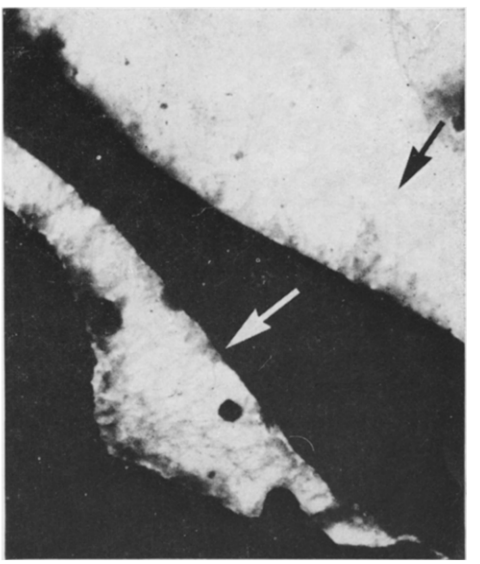


Fig 18 Electron microscope picture (80 kV) of the same preparation as in Fig 17 The elongated, densely stained mitochondria in the cytoplasm, are readily recognized Note the fine processes (arrows) extending out from the cell periphery Some (arrows) of these processes have a dense end Magnification, 6000 ×

Fig 19 Fine (about 1000–1400 Å diameter) processes (arrows) surrounding the surface of a wet melanoma cell They are similar to those seen in the Porter-type preparation of Fig 18 Magnification, 2400 ×

be detected in the light microscope because of edge diffraction (arrows in Fig. 17), but detail in them cannot be resolved. The diameter of most of the processes in wet preparations is about 1400 Å. These fine processes do not appear to have been observed in thin sections presumably because of the difficulty of reconstructing the three-dimensional picture from thin sections. Also, such a long thin process will only infrequently lie in the plane of the section, as shown in the electron micrographs of glutaraldehyde-fixed (Figs 12 and 13) and OsO_4-fixed cells (Fig. 18). However, they are visible in the Porter-type preparations. Fig. 20 shows a thin section through a long portion of such a process (unfortunately the end passes out of the section). Microtubules can be seen at the base of the process, but not in the interior. Most of the processes appear to be filled with amorphous densely staining material.

Fig 20 A portion of a long process of a normal 3T3 cell Microtubules are not visible in the process of this preparation, but are present at its base. The process contains mainly amorphous-looking, heavily stained material The process passes out of the section before the dense structure at its end is visible Magnification, 68400 ×

DISCUSSION

Effect of water scattering on image resolution and contrast

Our preliminary results suggest that even at high voltage (800 kV) relatively thin layers of water scatter strongly and reduce contrast and resolution. The contrast of small water drops (Figs 4 and 5) was higher than expected. Quantitation of the scattering of water films and drops will be reported later. However, present results (Figs 2 and 3) strongly emphasize the need to obtain strict control of the total thickness of water and wet cell material in developing this new technique. The need to define and reproduce the optimum wet layer thickness is analogous to the need to control the thickness of thin sections in conventional cytological electron microscopy. We have found that siliconization can prevent the trapping of water on the back side of the grid. We see no basic obstacles to the eventual standardization of a technique for providing reproducible optimum wet layer thickness of the specimen. A different technique, now being investigated in our laboratory is that of reducing water scattering to the minimum by depositing the wet objects to be studied on a dry (hydrophobic) surface.

Contrast of unstained structures

With respect to one of the major ultimate goals of biological electron microscopy (the microscopy of biological structures in the natural-hydrated, unstained and unfixed state) a basic set of data is the differential electron scattering between typical bioligical structures (viruses, membranes, whole mitochondria, nuclei, *etc.*) and the material in which they are immersed. Two situations arise. First, for intracytoplasmic structures the differential scattering will be with respect to the surrounding cytoplasmic fluid. Secondly, if the structures are isolated and handled in a saturated humidity environment, they may be deposited onto a hydrophobic surface, so that the scattering is measured with respect to the support film only. In some preparations, an intermediate situation can be produced whereby the isolated structure is mounted on a hydrophobic surface with a thin film of surrounding water. In this case, we find that care needs to be taken to adjust the thickness of the water layer. In work under way to standardize this procedure, we have found an interference microscope in the humidity box a useful aid. Also, measurement in the electron microscope of the water thickness by Faraday cage measurement of the relative intensity of wet material in comparison with an open grid square appears to also work satisfactorily.

The 3T3 mitochondria clearly show the importance of considering the scattering of the surrounding material for the overall contrast of any unstained wet cell structure. When surrounded by the cytoplasmic fluid (Figs 10 and 11) the mitochondria are less scattering than the surround. When the cells are glutaraldehyde-fixed, washed and air dried, so that no water surrounds the mitochondria, they are dense. In addition, a significant increase in density and scattering as a result of shrinkage of the mitochondria has to be taken into account. Since the low contrast situation where the structures are viewed with cytoplasmic fluid in place is more relevant to the viewing of structures in their natural state, a method of enhancing contrast electron optically (dark field, phase contrast, inelastic scatter filtering, *etc.*) is required. Recent work in our laboratory suggests that inelastic filtering is the most useful approach[25]

Special advantages of high-voltage microscopy of wet cells

The study of the cell periphery by this technique has made us aware of fine processes (about 1000 Å diameter) that were not evident in thin section photographs. In thin sections it would only be a rare section that traversed the length of the process In retrospect, the same processes were obvious in Porter-type preparations. We would not have accepted the fine processes as genuine cell structures on the basis of Porter-type preparations alone, because of the possibility of artifact from distortion during air drying. However, they are found also, in the wet whole cell mounts.

At the present state of the art with wet whole cell mounts, we cannot confirm that the ends of the fine processes contain dense structures as indicated by the Porter-type preparations. The fine processes are now being compared in normal and transformed 3T3 cells.

Another advantage of the whole wet cell approach is the ability to study the overlap of the thicker processes of normal and transformed cells. This cannot be conveniently studied by light phase microscopy because of the inadaquate resolution and the short depth of field.

A possible advantage of great interest would be to examine cell movements.

We have made a series of attempts to observe live cell processes in the hydration chamber, so far without success. These include an examination of *Escherichia coli* in the logarithmic phase of growth in an attempt to photograph the formation of the septum prior to division, an attempt to see movement of the tails of bovine spermatozoa, and an attempt to see cytoplasmic streaming of melanin granules in melanoma cells. In some, but not all, of these attempts we took special precautions to keep the radiation dose from the electron beam low (previously unexposed areas were photographed on high sensitivity X-ray film). However, much more can be done to reduce the specimen exposure, particularly with respect to use of an efficient image intensifier system for the high-voltage microscope. In another publication the difficulties of maintaining low radiation damage in the high-voltage microscope[26] have been discussed. We plan to reduce the exposure required for recording of images by optimization of the working magnification, film resolution and speed. A nomograph has been worked out for this purpose[21].

However, a problem of equal importance is to maintain physiological conditions when the cells are covered only by a thin film of medium instead of immersed in bulk quantity of medium. It appears unlikely that any useful imaging can be achieved through a thick layer of medium. A thin layer is likely to be rapidly depleted of nutrients or to rapidly accumulate toxic products. The answer may lie in the development of supplemented media, perhaps containing ATP, in which cytoplasmic movement is preserved even when only thin layers are present. In addition, special attention has to be paid to the composition of gas mixture (O_2,He,CO_2) used in the hydration chamber. So far we have not observed any cell bursting or other deleterious effect from maintaining the cell at a low total pressure (*e.g.* 24 torr of water vapor).

Since much more development is required in techniques, we wish to emphasize that the results reported here are, very probably, much less than the optimum with respect to visualizing wet whole cells at 800 kV acceleration voltage. It is hoped that futher improvement in the resolution and contrast of this technique will make possible the study of the structure of the polysaccharide surfaces of normal and transformed cells in contact. Because of the dependence of the polysaccharide structure on charge repulsion of sialic acid groups[27] while immersed in water, it seems unlikely that the structure can be visualized correctly in dehydrated specimens.

ACKNOWLEDGEMENTS

The award of time of the U.S. Steel Monroeville 1.0 MeV electron microscope was made by N.I.H. contract 70-4136. We are grateful to Dr R. M. Fisher, Director of the U.S. Steel High Voltage Microscope Laboratory, for his encouragement, to Mr Anderson of U.S. Steel for his design assistance and machining of the hydration chamber and to Dr J. Scott Lally, for assistance with many points about microscope operation. We thank Mr George Hausner, Jr of this laboratory for assistance in machining equipment used in this study. Also, we thank Dr H. Cox of R.P.M.I. for use of his cell culture facilities and Dr G. Tritsch of R.P.M.I. for use of his interference microscope.

REFERENCES

1 R C. Moretz and D F Parsons, in C J Arceneaux, *Proc 27th Annu Meet Electron Microsc Soc Am*, Claitor, Baton Rouge, 1969, p 418
2 R C Moretz, G G Hausner, Jr and D F Parsons, in C J Arceneaux, *Proc 28th Annu Meet Electron Microsc Soc Am*, Claitor, Baton Rouge, 1970, p 544
3 D F. Parsons and R C Moretz, *Proc 7th Int Congr Electron Microsc, Grenoble, France*, Vol 1, Soc Française de Microscopie Electronique, Paris, 1970, p 497
4 R C Moretz, G G Hausner, Jr and D F Parsons, in C J Arceneaux, *Proc 29th Annu Meet Electron Microsc Soc Am*, Claitor, Baton Rouge, 1971, p 44
5 V R Matricardi, G G Hausner, Jr and D F Parsons, in C J Arceneaux, *Proc. 28th Annu Meet Electron Microsc Soc Am*, Claitor, Baton Rouge, 1970, p 542
6 V. R Matricardi, D F Parsons, G Wray, R C Moretz and J Subjeck, *Prog Survey, Biomed Appl High Voltage Electron Microsc, November 16, 1971*, sponsored by National Institute of Health, U S Steel Research Center, Monroeville, Pa
7 V R Matricardi and D F Parsons, presented at the *Annu Meet Biophys Soc, Toronto, 1972*.
8 P R. Ward and R F Mitchell, *J Phys E (J Sci Instr)*, 5 (1972) 160
9 I G Stoyanova and G A Mikhailovskii, *Biophysics, U S S R* (Engl transl), 4 (1959) 116
10 G Dupouy, F Perrier and L Durieu, *C R Acad Sci Paris*, 251 (1960) 2836
11 H G Heide, *J Cell Biol*, 13 (1962) 147
12 H Hashimoto and T Naiki, *Jap J Appl Phys*, 7 (1968) 946
13 J. Escaig and C Lella, *C R Acad Sci Paris*, 268 (1969) 532
14 D L Allison, *Proc 7th Int Congr Electron Microsc, Grenoble, 1970*, Vol 1, Soc Française Microscopie Electronique, Paris, 1970, p 169
15 J A Swift and A C Brown, *J Phys E (J Sci Instr)*, 3 (1970) 924
16 F Nagata and K Ishikawa, in C J Arceneaux, *Proc 29th Annu Meet Electron Microsc Soc Am*, Claitor, Baton Rouge, 1971, p 464
17 V R Matricardi, R C Moretz and D F Parsons, *Science*, 177 (1972) 268
18 D F Parsons and V R Matricardi, reported at the *Annu Meet Biophys Soc, Toronto, 1972*
19 R C Moretz, G G Hausner, Jr and D F Parsons, reported at the *Annu Meet Biophys Soc, Toronto, 1972*
20 I G Stoyanova and T A Nekrasova, *Dokl Akad Nauk SSSR*, 134 (1960) 467
21 V R Matricardi, G P Wray and D F Parsons, *Micron*, 1972, in the press
22 K R Porter, A Claude and E F Fullam, *J Exp Med*, 81 (1945) 233
23 N E Dorsey, *Properties of Ordinary Water-Substance*, Hafner Publ Co, New York, 1968, p. 563
24 F H Moyer, *Am Zool*, 6 (1966) 43
25 J N Turner, H M Johnson and D F Parsons, presented at the *Annu. Meet Biophys. Soc, Toronto, Canada, February, 1972*
26 D. F Parsons, *J Appl Phys*, 43 (1972) 2885
27 D. F. Parsons and J R Subjeck, *Biochim Biophys Acta*, 265 (1972) 85