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ABSTRACT

Human erythrocytes exposed *in vitro* to low concentrations of uranyl ions are immediately changed in shape to stomatocytes. Electron microscope examination demonstrates that cellular damage is confined to the plasma membrane. Endocytosis of the cell membrane produces groups of inside out membrane-lined vesicles within the cell; lipid from the membrane enters the cell, giving rise to intracellular myelin figures, and breaks are seen in the cell membrane. The degree and type of change is shown to be a dose-dependent phenomenon. It is proposed that the lipid fraction of the cell membrane is the primary target for damage by uranyl ions.

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ACETATES; ANIMAL CELLS; CELL MEMBRANES; DOSE-RESPONSE RELATIONSHIPS; ELECTRON MICROSCOPY; ERYTHROCYTES; SHAPE; TOXICITY; URANYL COMPOUNDS

CONTENTS

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CONTENTS (continued)

1. INTRODUCTION

This report describes preliminary observations by electron microscopy of the changes which occur in human erythrocytes after exposure to uranyl acetate solutions. The work is part of a general investigation into the chemical toxicology of uranium compounds, and of the reaction of biological molecules with uranium.

Uranyl acetate is used widely as a stain in electron microscopy to reveal the structure of biomembranes (Pease 1964; Kay 1965; Sjöstrand 1967) and as a fixative for bacterial nucleoplasma (Ryter & Kellenberger 1958); more recently it has been recognised to have a valuable fixative effect on lipids (Biava & Shelly 1968; Silva 1968, 1971; Terzakis 1968). However, apart from the work of Hoffman (1972) who observed that erythrocytes underwent a change in shape from disc to sphere in low concentrations (1×10^{-7} *M*) of uranyl nitrate solution, the interaction between the unfixed cell and uranyl solutions has not been studied in detail.

Current work at the AAEC Research Establishment, Lucas Heights, has shown that exposure to low concentrations of uranyl ions (10^{-5} to 10^{-4} *M*) produces haemolysis of human erythrocytes (W.I. Stuart, AAEC private communication); this electron microscope work was undertaken as a parallel study to investigate ultrastructure changes induced in erythrocytes by uranyl acetate. The present results are preliminary and much work remains to be done; nevertheless they provide clear evidence of cellular changes induced by uranyl acetate.

2. METHOD

Uranyl acetate solutions in 0.9 per cent (physiologically normal) sodium chloride to give 0.005 *M*, 0.0005 *M*, and 0.00005 *M* concentrations were used. Erythrocytes washed three times in saline were added to the uranyl acetate solutions to give the following ratios of uranium/cell suspensions:

Sample (i)	30.0 fmol/cell
Sample (ii)	3.0 fmol/cell
Sample (iii)	0.3 fmol/cell.

A fourth sample in physiological saline only was used as a control. After 40 min at room temperature, the uranyl acetate solutions were removed from the cells and cacodylate-buffered glutaraldehyde fixative was added. Secondary fixation with osmium tetroxide was followed by dehydration in four changes of acetone. Specimens were embedded in Araldite resin (Spurr 1969) and sections (cut within the silver to gold interference colour range) were mounted unsupported on 400 mesh grids. Sections were stained with lead citrate (Reynolds 1963).

3. RESULTS

During the 40 min treatment with the uranyl acetate solutions, and during the steps of fixation, samples of cells were taken for optical microscopy by dark-ground and phase contrast in order to assess any changes and the possible production of gross artefacts by fixation. The initial changes observed were the clumping of cells and a change in shape from spherocytes to stomatocytes (Bessis 1972) or cup-shaped cells (Figure 1). Relative changes at different cell doses and time intervals are shown in Figure 2. These observations indicate that the change from sphere to stomatocyte is primarily dependent on the dose per cell rather than on the concentration of uranyl acetate in the solution. Further investigation has shown that uranyl acetate of the same concentration as Sample (iii) gave the same percentage cell change as Sample (ii) when the number of cells added to the solution was reduced by a factor of ten, effectively giving the same dose per cell. Similarly, a reduction in the number of cells in Sample (ii) by a factor of ten induced clumping and a few stomatocytes as in Sample (i). A second factor due to time may also be present since as, at 180 min, Sample (iii) produces stomatocyte changes.

The stomatocyte cells in these preparations could be returned to spherocytes by washing in phosphate buffer of pH 7.0, or by the addition of serum to the cell suspension. Reversal of shape change by buffer and serum has been noted previously by Hoffman (1972) and Deuticke (1968). All specimens were checked further after fixation and no change in appearance was seen by optical microscopy. The control cells in physiological saline showed no change throughout the experiment.

Electronmicrographs showed the following changes in uranyl-treated cells. None of these changes were seen in the control cells:

(a) Intracellular vacuoles were present at all three dose rates, more than 50 per cent of the cells in all preparations being vacuolated (Figures 3 and 4). Specific differences exist at different doses. High dose (30.0 fmol/cell) produces round vacuoles, whereas at the low dose (0.3 fmol/cell) short tubules are formed (Figures 3, 4 and 7). More individual vacuoles were present in each cell in the 0.3 fmol/cell preparation than in the 30.0 fmol/cell preparation. A well defined membrane is seen around the vesicles of the 30.0 fmol/cell preparation, but in the 0.3 fmol/cell preparation the membrane is ill defined (Figures 6, 8, 9 and 11). The vesicular membrane is identical with the plasma membrane (Figures 5 and 8). In the 30 fmol/cell specimen some of the vacuoles contain electron dense needle-shaped inclusions

(Figures 8 and 20). These have not been seen at lower doses or in any area outside the vacuoles.

(b) Myelin figures were seen in about 5 per cent of the 30.0 fmol/cell specimens (Figure 12), but they were not seen in the 0.3 fmol/cell specimens.

(c) Plasma membrane is well defined at 30.0 fmol/cell and, in some cases, shows a beaded appearance as if local concentration of material had taken place. At 0.3 fmol/cell the membrane is not well defined. Membrane breaks are present in some cells at all concentrations (Figure 19).

(d) Electron-dense inclusions occur in less than 1 per cent of the 30.0 fmol/cell preparation (Figure 11).

(e) Cells in the 0.3 fmol/cell preparations are slightly smaller than the 30.0 fmol/cells. In the 30.0 fmol/cell specimens the cell appears to be readily deformed by contact with other cells (Figures 3 and 4).

(f) Ghosts and extracellular haemoglobin are a common feature at the 30.0 fmol/cell dose rate. A few ghosts and a small amount of extracellular haemoglobin is present at the 0.3 fmol/cell dose. Cell wall invaginations and protrusions are common to both doses but are more common in the 30.0 fmol/cell specimen.

4. DISCUSSION

Stomatocyte (cup) cells have been shown to be produced by a variety of agents such as low pH (Jolly 1923; Bessis & Bricka 1950; Halpern & Bessis 1950) and chemicals including primaquine, chloromozine, triton X, 8 per cent ethanol (Deuticke 1968; Bessis 1972; Fujii, Sato & Nakanishi 1973; Weed & Bessis 1973). Intracellular ATP (adenosine triphosphate) also appears to be in some way implicated in the change (Fujii, Sato & Nakanishi 1973). The mechanism for stomatocyte formation has not been properly explained; it is however of interest that in the case of uranyl acetate there is a threshold dose below which the change is not produced although other cell changes, such as membrane invaginations and vacuoles, are still evident. Addition of uranyl acetate in any of the three concentrations used induces negligible changes in pH and osmolarity so that these two factors do not account for the observed cellular changes. The only erythrocyte shape change associated with haemoglobin is the sickle cell. In the electron microscope, the haemoglobin of these cells shows a characteristic rod-like structure in reversible sickling, whereas irreversible sickling appears to be caused by membrane damage (Bertles & Dobler 1969; Eaton *et al*. 1973). Microtubules or abnormal haemoglobin have not been seen in uranyl acetate treated cells.

Vacuoles have been demonstrated in circulating erythrocytes from splenectomised subjects by Kent *et al.* (1966), Schnitzer (1971) and Holroyde (1970). *In vitro* studies have shown that they are produced by antibody challenge to the blood of the new born but not to those of the adult (Blaton 1968; Preston 1970); vitamin A alcohol (Glauert *et al.* 1963); primaquine (Ginn, Hochstein & Trump 1969) and in erythrocyte ghosts only by ATP (Penniston & Green 1968).

Vacuolated erythrocytes are an abnormal form resulting from alterations in the membrane lipids, as with primaquine and vitamin A alcohol which also produce haemolysis, or they represent degenerative cellular processes consequent to cell ageing, or degeneration of the cell owing to trauma or toxic conditions.

Uranyl acetate-treated cell vacuoles are similar to those induced by vitamin A and primaquine. They appear to be formed by a process similar to pinocytosis in other cells. Deep invaginations of the membrane are formed (Figures 14 to 19) which become constricted at the cell surface and eventually close (Figures 15, 17 and 18), forming a membrane-lined vesicle within the cell. It can be seen from the micrographs that the membrane around the vesicle is an inside out plasma membrane. The electron-dense material which is normally seen just inside the membrane of erythrocytes is on the outside of the vacuole. A few vesicles contain material similar to the cell matrix. This most probably is external haemoglobin and other material which enter the vacuole while it is still an invagination (Figures 17 and 18).

Needle-shaped inclusions within the vacuoles were at first regarded as artefacts. Examination of many sections has shown that they form only within the vacuoles of the high dose specimens. It seems reasonable to suppose that they are not artefacts produced during specimen preparation, but are products of cellular damage at the highest dose. The possibility that they are recrystallised uranyl acetate was considered, but examination of unstained sections failed to show any electron-dense material within vacuoles. If the material was in fact recrystallised uranyl acetate, it should be quite evident in unstained material. A less homogeneous appearance would also be expected from uranyl acetate which has a crystal unit structure of about 10 Å (Figures 8 and 20). That the vesicles could represent cross sections of tubular systems within the cell rather than discrete vesicles has been considered, but no evidence has been found for this in the large number of cells examined. This observation is also supported by the literature, (Glauert *et al.* 1963; Ginn, Hochstein & Trump 1969). The vacuoles accumulate in clumps close to the membrane in a similar way to those produced by vitamin A and primaquine, and retain their typical shape and location even in haemoglobin-depleted ghosts (Figures 8 and 20).

A few cells > 1 per cent show electron-dense inclusions within the cytoplasma. These are probably formed by the breakdown of existing vesicles, the contents of which would be a uranyl acetate solution which would be released into the cell matrix, producing an area of increased density when it reacted with the cell contents (Figures 8 and 19).

Myelin figures are produced by bimolecular layers of lipid which alternate with layers of water (Finean & Robertson 1958; Stoeckenius 1962). The lipid of myelin figures is in the form of phospholipid. Their presence in cells is regarded as evidence of cellular damage and degeneration of the membranous components of the cell. As the plasma membrane is the only membrane system present in the erythrocyte, myelin figures must be regarded as an indication of damage involving transfer of phospholipids from the membrane to the interior of the cell, as no other source of phospholipid was available in our preparations.

The number of haemoglobin-depleted ghosts and the amount of extracellular haemoglobin is an indication of the relative haemolysis, but it cannot be regarded as a quantitative measure. Sampling, and the fact that processing for sectioning would be expected to produce some lysis, argues against any quantitative assessment. Allowing for this, the 30 fmol specimen quite clearly demonstrates a much higher haemolytic activity for this dose than that shown by the lower doses.

Membrane breaks are evident in some cells and one (Figure 19) shows haemoglobin being released from the cell. Membrane breaks can be shown to be transient holes in some forms of haemolysis (Seeman 1967). These are said to occur within the first 15 to 150 s of the reaction, so the number seen in our preparations at a much longer time have no numerical significance. Cells exposed to uranyl acetate, and then fixed in glutaraldehyde, which does not affect electron density, show an electron dense membrane area in the electron microscope (Figure 21). This electron density can only be produced by the uranium located in the membrane and is not seen in cells which have not been exposed to uranyl acetate. Goodford & Wolowyk (1972) show a similar result.

5. CONCLUSIONS

There is a distinct difference in the appearance of cells at varying doses. High doses produce more membrane change, the low dose membrane appearing normal whereas the high dose membrane shows thickening and a beaded appearance, as if there had been a condensing of material into globular units. Myelin figures in the high dose specimen are an indication of disruption of the plasma membrane and internalisation of phospholipid. The primary site of cellular damage resides in the plasma membrane and the indication is that the lipids of the membrane are the most likely part affected. It has been proposed by Ginn, Hochstein & Trump (1969) and other investigators that the invagination of membrane to form vesicles will effectively decrease the surface area of the cell, thus reducing the critical haemolytic volume. Taking the surface area of the human erythrocyte to be about 140 µm and the average vacuole to have a diameter of 0.25 µm, it can be calculated that there is sufficient surface membrane in the cell to form about 780 separate vacuoles. On this assumption, the vacuolated cells considered in this report have probably lost between 0.07 and 0.14 per cent of their cell membrane to the vacuoles.

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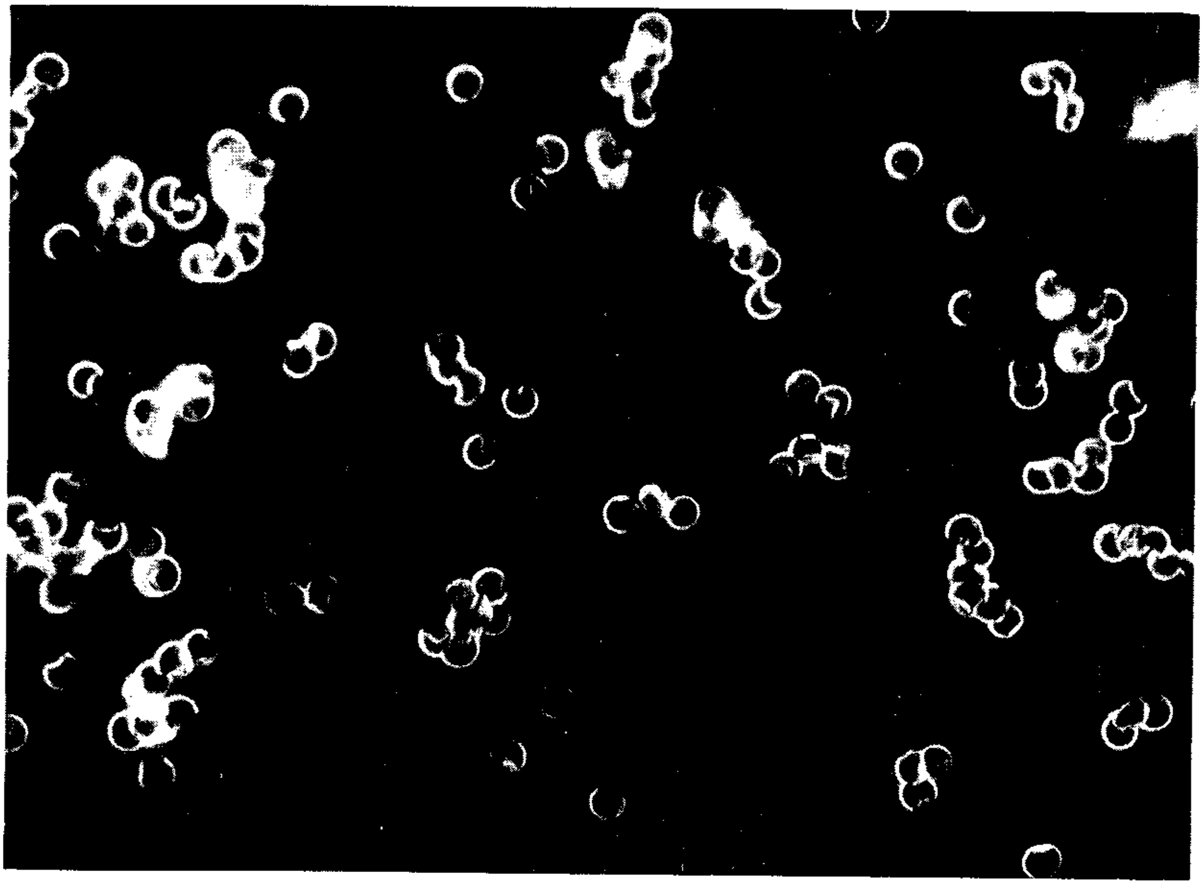


FIGURE 1 ERYTHROCYTES EXPOSED TO 3.0 fmol/cell URANYL ACETATE. DARK-GROUND OPTICAL MICROSCOPY SHOWS SMOOTH SPHEROCYTES AND STOMATOCYTES

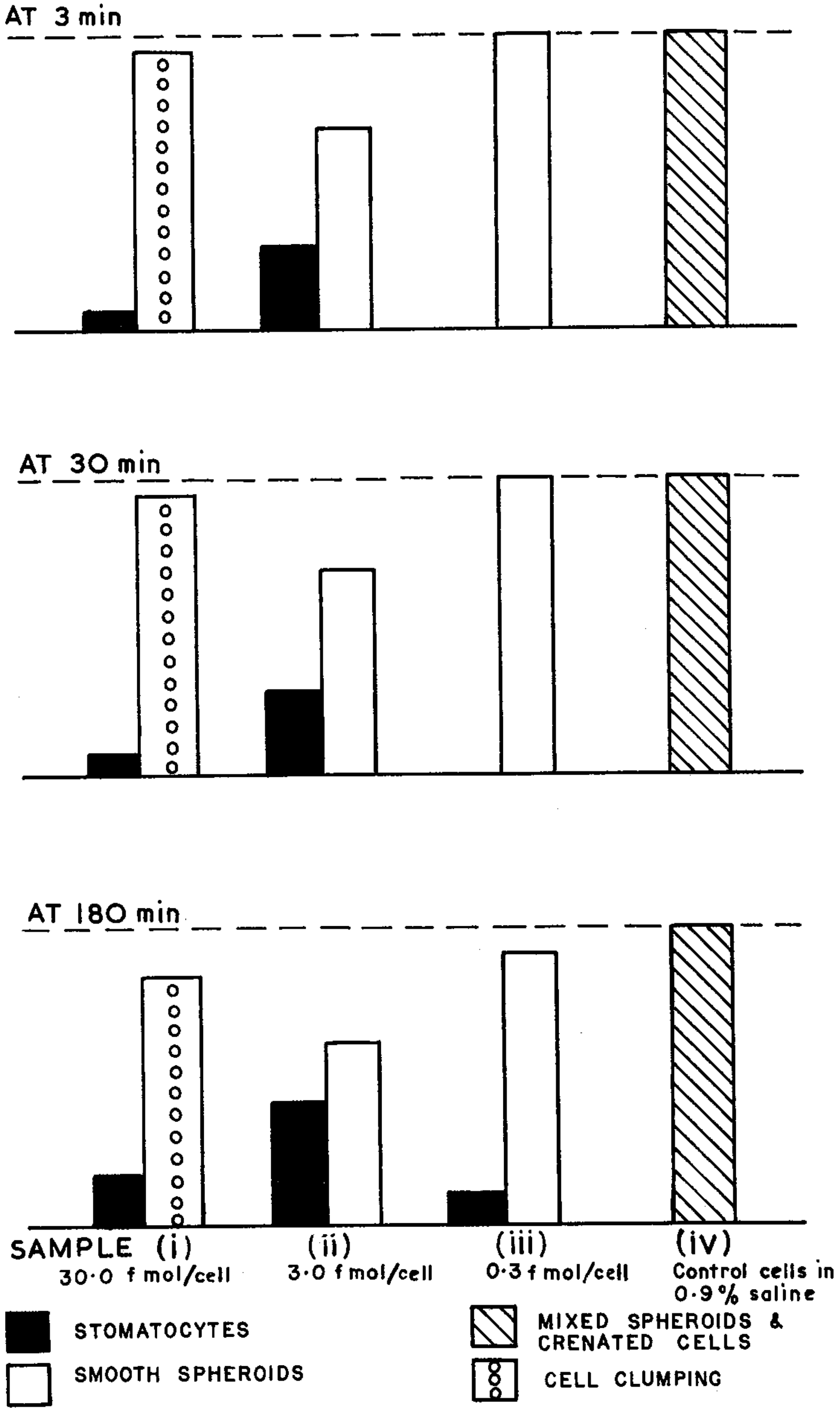
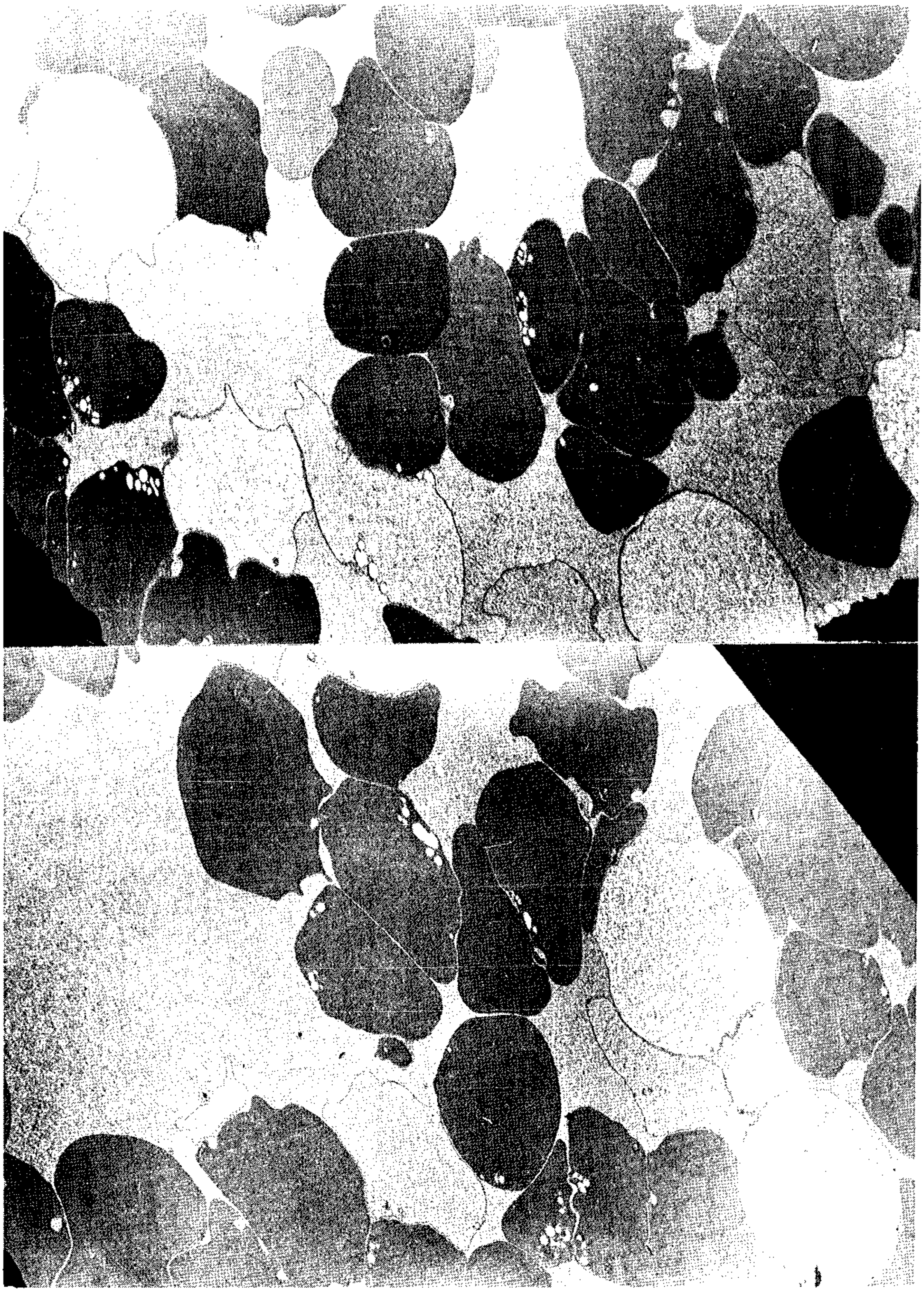


FIGURE 2 DISTRIBUTION OF CELL SHAPES RELATIVE TO VARYING DOSE LEVELS AND TIME INTERVALS

X5,000

FIGURE 3 LOW MAGNIFICATION ELECTRON–MICROGRAPH OF 0.3 fmol/cell PREPARATION.
NOTE CELL SHAPE, VACUOLES AND MEMBRANE INVAGINATION



X5,000

FIGURE 4 LOW MAGNIFICATION ELECTRON–MICROGRAPH OF 30.0 fmol/cell PREPARATION. NOTE CELL SHAPE, VACUOLES, MEMBRANE INVAGINATIONS AND EXTRACELLULAR HAEMOGLOBIN

X120,000

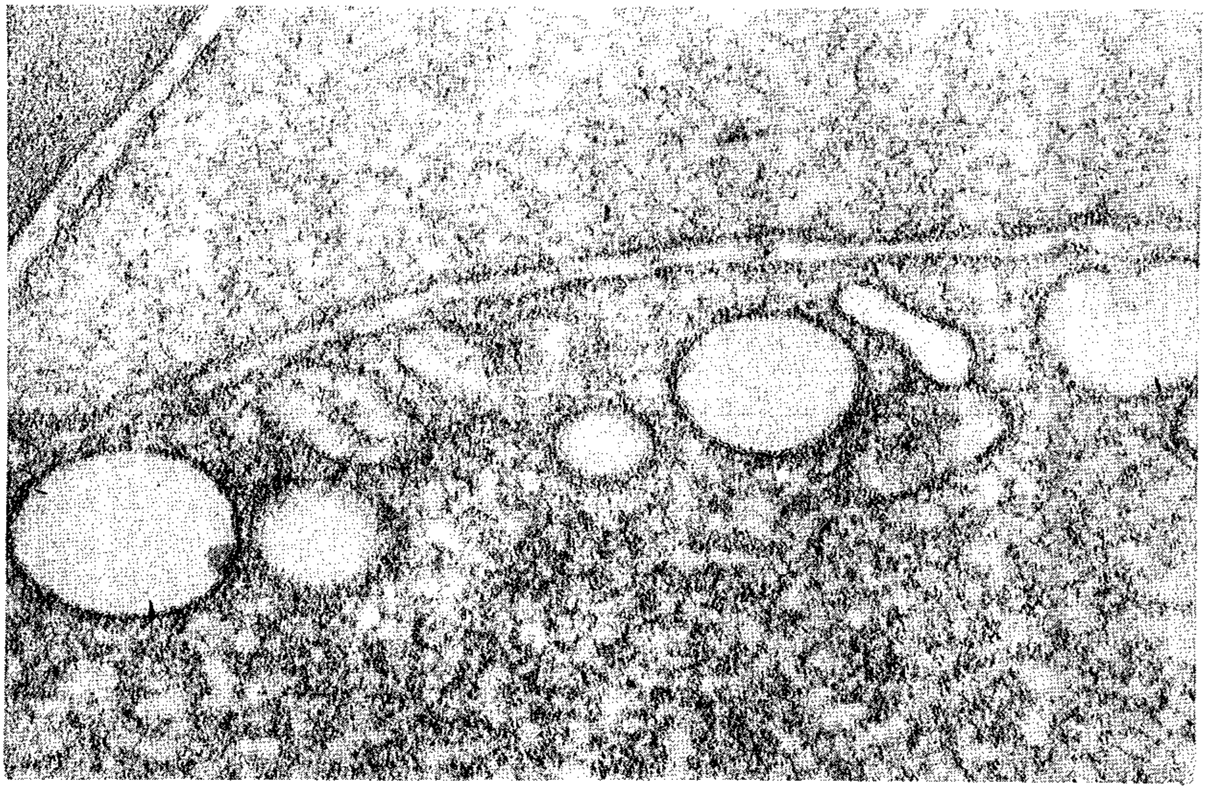
FIGURE 5 0.3 fmol/cell PREPARATION SHOWING MEMBRANE-LINED VESICLES

X120,000

FIGURE 6 0.3 fmol/cell PREPARATION SHOWING MEMBRANE-LINED VESICLES

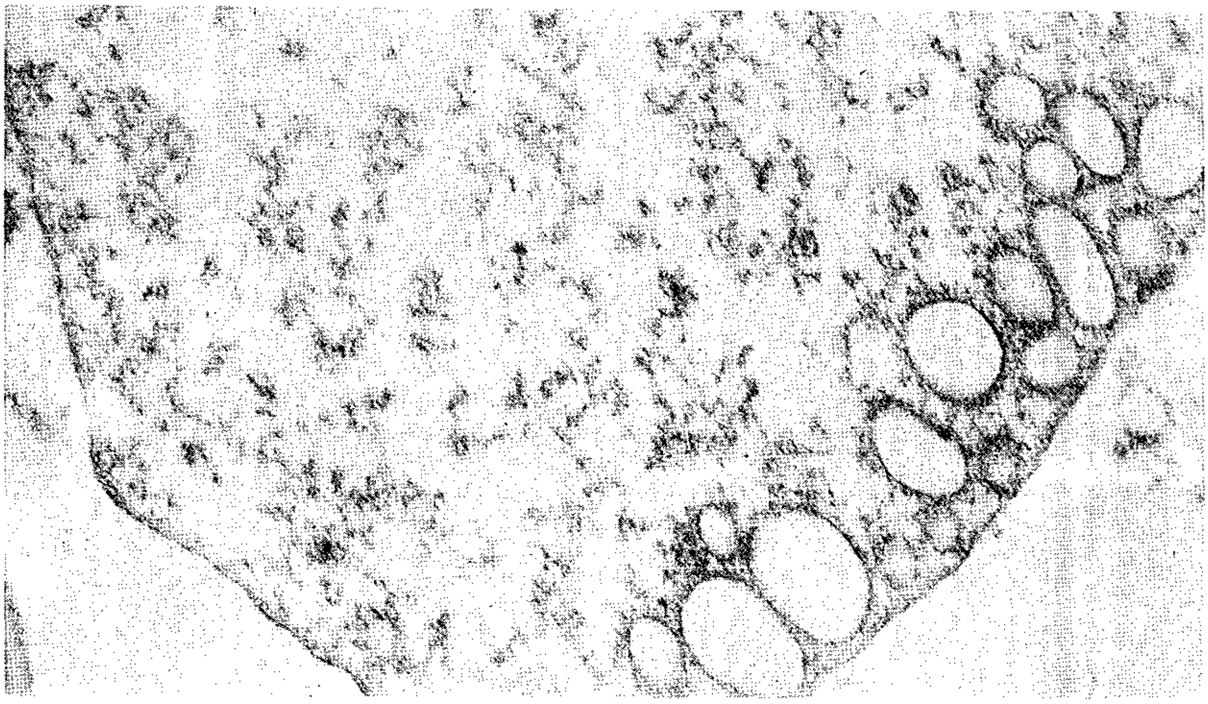
X120,000

FIGURE 7 0.3 fmol/cell PREPARATION. NOTE MEMBRANE–LINED VESICLES AND EXPANDED CELL MEMBRANE



X120,000

FIGURE 8 30.0 fmol/cell PREPARATION. MEMBRANE-BOUND VESICLES WITH DENSE NEEDLE-SHAPED INCLUSIONS



X120,000

FIGURE 9 0.3 fmol/cell PREPARATION

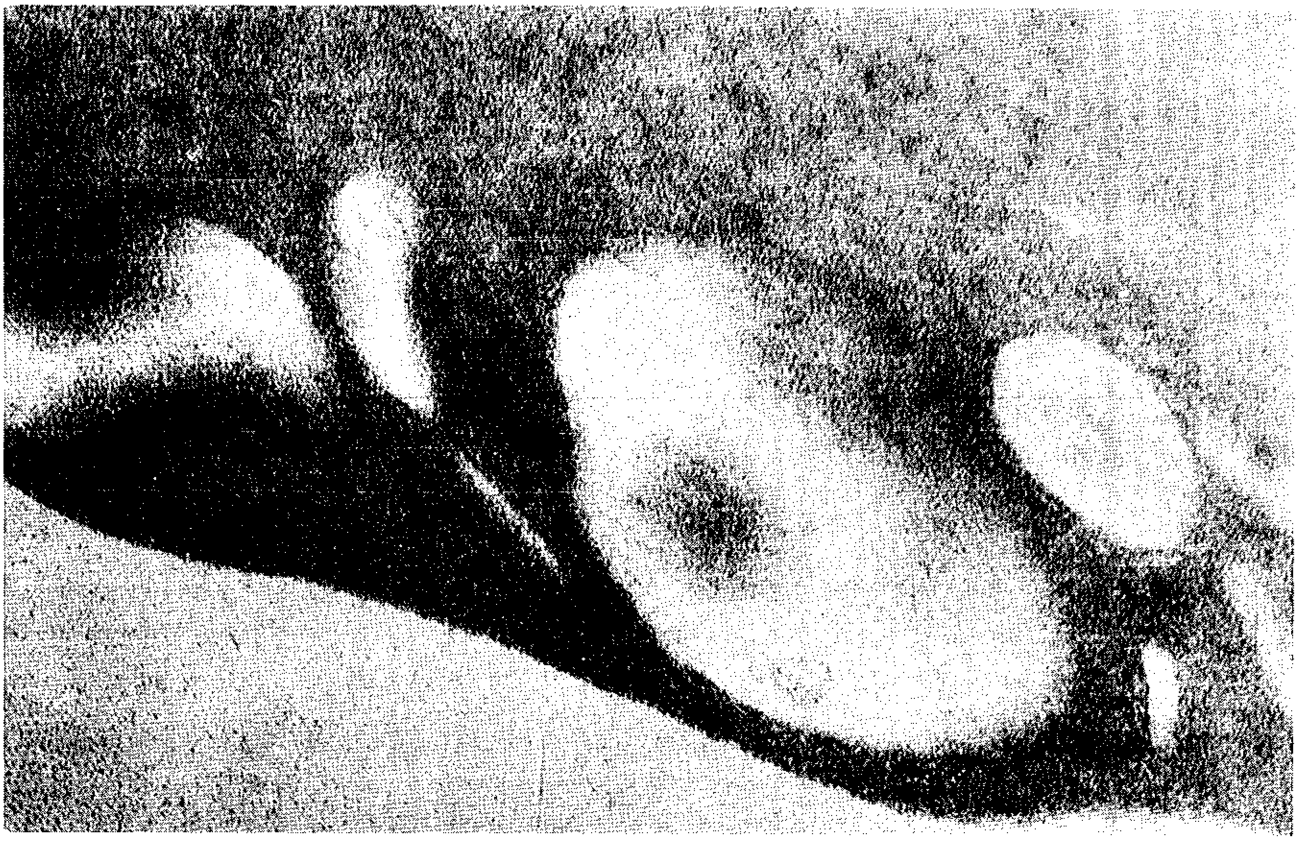
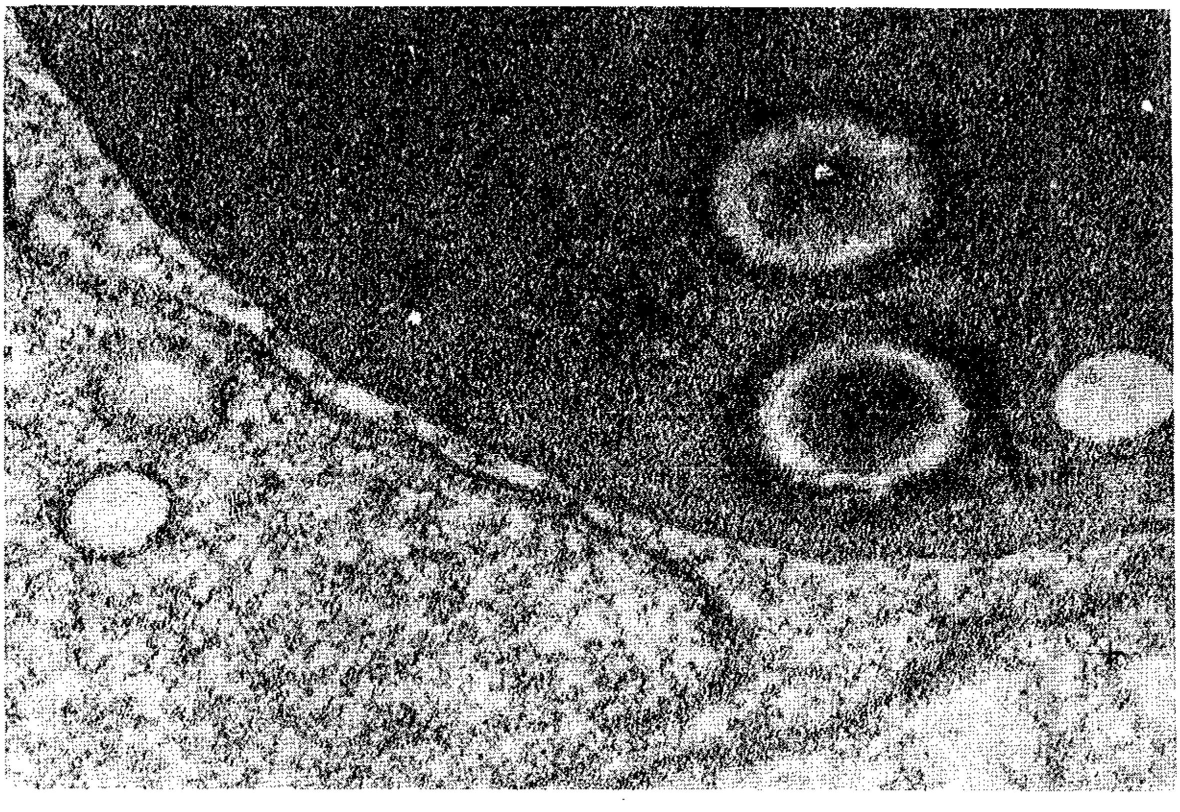


FIGURE 10 0.3 fmol/cell PREPARATION. NOTE PENETRATION OF CELL MATRIX THROUGH MEMBRANE OF LARGE VESICLE



X120,000

FIGURE 11 30.0 fmol/cell PREPARATION WITH TWO ELECTRON-DENSE INCLUSIONS

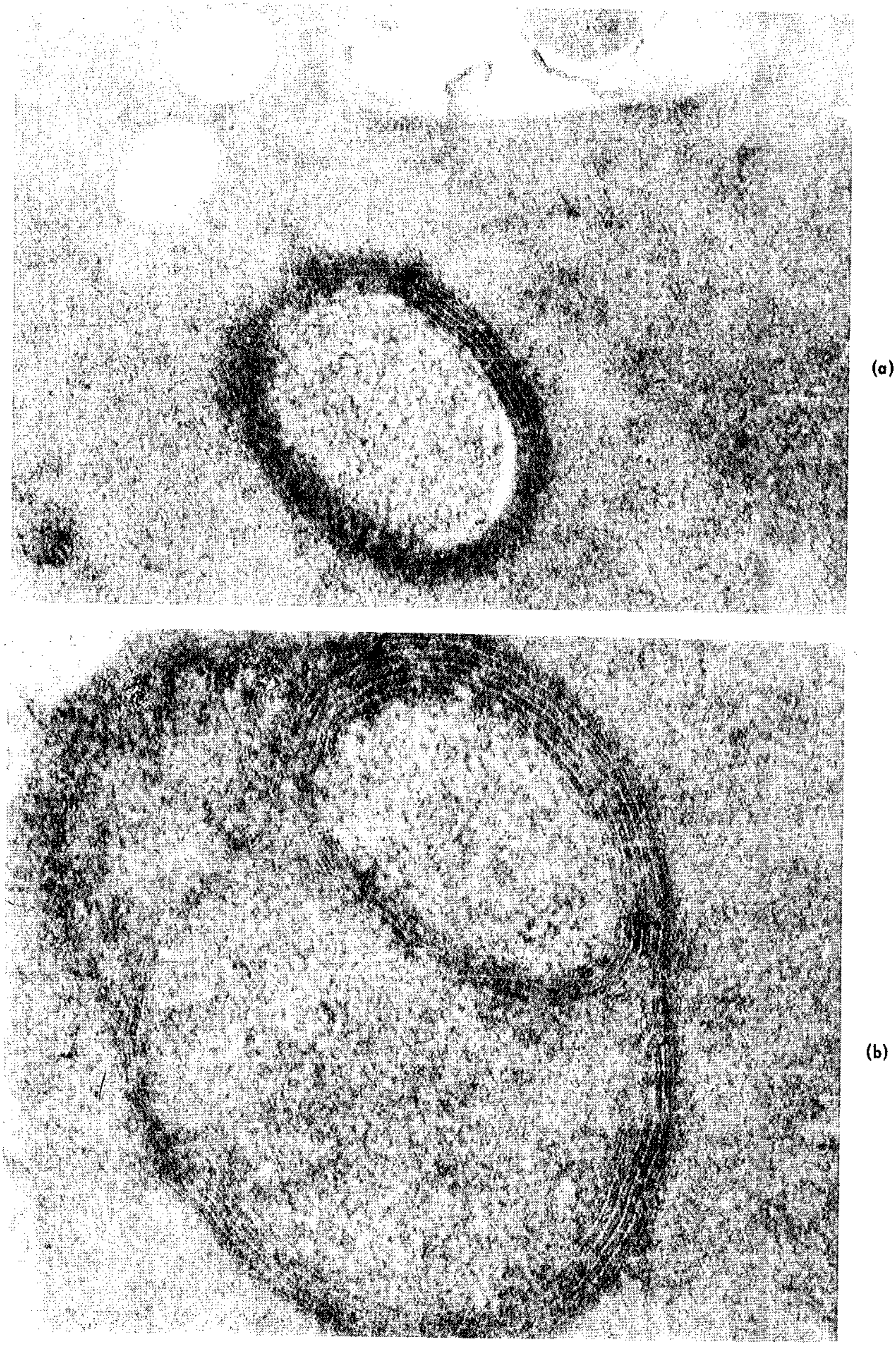


FIGURE 12 (a) & (b) 30.0 fmol/cell PREPARATION MYELIN FIGURES

(a) X25,000
(b) X20,000

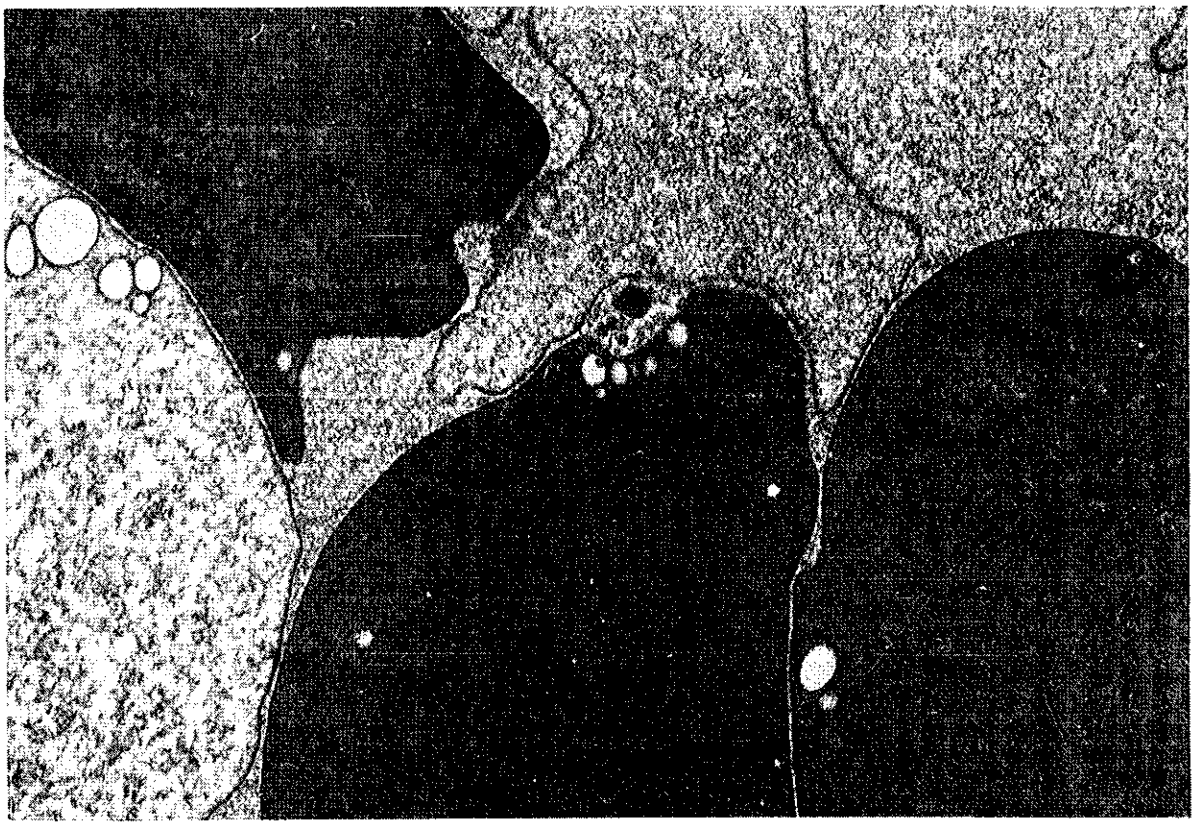


FIGURE 13 30.0 fmol/cell PREPARATION. NOTE MYELIN FIGURE, VESICLES AND GHOST CELL

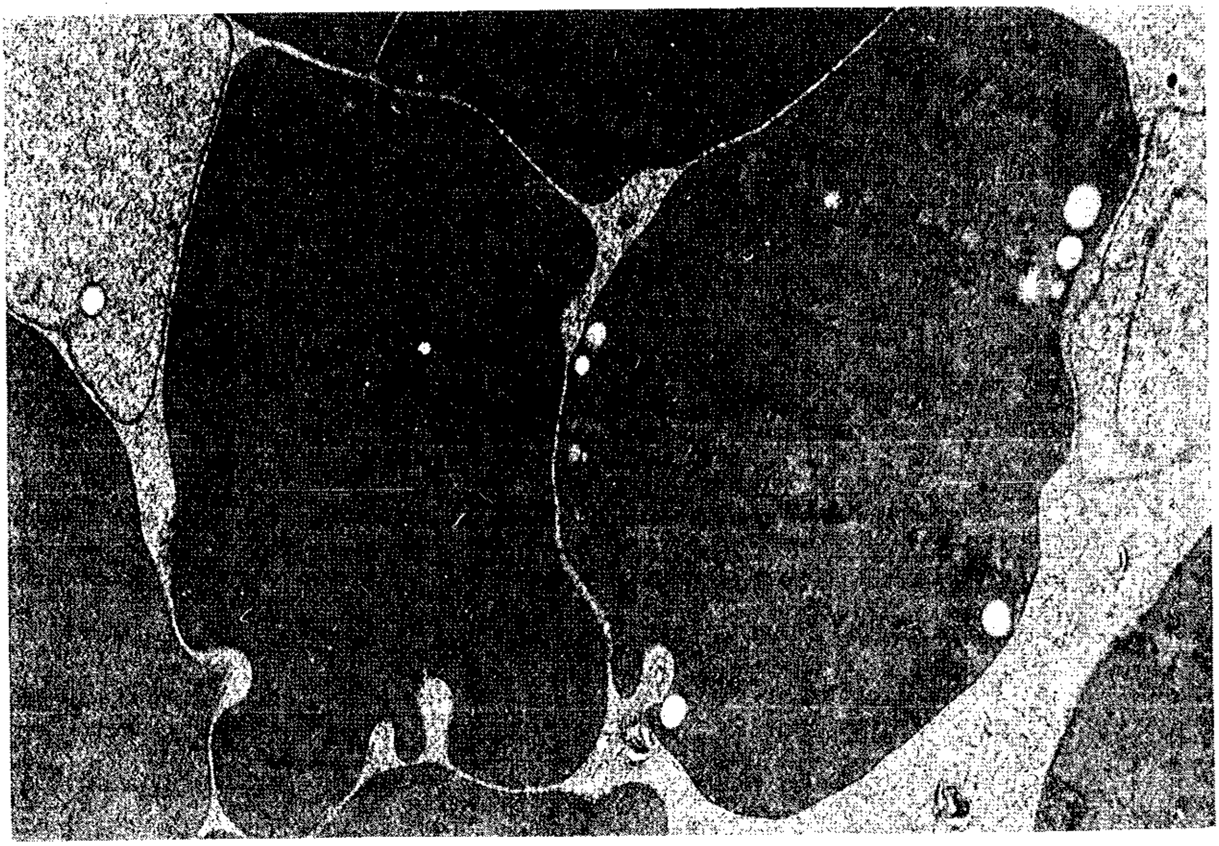
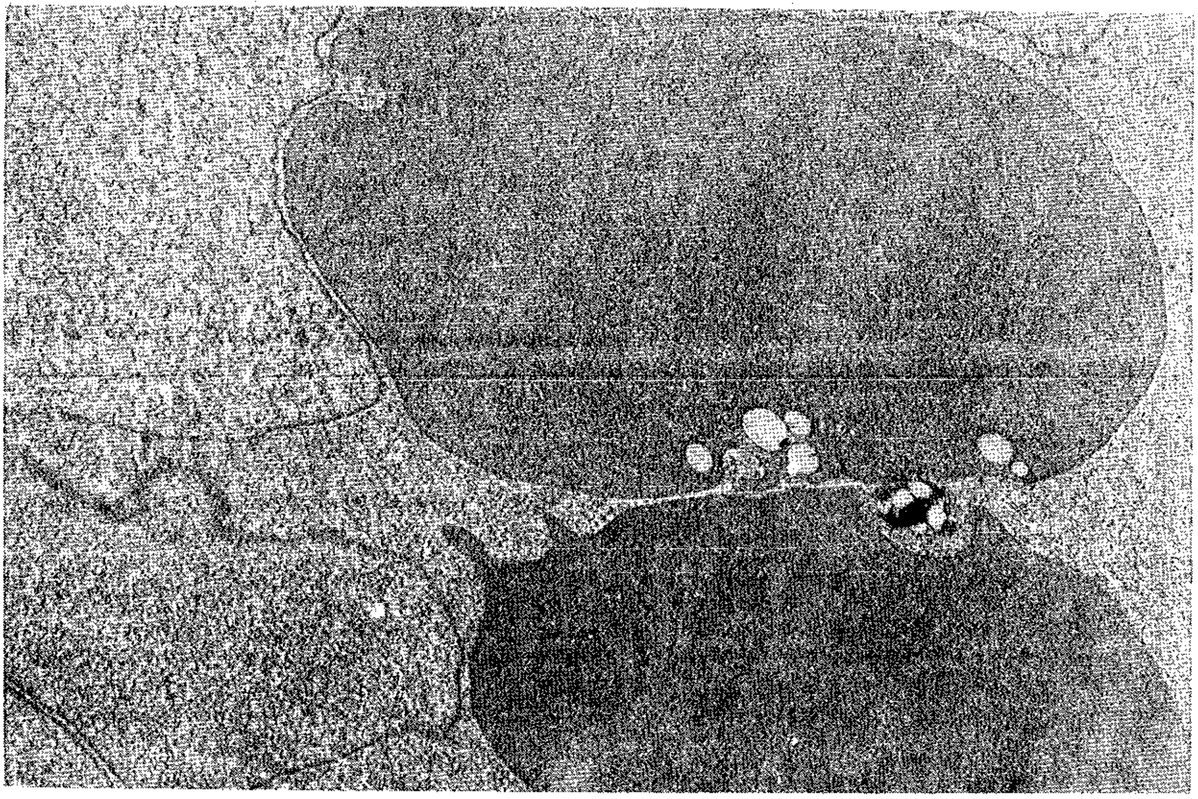
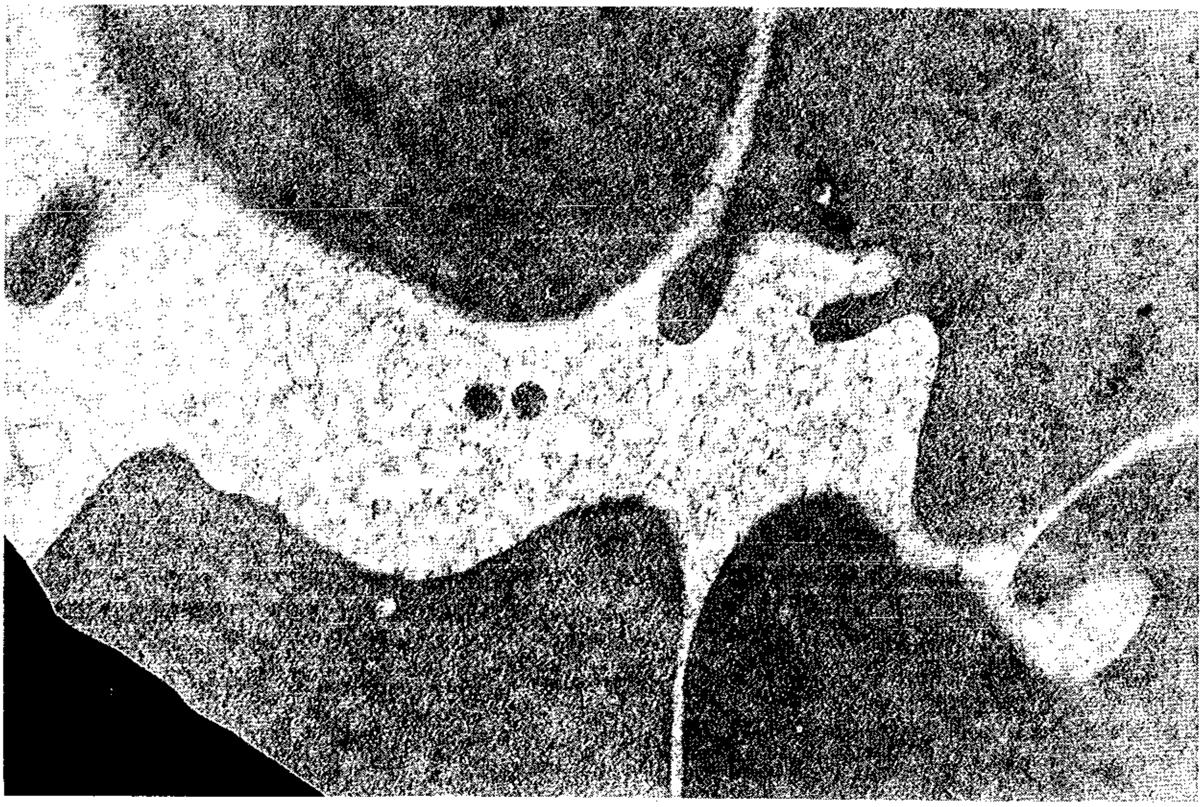


FIGURE 14 30.0 fmol/cell PREPARATION. NOTE INVAGINATED MEMBRANES



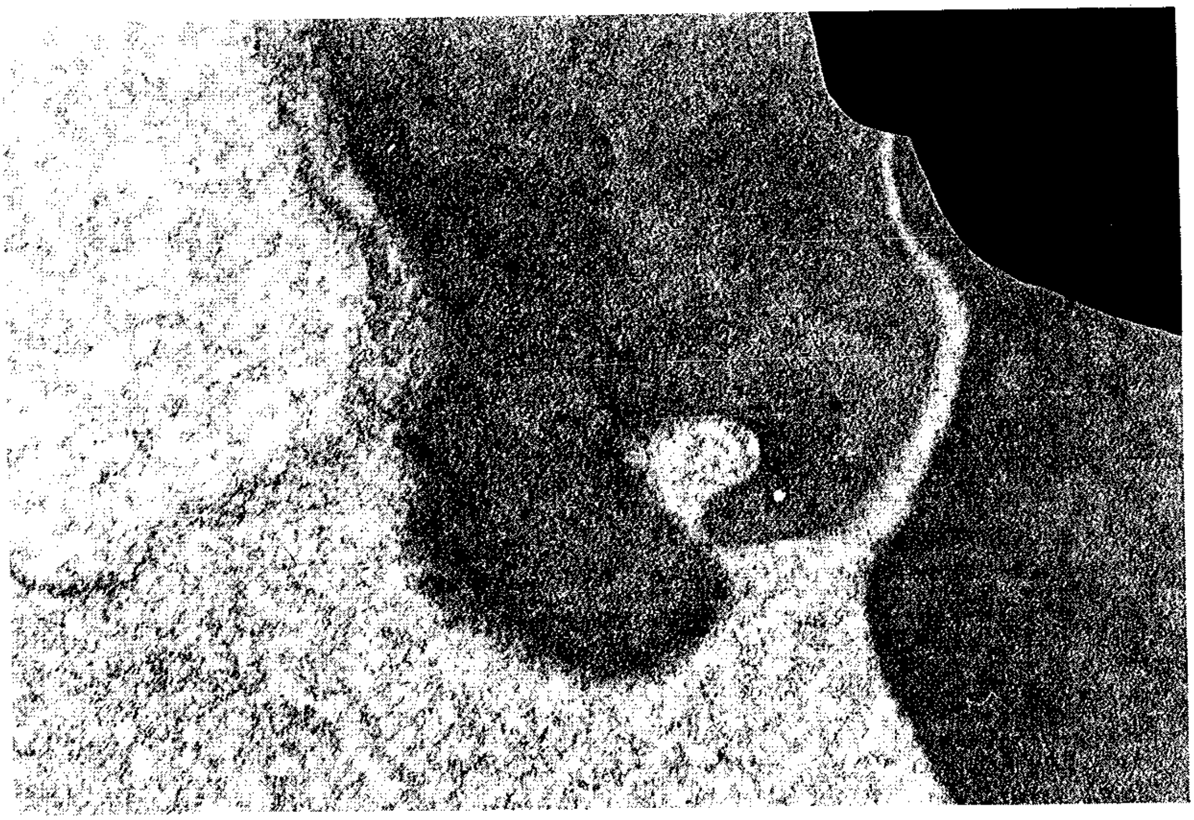
X60,000

FIGURE 15 30.0 fmol/cell PREPARATION. PARTLY CLOSED VESICLE CENTRE OF FIELD



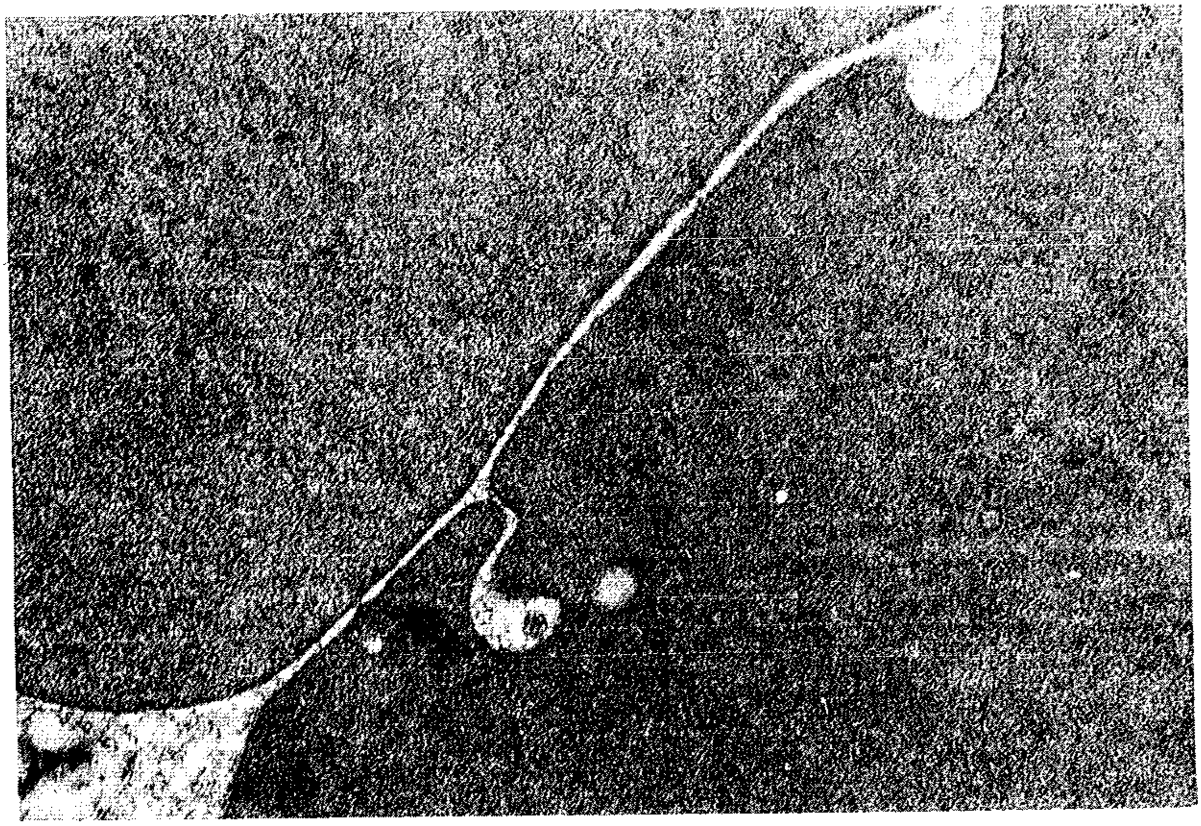
X120,000

FIGURE 16 30.0 fmol/cell PREPARATION. PARTLY CLOSED VESICLE LOWER RIGHT OF PICTURE



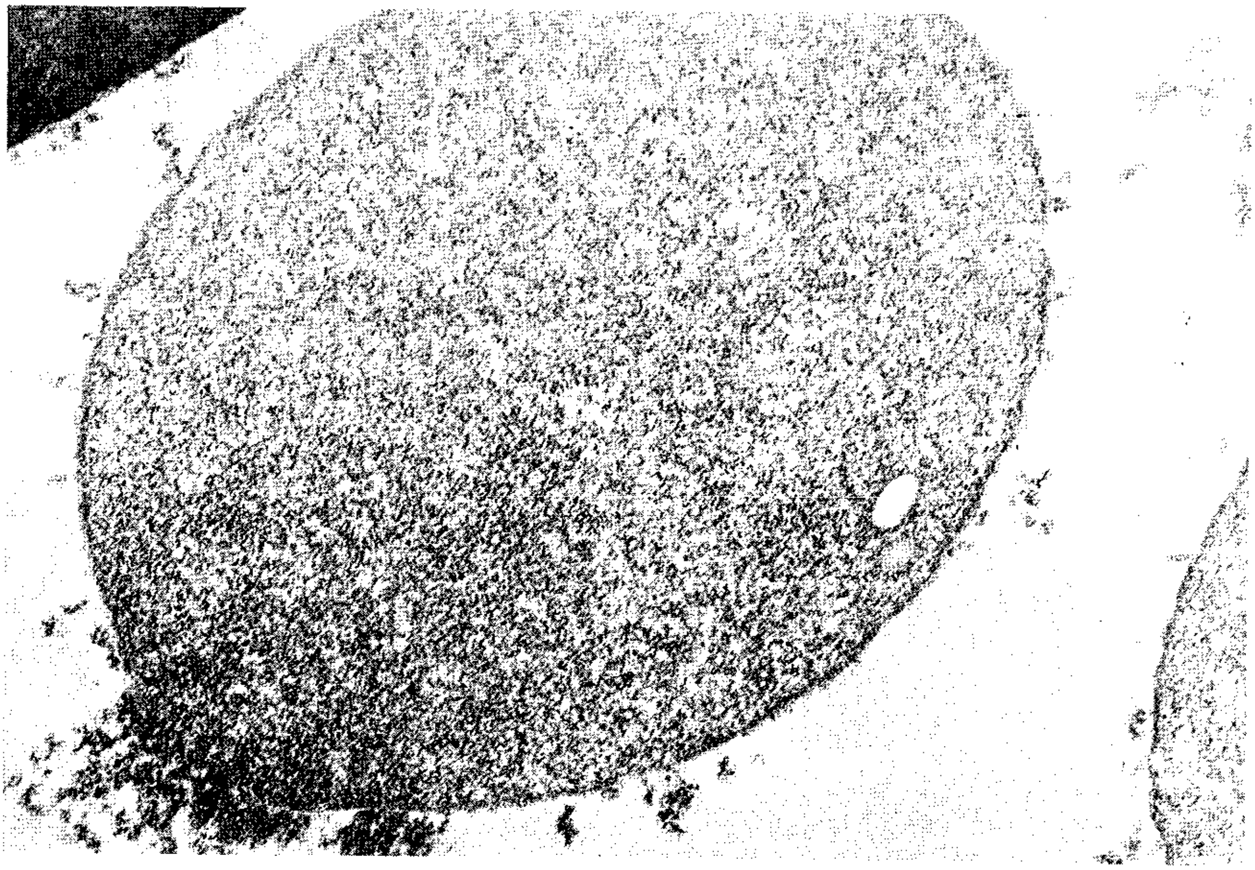
X120,000

FIGURE 17 30.0 fmol/cell PREPARATION WITH PARTLY COMPLETED VESICLE CONTAINING EXTRACELLULAR HAEMOGLOBIN



X120,000

FIGURE 18 30.0 fmol/cell PREPARATION. BROAD INVAGINATION AT TOP RIGHT; DEEP INVAGINATION WITH ALMOST CLOSED NECK AND ENTRAPPED EXTRACELLULAR MATERIAL AT CENTRE



X120,000

FIGURE 19 30.0 fmol/cell PREPARATION. PARTIAL GHOST WITH HAEMOGLOBIN ESCAPING

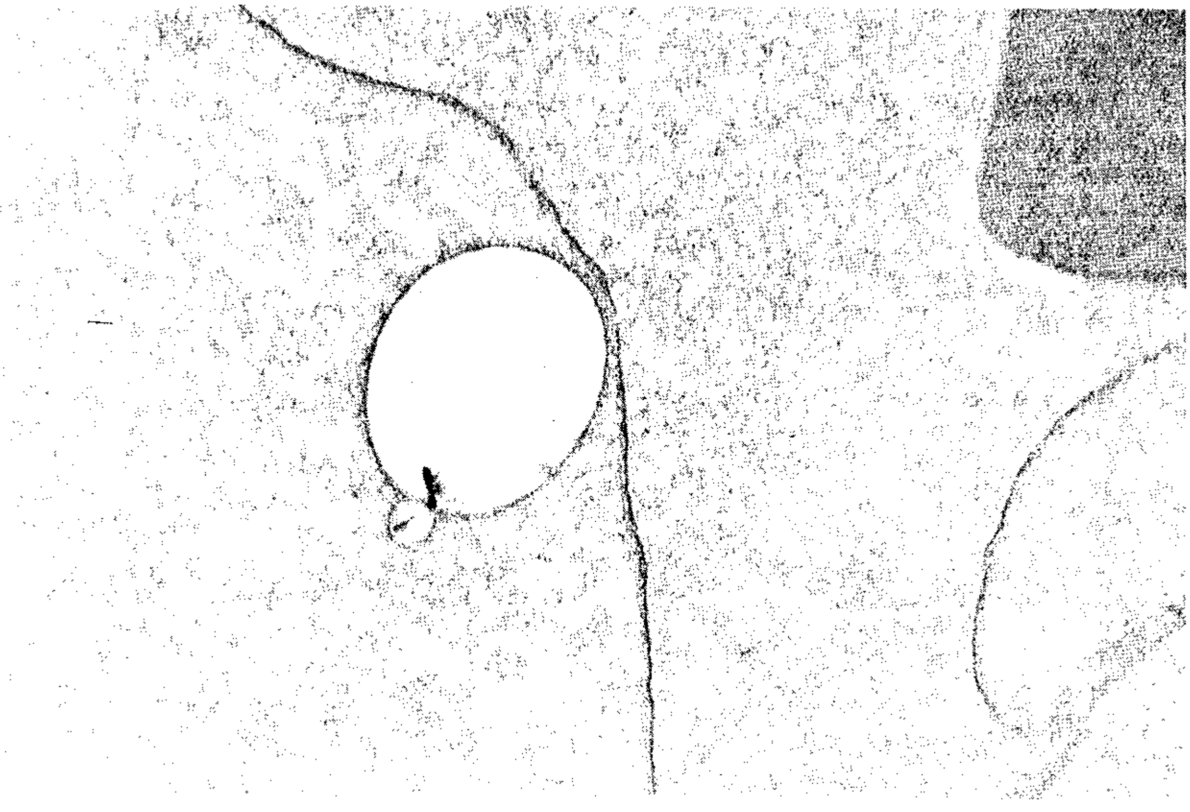


FIGURE 20 30.0 fmol/cell PREPARATION. VACUOLE IN GHOST CONTAINING INCLUSION

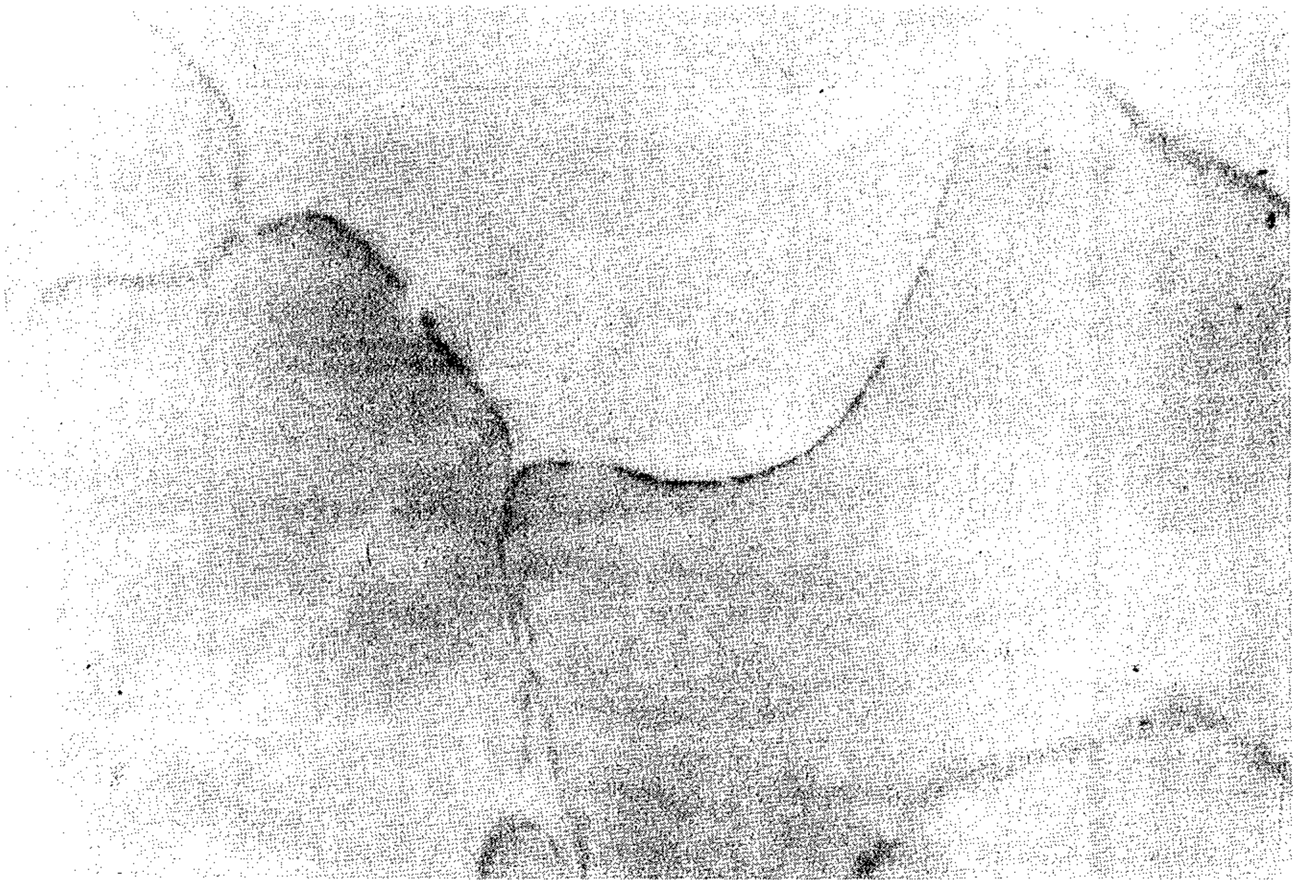


FIGURE 21 ERYTHROCYTES EXPOSED TO URANYL ACETATE SOLUTION, FIXED IN GLUTARALDEHYDE ONLY AND SECTIONED. UNSTAINED SECTION SHOWING INCREASED ELECTRON DENSITY WITHIN THE MEMBRANE