Structural Changes in Mammalian Cells Associated with Cooling to −79°C.

By L. WEISS,* M.D., and J. A. ARMSTRONG, M.B.

(From the National Institute for Medical Research, Mill Hill, London)

PLATES 331 TO 334

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ABSTRACT

Suspensions of HeLa and S37 cells, with and without added glycerol, were cooled in stages to −79°C. and held at that temperature for 30 minutes. After warming to room temperature the cells were fixed, sectioned, and compared by phase contrast and electron microscopy with similar specimens kept at room temperature. Correlated viability tests were made.

Abnormal cytological characteristics, visible with the phase contrast microscope, were clearly related to the sequence of freezing and thawing, and the proportion of altered cells was highest in specimens cooled without glycerol. Electron microscopy showed that even in the presence of glycerol all cells were markedly altered, with distinctive vesiculation and disruption of the various intracellular membranes. There is evidence that much cytoplasmic damage is compatible with survival, but it seems likely that separation of the two layers of the nuclear envelope and rearrangement of the nuclear contents are signs of irreversible damage.

The findings lend some support to the belief that cell death on cooling is due largely to denaturation of semipermeable membranes, caused by the increasing concentration of electrolytes.

INTRODUCTION

The preservation of some vertebrate tissues at low temperatures became a practical possibility when it was shown by Polge, Smith, and Parkes (17) that under suitable conditions glycerol can protect cells from the lethal damage produced by cooling. The technique for cold storage of mammalian spermatozoa is now well established (15) and recently it has been shown that monkey kidney cells after similar treatment are suitable for use in virus propagation experiments (20).

In spite of this progress the nature and origin of damage to unprotected cells during freezing and thawing constitute a problem which has not yet been fully resolved. Mechanical disruption due to the formation of ice crystals around and within the cell was postulated, and microscopical evidence of intracellular crystallization has been obtained on several occasions (4, 7, 8). In the presence of glycerol ice crystals form more slowly and are smaller (18). However, it is now recognized that other physicochemical changes which emerge as a consequence of cooling are of the greatest importance in relation to cell viability. Lovelock (11, 12) has pointed out that one effect of the increasing concentration of electrolytes which occurs when ice forms in the suspending medium would be the denaturation of lipoproteins in the various cell membranes, leading probably to irreversible permeability changes.

In the report which follows, structural changes are described involving the intracellular membrane systems in two kinds of mammalian cells, which had been cooled to −79°C. for 30 minutes and then thawed.

Materials and Methods

Four separate experiments were performed. HeLa cells in suspension were utilized on 3 occasions, and murine Sarcoma 37 (ascites) cells mixed with erythrocytes were used in the fourth experiment. In each case the cell suspension was divided into 4 parts and subjected to the following treatments:—

* The Strangeways Laboratory, Cambridge, England.

Treatment 1.—Cells suspended in a mixture of Hanks' balanced salt solution (B.S.S.)—70 parts, and human serum (H.S.)—15 parts, were cooled to −79°C.

Treatment 2.—Cells suspended in B.S.S.—70 parts, H.S.—15 parts, and glycerol—15 parts, were cooled to −79°C.

Treatment 3.—Cells suspended in B.S.S.—70 parts, and H.S.—15 parts, were kept at room temperature for 75 minutes.

Treatment 4.—Cells suspended in B.S.S.—70 parts, H.S.—15 parts, and glycerol—15 parts, were kept at room temperature for 75 minutes.

It will be seen that Treatments 3 and 4 acted as controls for Treatments 1 and 2 respectively. Cell suspensions for cooling were put into glass ampoules and sealed. They were cooled to −50°C. at a rate of about 1°C. per minute in the "slow cooler" described by Polge and Lovelock (16), and thence rapidly to −79°C. by plunging the ampoules into Dewar flasks containing methanol and solid CO_2 at temperatures of −60°, −70°, and −79°C. After 30 minutes at the lowest temperature, the ampoules were placed in a water bath at 37°C. and their contents thawed in 20 to 30 seconds.

In each experiment viability checks were made on samples of the cells in each treatment category. The HeLa cells were cultivated *in vitro*; the Sarcoma 37 cells were injected subcutaneously into mice, approximately 50,000 cells being introduced into each site. Evidence for viability comprised growth in tissue culture or the development of typical tumours.

Samples of each cell suspension were pipetted into a 1 per cent solution of osmium tetroxide in veronal-acetate buffer at pH 7.4 (14) and into a similarly buffered 0.6 per cent solution of potassium permanganate (13). After fixation for 30 minutes at room temperature the cells were washed once in Hanks' solution and centrifuged to form a pellet. Part of the material was then dehydrated in the usual way, embedded in a 6:1 mixture of butyl- and methylmethacrylate, and sectioned with a Porter-Blum microtome for examination with a Siemens UM 100 electron microscope. The rest of the cells, after standing in 70 per cent alcohol for about 18 hours were processed by double embedding in celloidin and paraffin wax: sections were cut at a thickness of 1 to 3 μ, and after removal of the embedding media with xylene and alcohol-ether they were mounted in water for examination with the phase contrast microscope.

RESULTS

Viability Tests.—Of the HeLa cell suspensions only those which had been cooled to −79°C. without glycerol (Treatment 1) proved to be wholly non-viable. Samples from the other treatment categories all showed growth on subsequent culture.

The same result was obtained with the Sarcoma 37 cells. In undiluted ascitic fluid these cells are known to be unusually tolerant of cooling to low temperatures (3) but their resistance disappears on dilution in physiological salt solutions as in the present experiments. No tumours developed in the mice at the sites of subcutaneous injection of cells which had been frozen and thawed without glycerol. As approximately 2000 viable cells will induce tumours in some 50 per cent of mice injected (21) it is probable that fewer than this number of cells were viable out of the 50,000 injected in the tests, *i.e.* less than 4 per cent.

Phase Contrast Microscopy.—In both the HeLa and Sarcoma 37 cell suspensions two kinds of cells with distinctive cytological characteristics could be recognized, and were designated Types A and B respectively.

In Type A cells there is marked cytoplasmic vesiculation, but the plasma membrane appears to be intact (Fig. 1). The nucleus has a pale "empty" appearance and its membrane is sharply defined. Some nuclei are deformed or obscured by large paranuclear vesicles. The nucleoli are very prominent. The Type B cells (Fig. 2) more nearly resemble free, rounded living cells as seen with a phase contrast microscope. Cytoplasmic granules, filaments, and some osmiophilic droplets are present but vesiculation is minimal or undetectable. The nucleus has a rather uniform greyish appearance, and in the thicker (3 μ) sections its limiting membrane is not always easy to define. The nucleoli though visible are not so obvious as in the Type A cells.

Essentially the same features were seen whichever fixative was employed. It was noticeable, however, that vesiculation of Type A cells was usually more prominent in those fixed with potassium permanganate than in the osmium-fixed preparations. Cell counts on sections prepared from the suspensions cooled to −79°C. without glycerol, indicated that between 60 and 65 per cent of the cells were of Type A; whereas in sections made from the suspensions frozen in the presence of glycerol, 15 to 25 per cent of cells were of Type A. Material from the unfrozen control suspensions contained only occasional cells of Type A. As expected, no intact erythrocytes were found in specimens which had been cooled without glycerol.

Electron Microscopy.—Sections of the control material in Treatment category 3 had an essen-

tially normal appearance (Fig. 3) although, as is usual, certain details were dependent on the method of fixation used. Permanganate fixation was regularly associated with a minor degree of mitochondrial swelling and a fine granular pattern of the intranuclear substance, the latter contrasting with the more dense and somewhat aggregated pattern of nucleoplasm which is a familiar feature of osmium-fixed cells. All cells in Treatment category 4 showed obvious mitochondrial swelling; and this, although more marked after permanganate fixation, was also quite definite in the osmium-fixed material. It is inferred from this that a moderate degree of mitochondrial swelling results from the addition of glycerol to the medium even at room temperature.

Sections of the frozen and thawed cells subjected to Treatments 1 and 2 revealed substantial intracellular damage, and no cells of completely normal appearance were seen even in the specimens to which glycerol had been added before cooling. Cytoplasmic swelling had resulted in a general smoothing out of the folds and villous projections of the plasma membrane; and there was widespread disruption of normal cytoplasmic structure (Fig. 4). Mitochondria, endoplasmic reticulum, and the Golgi zone were often difficult to identify, and a zone just inside the plasma membrane frequently seemed to be quite empty. The cytoplasm contained many large rounded or cleft-like profiles, bounded by either single or double membranes. Some were of mitochondrial origin as evidenced by torn but recognizable remnants of the membranous cristae, whilst others seemed to be the product of swelling in the endoplasmic reticulum. Most of the latter were partially filled with an amorphous material of low density. Large cavities of a similar kind could be seen in the perinuclear area and in some sections their limiting membrane was continuous with the outer layer of the double nuclear envelope, and they had clearly arisen by a process of localized ballooning within the nuclear envelope (Fig. 5). Occasionally, small extensions of the nuclear contents projected into the bleb-like cavities. It was remarkable that in spite of extreme intracellular changes the plasma membrane was usually intact, at least in the plane of section.

In the osmium-fixed specimens nearly all cells showed some degree of altered consistency within the nucleoplasm. Irregular condensation of the chromatin substance along the inner surface of the nuclear envelope and in small clumps throughout the nucleus, was accompanied by the development of large intervening areas of low density (Fig. 4). The most severely affected nuclei had a strangely pallid appearance, apart from the dense and usually intact nucleoli. Although it seemed possible that some of the nuclear contents had been lost into the surrounding cytoplasm, this could not be determined with certainty. Intranuclear structural changes were less clearly indicated in the corresponding permanganate-fixed specimens, in which the nucleoplasm of most cells retained the rather homogeneous granular consistency that is a feature of the normal nucleus after this method of fixation.

It would probably be of little value for comparative purposes to classify the cells in specimens cooled with and without glycerol into arbitrary groups of severely and less severely damaged forms. Also the large numbers of separate cells necessary for a significant analysis would be difficult to obtain from the ultrathin sections required for electron microscopy. It was a constant impression, however, that nuclear damage was less extensive in the presence of glycerol (Fig. 6). The most abnormal nuclei occurred only in the cells showing maximum over-all damage, and these appeared to be less numerous when glycerol had been added before cooling.

DISCUSSION

It is well known that if most kinds of living mammalian cells are cooled to −79°C. without glycerol almost all of them are killed, and in our experience no evidence of viability was obtained after such treatment. Even when protection is afforded by the addition of glycerol the survival rate for HeLa cells rarely exceeds 75 per cent (19). From observations with the phase contrast microscope, it might reasonably be concluded that the cytological pattern associated with this mode of death is represented in the vesiculated Type A cells. Fewer Type A cells were present in suspensions cooled with added glycerol (15 to 25 per cent) than that without it (60 to 65 per cent); but as cell death is virtually the rule without glycerol protection, it would be wrong, in spite of their relatively normal appearance, to assume that all of the cells designated Type B would have been viable.

By electron microscopy it was possible to observe more precisely the character of the structural

damage suffered by individual cells. At these magnifications it appeared that no cells with completely normal morphology remained after freezing and thawing, even when glycerol had been added to the system. Since we know that many cells are still viable after this treatment it follows that a certain, not inconsiderable, degree of swelling and disruption of mitochondria and endoplasmic reticulum is consistent with survival. It is quite likely that the viability of a particular cell depends ultimately on the sum total of structural and biochemical injury sustained by its component parts; but the possibility remains of there being some critical site such as the nuclear membrane, the integrity of which is essential if the cell is to have any chance of recovery.

There is no reason to suppose that the damage revealed by the electron microscope in cells cooled to −79°C. was merely a technical artefact due, for example, to destructive polymerization of the methacrylate monomer; it was consistently absent from the control specimens. It was noted that the structural changes, when present, seemed to be rather more extensive after permanganate fixation than in the corresponding osmium-fixed specimens; and even in the control cells of Treatment category 3 permanganate fixation had induced slight mitochondrial swelling. This is not altogether surprising as a tendency to cause swelling of normal mitochondria was mentioned by Luft (13) and has been noted by others (5). Nevertheless, most membranous structures are particularly well revealed after this method of fixation, which was the main reason for employing it along with osmium tetroxide in this investigation.

Structural changes like those which result from freezing and thawing have been reported under certain quite different circumstances. The pattern of cell damage cannot therefore be regarded as necessarily indicative of a particular mode of cell death. Anderson (1) using phase contrast microscopy, studied the effects of various solutions on isolated rat liver cell nuclei. Water and dilute solutions of certain salts caused visible blebs or blisters to rise from the nuclear surface; but the findings suggested that bleb formation was not due to osmotic pressure changes *per se*, and the author concluded that it was unlikely that the blebs were formed from a preexisting continuous surface sheet. The fixation and dehydration required for electron microscopy caused the blebs to disappear, and it is not certain, therefore, that the nuclear phenomenon observed by Anderson was the same as that seen in the present experiments. During a so called "destructive" phase of influenza infection of the respiratory epithelium of ferrets, Hotz and Bang (9) observed with the electron microscope both cytoplasmic vesiculation and blebs on the nuclear membrane. More recently, Goldberg and Green (6) have described electron microscopic appearances of Krebs ascites tumour cells treated with rabbit immune γ-globulin, and Latta (10) the appearance of cultured embryonic chick heart cells after treatment with specific guinea pig antiserum. In both of these reports swelling and rupture of the mitochondria and endoplasmic reticulum are described; in addition, Latta found separation of the outer layer of the nuclear membrane to form prominent blebs, and suggested that the changes were due to osmotic pressure differences between the various cell compartments.

Lovelock (11) has proposed that the electrolyte disturbances associated with the freezing of cell suspensions can result in dispersion of the lipids and lipoprotein from cell membranes, leading to permeability changes and finally cellular dissolution. Thus, the cytoplasmic and nuclear membrane changes seen in the present experiments could reasonably be interpreted as evidence of osmotic phenomena, arising probably during the period of thawing at sites of altered permeability. However, it is remarkable that disruption of the plasma membrane itself was rarely observed. It is conceivable that all cells with damaged plasma membranes had disintegrated, and their contents been lost during subsequent processing; but the fact remains that many cells with apparently intact plasma membranes had suffered extensive intracellular damage, which seems to indicate a greater resilience on the part of the plasma membrane (2). It is well known that repeated freezing and thawing of unprotected cell suspensions is necessary to ensure their complete break up when this is required for experimental purposes; this suggests that damage to the membranous elements of the cells during the initial cooling and rewarming to room temperature may render the cells more susceptible to the physical stresses of subsequent cycles of freezing and thawing.

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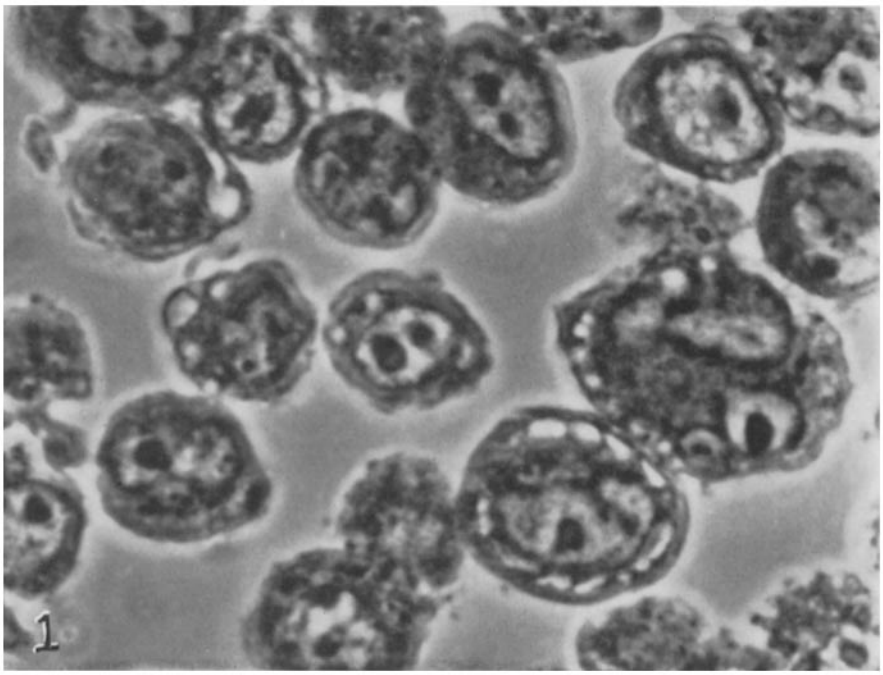
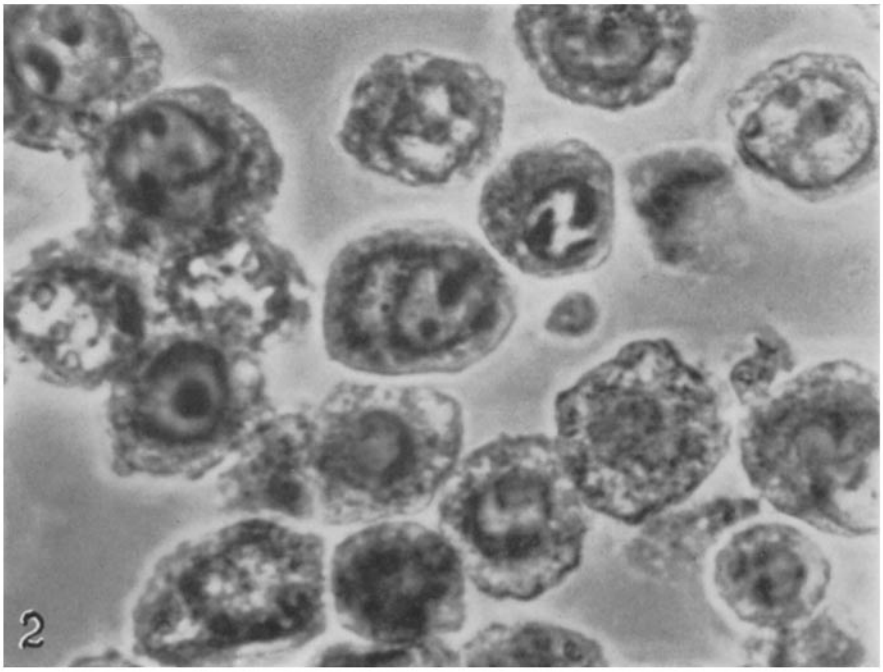
Explanation of Plates

Plate 331

Fig. 1. Phase contrast photomicrograph of sectioned Sarcoma 37 cells, frozen and thawed without glycerol (Treatment 1). They are mostly of the Type A described in the text. OsO_4 fixation. × 2,000.

Fig. 2. Similar photograph of Sarcoma 37 cells from a control suspension (Treatment 3). The cells are of Type B. OsO_4 fixation. × 2,000.

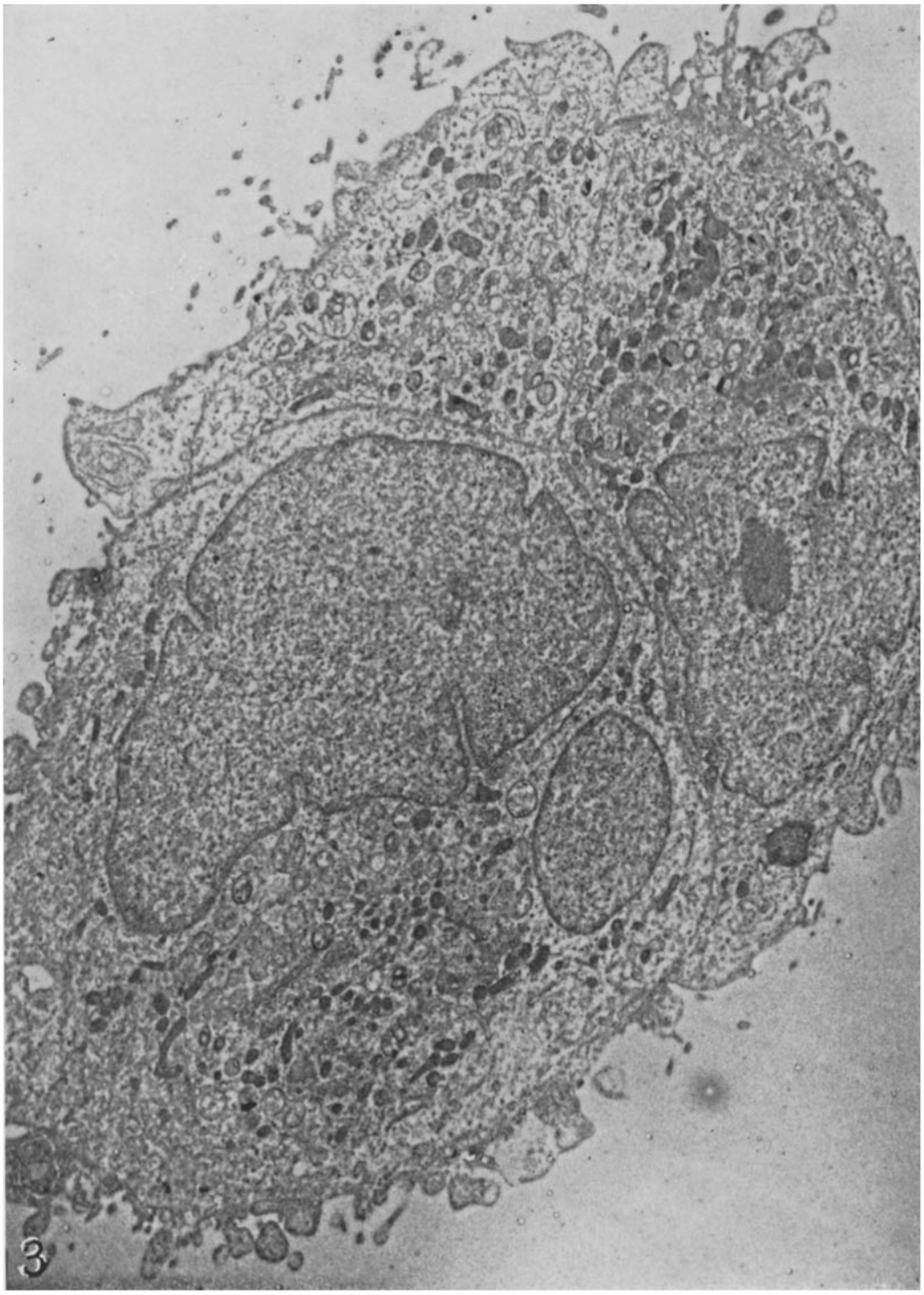




(Weiss and Armstrong: Structural changes in mammalian cells)

PLATE 332

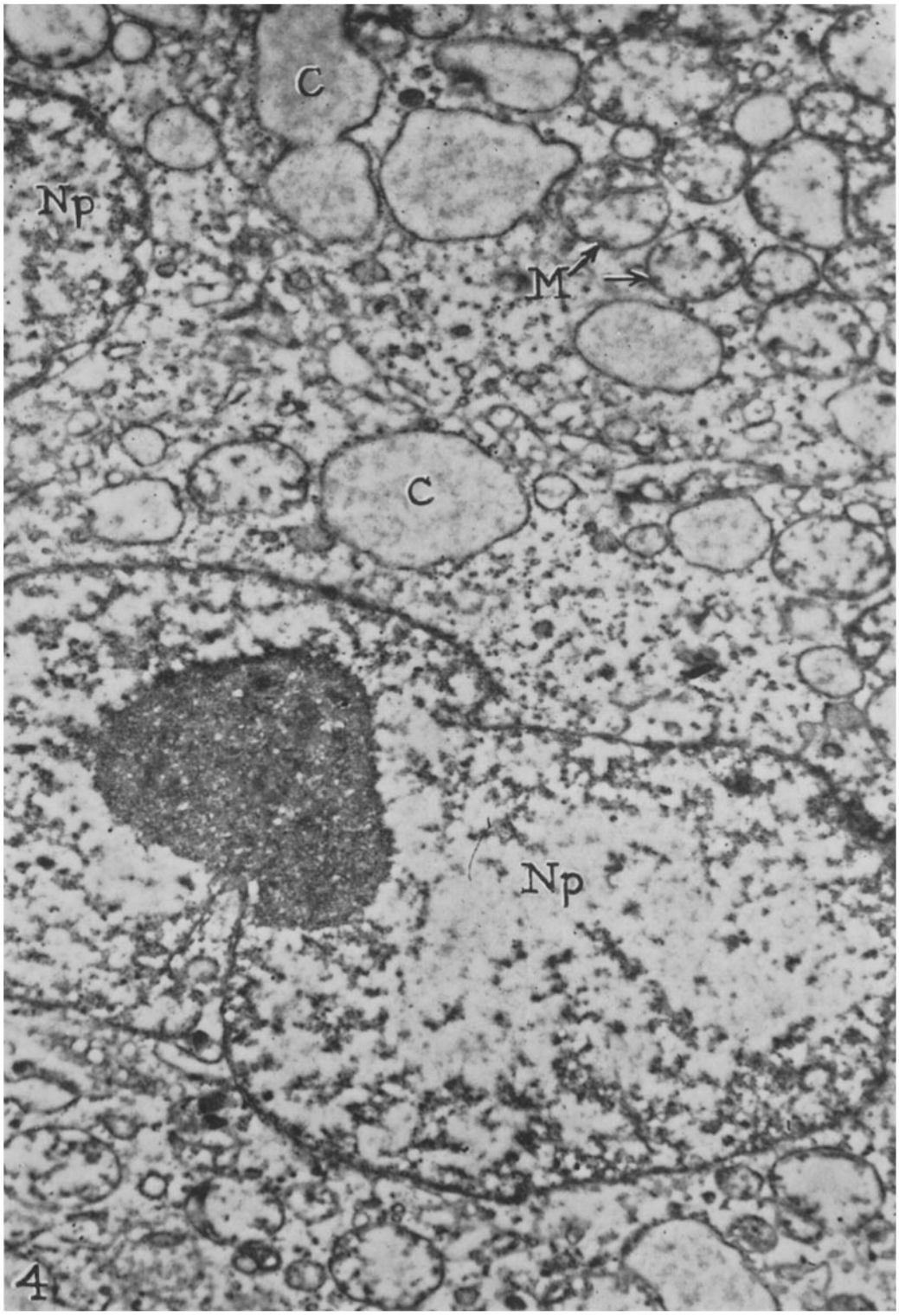
FIG. 3. Electron micrograph showing parts of three sectioned HeLa cells from a cell suspension kept at room temperature for 75 minutes (Treatment 3). OsO_4 fixation. × 7,000.



(Weiss and Armstrong: Structural changes in mammalian cells)

PLATE 333

FIG. 4. Section of the nucleus and adjacent cytoplasm in a HeLa cell frozen and thawed without glycerol (Treatment 1). Conspicuous in the cytoplasm are swollen mitochondria (*M*), and cavities (*C*) probably derived from the endoplasmic reticulum. Nuclear damage is indicated by irregular clumping and dispersion of the nucleoplasm (*Np*). OsO_4 fixation. × 18,000.

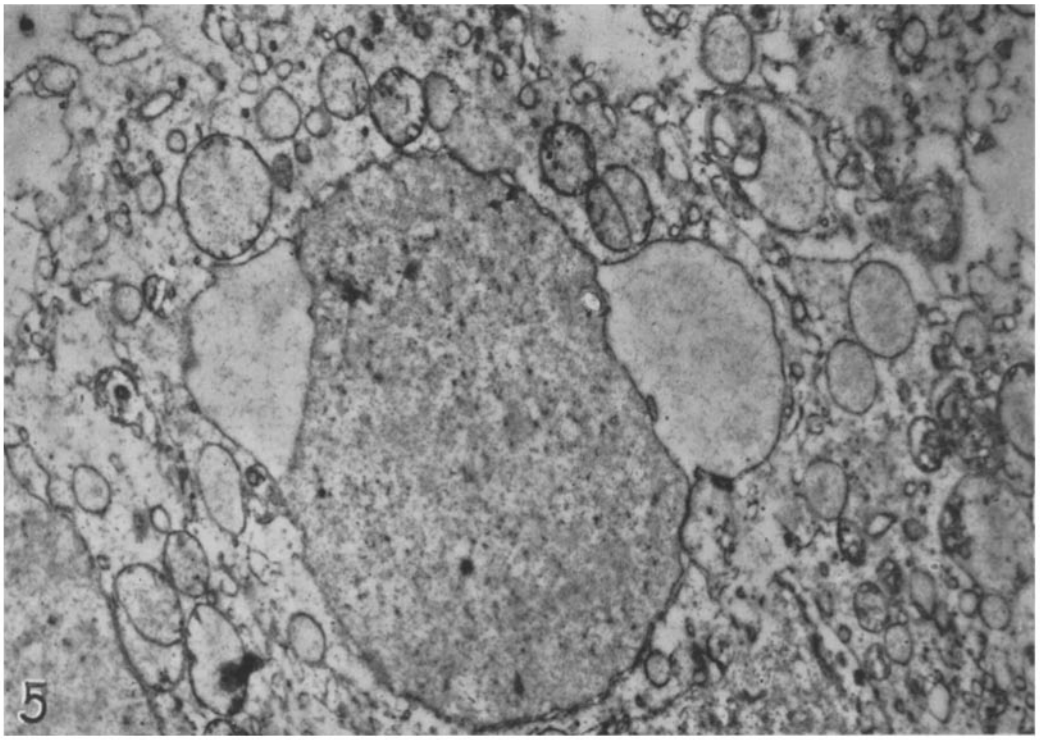


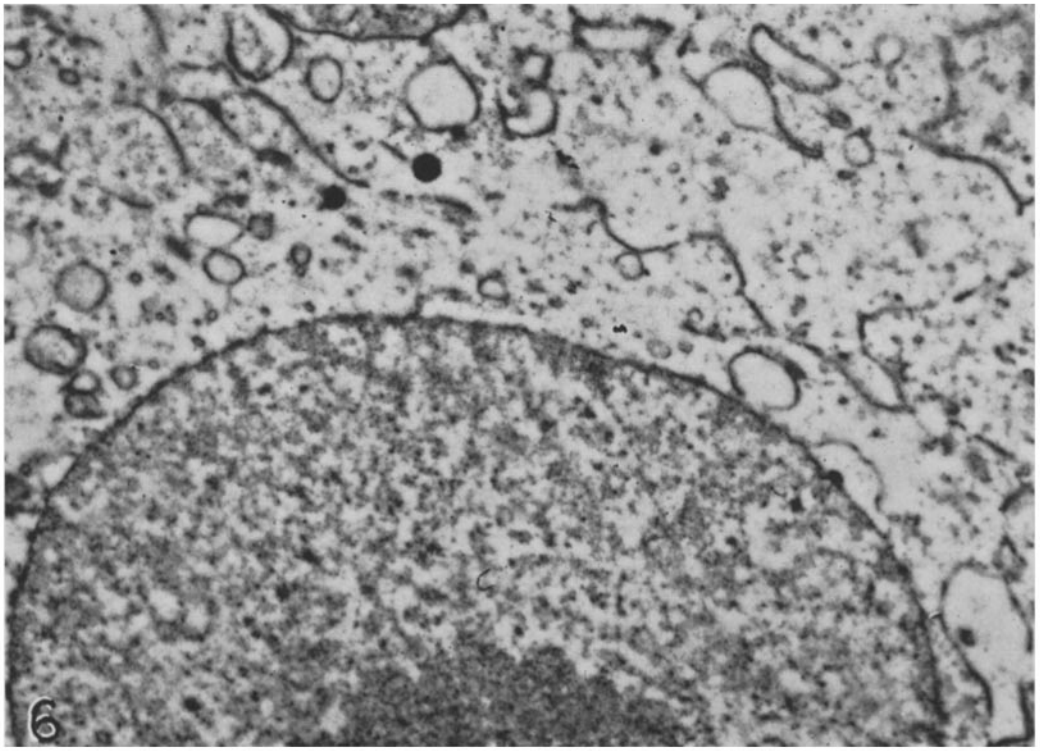
(Weiss and Armstrong: Structural changes in mammalian cells)

PLATE 334

FIG. 5. Nuclear blebs formed by separation of the two layers of the nuclear envelope in a HeLa cell frozen and thawed without glycerol. Remains of the membranous cristae show the mitochondrial origin of some of the cytoplasmic vesicles. $KMnO_4$ fixation. × 13,000.

FIG. 6. Nucleus and adjacent cytoplasm in a HeLa cell frozen and thawed in a medium containing 15 per cent glycerol (Treatment 2). Cytoplasmic vesiculation is present; but minimal alteration of the nucleoplasm suggests a degree of nuclear protection. OsO_4 fixation. × 14,500.





(Weiss and Armstrong: Structural changes in mammalian cells)