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AUSTRALIAN ATOMIC ENERGY COMMISSION

RESEARCH ESTABLISHMENT

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March 1977

ISBN 0 642 99780 2

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ABSTRACT

Phase contrast and dark field microphotographs were made to record variation of the shape and size changes seen when human erythrocytes are exposed in a number of ways to uranyl acetate *in vitro*. The degree of hemolysis produced by varying the tonicity of the uranyl acetate solutions was measured, and the results are discussed with particular reference to the possible influence of pH.

National Library of Australia card number and ISBN 0 642 99780 2

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ACETATES; CELL MEMBRANES; ERYTHROCYTES; HEMOLYSIS; IN VITRO; OSMOSIS; PH VALUE; SWELLING; URANYL COMPOUNDS;

CONTENTS

(continued)

CONTENTS (Continued)

All figures with the exception of Figures 5 and 6 are at 460x magnification. Figures 5 and 6 are at 660x magnification.

1. INTRODUCTION

This report describes a light microscope study of the changes seen when human erythrocytes are exposed to uranyl acetate *in vitro*. A previous report (Wyatt 1975) examined the ultrastructural changes occurring at similar dose levels.

Human erythrocytes were exposed to varying concentrations of uranyl acetate in physiological saline in the following ways:

- as a free floating suspension of cells in uranyl acetate NaCl solutions;
- by allowing the cells to attach to a microscope slide and then exposing them to uranyl solutions; and
- as a cell suspension in uranyl acetate solutions of graded Na^+ ion tonicities similar to those used in the clinical fragility test.

Phase contrast and dark field microphotographs were made to record variations in shape and size. The degree of hemolysis produced by varying the tonicity of the uranyl acetate solutions was measured, and the results are discussed with particular reference to the possible influence of pH.

2. MATERIALS AND METHODS

Erythrocytes separated from whole blood were washed three times in phosphate buffered saline (310 mosm, pH 7.4) then given a final wash in 0.9% saline. For all tests one packed volume of these cells was resuspended in an equal volume of saline. The standard suspension used throughout was 0.1 mℓ of packed cells to 10 mℓ of the test solution. Three standard solutions of uranyl acetate in 0.9% NaCl were used, 5×10^{-3}M, 5×10^{-4}M and 5×10^{-5}M. These concentrations were also used for the tests with varying tonicity in graded NaCl solutions from 0.1 to 0.8% in steps of 0.1%. After 60 min these preparations were centrifuged at 2000 rev./min for 5 min and comparative hemolysis measurements were recorded on a Bausch and Lomb spectronic 20 photometer against a 100% lysed (0.1% NaCl) specimen. For experiments with cells attached to microscope slides a few drops of cell suspension were placed on a slide and after 30 s the slide was washed in a gentle stream of 0.9% NaCl to remove unattached cells.

3. RESULTS

3.1 Cells as Free Suspensions in Uranyl Acetate Solutions

Cells were suspended in the three concentrations of uranyl acetate for 10 min before the photomicrographs were made. The relative dose in these preparations was 30 fmol/cell, 3.0 fmol/cell and 0.3 fmol/cell.

The saline washed cells used in these preparations were all spherocytes (Figure 1). After exposure to 5 x 10^{-3}M uranyl acetate the cells varied considerably in size, some cup cells were present and there was a marked tendency for the cells to aggregate in clumps surrounded by finely particulate material (Figure 2). In the 5 x 10^{-4}M preparation there were many cup cells and a few swollen cells. Size variation and cell aggregation was not as marked as in the previous preparation (Figure 3). The 5 x 10^{-5}M dose produced smooth spheroids with only an occasional cup cell. No cell clumping occurred. A further sample exposed to 5 x 10^{-6}M uranyl acetate was similar to the original washed cells (Figure 4).

To indicate the degree of swelling involved, cell diameters were measured directly from photographic prints and by projection of the negative image, the few extremely swollen cells present at the high dose being disregarded. The scale of measurement shown in the table is empirical and intended only to give relative size comparisons within the series (Table 1).

3.2 Cells Attached to Microscope Slides before Exposure to Uranyl Acetate

Figure 5a shows cells attached to a slide. Figure 5b is the same group of cells after 1 min exposure to 5 x 10^{-4}M uranyl acetate. The irregular shape of the cells in the saline sample is caused by their points of attachment to the slide. However it can be seen that even under these restrained conditions, 5 x 10^{-4}M uranyl acetate produces smoother, rounder cells suggesting that they have become spheroidal. Many of these cells also show cupping.

Figure 6 shows another similarly treated set of cells. There is an indication of fusion between cells in this preparation.

Uranyl acetate has been shown to cause cell fusion under certain conditions [Pertz *et al*. 1974; Toister & Loyter 1973; Zakai, Loyter & Kulka 1974]. Exposure to a higher concentration of uranyl acetate produces a loss of optical density in the cells indicating hemolysis and the production of particulate material around the cells. Because dose per cell cannot be estimated, changes seen in these preparations can be related only to concentration of uranyl acetate.

3.3 Cells Exposed to Uranyl Acetate at Varying Tonicities

Washed erythrocytes were exposed to constant levels of uranyl acetate in solutions containing NaCl of various ionic strengths. Table 2 shows the principal changes in shape, size and clumping at the different levels.

Photomicrographs (Figures 7 to 11) show the appearance of the cells and the graph (Figure 12) indicates the degree of hemolysis at the various ionic strengths.

4. DISCUSSION

In any discussion of the results, such factors as osmolarity and pH must be taken into account. Addition of uranyl acetate to NaCl solutions would be expected to increase the osmolarity, which would in effect move the fragility curve to the left. Any alteration in tonicity then would be proportional throughout the curve for any individual dose and the general shape of the curve should be unchanged. There is no suggestion that the results (Figure 12) are caused by such a situation. Therefore the effect of the uranyl acetate on tonicity can be ignored in this context.

Parapart *et al.* [1947] have shown that a shift of 0.1 pH is equivalent, in a fragility test, to altering the tonicity of the NaCl solution by 0.01% NaCl. Since the uranyl acetate solutions used are in the range pH 5.0 - 4.5, the effective tonicity of the solutions should be increased by the equivalent of 0.3% NaCl. This expected increase could account for the occurrence of hemolysis in 0.85% NaCl when uranyl acetate is present. As with tonicity, pH would be a constant factor at each point and would serve only to move the whole curve to the right.

More important is the effect of pH on the cell. Intracellular pH of the erythrocyte is controlled by a number of factors, one of which is the pH of the surrounding medium. Hilpert *et al.* [1963], Waddell & Bates [1969], Desforges & Slawsky [1972] and Warth & Desforges [1975] have shown that, both *in vitro* and *in vivo*, the intracellular pH tends to change in the same direction as that of the surrounding medium.

At physiological pH 7.4 each hemoglobin molecule has about three negative charges; at pH 6.95 the isoelectric point of the hemoglobin is reached and the net charge falls to zero. As the pH decreases further the hydrogen ion concentration of the external solution increases and the hemoglobin begins to acquire positive charges. If it is accepted that the cell must preserve electroneutrality, this change induces an anionic gradient into the cell, requiring a readjustment of water balance to maintain osmotic equilibrium with the external medium. This phenomenon could explain the initial swelling seen in the cells.

At the lowest dose the uranyl acetate NaCl fragility curve deviates from normal at the top and bottom of the curve. Microscopically the cells differ from the NaCl control only in that an occasional cup cell is seen

and that the initial swelling takes place at a higher tonicity (Table 2).

At the two higher doses the curve flattens out and only about 50% hemolysis occurs at the tonicity where standard hemolysis is complete. Considerable progressive swelling is present with more variation in cell size in the highest dose. These curves suggest that in the presence of uranyl acetate the hemoglobin is released by a process more gradual than the catastrophic disruption which occurs when NaCl alone is used.

Cup cells are a regular feature of exposure to uranyl acetate. They form rapidly, within 2 to 3 s, preceded by the cell swelling and spherocytosis. To estimate the approximate volume lost when a cell membrane invaginates to form a cup cell, a wax sphere was formed, sufficient wax removed to make a cup cell shape similar to that seen microscopically, and the wax masses weighed. This procedure indicated that the cup invagination is equivalent to 20% loss in volume when compared with a spherical cell of the same diameter. The size measurements in Table 1 can be used to show that the normal spheres produced by washing in NaCl have a surface area of 38 and a volume of 22, and that the cells swollen by uranyl acetate have an area of 50 and a volume of 33. However if the swollen cell figures are adjusted to allow for cupping, an area of 50 and volume of 26 result, indicating that a cup cell has an increased surface area of 20%. This suggests that the membrane is expanding within itself rather than being stretched by internal pressure. In hypotonic solutions cup cell formation ceases at 0.5% NaCl. As this is the tonicity at which cells normally swell and hemolyse, an osmotically induced pressure can be reasonably expected to occur within the uranyl acetate treated cells at this point also. This expected event combined with swelling of the cell matrix, could be sufficient to prevent the expanded cell membrane from invaginating to form a cup cell.

The sequence of events shown in the graph and photomicrographs can be explained by expansion of the cell membrane by uranyl acetate, which, in the case of cup cells, is relatively greater than the swelling of the cell. In the swollen cells this expansion leads to increased membrane permeability without any increase in the internal pressure of the cell. Rand & Burton [1963] showed that an increase in membrane without any significant increase in volume can cause hemolysis. The very large cells seen at high dose most probably result from cell fusion.

5. CONCLUSION

- There is a marked change in the mechanism of osmotic hemolysis in the presence of uranyl acetate.
- Hemolysis by uranyl acetate may be produced by an increase in membrane area with increased permeability rather than by volume increase with rupture of the membrane.
- After hemolysis by hypotonic solutions in the presence of uranyl acetate the cells are intact and swollen indicating that hemoglobin leaks through the membrane owing to expansion and thinning of the membrane, rather than pressure and membrane rupture.

6. ACKNOWLEDGMENT

The author acknowledges the assistance of Mr. H.E. Smith with the hemoglobin estimations on the fragility curves.

7. REFERENCES

Desforges, Jane, F. & Slawsky, P. [1972] - Red cell 2, 3-diphosphoglycerate and intracellular arterial pH in acidosis and alkalosis. *Blood*, 40, 740.

Hilpert, P., Fleischmann, R.G., Kempe, D. & Bartels, H. [1963] - The Bohr effect related to blood and erythrocyte pH. *Am.J. Physiol.*, 205, 337.

Parpart, A.K., Lorenz, P.B., Parpart, E.R., Gregg, J.R. & Chase, A.M. [1947] - The osmotic resistance (fragility) of human red cells. *J. Clin. Invest.*, 26, 636.

Pertz, H., Toister, A., Laster, Y. & Loyter, A. [1974] - Fusion of intact human erythrocytes and erythrocyte ghosts. *J. Cell. Biol.*, 63, 1.

Rand, R.P. & Burton, A.C. [1963] - Area and volume changes in hemolysis of single erythrocytes. *J. Cell. Comp. Physiol.*, 61, 245.

Toister, Z. & Loyter, A. [1973] - The mechanism of cell fusion II. Formation of chicken erythrocyte polykaryons. *J. Biol. Chem.*,248,422.

Waddel, W.J. & Bates, R.G. [1969] - Intracellular pH. *Physiological Reviews*, 49, 285.

Warth, J. & Desforges, Jane F. [1975] - Determinants of intracellular pH in the erythrocyte. *Brit. J. Hematol.*, 29, 369.

Wyatt, J.H. [1975] - Ultrastructure changes produced by the action of uranyl acetate on the human erythrocyte *in vitro*. AAEC/E346.

Zakai, N. Loyter, A. & Kulka, R.G. [1974] - Fusion of erythrocytes and other cells with retention of erythrocyte cytoplasm. *J. Cell. Biol.*, 61, 241.

TABLE 1

COMPARISON OF CELL DIAMETER AND CONCENTRATION

Uranyl acetate in 0.9% NaCl	Cell Diameter
5×10^{-3}M uranyl acetate	4.2
5×10^{-4}M uranyl acetate	4.0
5×10^{-5}M uranyl acetate	3.5
Saline washed cells	3.5

TABLE 2

CHANGES IN CELL SHAPE, CLUMPING AND SIZE AT DIFFERENT LEVELS OF URANYL ACETATE

NaCl (%)	5×10^{-3}M Uranyl acetate			5×10^{-4}M Uranyl acetate			5×10^{-5}M Uranyl acetate			NaCl only		
	Cell Shape	Cell Clumping	Cell Size	Cell Shape	Cell Clumping	Cell Size	Cell Shape	Cell Clumping	Cell Size	Cell Shape	Cell Clumping	Cell Size
0.7	Cups & smooth spheres	+	Variable up to 50% swelling	Cups 20% & smooth spheres	-	As in NaCl	Smooth spheres very occasional cup	-	As in NaCl	Smooth spheres	-	Normal
0.6	Increased cupping spheres	+	Variable with more small cups	Numerous cup cells	-	Swollen by 20%	Occasional cup cell smooth spheres	-	Swollen by 20%	Smooth spheres	-	Normal
0.5	Few cups large ghosts spheres	+	Swollen by 50% very large ghosts	Fewer cup cells most spheres	-	Swollen by 20%	Occasional cup cell smooth spheres	-	Swollen by 20%	Slightly crenated spheres	-	Swollen 15 to 20%
0.4	All spheres No cups	+	Variable some extreme swelling	All spheres No cups	-	Swollen by 20%	Spheres mainly ghosts	-	Mostly ghosts swollen 50%	Nearly all ghosts spheres	-	Swollen 50%
0.3	Spheres No cups	+	Extremely swollen	All spheres	-	Swollen 30%	No cells seen, only ghosts			No cells seen, only ghosts		
0.1	Spheres only	+	Variable swollen ghosts	All spheres	+	Swollen by 50%	General amorphous material only			General amorphous material only		

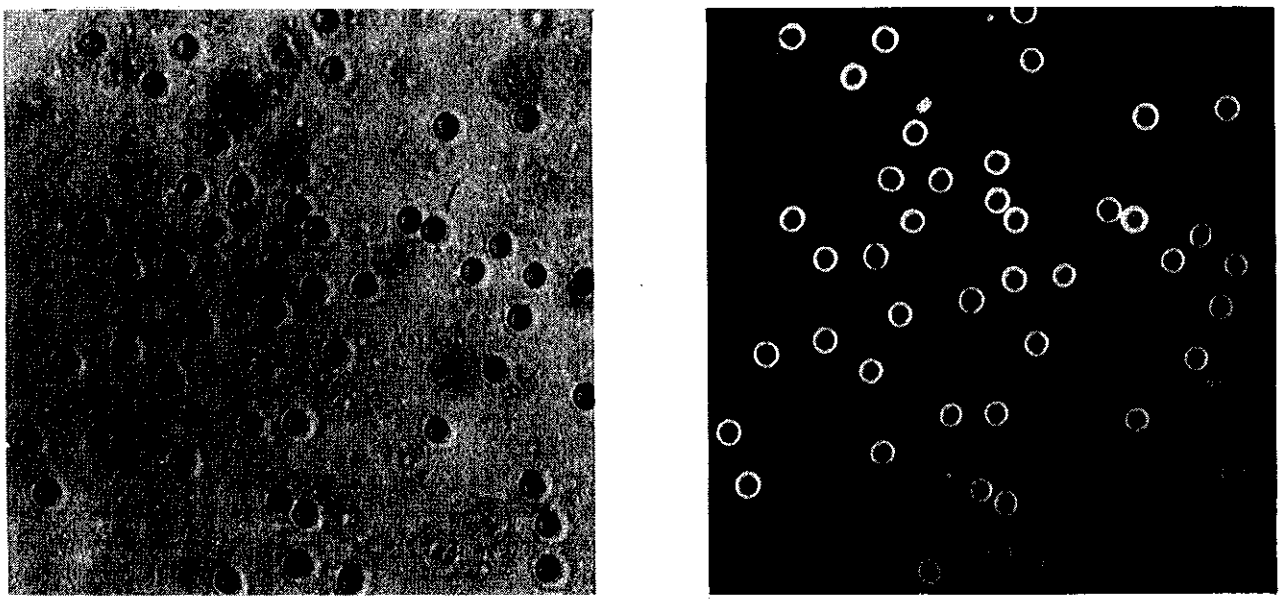


Figure 1 Saline washed cells

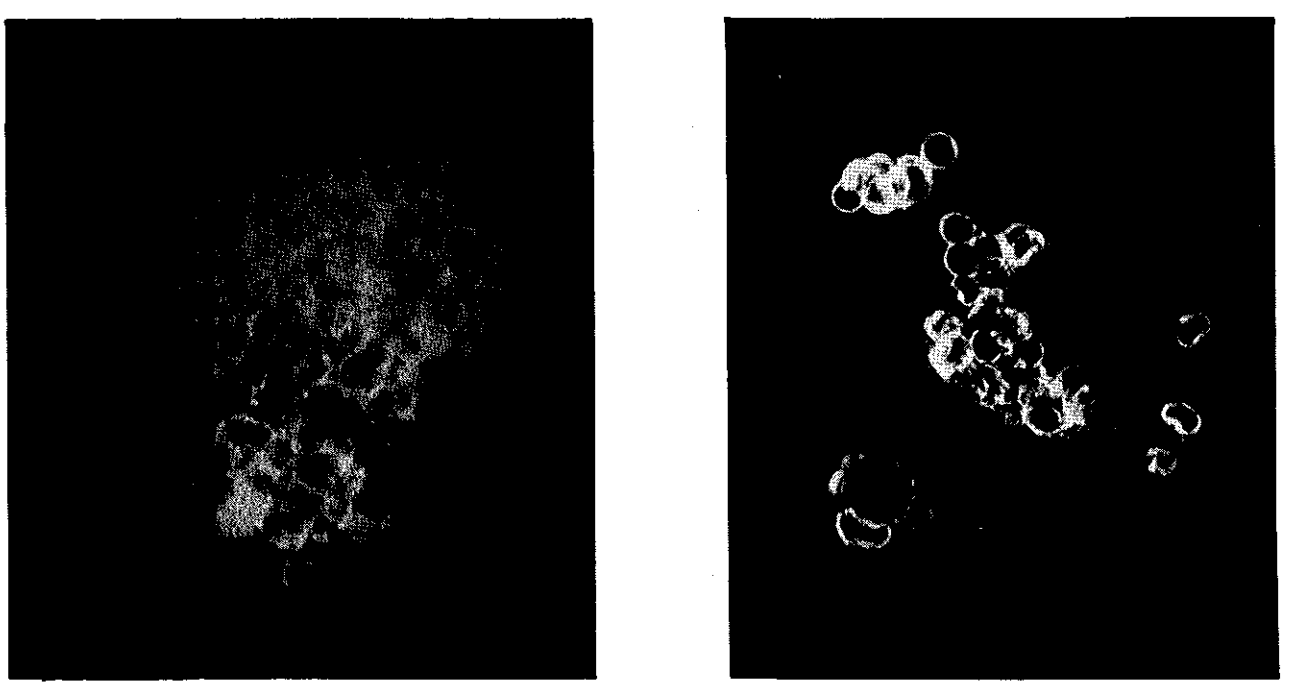
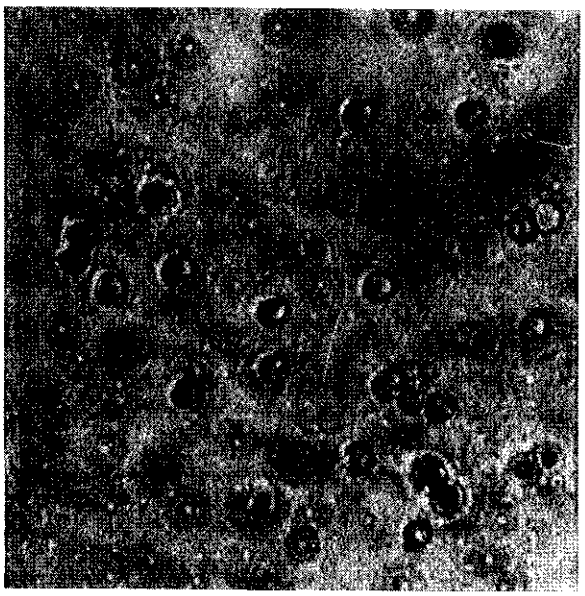


Figure 2 Cells exposed to 5×10^{-3} *M* uranyl acetate in 0.9% NaCl.



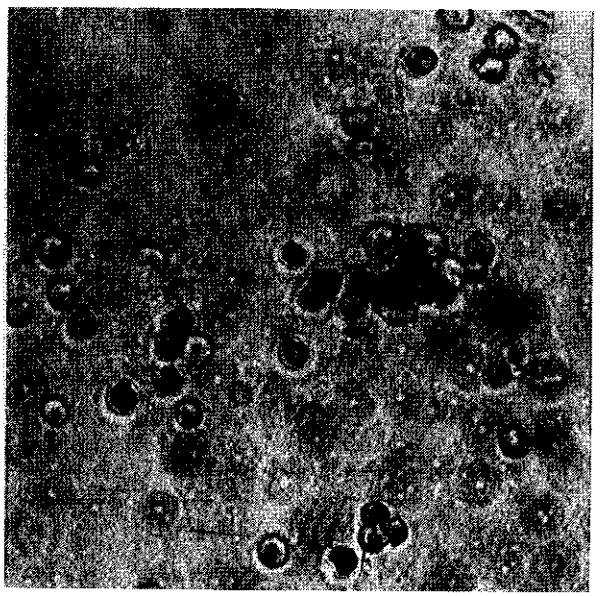


Figure 3(a-b) Cells exposed to 5×10^{-4} *M* uranyl acetate in 0.9% NaCl.

Figure 3(c) Cells exposed to 5×10^{-4} *M* uranyl acetate in 0.9% NaCl.

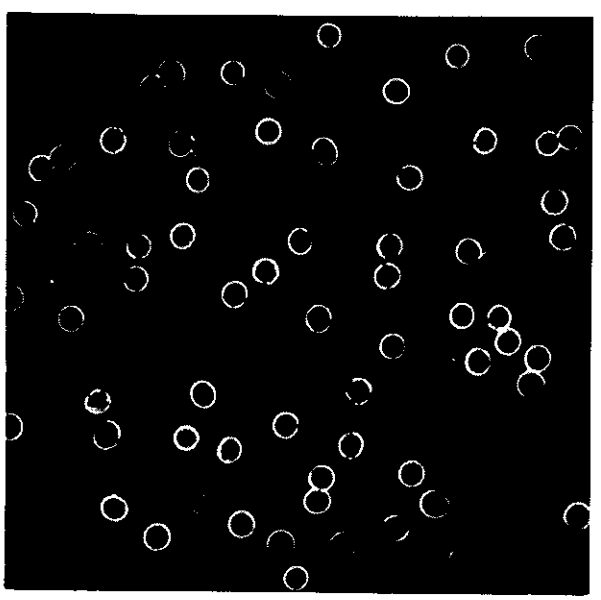


Figure 4 Cells exposed to 5×10^{-6} *M* uranyl acetate in 0.9% NaCl.

Figure 5(a) Cells attached to a microscope slide in 0.9% NaCl.

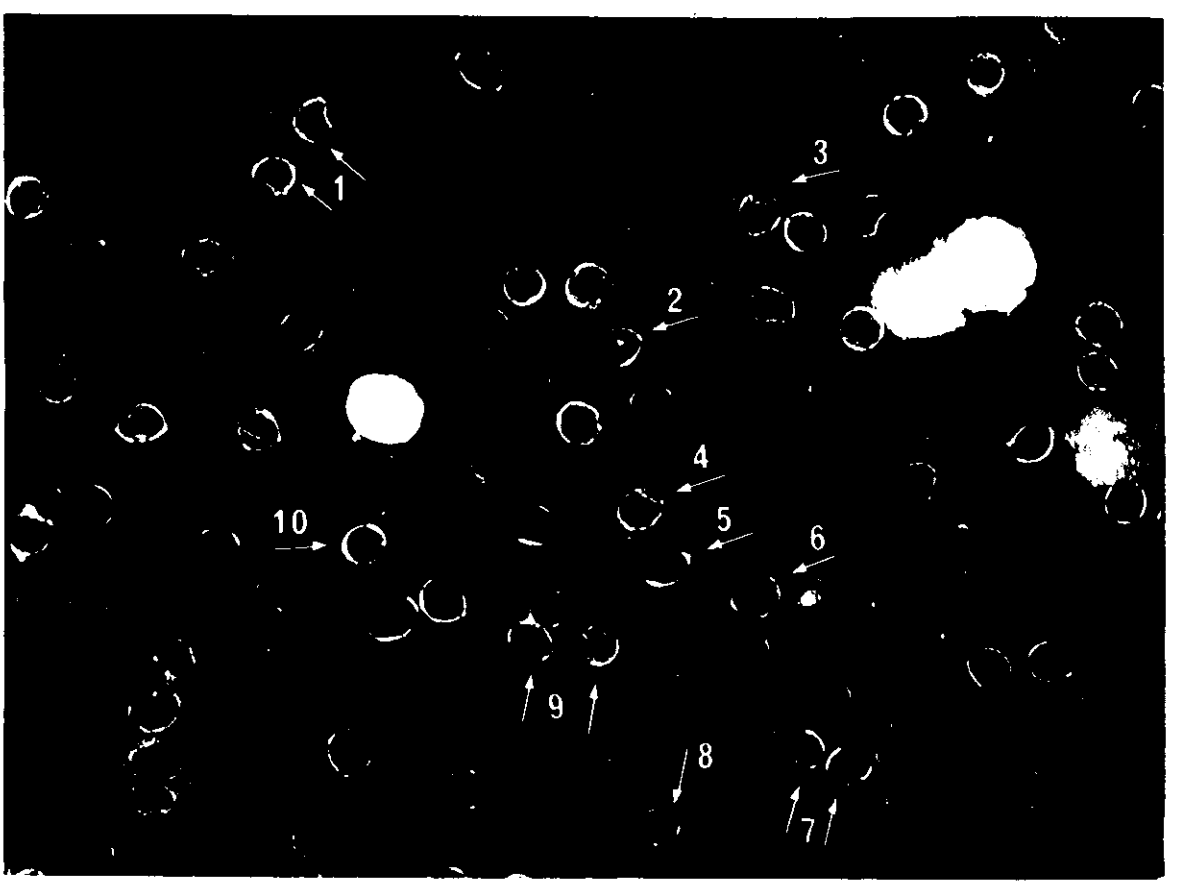


Figure 5(b) The same cells exposed to 5 x 10^{-4} *M* uranyl acetate in 0.9% NaCl for 1 minute. Marked cells form cups in the uranyl acetate.

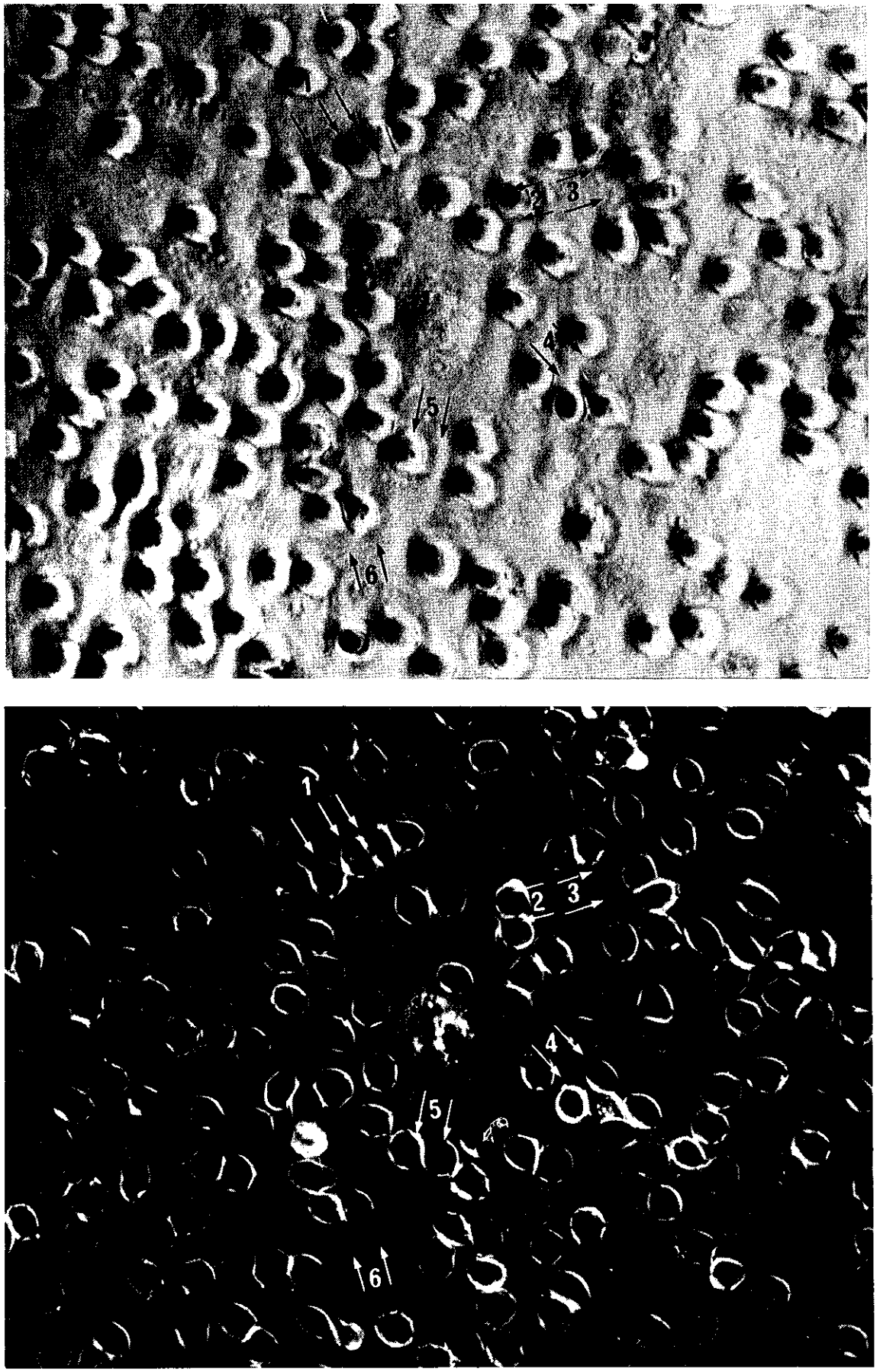


Figure 6(a) Cells attached to a microscope slide in 0.9% NaCl.

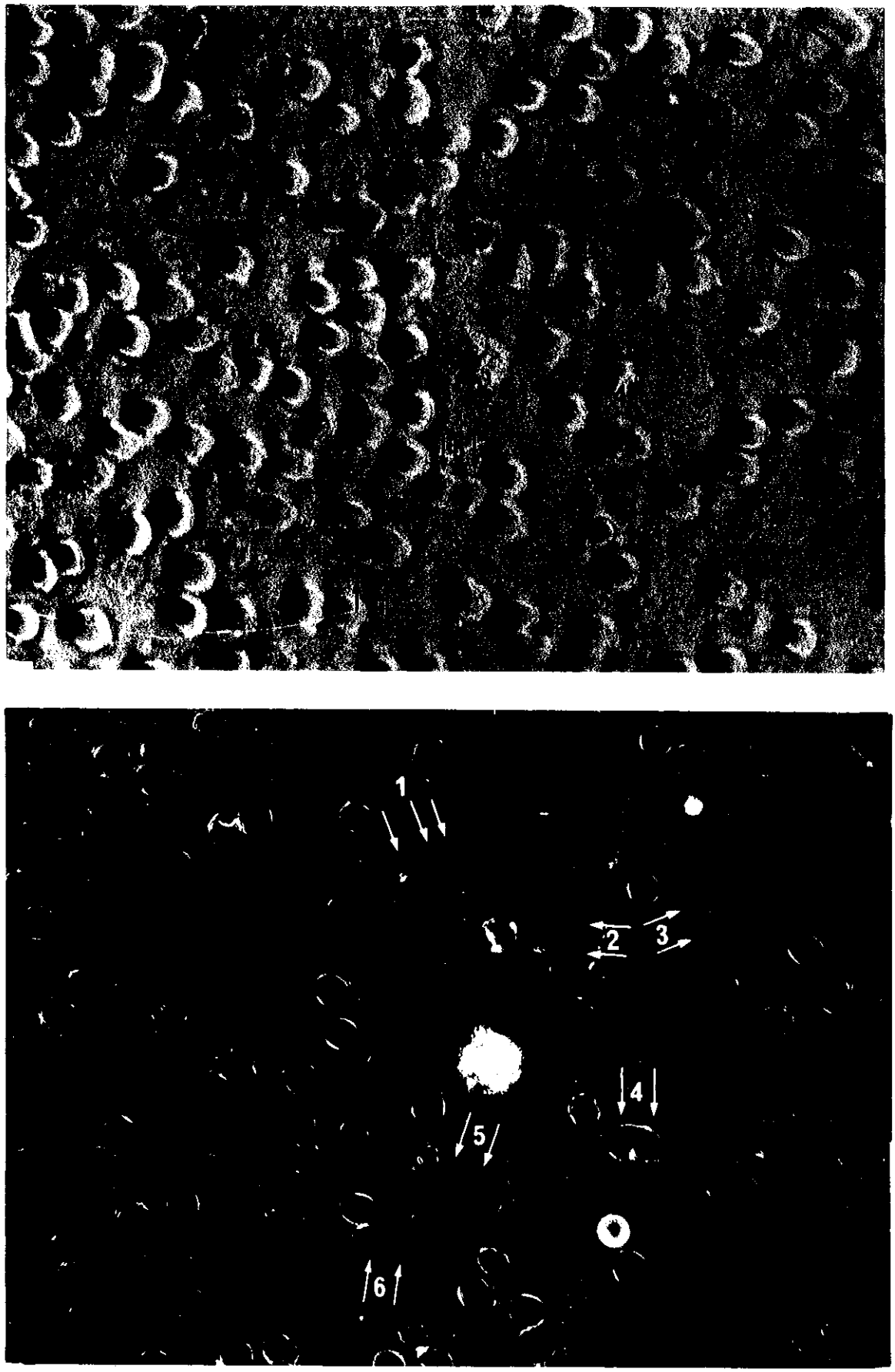


Figure 6(b) The cells in Figure 6(a) after 1 minute exposure to 5×10^{-4} *M* uranyl acetate. Groups of marked cells in Figure 6(a) appear to have fused in this figure.

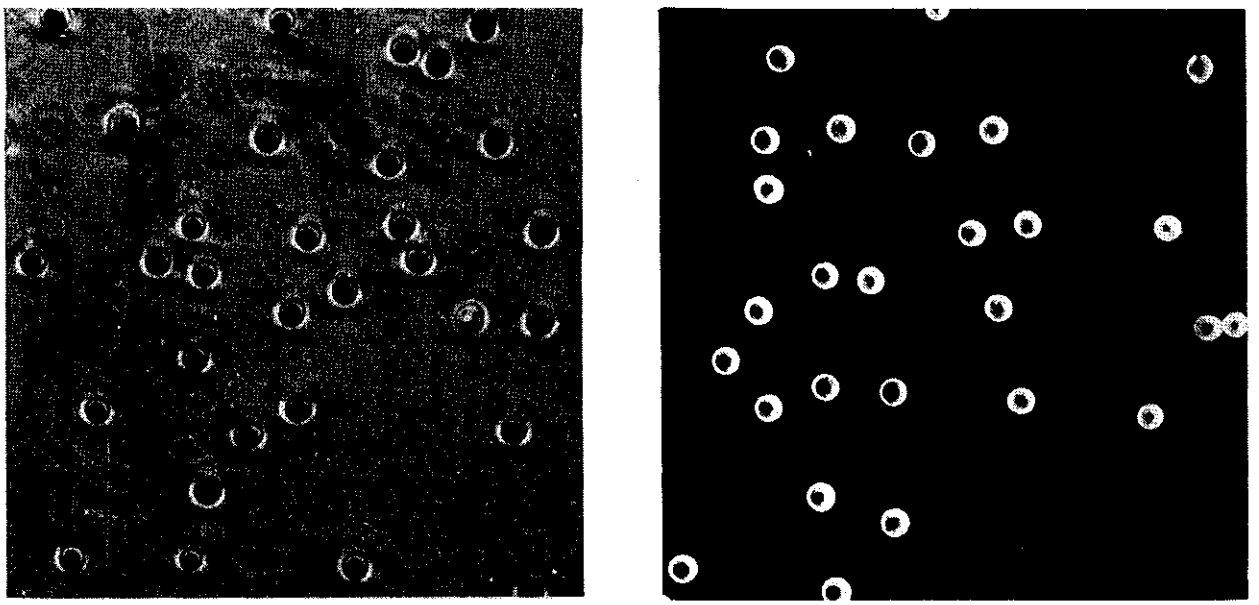


Figure 7(a) Cells in 0.7% NaCl.

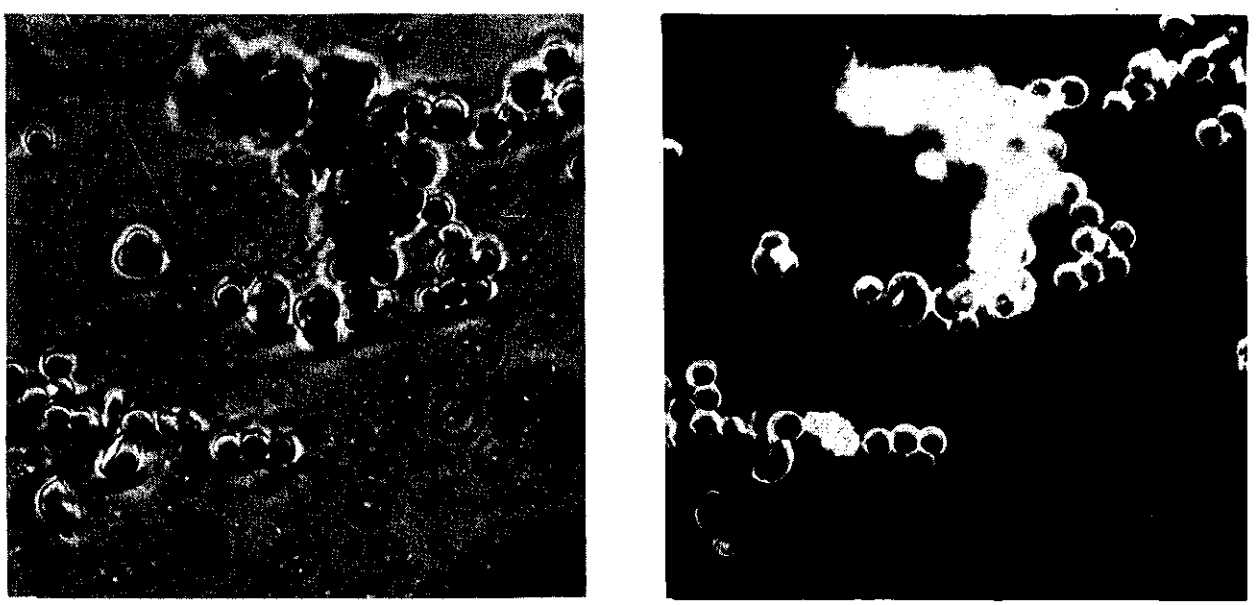


Figure 7(b) Cells in 0.7% NaCl plus 5 x 10^{-3} *M* uranyl acetate.

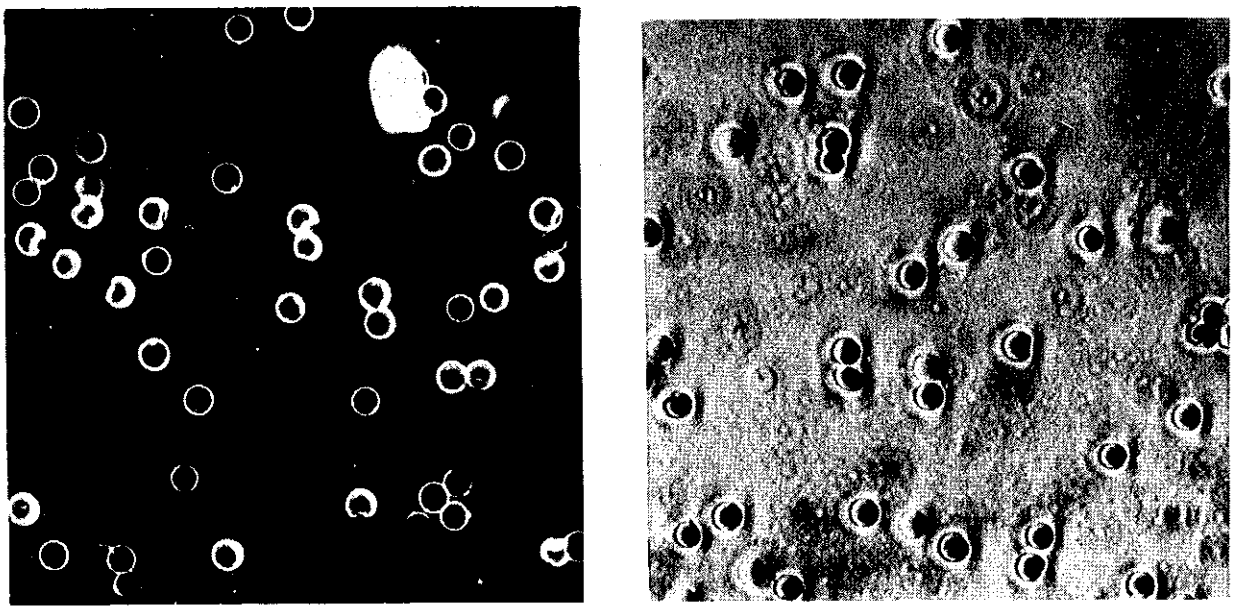


Figure 7(c) Cells in 0.7% NaCl plus 5 x 10^{-4} *M* uranyl acetate.

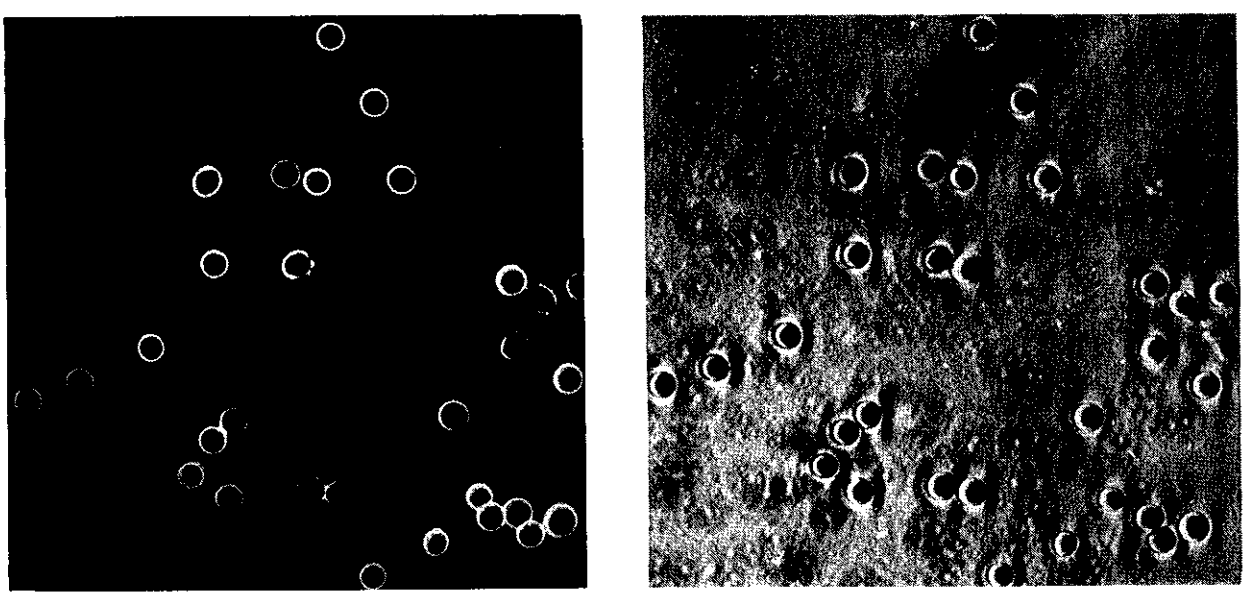


Figure 7(d) Cells in 0.7% NaCl plus 5 x 10^{-5} *M* uranyl acetate.

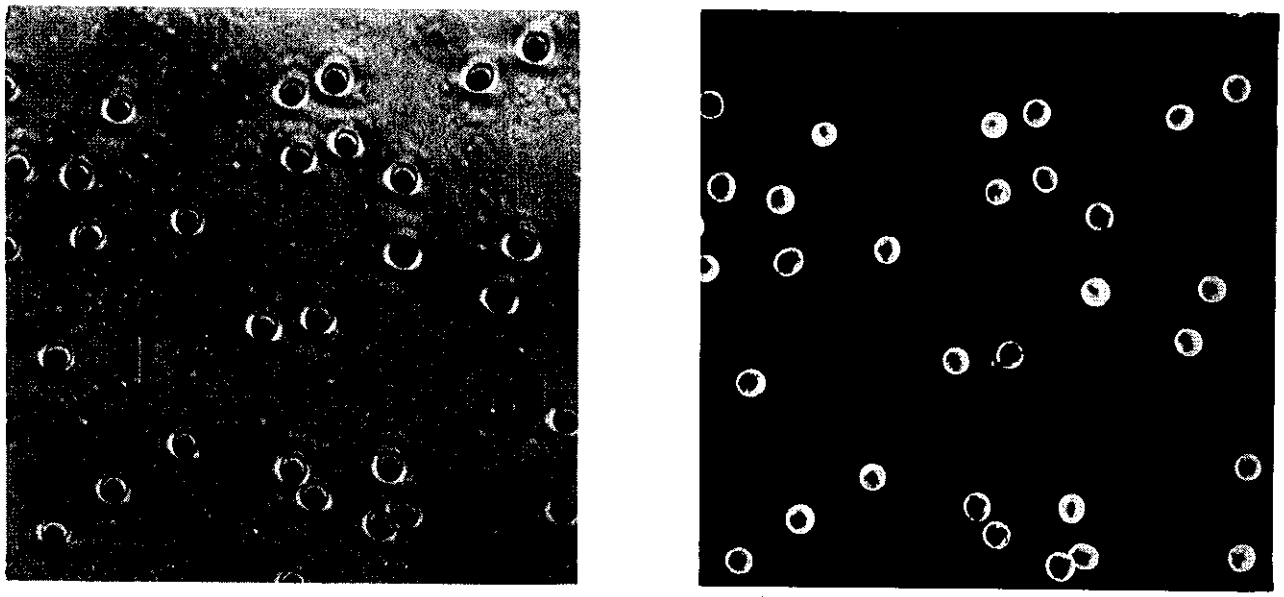


Figure 8(a) Cells in 0.6% NaCl.

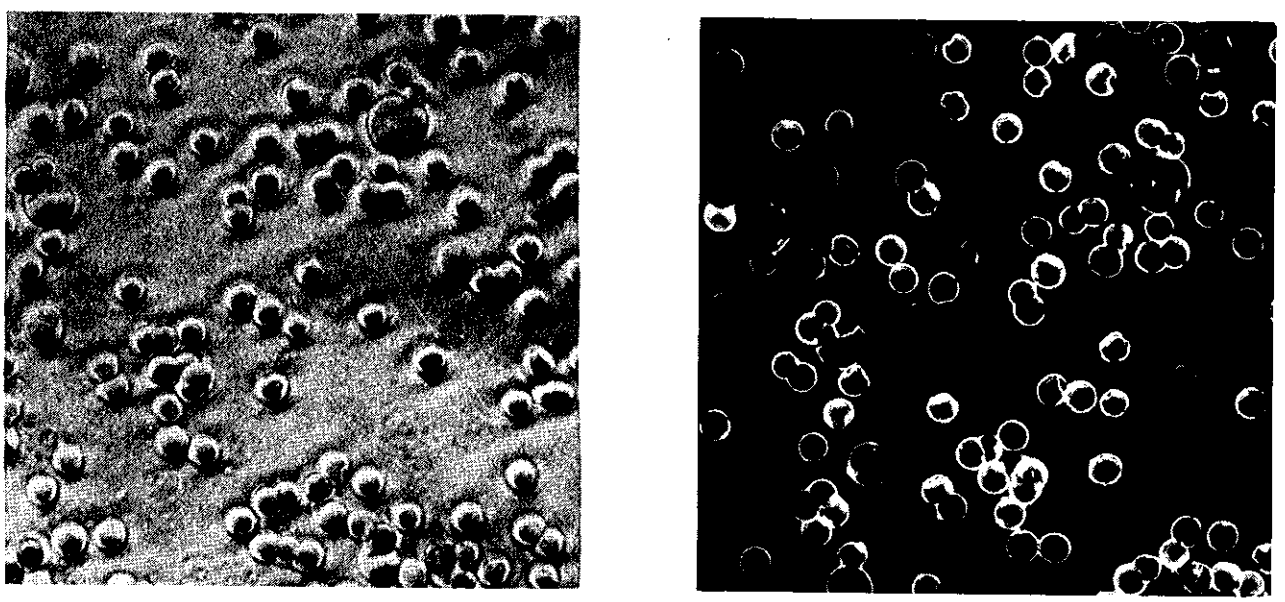


Figure 8(b) Cells in 0.6% NaCl plus 5 x 10^{-3} *M* uranyl acetate.

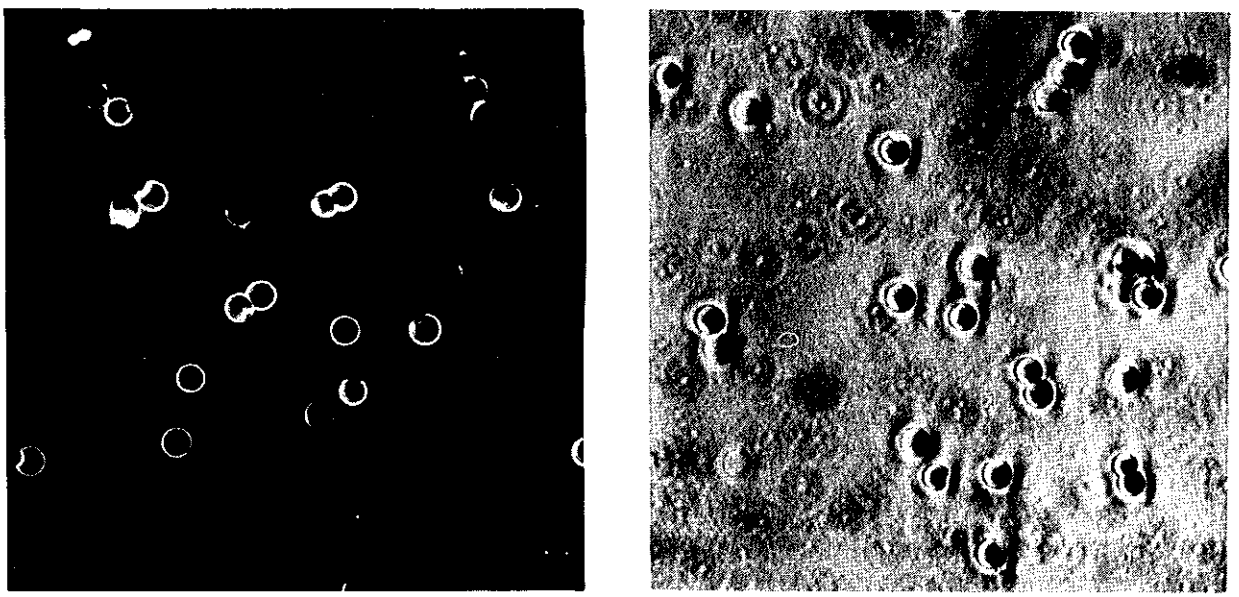


Figure 8(c) Cells in 0.6% NaCl plus 5 x 10^{-4} *M* uranyl acetate.

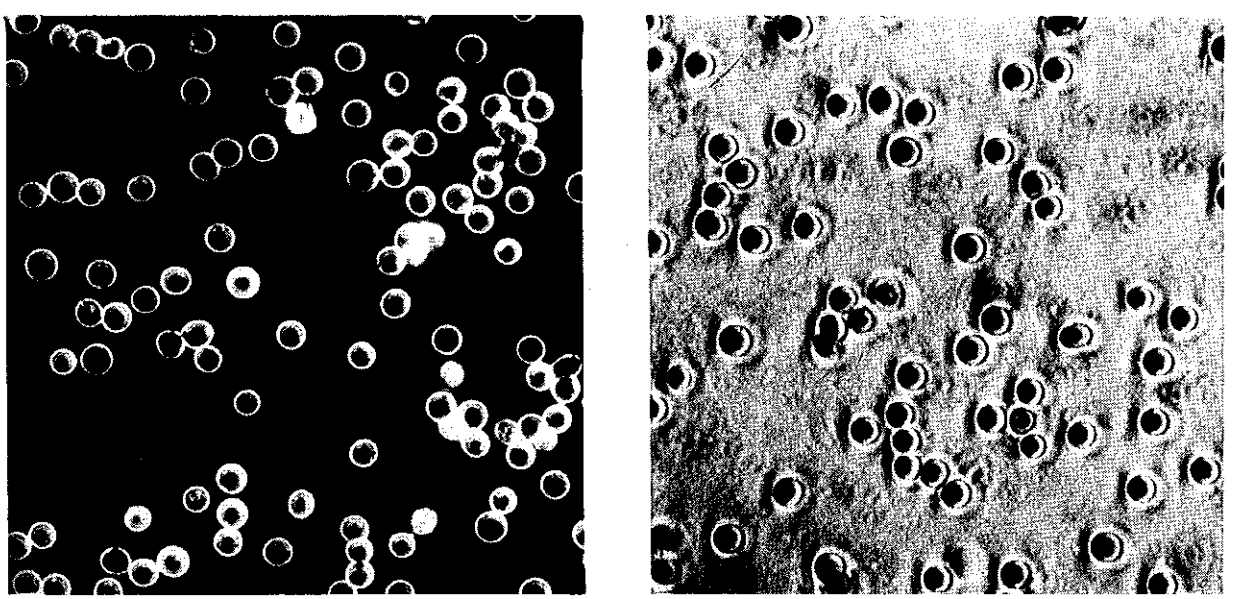


Figure 8(d) Cells in 0.6% NaCl plus 5 x 10^{-5} *M* uranyl acetate.

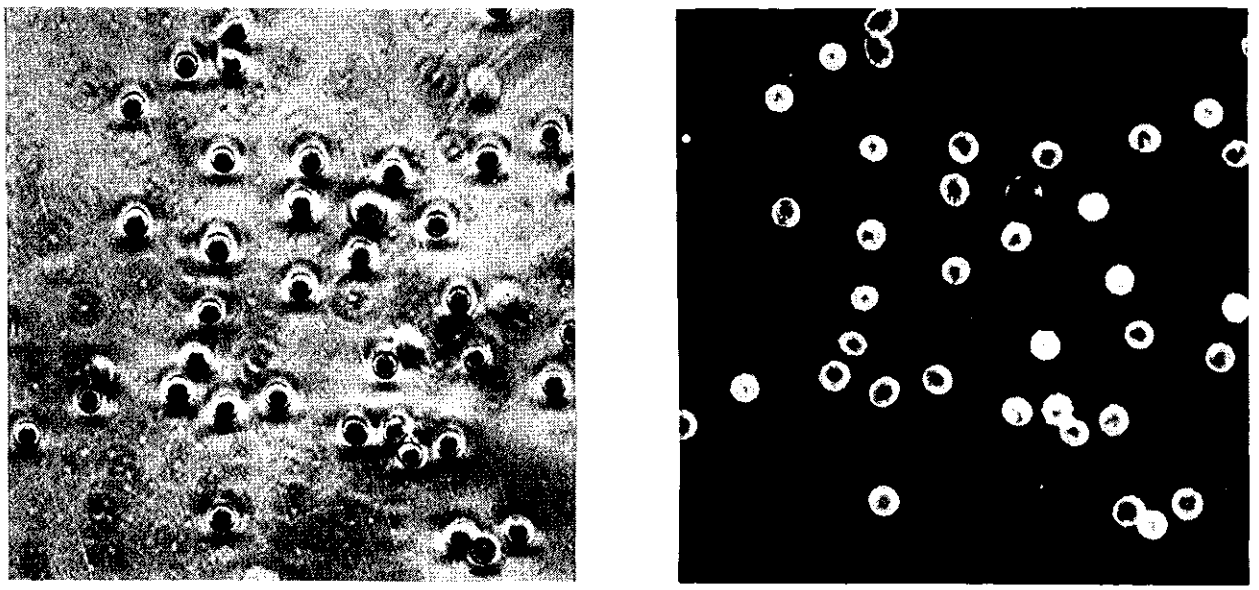


Figure 9(a) Cells in 0.5% NaCl.

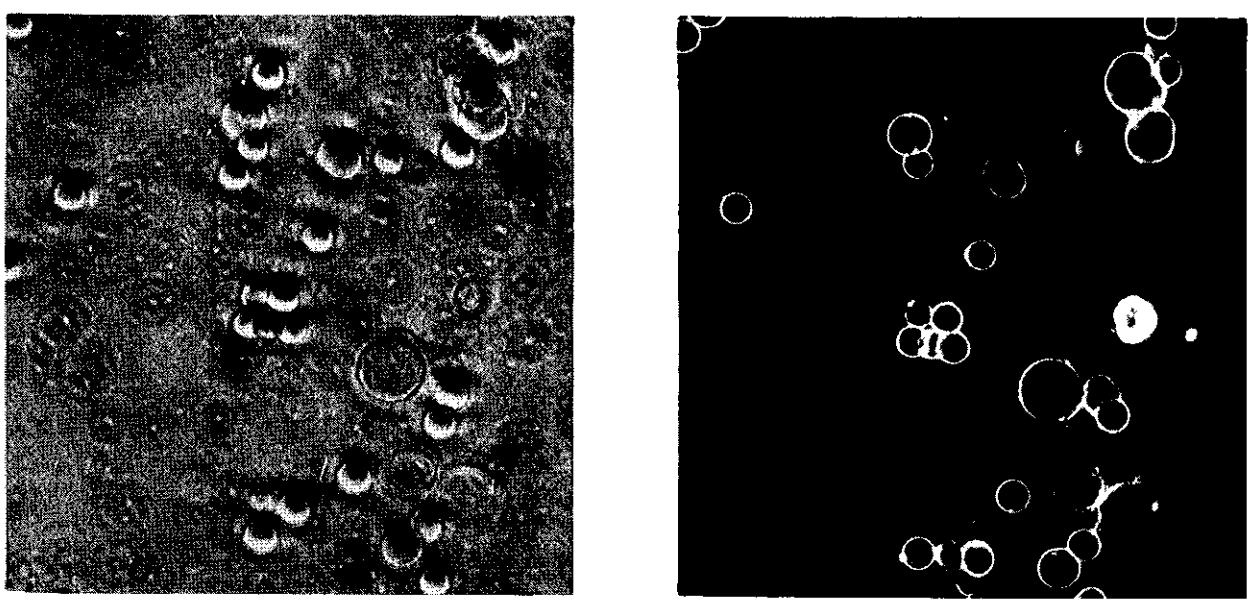


Figure 9(b) Cells in 0.5% NaCl plus 5 x 10^{-3} *M* uranyl acetate.

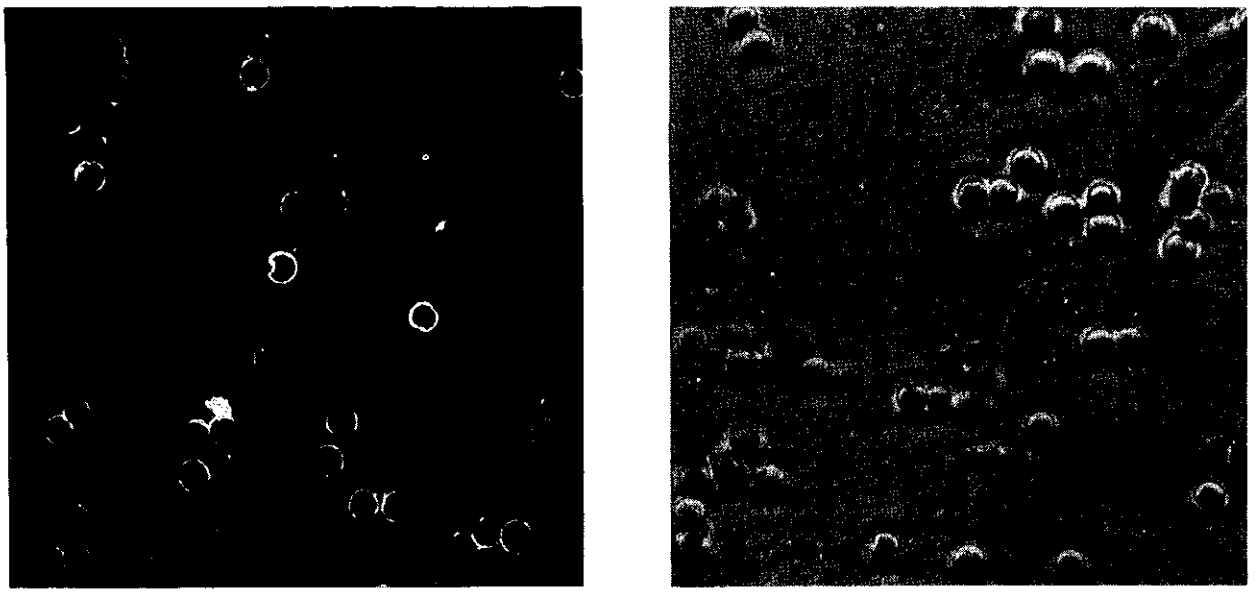


Figure 9(c) Cells in 0.5% NaCl plus 5 x 10^{-4} *M* uranyl acetate.

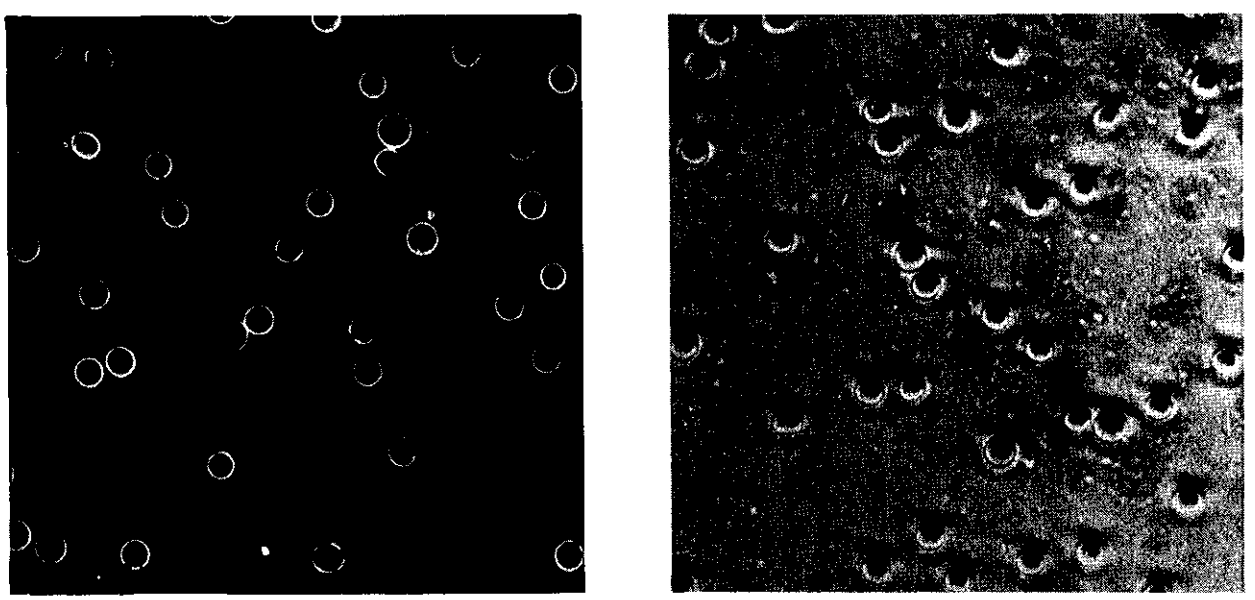


Figure 9(d) Cells in 0.5% NaCl plus 5 x 10^{-5} *M* uranyl acetate.

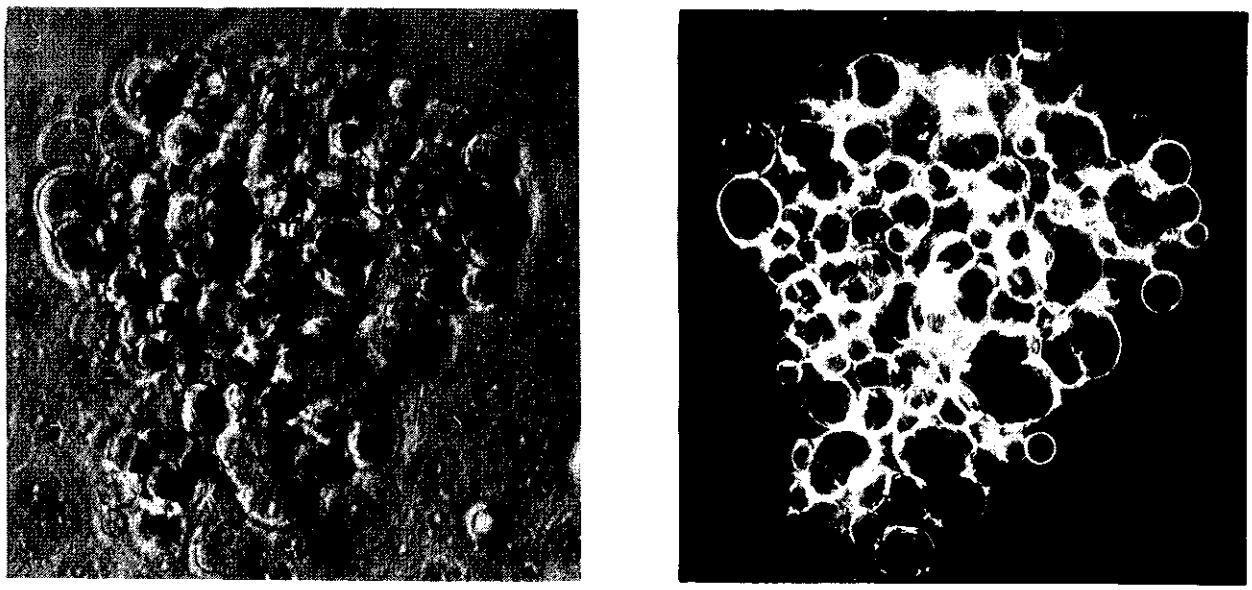


Figure 10(a) Cells in 0.4% NaCl plus 5 x 10^{-3} *M* uranyl acetate.

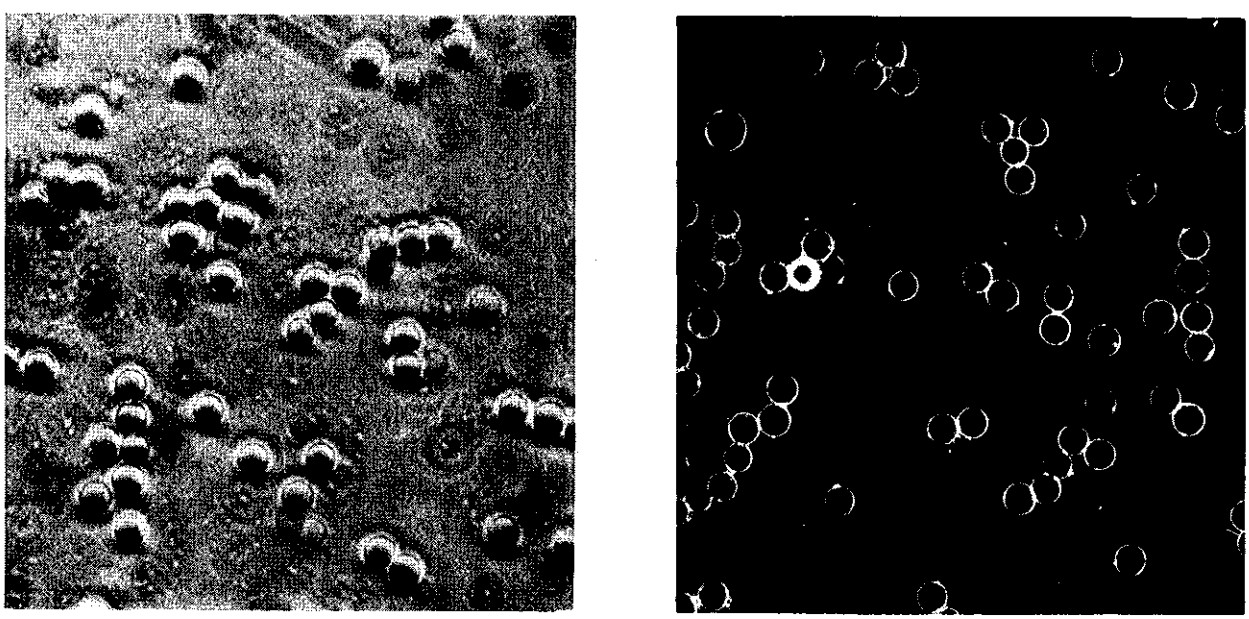
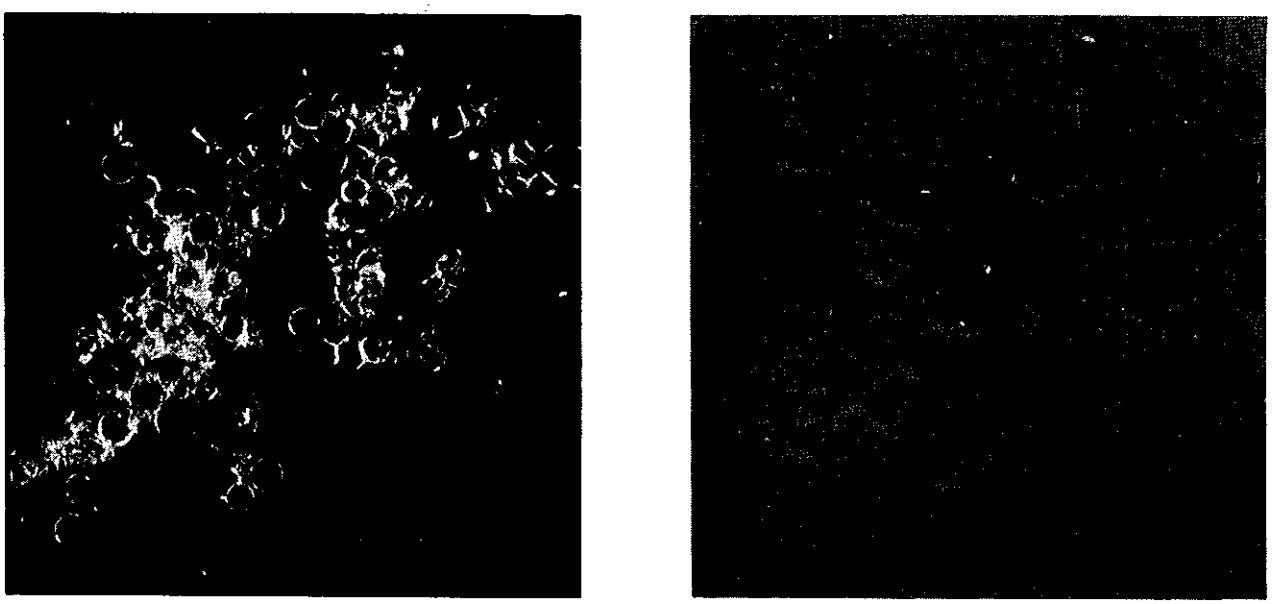
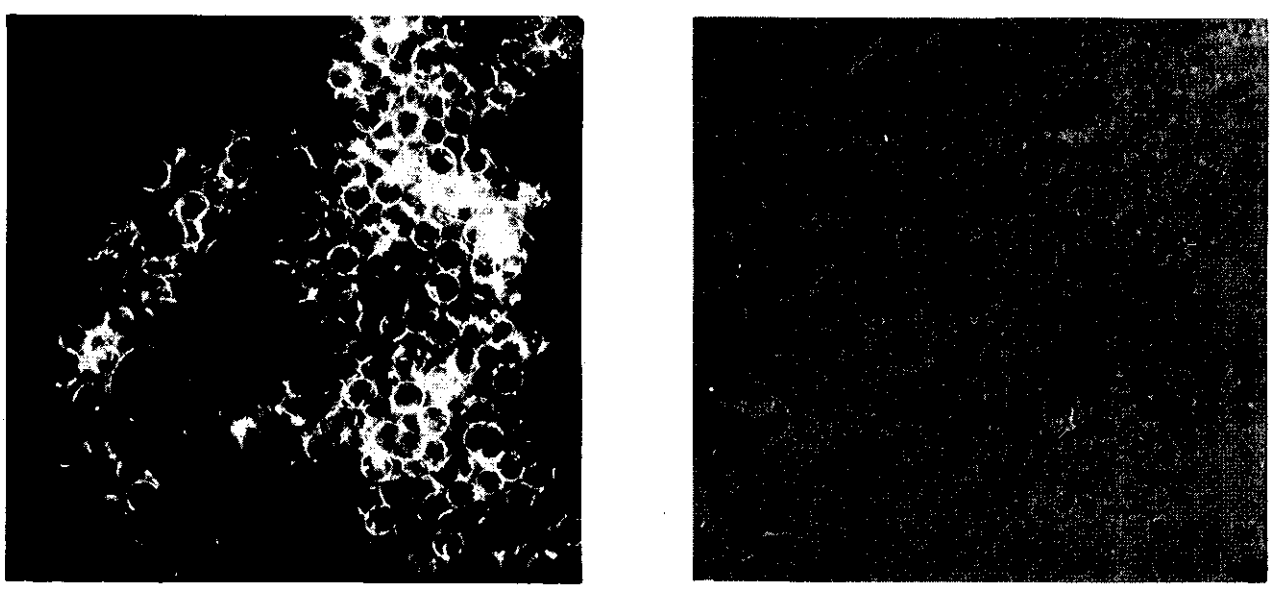


Figure 10(b) Cells in 0.4% NaCl plus 5 x 10^{-4} *M* uranyl acetate.

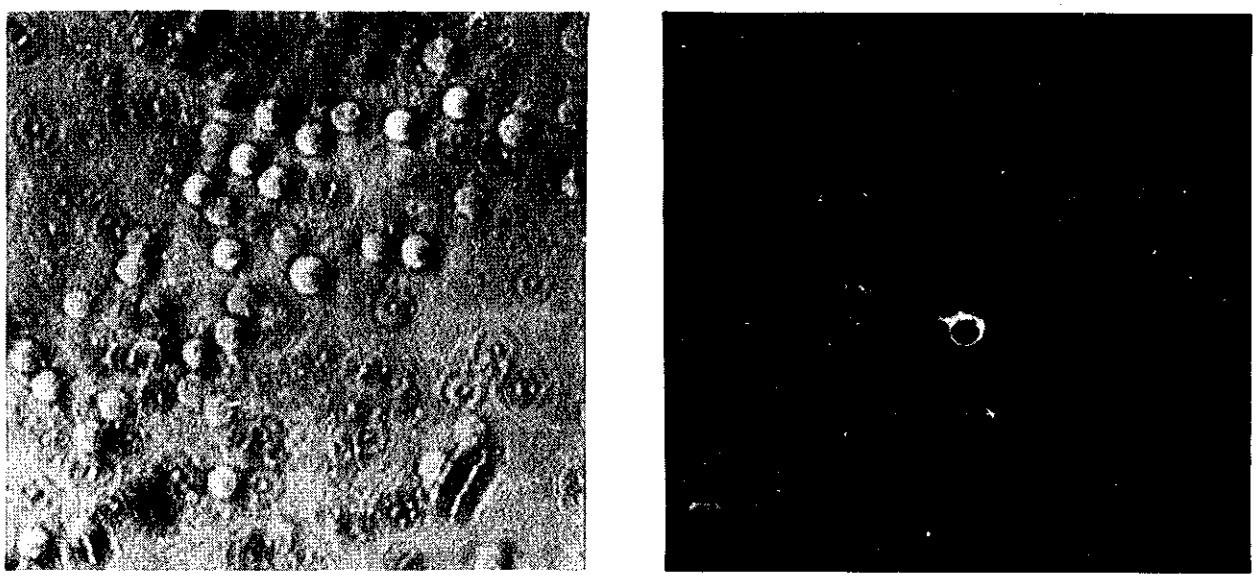


11a(i)

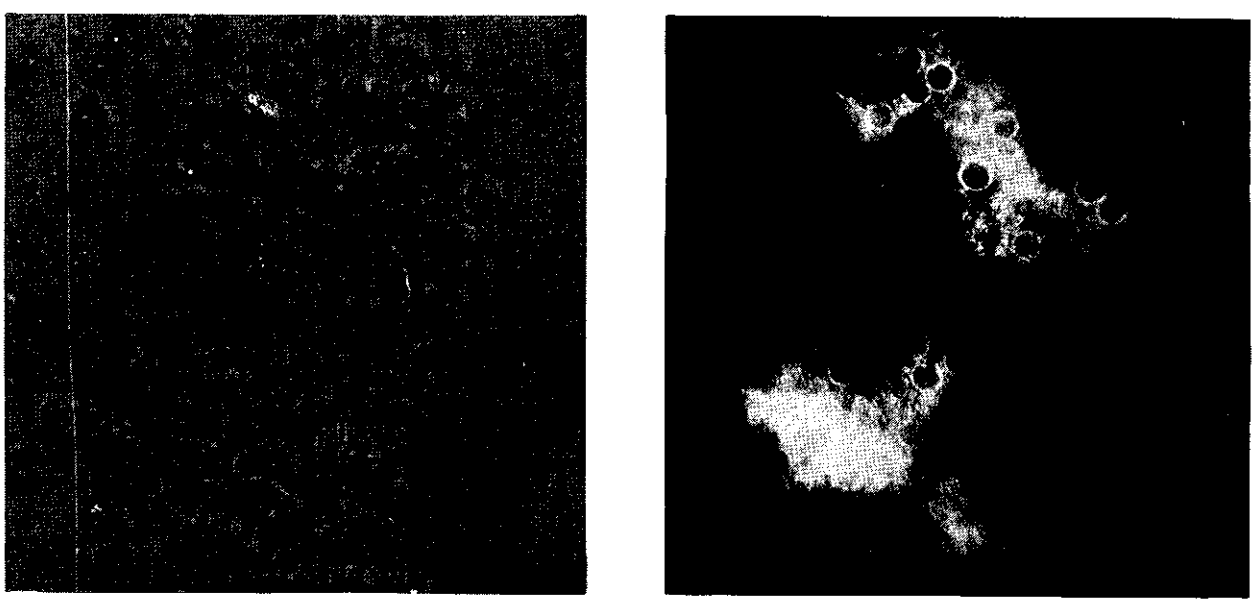


11a(ii)

Figure 11(a) Cells in 0.1% NaCl plus 5 x 10^{-3} *M* uranyl acetate



11b(i)



11b(ii)

Figure 11(b) Cells in 0.1% NaCl plus 5 x 10^{-4} *M* uranyl acetate.

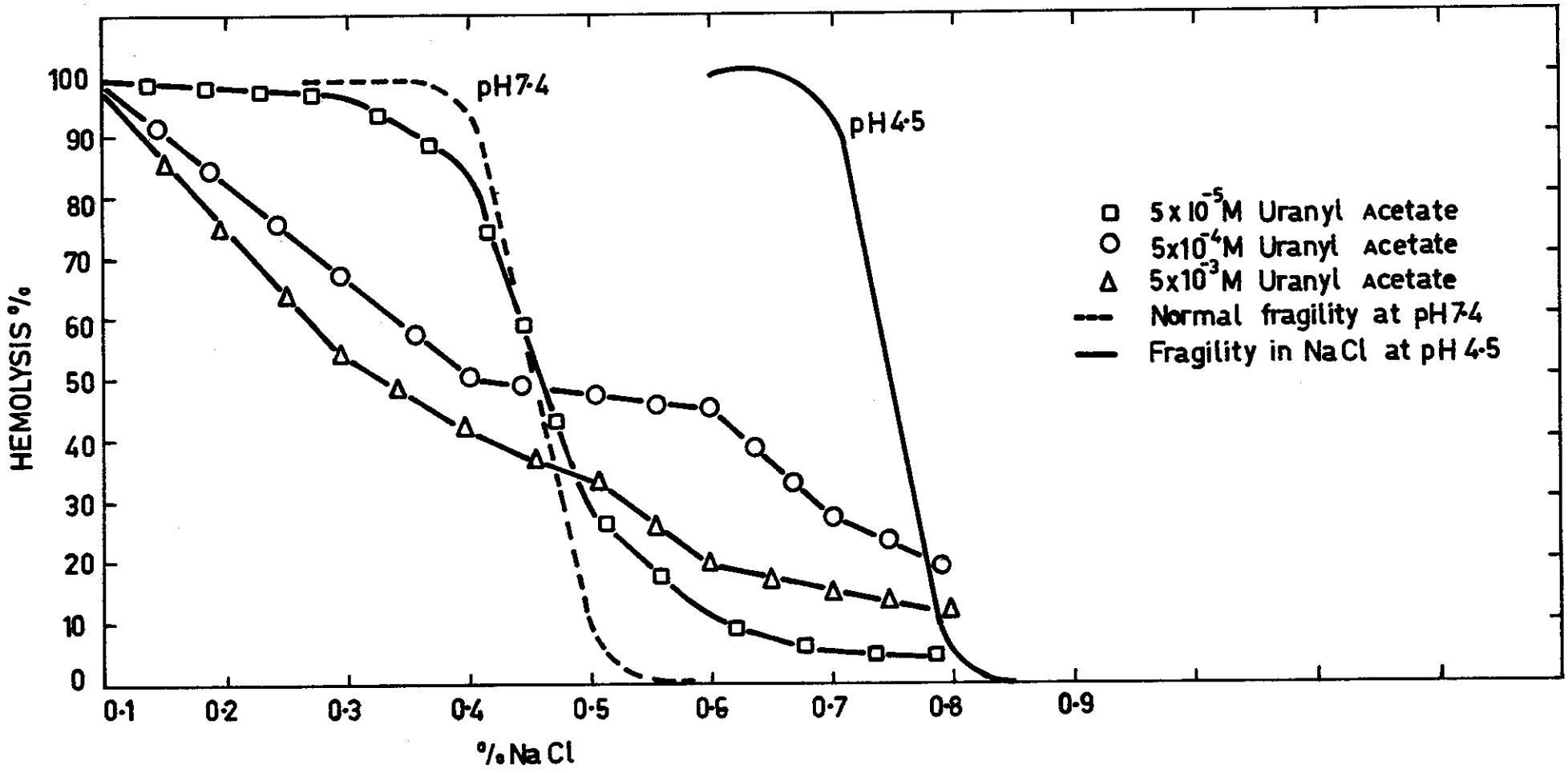


Figure 12 Osmotic fragility curves for cells exposed to graded NaCl solutions with uranyl acetate added to them. A normal fragility curve at pH 7.4 and a curve for NaCl solutions at pH 4.5 are included for comparison