CELL DEATH

III. The Effect of Injury on Water and Electrolytes of Ehrlich Tumor Cells *

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The role of ions in the cell has not been fully understood. They have been thought to participate as hydration regulators, as integral parts of the structure of the cell,[1] associated with the transport of adenosine triphosphate,[2,3] and as inhibitors or accelerators in specific enzymatic reactions.[4-9] Most studies on cell electrolytes suggest that cells are iso-osmotic although much evidence has been presented to indicate an intracellular hypertonicity.[10] It is also accepted by many that sodium and potassium transport systems are linked and reciprocal in nature;[11] however, many reports are available on the independence of ion and water movements.[12-14] Many of the exchange studies available are concerned with only one of 3 major components: sodium,[15] potassium,[16-18] or water,[19-21] although others include two or more components.[22]

The system of Ehrlich tumor cells used in these experiments offers the opportunity of measuring not only sodium and potassium but, indirectly, the water content as well. It also eliminates two errors of *in vitro* studies using tissue slices, namely, miscalculation of extracellular space and the presence of large amounts of dead or dying cells at the edge of the slice.

Experiments

The cells were obtained by methods described in preceding papers.[23,24] In addition to those already described, the following measurements were made.

Cell Volume

The cells are generally considered to be spheres in the free floating state. In order to maintain this living form uninjured, a few drops of the cell suspension were pipetted on a blood counting chamber. Multiple photographs were taken with a phase microscope at a magnification of 970 times. Since only 6 to 8 cells were visible in each photograph, it was necessary to select 8 to 10 random fields throughout the slide in order to have at least 50 cells per sample. Later, circumferences of the

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cells in the enlarged photographs were plotted by means of a planimeter, and from these, cell volumes were calculated and taken as an indication of water content.

Nuclear Volume

Since there is a very high nuclear cytoplasmic ratio in tumor cells, it is difficult to distinguish the nucleus from the cytoplasm accurately in unstained, uninjured cells. The nuclear size was obtained by planimeter measurements of camera lucida drawings of fixed cells stained with Feulgen or hematoxylin and eosin stains.

Electrolytes

At each time period, five 2-cc. samples were withdrawn from each of 3 Erlenmeyer flasks for sodium and potassium determinations. The cell suspensions, in graduated centrifuge tubes, were spun at 3,000 r.p.m. for 5 minutes in a Clay-Adams centrifuge and the supernatant decanted. The sediment was allowed to drain for 8 hours. At this time 0.5 cc. of concentrated nitric acid was added for digestion, and the tops of the tubes were covered with parafilm. Forty-eight hours later, 1.5 cc. of distilled water was added to restore the samples to their original volume, and the sodium and potassium content was determined in a Model B Beckman Flame Spectrophotometer. Five readings were taken on each specimen.

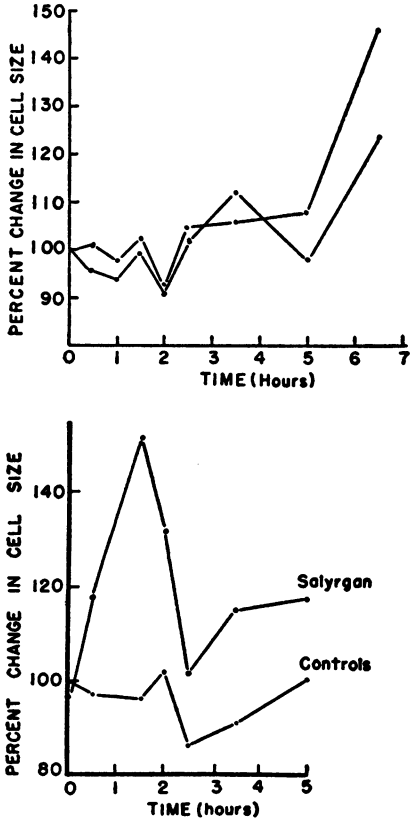
RESULTS

There are several methods of determining cellular volume, none of which are accurate. Perhaps the best is that of Lucké and Parpart,[25] who recorded the changes in light transmitted through a cell suspension directly on a galvanometer. Changes in volume of perfect osmometers will be measured by changes in light transmitted. However, this method demands that the cells always swell homogeneously and that they do not contribute their content to the extracellular suspension. Neither of these stipulations can be met in experiments of long duration. Cell size in these experiments was originally computed by two methods: the microhematocrit method employed by Ponder[26] and others, and a photographic planimetry method described earlier. It was surprising how closely the two methods checked each other in one experiment. However, a word of caution should be voiced concerning the packed centrifuged cells in the microhematocrit chamber. There is little doubt that centrifugation results in damage to the cell membrane which may not show up immediately. In one experiment, cells were initially examined and volume calculated by the planimetry method. A second aliquot was centrifuged in a microhematocrit tube. Follow-

ing centrifugation, the packed cells were resuspended in Krebs-Ringer solution, photographed, and the size of 50 cells measured by means of a planimeter. A third aliquot from the original uncentrifuged suspension was taken for the final control sample and the cell size measured with the planimeter. The membranes of the centrifuged cells were sufficiently damaged so that, on resuspension after centrifugation, they took in 45 per cent more water than the two control samples, before and after centrifugation.

Changes in Cell Size in Injured Cells

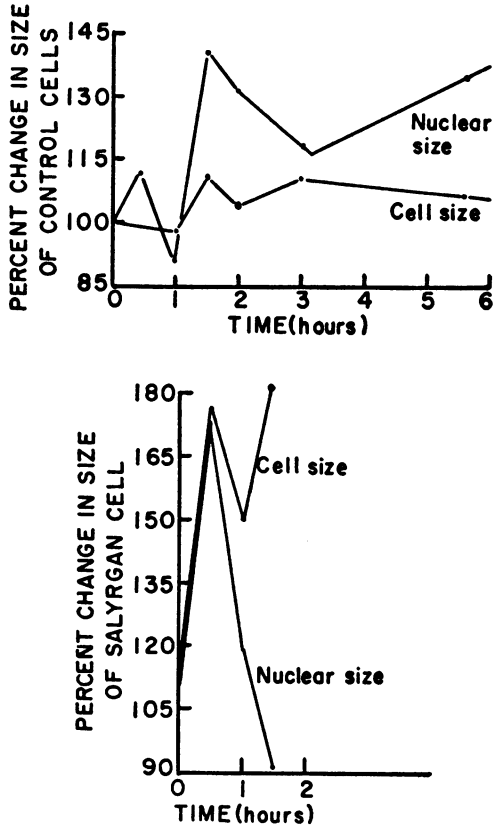
The cells changed their size in a fairly characteristic fashion, regardless of the form of injury. These changes in size were assumed to represent changes in the water volume. The characteristic curves of initial shrinkage followed by a terminal swelling of the cell were found



Text-figure 1. Effect of irradiation and salyrgan on the size of Ehrlich tumor cells. Cells suspended in Krebs-Ringer solution (pH 7.46) with glucose added to a final concentration (0.0014 M). The top graph represents the change in size in the irradiated group, and the bottom graph, in the salyrgan-treated group. The bottom line in each group represents the control sample. Each point represents the average of 50 cell planimeter measurements and the percentage of change from the original cell size plotted.

in all 3 samples: control, irradiated and salyrgan-treated (Text-fig. 1). The only difference was the time element. The salyrgan-treated cells, as expected, started to swell almost immediately, while the irradiated cells had delayed swelling and paralleled the control cells until the terminal phase.

The nuclear size may have no relation to the size of the cell. It varied over a wide range when measured by a planimeter (Text-fig. 2).

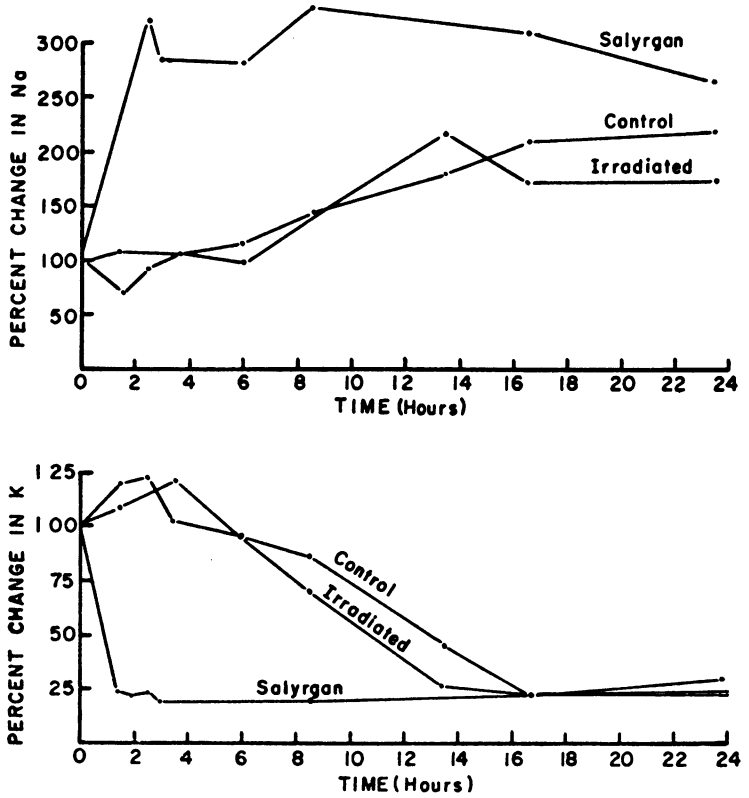


Text-figure 2. Relation of nuclear to cytoplasmic size of Ehrlich tumor cells. Each point represents the average of 50 planimeter measurements. The depression in nuclear size between 1 and 3 hours in the control cells is significant to the 0.05 level. There is no significant change in cell size in the control group by analysis of variance. The changes in nuclear and cell size in the salyrgan-treated cells are significant to the 1 hundredth level.

The nucleus appeared more labile and became significantly smaller or larger without corresponding alterations in the cytoplasm when cells were treated with salyrgan. The nucleus would swell proportionately with the rest of the cell, but the shrinkage of the nucleus might occur independently of changes in the cytoplasm. The latter phenomenon took place dramatically and irreversibly.

Changes in Sodium and Potassium Content in Injured Cells

The sodium and potassium in general maintained a reciprocal relationship to each other; in addition, the sodium closely paralleled the changes in water content. The control tumor cells maintained their normal concentrations for a period of 3 to 6 hours. At this time, concurrently with a decrease in respiration, fermentation, and the production of energy, there were a gradual loss of potassium and an associated increase in the amount of sodium and water contained in the cells (Text-fig. 3). The irradiated cells followed this pattern with slightly



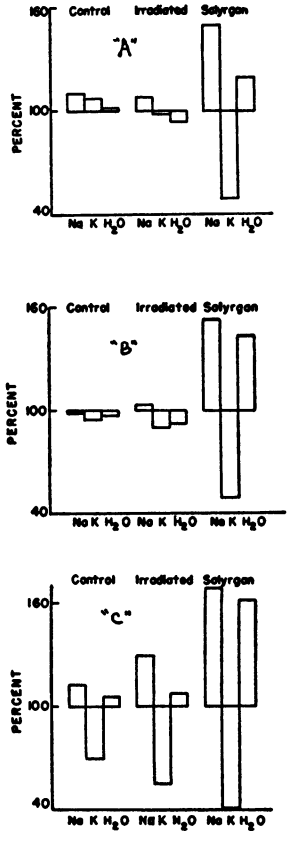
Text-figure 3. The effect of irradiation and salyrgan on the sodium and potassium content of Ehrlich tumor cells. Each point represents the average of 5 samples (5 readings on each sample) in a typical experiment.

earlier terminal alterations. The salyrgan-treated cells immediately reversed the normal intracellular ion content. When the cell had taken up 1.5 to 2.0 times its original content of sodium, it burst, and there was a loss of sodium and water. In experiments of longer duration, the loss of sodium following disruption of the membrane was followed by a secondary uptake of sodium. This was presumably due to the

attachment of hydrated ions to the newly released reactive groups of the denatured protein molecules. The sodium attached to the residual cytoplasmic structure remained for several hours until the protein was gradually dissolved into the surrounding medium. The irradiated and control cells ultimately followed a pattern very similar to that seen in the salyrgan-treated cells.

Correlation of Sodium, Potassium, and Water in Injured Cells

Text-figure 4 provides a summary of 8 experiments, representing hundreds of determinations in the study of electrolyte and water changes in injured cells. The pattern followed was the same, regardless



Text-figure 4. Effect of irradiation and salyrgan on Ehrlich tumor cells. The results are plotted as percentage of change in sodium, potassium, and water content, with the original cell samples given the value of 100 per cent. Part A represents changes during the first hour of incubation. Each bar represents 185 determinations from 7 different experiments. Part B represents the changes during the 1 to 6 hours of incubation. Each bar represents the average of 150 determinations from 6 different experiments. Part C represents changes from the terminal phase of cell death. Each bar represents the average of 150 determinations from 6 different experiments.

of the form of injury. The graph is divided into 3 portions. The top section (A) represents the changes occurring in the initial transfer of cells into cold Krebs-Ringer solution. The salyrgan-treated cells immediately gained sodium and water and lost potassium, whereas the control and irradiated cells underwent an equilibratory period with minor fluctuations of usually less than 10 per cent. The middle section (B) of the graph represents the major portion of the experiment, lasting 5 to 6 hours. While the control cells were maintaining a very constant sodium, potassium, and water content, the irradiated cells were beginning to lose potassium and to undergo the slight shrinkage previously described. In the bottom section of the graph (C) the final picture is plotted. After 5 to 6 hours of incubation, all 3 samples gained sodium and water and lost potassium in amounts reflecting the state of injury to the cell.

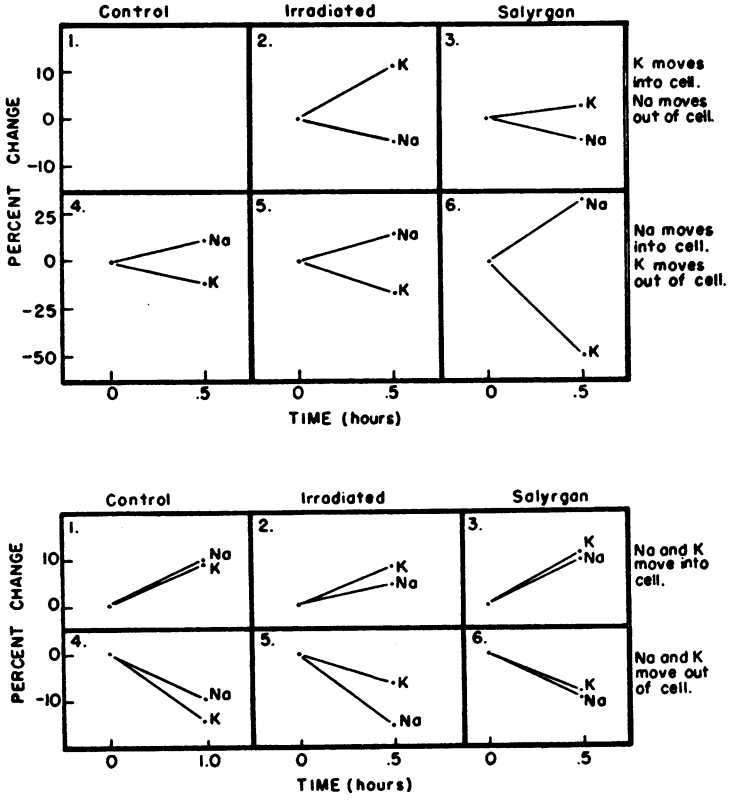
Independence of Sodium, Potassium, and Water Mechanisms

We believe the figures cited in Text-figure 4 show the general picture of sodium, potassium, and water changes in injured cells. However, these very cells made every effort during the process of death to compensate for the irregularities. During this period of stress, the complete independence of the sodium, potassium and water regulating mechanisms was clearly illustrated. Subsequent figures were taken from different experiments at different time periods in successive 30 or 60 minute periods. All results were statistically evaluated by analysis of variance and found to be significant to the 1 per cent or the 5 per cent levels, as indicated. The alterations took place in the control and irradiated groups while trypan blue indicated 95 to 100 per cent viability.

Text-figures 5 and 6 are concerned with the movements of sodium and potassium. As was noted in Text-figure 4, the overall trend was one of a reciprocal nature between these two ions, and, indeed, in the top portion of Text-figure 5 this is easily illustrated. However, in Text-figures 5 and 6 it is also clearly shown that sodium and potassium might move together, or one might not change while marked changes were noted in the other ion.

Text-figures 7 and 8 are concerned with the movements of all 3 major components, sodium, potassium, and water. Again, as previously shown in Text-figure 4, sodium and water usually moved together in the opposite direction from potassium. However, under periods of stress, the 3 components might move completely independently of one another.

The final Text-figure 9 merely indicates that the sodium and potassium changes shown to be associated with cell death occurred earlier and to a more severe degree in those cells maintained under strict anaerobic conditions.

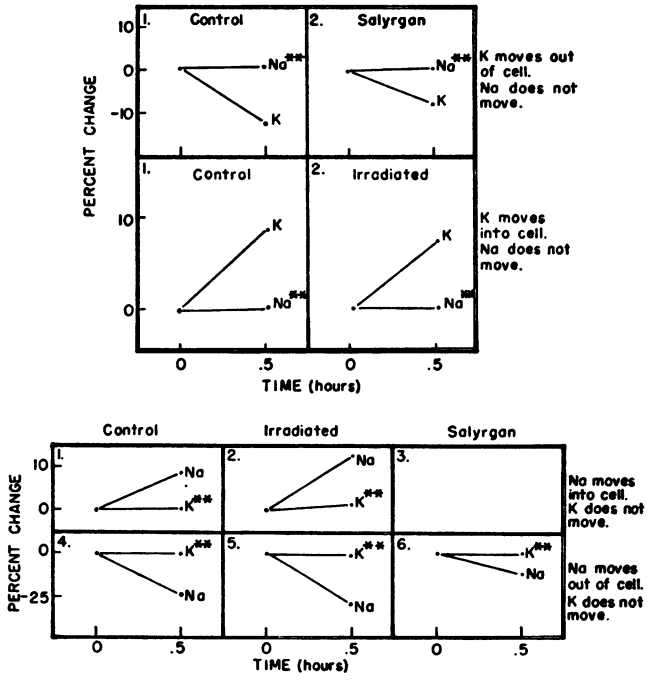


Text-figure 5. Effect of irradiation and salyrgan upon Ehrlich tumor cells. The graphs show that sodium and potassium may either move in a reciprocal fashion or move together concurrently in or out of the cell. The 2 points taken for each ion represent 5 samples taken from the same flask at 2 consecutive time periods 30 minutes apart. The flasks contained control, irradiated, or salyrgan-treated cells from 7 similar experiments, and the samples were taken after varying periods of incubation. The results are plotted as percentage of change in ion content from the original. All results are significant at the 1 hundredth level by analysis of variance except when starred; these are significant at the 5 hundredth level. All cells in the control and irradiated samples show 95 to 100 per cent viability as measured by the entrance of trypan blue into the nucleus.

Discussion

The subject of the passage of ions and water across a cell wall has been extensively reviewed recently.[11,27,28] Although the terms diffusion, active transport, pinocytosis, and phagocytosis are used to describe transport mechanisms, the definitions of these terms are vague, and considerable confusion and overlap result.

Many believe that while sodium is an active transport process,[29] potassium is passive,[30,31] whereas others believe potassium represents active transport also.[20,32-34] Our experiments did not elucidate any new information on this subject but merely confirmed a gain in sodium and loss in potassium under anaerobic conditions.

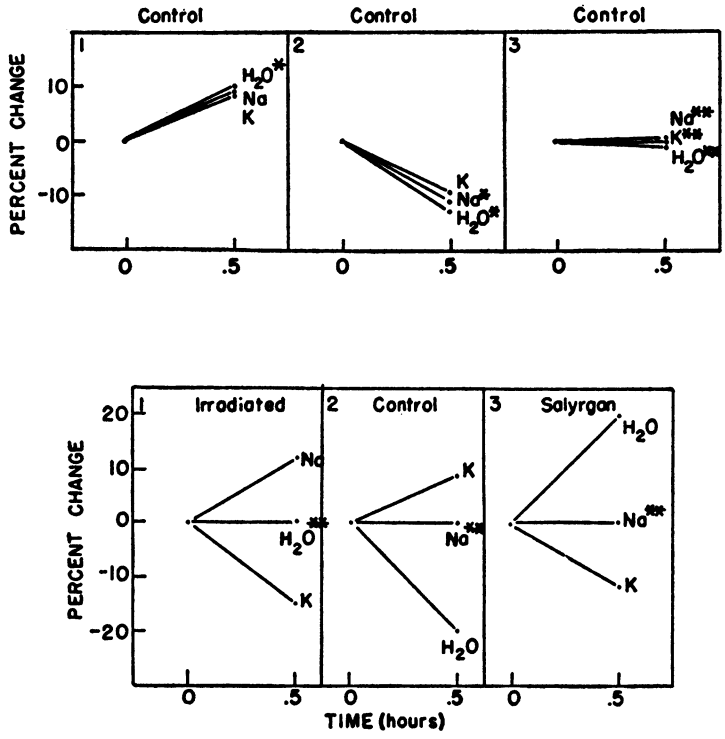


Text-figure 6. Effect of irradiation and salyrgan upon Ehrlich tumor cells. The graphs show that one ion may remain constant while the other ion moves in or out of the cell. The taking of samples, statistical evaluation, and viability tests were done as described in Text-figure 5.

It has been claimed that in all carefully controlled studies of the cell, there is a reciprocal relationship between sodium and potassium.[11] Others have failed to find this relationship constantly.[12,13] It has also been claimed that the sum of the sodium and potassium remains constant regardless of the treatment imposed.[35] Our experiments confirm, in general, the reciprocal nature of sodium and potassium over a long period of time. However, we believe that there is unequivocal evidence, supported by more than adequate statistical evaluation, that in periods of stress with disturbance of the regulatory mechanisms of the cell, sodium, potassium, and water may move in and out of the cell independently of each other. They can even move against concentration gradients and in a manner suggesting that many other factors not usually considered are important in regulation of ion concentrations.

These may include regions of varying metabolic activity,[33,36-39] hormones,[40-42] special tissue characteristics,[43,44] bound ions, inorganic metabolites,[45] or organic metabolites such as amino acids.[16]

We suggest that there are continued changes in the osmotically inactive forms of base in the cell. The water content of particulate components of the cell, such as mitochondria and nucleus, as well as the



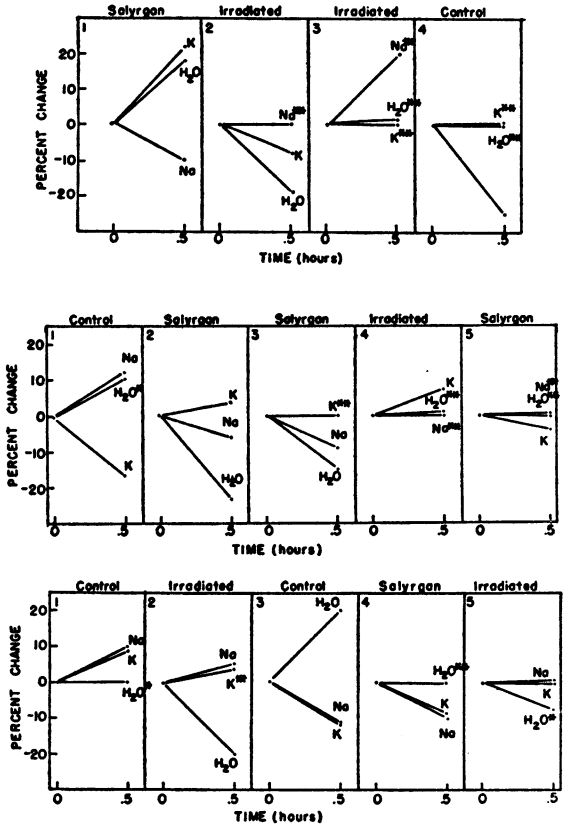
Text-figure 7. Effect of irradiation and salyrgan upon Ehrlich tumor cells. The graphs show that all 3 components measured (sodium, potassium, and water) may move concurrently and also independently. The taking of samples, statistical evaluation, and viability tests were done as described in Text-figure 5.

intramolecular water in corpuscular proteins, may respond primarily to local changes in cell metabolism and secondarily to the regulating influences of the complete cell. The whole series of experiments, including the specific effects on the structure (the variance in nuclear cytoplasmic ratio) as well as function (inhibition of division only), further confirmed this impression.

Effect of Injury on Electrolytes and Water

There are many publications concerning the effect of irradiation on sodium and potassium *in vivo* in the mammalian body,[28,29] and *in vitro*

on yeast and mammalian red blood cells[46] and sarcoma cells.[47] In general, they all show a loss of potassium and increase in sodium. One investigator irradiating amphibian erythrocytes also noted the preliminary shrinkage of the cell before swelling,[48] as we did. Our experiments are interesting in this regard only in that they showed that the control and salyrgan-treated cells exhibited shrinkage before the final swelling similar to that of the irradiated cells at different time intervals.

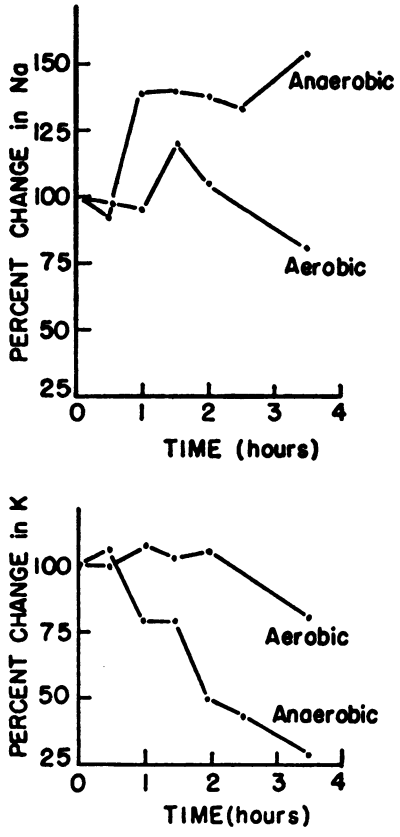


Text-figure 8. Effect of irradiation and salyrgan upon Ehrlich tumor cells. The independence of sodium, potassium, and water is well illustrated in this graph. The taking of samples, statistical evaluation, and viability tests were done as described in Text-figure 5.

Summary

Ehrlich tumor cells, when injured, immediately show small changes (usually not over 10 per cent) in sodium, potassium, and water content. These changes are reversible and are not fatal or detrimental to the fermentative or oxidative metabolism of the cell, although it is possible that they may have a deleterious effect on mitosis. A constant rela-

tionship between sodium, potassium, and water is maintained, but eventually, regardless of the form of injury, the cell swells, loses potassium, and incorporates large amounts of sodium and water. During periods of stress, the mechanism governing sodium, potassium, and water may be altered so that these 3 components may move independently of each other.



Text-figure 9. Effect of irradiation and saylrgan upon Ehrlich tumor cells. Effect of anaerobic conditions on the sodium and potassium content of Ehrlich tumor cells. Each point represents an average of 25 determinations from a typical experiment.

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