4. Pocidalo, J. J., Lissac, J., Amiel, J. L., Blayo, M. C., *Rev. fr. clin. Biol.*, 1958, v3, 1079.

5. Mithoefer, J. C., Davis, J. S., PROC. SOC. EXP. BIOL. AND MED., 1958, v98, 797.

6. Amiel, J. L., Pocidalo, J. J., Lissac, J., Blayo, M., *Rev. fr. clin. Biol.*, 1959, v4, 44.

7. Lissac, J., Amiel, J. L., Cophignon, J., Pocidalo, J. J., *ibid.*, 1959, v4, 157.

8. Mithoefer, J. C., *J. Appl. Physiol.*, 1959, v14, 109.

9. Chinard, F. P., Enns, T., Nolan, M. F., *Am. J. Physiol.*, 1960, v198, 78.

10. Spratt, J. L., LeRoy, G. V., in preparation.

Received March 20, 1961. P.S.E.B.M., 1961, v107.

Factors Influencing Experimental Modification of Ehrlich Ascites Tumor Cells.* (26634)

JOHN PAUL KALTENBACH AND W. B. LYONS (Introduced by R. B. Jennings)
Dept. of Pathology, Northwestern University Medical School, Chicago, Ill.

Previous studies have shown that the dye nigrosin can be used to differentiate unmodified cells, whose membrane is intact (unstained cells), from modified cells whose membrane permeability has been altered experimentally or by death of the cell (stained cells)(1). This differential staining technic has been used to quantitate metabolic data of ascites tumor cells and suspensions of cells obtained from solid tumor as to the proportion of activity due to stained or unstained cells(2). Recently, a modification procedure was described to increase experimentally the permeability of ascites tumor cells by brief exposure of the cells to a hypotonic medium (1). It is of considerable interest that the modification procedure does not result in uniform modification of all cells present, *i.e.*, some cells are apparently resistant to such injury-provoking conditions as hypotonic shock. The following experiments were done to determine whether or not any of the variables involved in experimentally increasing the permeability of Ehrlich ascites tumor cells would influence the resistance and/or lack of resistance of these cells to these experimental conditions.

Material and methods. Preparation of ascites tumor cells. A hypotetraploid subline of Ehrlich ascites tumor cells (EAT cells), carired in Strong A mice, was used in all experiments. The ascites tumor from each animal was collected in 15 ml centrifuge tubes containing 2 to 4 ml of cold buffered saline (9 parts of 0.9% NaCl + 1 part M/15 phosphate buffer). Cold saline was used because it partially prevents coagulation of the ascites fluid. The cells were centrifuged from the ascitic fluid, and twice washed in buffered saline. Centrifugation was carried out at about 125 to 150 $\times$ gravity for 3 minutes, then increased to about 200 $\times$ gravity for an additional 2 to 3 minutes. This centrifugation procedure aided in separating the tumor cells from the red blood cells and white cells; the latter tended to remain in the supernatant fluid. Tumors that were grossly contaminated with erythrocytes were discarded.

Preparation of modified tumor cells (MA cells). The ascites tumor cells were modified according to the following procedure: 0.5 ml of cells (about 20 $\times$ 10^6 cells) in buffered saline were diluted with distilled water to 4.0 ml. The cells were allowed to stand at room temperature for 4 minutes, then 4.0 ml of double strength saline (1.8%) were added to stop the modification. This procedure was altered only in those experiments described in the text that were designed to test the effect of different salt concentrations on the modification procedure.

Staining procedure: Cells were diluted with 0.2% water-soluble nigrosin dissolved in buffered saline. Differential cell counts of stained and unstained cells were made in

* This investigation was supported by PHS research grant, Nat. Cancer Inst., U.S.P.H.S.

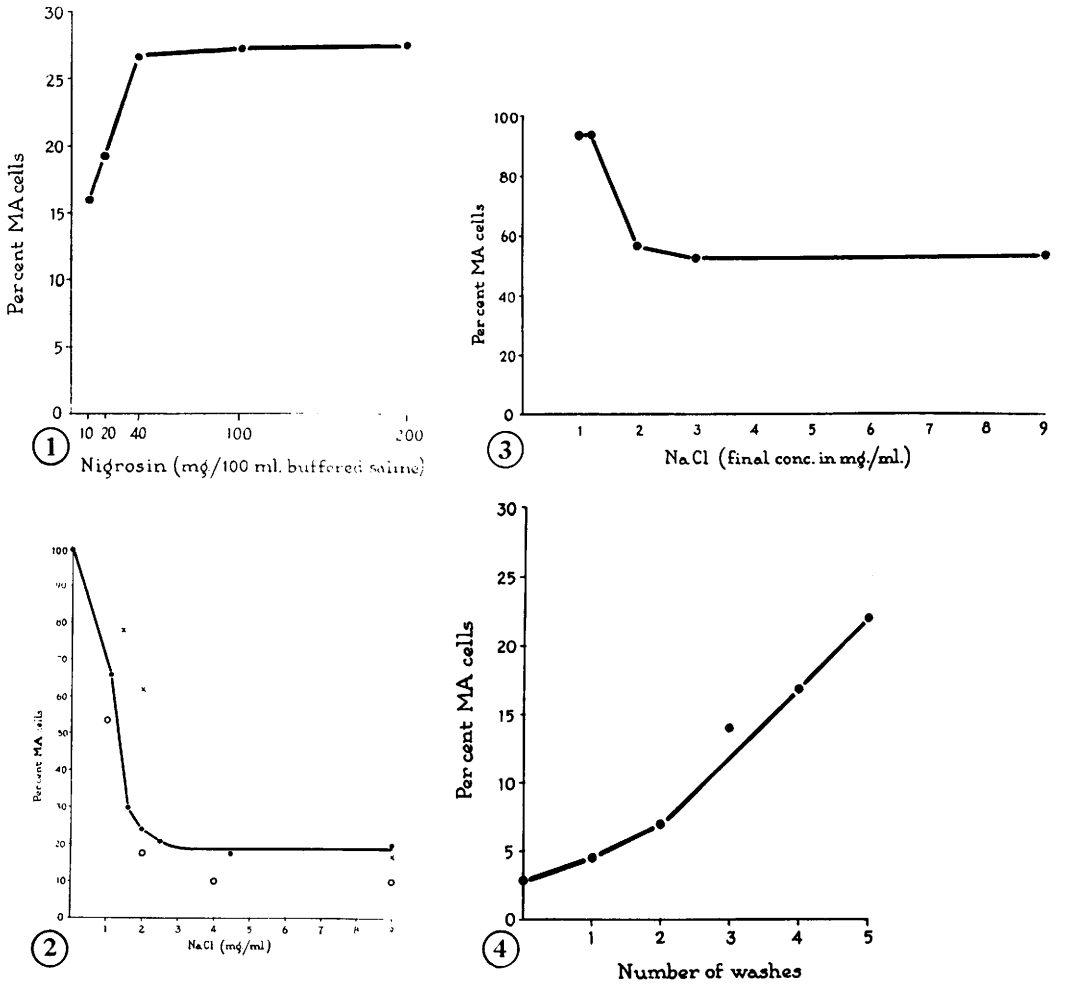


FIG. 1. Relationship between nigrosin concentration and number of cells stained.
FIG. 2. Resistance of EAT cells to low concentrations of NaCl (solid circles), buffered NaCl (open circles), and KCl (crosses).
FIG. 3. Concentration of NaCl required to stop modification of EAT cells.
FIG. 4. Relationship between number of times EAT cells are washed (centrifuged and resuspended) and increase in modified cells.

a hemocytometer according to the procedure described previously(1). Results are presented as percent modified cells (percent MA cells), as total cell count did not change appreciably during these experiments. Cell counts were done in duplicate and the average recorded.

Results and discussion. Effect of nigrosin concentration. Various concentrations of nigrosin were tested to determine the range of optimum dye concentration that would give reproducible results for quantitative staining of the modified tumor cells. Dye concentrations of 0.2% and less were used because previous work has shown that 0.2% nigrosin had no deleterious effect on cell membrane permeability or cell metabolism(1).

EAT cells (20×10^6 cells) were modified as described in Methods. Differential cell counts were made using concentrations of nigrosin from 0.01 to 0.2%. Fig. 1 shows that the number of stained cells increases with increasing concentrations of nigrosin, and that at least 0.1% nigrosin is required to obtain maximum and consistent stained-cell counts.

The importance of determining optimum dye concentration for quantitative staining of

modified EAT cells is shown by the above results. Hanks and Wallace(3) have also indicated the importance of determining optimum dye concentration in studies using the more fragile tissue culture cells. Dye inclusion in these cells apparently suggests loss of viability.

Effect of NaCl concentration. The purpose of these experiments was to determine the lowest concentration of NaCl required to prevent modification of EAT cells. The effect of buffered saline, KCl, and cell concentration was also investigated.

The EAT cells were modified as described in Methods, except that the cells were diluted with various concentrations of NaCl, buffered NaCl, or KCl instead of distilled water. Fig. 2 shows that the number of cells modified did not change appreciably until NaCl concentration was decreased to less than 3 mg/ml. Cell lysis occurred following exposure to solutions containing less than 1.0 mg NaCl/ml. Similar results were obtained when buffered saline was substituted for NaCl. Substitution of KCl in the modification procedure resulted in a somewhat greater number of modified cells. The results are similar whether based on absolute or equivalent ionic concentration. Varying the total number of cells in the aliquot to be modified from 10^7 to 40^7 did not affect the results.

These findings show that the final concentration of sodium chloride in the cell suspension is a primary factor influencing modification of EAT cells under the conditions employed, *viz.*, brief hypotonic shock. Moreover, the abrupt increase in number of modified cells at low concentrations of sodium chloride lends to the difficulty of standardizing the modification procedure. Also indicated is the apparent stability of the semipermeable character of the cell membrane of EAT cells over a relatively large range of sodium chloride concentrations. It is noteworthy that certain other cells, in contrast to EAT cells, are apparently adversely affected by even 0.85% (normal) saline, *e.g.*, tissue culture cells(4). The results obtained with EAT cells may be due, in part, to the relative impermeability of the tumor cells to sodium chloride(5).

Concentration of NaCl to stop modification. In the previous experiment, cells were modified in various concentrations of sodium chloride. In this experiment, the cells were modified in distilled water and various concentrations of sodium chloride were added to stop the modification of the cells. The purpose was to establish the fact that a certain minimal concentration of sodium chloride is required to prevent modification. Positive results would confirm the findings of the previous experiment.

Cells were modified as described in Methods, except that various concentrations of sodium chloride were used to stop the modification of cells, in place of double strength saline. Fig. 3 shows that a concentration of 3 mg/ml of sodium chloride was sufficient to stop the modification of the cells. This corresponds to the minimum concentration of sodium chloride that prevented modification in the previous experiment. These results also indicate that gross changes in the semipermeable nature of the EAT cell are manifested at, or below, a concentration of 3 mg/ml sodium chloride.

The ability of salt solutions to prevent modification of cells has also been shown to occur with plant cells. Thin slices of red beet root (*Beta vulgaris*) subjected to distilled water continued to leak red pigment for several hours, but the leakage stopped as soon as the slices were transferred to 0.1 to 0.2 molar sodium or potassium chloride solution(6).

Effect of time on modification. As would be expected, more and more cells are modified the longer the cells are kept in water during the modification procedure. However, the relationship between time and percent of modified cells is not linear, apparently because the amount of protein (ascitic fluid) and salts contained in the aliquot of cells to be modified tempers the number of cells that become modified in any period of time. Thus, it is necessary to wash the cells to obviate the variable effects of ascitic fluid carried with the cells.

Effect of washing the cells. Manipulation of the ascites tumor cells may also cause a change in cell membrane permeability. This

is probably due to the elution of low molecular weight cofactors and substrates from the cells so that the cells can no longer maintain the integrity of their semi-permeable membrane. Le Page(7) has shown that 5 washes (centrifuging and resuspending the cells) of EAT cells will deplete all or almost all of the intracellular free glycine. Fig. 4 shows that repeated washings of EAT cells in buffered saline result in an increasing number of cells which take up the nigrosin dye indicating a change in membrane permeability. The effect of time on modification of the cells and the effect of repeated washing on the cells remain empirical factors in the modification procedure, since both are influenced by the amount of protein and salts present in the aliquot to be modified. Studies are in progress to attempt control of these factors through use of a relatively inert suspending medium.

Of particular interest with regard to previous studies and to the results herein obtained is the fact that all cells in a given population were not similarly nor simultaneously affected by the experimental conditions employed. Inspection of the figures shows that only a certain percentage of the cells were affected as measured by dye (nigrosin) uptake, although all cells were simultaneously subjected to the same environment. The change in membrane permeability or the reaction of these cells to a particular environment appears to be dependent upon the cell *per se* and seems to vary from cell to cell. It is not possible at this time to definitely ascribe this phenomenon to any particular biochemical or physiological characteristic or function of the cell.

Summary. A study was made of factors involved in modifying the semi-permeable nature of the cell membrane of Ehrlich ascites tumor cells. It was found that a minimum concentration of 0.1% nigrosin was necessary for accurate differential quantitation of modified and unmodified tumor cells. The cells were not visibly affected by hypotonic saline solutions as low as 3.0 mg NaCl/ml. Lower concentrations resulted in changes in membrane permeability, and lysis occurred at concentrations of 1.0 mg/ml, or less. Not all cells of a given population were affected similarly by experimental hypotonic shock.

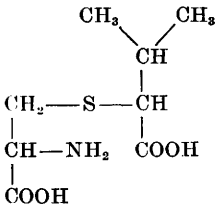
1. Kaltenbach, J. P., Kaltenbach, M. H., Lyons, W. B., *Exp. Cell Research,* 1958, v15, 112.
2. Kaltenbach, J. P., Kaltenbach, M. H., *Ann. N. Y. Acad. Sci.,* 1956, v63, 977.
3. Hanks, J. H., Wallace, J. H., PROC. SOC. EXP. BIOL. AND MED., 1958, v98, 188.
4. Pomerat, C. M., Overman, R. R., *Z. f. Zellforsch.,* 1956, v45, 2.
5. Lucke, B., Parpart, A. K., *Cancer Research,* 1954, v14, 75.
6. Ginzburg, B. Z., *Nature,* 1959, v184, 1073.
7. Le Page, G. A., *Cancer Research,* 1953, v13, 178.

Received March 30, 1961. P.S.E.B.M., 1961, v107.

A Convenient Preparation of Isovalthine.* (26635)

LEMUEL D. WRIGHT
Graduate School of Nutrition, Cornell University, Ithaca, N. Y.

Mizuhara *et al.* and Oomori and Mizuhara have recently described the discovery(1,2), isolation(1,2), identification(3), and synthesis(3) of a new amino acid, S-(isopropyl-carboxymethyl)-cysteine, to which they gave the name isovalthine. This amino acid was first observed in the urine of hypercholesterolemic individuals.



* Supported by research grants from Nat. Science Foundation and Nat. Inst. Health, and by funds made available through State Univ. of New York.