CELL DEATH
IV. The Effect of Injury on the Entrance of Vital Dye in Ehrlich Tumor Cells*

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In 3 preceding papers[1-3] the changes occurring in injured Ehrlich tumor cells have been discussed. In this final paper the use of a vital stain, trypan blue, and its relative importance as an indication of the presence of dead and dying cells will be described. In addition, the general pattern of death of cells will be discussed. Although Lepeschkin and Goldman[4] emphasized the early degenerative changes in cellular protein, Rahn[5] demonstrated that coagulation of protoplasm was the very terminal event in cell death. We believe Lepeschkin and Goldman's observations were indicative of coagulative conglomeration of protein following rupture of cell membranes and had no relation to the early unfolding or denaturation of the globular proteins, a phenomenon which occurs much earlier in the degenerative process.

Vital dyes have long been used to determine cell viability. It is an old dictum of biologists that dyes will never enter the living nucleus.[6] Rahn used methylene blue as an indicator of alterations in cellular permeability and found that injured cells took up the dye long after they had lost the capacity to divide and to ferment glucose. However, methylene blue undoubtedly enters the cell and is present in a reduced form long before it can be seen.

Evans and Schulemann[7] concluded that the entrance of trypan blue into the cell was purely a physical phenomenon based on the size of the particle and was not due to the chemical union between dye and protoplasm. In experiments with Ehrlich tumor cells, it proved to be an excellent guide as to both structural and metabolic activity but a poor indicator of changes in cellular permeability. Marked alteration in the amount of cellular protein, sodium, potassium and water occurred before the dye was taken up by the cell.

Experiments

The incubation experiments were carried out as described in the previous papers.[1-3] Cells were incubated with .0014 M glucose or

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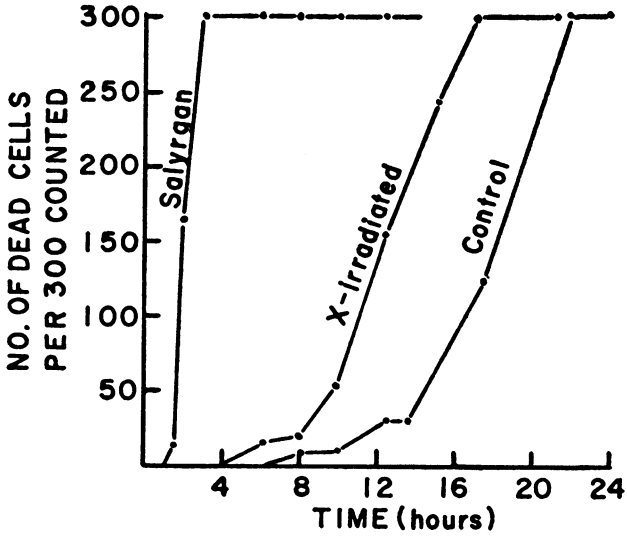
pyruvate, maintained under air or nitrogen and irradiated with 1×10^6 r.

An aqueous solution (0.01 per cent) of trypan blue was used as a vital stain. One drop was added to 3 drops of cell suspension under the cover slip of a standard blood counting chamber. Three hundred to 500 cells were counted at each time period, and the percentage of cells taking the stain into the nucleus was noted.

Results

Trypan blue was used as a test for the viability of Ehrlich tumor cells. The entrance of trypan blue into the cell occurred later than the derangement of the other processes measured (Text-fig. 1). One hundred per cent of the salyrgan-treated cells usually completed the uptake of trypan blue in less than one hour. The irradiated and control cells had similar curves at a later hour. In many experiments of 6 to 8 hours duration, the control and irradiated cells never took up trypan blue.



Text-figure 1. The effect of x-irradiation (1×10^6 r.) and salyrgan (0.001 M) on the viability of Ehrlich tumor cells (stained with trypan blue) in a typical experiment.

Following rupture of the cell as the cytoplasmic and nuclear protein gradually dissolved in the surrounding medium, the dye became lighter and lighter until the final remnants failed to stain.

There were, however, histologic alterations noted in the cell structure before the entrance of trypan blue and the subsequent rupture of the cell. M. Lewis[8] found a brightening and accentuation of the nuclear membrane in tissue culture fibroblasts. In Ehrlich tumor cells,

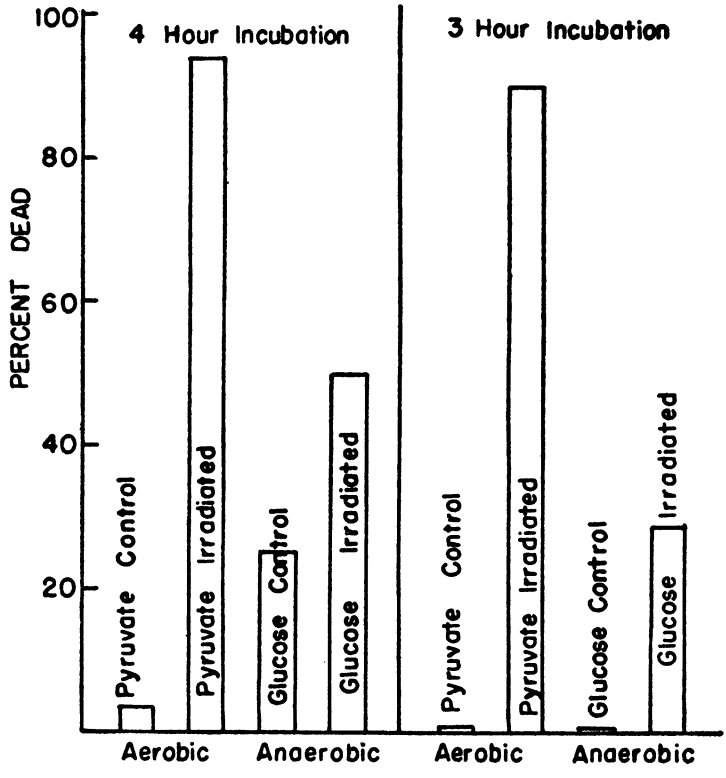
this occurred principally after the shrinkage of the nucleus and before rupture of the cytoplasmic membrane.

The formation of cytoplasmic blebs similar to pseudopods has been seen regularly in tissue cultures.[9,10] Although they were occasionally observed in control cells, they were found principally in the irradiated cells in our experiments (Figs. 3 and 4). The salyrgan-treated cells usually swelled regularly and homogeneously without exhibiting these localized irregularities in outline (Figs. 5 to 10). The blebs in the irradiated cells sometimes appeared to be clear, in the nature of vacuoles. Protein was not apparent in the fluid in hematoxylin and eosin stained sections. The blebs could represent localized weaknesses in the cell membrane. In other instances, they were opaque and continuous with cytoplasmic structure. Cloudy swelling, a term first used by Virchow, has long been thought to represent an early stage of degeneration. The cells in this condition were described as being large and cloudy, showing fine granularity of the cytoplasm. Virchow thought that injury to the cell impaired the ability of its cytoplasm to assimilate protein brought to it by the blood.[11] In recent years, pathologists have justly attached little diagnostic significance to this term. It is impossible to distinguish between late pre-mortem and early post-mortem alterations. Injury to the cell obviously occurs following removal from the organism.

A similar but more marked process has been described by a number of investigators working with tissue cultures. W. H. Lewis[12] found that the first indication of death of the cell was a hazy granulation of the nucleus and later granulation in the clear peripheral processes of fibroblasts. Later the cytoplasm and nucleus became filled with fine, white granules which at first showed brownian movement. Since they were separate and distinct from the mitochondria, they may have been conglomerate, denatured, corpuscular proteins. Present workers in tissue culture have described this latter phenomenon.[13] Photographs taken in this laboratory of unfixed Ehrlich tumor cells injured with salyrgan and roentgen rays (Figs. 5 to 10) show the typical features of cloudy swelling.

Trypan blue was also of value in attempts to measure the effect of injury on the aerobic and anaerobic parts of the glucolytic cycle. Heinmets and Kathan[14] thought from their studies with bacteria that the fermentative cycle was more affected than the aerobic cycle. In experiments in this laboratory glucose and pyruvate were added to two aliquots of the same sample of cells to a final concentration of .0014 M respectively. They each received equal doses of irradiation (1×10^6 r.) simultaneously under aerobic conditions. Examination

of the cells by vital stain techniques (trypan blue) showed that the control cells incubated in pyruvate under aerobic conditions and glucose under anaerobic conditions maintained their integrity without damage (Text-fig. 2). However, cells irradiated in pyruvate and



Text-figure 2. The effect of irradiation (1×10^6 r.) on cells incubated under anaerobic and aerobic conditions as measured by the entrance of trypan blue into the cell.

maintained under aerobic conditions had a 90 per cent uptake of vital stain in 3 hours, while only 29 per cent of those irradiated in glucose and maintained under anaerobic conditions took up trypan blue. In a later experiment, 4 hours in duration, the control pyruvate-incubated cells again maintained 100 per cent viability as measured by this technique, and the glucose control cells had 97 per cent viability. The pyruvate-irradiated cells showed evidence of much more damage than the glucose-irradiated cells. In the former group 98 per cent took the stain, while in the latter only 49 per cent did so (Text-fig. 2).

In earlier experiments with two groups of cells not irradiated but given equal amounts of glucose and studied under anaerobic or aerobic conditions, it was shown that the cells in the anaerobic group were unable to maintain the proper electrolyte concentrations, even though

they did not take up vital stain. Control cells given pyruvate maintained normal contents of sodium, potassium, and water. Control cells given glucose and incubated anaerobically failed to maintain normal sodium and potassium levels, although appreciable numbers did not take in vital stain. Irradiated cells given glucose did less well than the two control groups, but did appreciably better in maintaining normal sodium and potassium levels than the cells irradiated in pyruvate.

Thus, from evidence gained by the entrance of vital stain, the maintenance of proper sodium and potassium values, and from the cessation of respiration while lactic acid production continued, it is concluded that in these cells one enzyme or a group of enzymes in the oxidative cycle is more sensitive to radiation in the presence of substrate than the enzymes in the fermentative part of the cycle. Although both groups were irradiated in air, it might be postulated that hydrogen peroxide formed in sufficient quantities would be effective over a longer time period in continually aerated solutions than in solutions kept under anaerobic conditions.

Discussion

The purpose of this investigation was to follow the processes leading to death of a cell and to determine, if possible, which physiologic features of the cell were most susceptible to injury. The results of many experiments on the metabolic processes of degenerating control, irradiated, and salyrgan-treated cells indicated that the sequence of events in Ehrlich tumor cells followed a typical pattern for all 3 forms of injury. This may be divided into 4 stages (Table I).

Few investigators would maintain that the nucleus was essential for short term survival of cells. Red blood cells maintain an active metabolism for 120 days without benefit of a nucleus, and the functions of the nucleus are not well defined.[15] Many investigators have believed that the nucleus was the center of protein synthesis, and Gale and Folkes[16] have demonstrated the importance of deoxyribonucleic acid (DNA) on amino acid incorporation and the formation of adaptive enzymes. However, Brachet and Chantrenne[17] showed that the incorporation of amino acid and C^{14} in the giant alga (*Acetabularia mediterrania*) was not stopped when the cells were enucleated, and Malkin[18] observed similar results with sea urchin eggs. It appears likely that the nucleus will prove important in the general metabolism of the cell although it was no surprise to workers in this laboratory to find that some constituents of the nucleus could be badly damaged without untoward effects on other aspects of the cell's metabolism.

The nucleus became granular, hazy, swollen, and finally pyknotic earlier than its associated cytoplasmic component. It (the nucleus) lost appreciable amounts of DNA, and the mitotic index dropped precipitously before there was any demonstrable alteration in mito-

TABLE I

Stages of Cell Death

Stage 1
1. Division stops, as the cell fails to undergo mitosis.
2. Deoxyribonucleic acid is lost from the cell nucleus.
3. Protein is lost concurrently with the first two steps, probably from both the nucleus and the cytoplasm.
4. The formation of blebs, granules, and minor changes in the size of the nucleus and cytoplasm occur.
Stage 2
5. Respiration ceases several hours later.
6. Lactic acid production continues for a short time following cessation of respiration.
7. Total dehydrogenase activity is lost, with interruption of complex oxidative and fermentative cycles.
8. Mitochondria decrease in number and increase in size throughout the experiment, but they do not disappear completely with the loss of metabolic functions.
Stage 3
9. Potassium loss from the cell is concurrent with the entrance of sodium and water. Although small variations in these constituents may occur earlier, the terminal changes appear to be associated with the loss of energy production following breakdown of the glucolytic cycle.
10. Trypan blue enters the cell and stains the nucleus and cytoplasm.
11. At a critical point, in a rather narrow range, the cell bursts, releasing large amounts of sodium and water. This results in contraction in size, although a protein framework in the cytoplasm and nucleus remains intact.
Stage 4
12. The denatured protein of the cytoplasm takes up additional amounts of sodium and water. Later, as the protein is dissolved into the surrounding medium, the total content of sodium and water is reduced.
13. The terminal picture is that of a few irregularly stained nuclei embedded in a conglomerate mass of precipitated protein.

chondrial structure, plasma membrane structure, amount of ions or water, dehydrogenase activity, respiration or lactic acid production. The only concurrent phenomenon affecting the cell as a whole, contemporaneous with the drastic changes in the nucleus, was the loss of protein from the cell.

Three to 14 hours following alterations in the protein of the nucleus and cytoplasm, there were noted the first changes in some of the cytoplasmic physiologic processes. The complete breakdown of any one of the 4 major processes of the cell (biosynthesis of enzymes, production of energy, repair and restoration of structural deficiencies, and maintenance of a satisfactory ionic environment) will immedi-

ately result in alterations, usually irreversible, in the other 3 functions. Indeed, when cells were injured with salyrgan, which inhibits the action of many enzymes, there followed in quick succession a loss of energy, a falling apart of the protein structure, and rapid and highly deleterious changes in the ionic constituents of the intracellular fluid. With the destruction of the oxidative and fermentative cycles in the control and irradiated cells, there was a loss of energy available to maintain cellular structure and ionic balance. Protein architectural destruction was increased, or the rate of resynthesis decreased. This inevitably resulted in membrane alterations. A lack of energy to maintain cellular sodium and potassium equilibrium against the normal gradients resulted in a loss of potassium and a gain in sodium. The latter phenomenon was accompanied by a movement of water into the cell.

Denatured protein swells and takes on hydrated sodium ions resulting in further loss of the spatial relationships of the cell architecture. At the very end stage, shortly before the bursting of the cell, trypan blue dye entered both the cytoplasm and the nucleus. At this stage no metabolic activity exists, and the cell is completely disorganized. Examination of cells following rupture of the membrane usually revealed intact cells for many hours. Occasionally a tear in the membrane was seen, but often it was not. After a short initial contraction with release of water and sodium ions, the denatured residual protein structure again became swollen to its initial bursting size, or occasionally a little greater. Gradually over a period of time, protein was dissolved from both the nucleus and the cytoplasm, and the cell became shrunken to an irregular shell of a nucleus with a thin rim of cytoplasm surrounding it. The extravasated protein coagulated into a conglomerate gelatin-like mass.

Some would consider it unrealistic from a biochemical point of view to try to separate the metabolic processes, since the present knowledge concerning their mechanisms shows how intimately different processes are interrelated. For example, coenzyme A appears to be concerned with the metabolism of the 4 major constituents of the cell. A lack of coenzyme A might theoretically result in such widespread and apparently unrelated changes as inability to revise the protein or lipid portion of the plasma membrane or inability to provide the energy for the formation of DNA or the transportation of ions.

Despite the fact that essential key enzymes, coenzymes, vitamins, and hormones may be involved in numerous interrelated metabolic pathways, it nevertheless has been shown in these experiments that the injured cell passes through 4 fairly discrete stages. It cannot be said that all forms of injury require these discrete stages. Cells sub-

jected to large toxic doses of salyrgan passed through the degenerative phases so quickly as to make recognition of each stage almost impossible. It would also appear likely that some toxins and many chemicals would have a lytic effect on the membrane which would result in disorganization of cellular structure and function due solely to ionic imbalance. Competitive analogues may selectively inhibit one stage of protein synthesis without affecting the mitotic process. The discovery and identification of these processes in disease states will constitute the pathology of the future.

In this laboratory we have investigated some of the events leading up to cell death. From the studies described, cellular functions appear to be lost in a reproducible order, regardless of the form of injury. Most of these functions depend on energy production and biosynthesis of enzymes, and these two processes, in turn, are interdependent on each other. Probably the most important and unique property of protoplasm is the ability to duplicate its constituents exactly, and the loss of this quality may be the primary defect when cells fail to recuperate from injury. The delayed end result and sequence of events thenceforth will depend on how much of a given essential metabolite was present before the damage, the amount needed, the natural turnover rate, and the possible alternative paths of synthesis. If this property of self-duplication is that most susceptible to injury, this would explain why mitosis is the process most easily depressed. In no other function of the cell is a more complicated or vital reduplication necessary than in the structure of the genic pattern of the chromosomes. Later, despite the reserve capacities of the cytoplasm, the cell would be unable to duplicate the enzyme pattern essential for the completion of the energy producing reactions. Once energy production is lost, the cell is unable to maintain a normal concentration of ions and water, and the structural organization necessary for life is forever lost.

SUMMARY

Ehrlich tumor cells, when injured by irradiation and a mercurial metabolic inhibitor, pass through 4 discrete, recognizable stages. The fate of the cell is determined by its ability to repair those structures destroyed as well as to maintain synthesis of those molecules normally undergoing a fast turnover in the dynamic equilibrium of the cell.

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[*Illustrations follow*]

LEGENDS FOR FIGURES

Figures 1 and 2 represent unstained control Ehrlich tumor cells maintained in Krebs-Ringer solution.

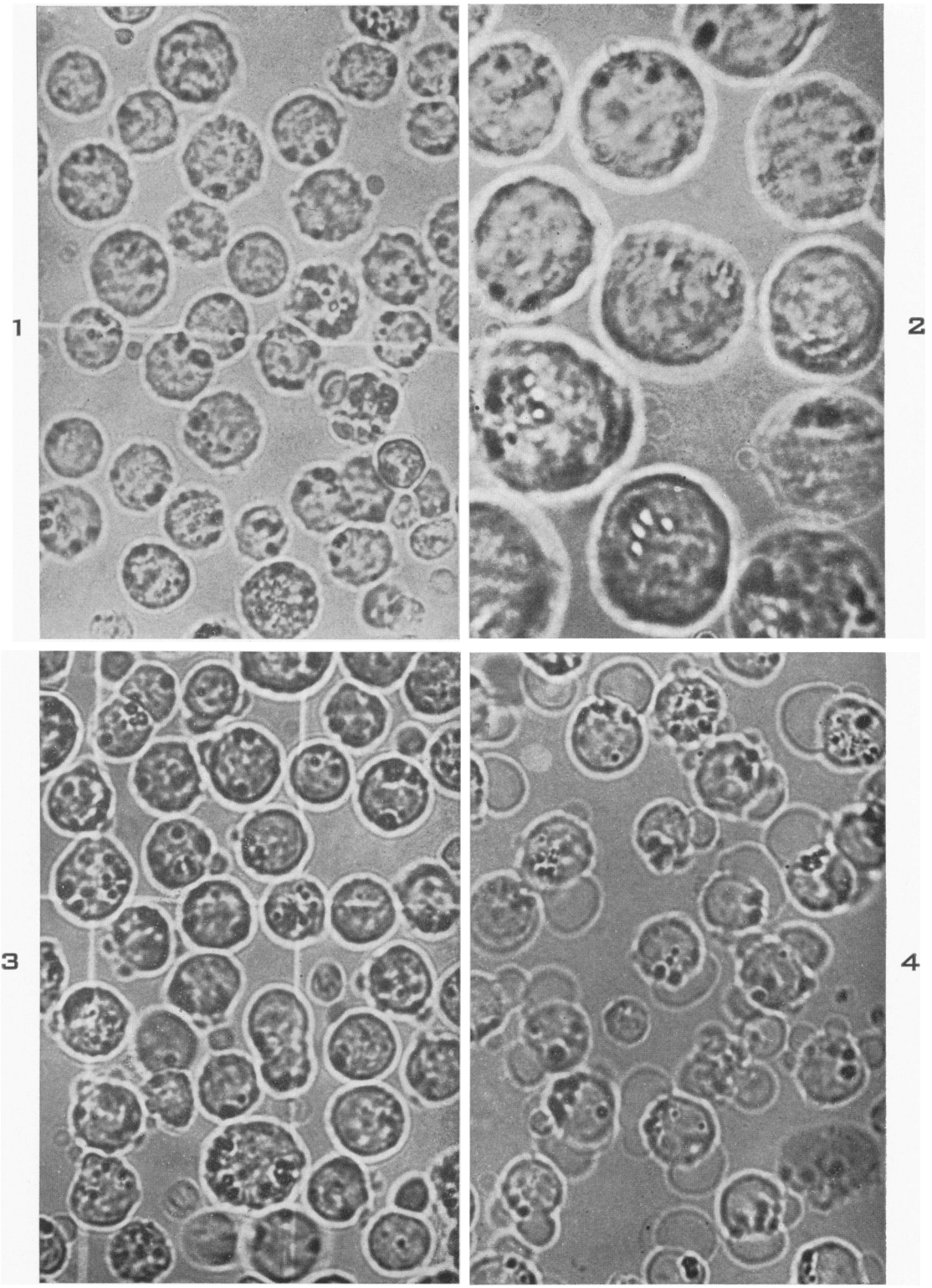
FIG. 1. Control cells immediately after incubation. The cells are spherical, regular in outline and contain numerous refractile granules or vacuoles. Very little cytoplasm is seen, as there is a very high nuclear cytoplasmic ratio in these tumor cells. × 430.

FIG. 2. Control cells 8 hours after incubation. Note the absence of staining with trypan blue. Although they have lost small amounts of protein and DNA and the mitotic index has been reduced, the cells have maintained a normal size, shape and appearance. When .1 cc. of tumor suspension containing 5×10^5 cells was re-injected into mice, 100 per cent of the animals developed tumor. × 970.

Figures 3 and 4 represent irradiated Ehrlich tumor cells maintained in Krebs-Ringer solution.

FIG. 3. Cells immediately after irradiation with 1×10^6 r. The unstained cells are regular in outline and resemble the control cells in every way. × 430.

FIG. 4. One hour following irradiation. The cells have developed irregular cytoplasmic blebs. Some of these appear continuous with the cytoplasm, while others are merely small pockets which do not appear to have protein when stained with hematoxylin and eosin. Although the cells are losing small amounts of DNA and protein, they are maintaining their normal respiration and lactic acid production. There are no marked changes in the electrolyte pattern, and those seen are apparently reversible. × 430.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Figures 5 to 10 represent salyrgan-treated Ehrlich tumor cells.

FIG. 5. Unstained Ehrlich tumor cells immediately after the addition of salyrgan. The cells are small, spherical and regular in outline, resembling the control cells. × 430.

FIG. 6. Fifteen minutes after incubation. The cells have lost large amounts of DNA and protein, but their respiration and lactic acid production have not ceased. The nuclei and cytoplasm are losing potassium, gaining sodium and water, and starting to swell, exhibiting irregular cell membranes and partial clumping of the granules. × 970.

FIG. 7. Forty-five minutes following incubation. Some of the nuclei have stopped swelling and begun to contract. The cytoplasmic protein has a cloudy appearance which probably indicates denaturation or unfolding of the globular proteins. Respiration, lactic acid production and dehydrogenase activity have ceased. × 970.

FIG. 8. One hour following incubation. Two cells show rupture of the cell membrane with resultant loss of protein, sodium and water. × 970.

FIG. 9. Two hours following incubation. Following rupture of the cell membrane, the cell maintains an intact structure. Both the nucleus and the cytoplasm stain avidly with trypan blue. For a short time the denatured protein again takes up sodium and water, but this is lost along with the stainability as the protein gradually goes into the surrounding medium. × 430.

FIG. 10. Two hours following incubation. Note the variation in nuclear size and the presence of a few granules. × 970.

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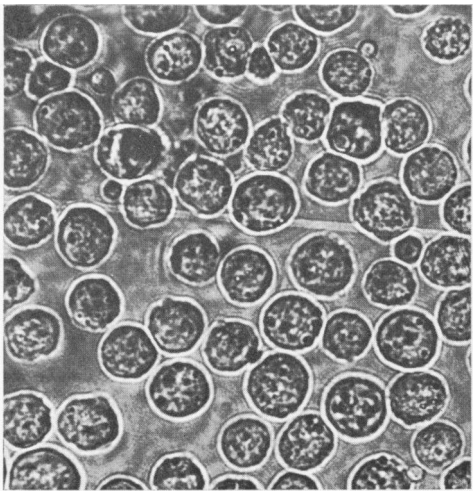

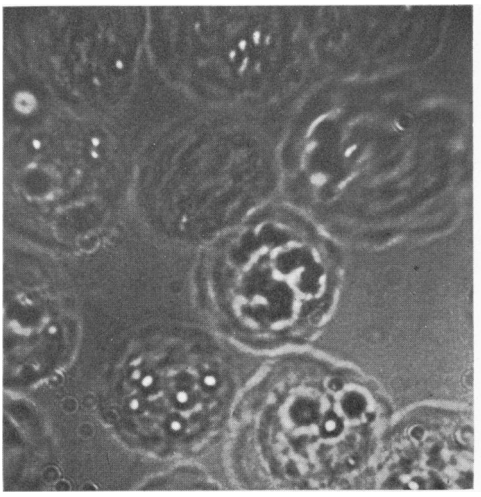

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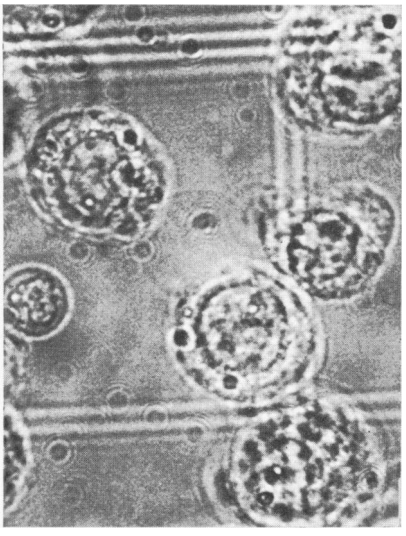

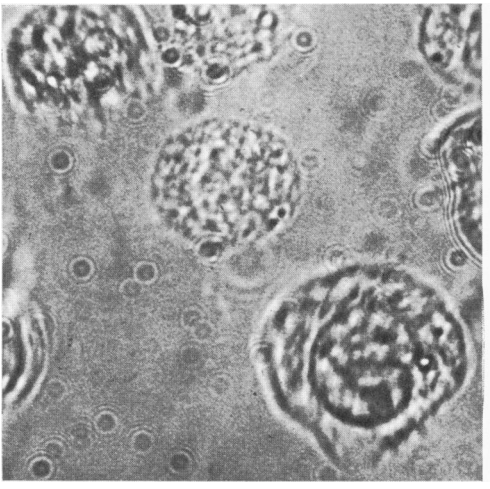

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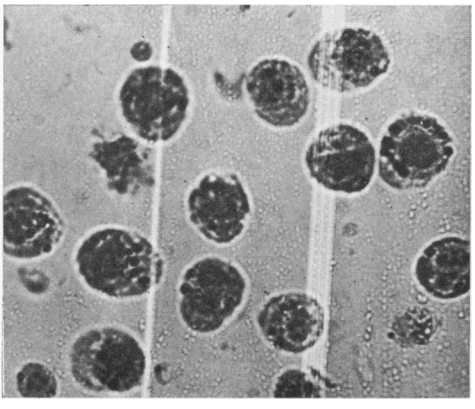

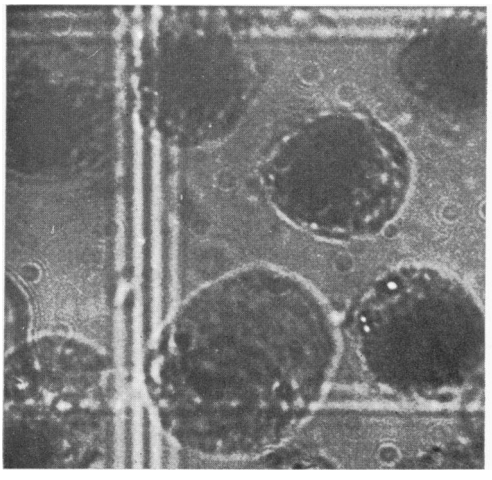

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