BIOCHEMICAL MEDICINE (1975) **13**, 164–177

Measures of Viability in Isolated Cells

FEDOR MEDZIHRADSKY AND MICHAEL J. MARKS

Departments of Biological Chemistry and Pharmacology, and Upjohn Center for Clinical Pharmacology, The University of Michigan Medical School, Ann Arbor, Michigan 48104

Received March 17, 1975

A well-recognized essential requirement of most biological investigations using cellular preparations is the assertion of cell viability. However, there seems to be little agreement on the criteria to be applied in establishing this cellular property. Cell viability has been assessed as morphological integrity and metabolic capacity, or interpreted as a specific cellular function, e.g., phagocytosis. If viability is identified as the ability of the cells to carry out a particular metabolic function, e.g., a biosynthetic reaction, cell integrity is of lesser importance in characterizing these cells. On the other hand, whenever the biological event under investigation is dependent upon an intact plasma membrane, assertion of this condition during the experiment is of primary importance in assuring an unequivocal interpretation of the data. The most widely applied criterion for investigation of cell permeability is the exclusion by cells of dyes with high molecular weights ("vital stains"), e.g., trypan blue or eosin (1,2).

In the course of our investigations of mechanisms of cellular drug transport (3–6) and in conjunction with recently developed procedures for the separation of human and rat blood cells (7,8), estimation of viability was carried out considering various criteria. Results of these experiments suggested that trypan blue was a relatively poor and insensitive indicator of the presence of a morphologically intact, nonleaking plasma membrane. In view of these findings and considering the increasing utilization of isolated cells in clinical investigations, it was of interest to reevaluate frequently applied measures of cell viability.

MATERIALS AND METHODS

Materials. The biochemicals used for determination of lactate dehydrogenase (LDH) were products of Boehringer-Mannheim Corp., New York, NY. Trypan blue and bovine serum albumin were obtained from Sigma Chemical Co., St. Louis, Mo. Plasmagel, a modified gelatin solution, was purchased from HTI Corp., Buffalo, NY. All other chemicals were of reagent grade.

Buffer medium. Throughout this study a buffer medium with the following composition (mM) was used: NaCl, 133; KCl, 5.3; $MgSO_4$, 1.3; Na_2HPO_4, 13.3; glucose, 10. The pH of the solution was adjusted to 7.4 with HCl.

Isolation of cells. Leukocytes were isolated from blood of male Sprague–Dawley rats weighing 300 g by a procedure described previously (7). Briefly, erythrocytes were separated from other blood cells by sedimentation in the presence of Plasmagel. Further separation of leukocytes and platelets was carried out by differential centrifugation.

Suspensions of rat spleen cells were obtained by mechanical disruption of the tissue. The tissue was precut with scissors, placed on 200 mesh stainless steel wire gauze, and gently "teased" through into ice-cold buffer medium using a smooth porcelain pestle.

Ehrlich ascites tumor cells were kindly provided by Dr. H. N. Christensen, Department of Biological Chemistry, The University of Michigan. These cells were collected from the abdominal cavity of mice 3 days after inoculation and washed thoroughly by resuspension and centrifugation (9).

Perturbation of cells. The effects of three different disruptive treatments on viability of the isolated cells were investigated: incubation with metabolic inhibitor, exposure to hypertonic conditions, and exposure to heat. In one line of experiments, cell suspensions were incubated with an equal volume of a NaF solution, prepared twice as concentrated as desired during incubation using the standard incubation medium in which the concentration of NaCl was decreased proportionally to the amount of NaF included. Final concentration of the inhibitor ranged from 2 to 60 mM; incubation time was 2 hours at 37°. In a different series of experiments, isolated cells were incubated at 37° for 15 minutes in the standard buffer medium made hypertonic by inclusion of appropriately higher concentrations of its components without changing their relative ratio. In selected experiments, suspensions of leukocytes were heated for 8 minutes at 80°, cooled to room temperature, and subjected to further analysis.

Uptake of trypan blue. The isolated cells, suspended in the standard buffer medium were incubated at 37° for 15 minutes, at which time a solution of trypan blue in the standard medium was added to a final concentration of 0.2%. The cells were subsequently incubated for an additional 15 minutes at 37°. Aliquots of the cell suspension were then subjected to microscopic examination and cytographic analysis.

Microscopic examination and cytographic analysis. Prior to and after incubation with trypan blue, the cells were examined microscopically under phase contrast using a Phasestar series 10 microscope, American Optical Co., Buffalo, NY. Quantitative characterization of the cells, i.e., determination of cell counts, including the percentage of stained cells,

was carried out electronically using a Cytograf, model 6301, Bio/Physics Systems, Inc., Mahopac, NY. Good correlation was obtained between the electronic and manual cell counts. The principle of operation of the Cytograf is the diversion of a laser beam focused on a capillary flow cell through which the suspension of cells passes in a monolaminar sheet (10,11). In providing cell counts, the Cytograf differentiates between cells excluding trypan blue and those stained by the dye. In addition to display of the cell counts of both normal and stained cells, the two cell populations are characterized on the oscilloscope by their different coordinates, resulting in two distinct clusters of dots, each dot representing a cell (Fig. 4). Typical settings of the Cytograf were: total population threshold, 20; radius (outer), 15; radius (inner), 100; radius balance, 50; slope (outer), 15; slope (inner), 100; slope balance, 50; horizontal gain, 55; vertical gain, 40. Minor changes of the above values were required in counting the individual cell lines and particularly when estimating stained cells within the total population. The selection of optimal settings for counting of a given dot cluster was considerably facilitated by use of the particle simulator, an integral part of the utilized instrument.

Determination of protein. Protein was determined according to Lowry *et al.* (12), using bovine serum albumin as standard.

Cellular contents of K^+ and Na^+. The cellular concentrations of these ions were determined by flame photometry as described previously (7,8). Briefly, aliquots of cell suspensions corresponding to 1 mg protein were centrifuged at 3000*g* for 3 minutes, the supernatants decanted, and the pellets resuspended with 0.32 M sucrose, the pH of which had been adjusted to 7.4 with Tris base. These suspensions were centrifuged at 8000*g* for 3 minutes, the washing procedure was repeated, and the cellular pellets digested with concd HNO_3. After the addition of LiCl as internal standard, K^+ and Na^+ were determined in an IL flame photometer, model 143, Instrumentation Laboratories, Inc., Lexington, Mass.

Release of cellular LDH. For determination of released enzyme activity subsequent to the various treatments described above, aliquots of the cells suspensions were centrifuged at 3000*g* for 7 minutes and the activity of LDH in the resulting supernatant determined. In control experiments the activity of total cellular LDH was estimated by subjecting cell suspensions to repeated freezing at −70° and thawing, followed by centrifugation and measurement of enzyme activity in the supernatant. The released enzyme activity was expressed as percentage of the total activity present in control cells which were not exposed to the outlined adverse conditions. The activity of LDH was determined by measuring the initial decrease of absorbance, reflecting the oxidation at 340 nm of reduced nicotinamide adenine dinucleotide (NADH). Final concentrations in the assay were (mM): phosphate buffer (pH 7.0), 95; sodium pyruvate, 0.8; NADH, 0.2. The total volume of the assay was 1 ml (8).

RESULTS

In this study, suspensions of isolated leukocytes, spleen cells, and Ehrlich cells were exposed to various concentrations of F^-, to hypertonic conditions, and in selected experiments to heat. The effects of these treatments on cell permeability were investigated by estimating cell count, uptake of trypan blue, release of cellular LDH, and by determining the cell contents of K^+ and Na^+.

Cell permeability after exposure to NaF. Incubation of the above listed cells with up to 60 mM NaF for 2 hours had little or no effect on the concentration of the cells as estimated by the electronic cell counts (Figs. 1–3).

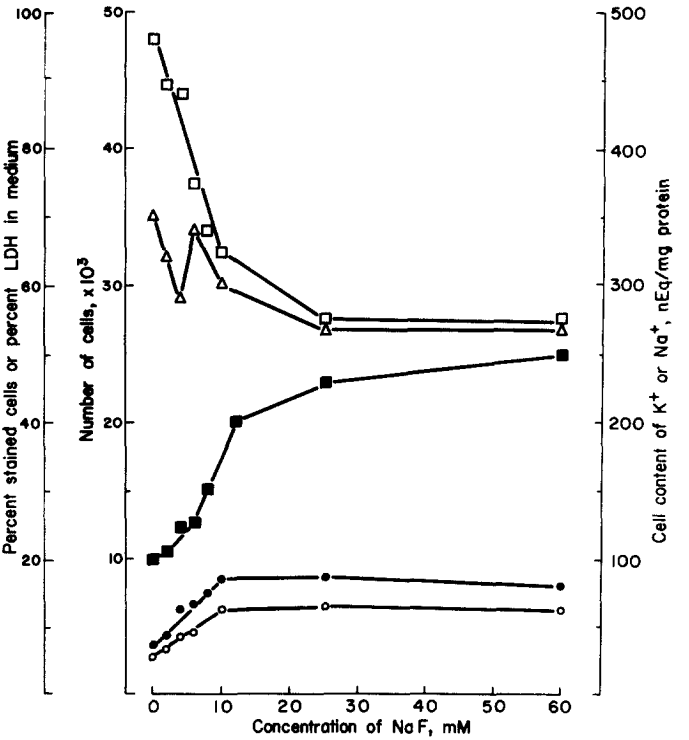

FIG. 1. Effect of NaF on leukocyte permeability. Rat leukocytes, suspended in the incubation medium described in the text, were incubated at 37° for 2 hours in the absence and presence of increasing concentrations of NaF as indicated. The tonicity, as well as the Na^+ content of the medium were kept constant by proportionally decreasing the NaCl concentration. Subsequently, the cell count (△), extent of cell staining by trypan blue (○), activity of LDH (●), and cellular concentrations of K^+ (□) and Na^+ (■) were determined as described in the text. Enzyme activity was measured in supernatants of the incubated cells separated by centrifugation, as well as after disruption of the cells by repeated freezing at −70° and thawing (total LDH activity). The enzyme activity in the supernatant of pretreated cells was expressed as percentage of the total activity of control cells. Presented are mean values of four experiments in which different preparations of cells were used. The standard deviation of the mean was less than ± 10%.

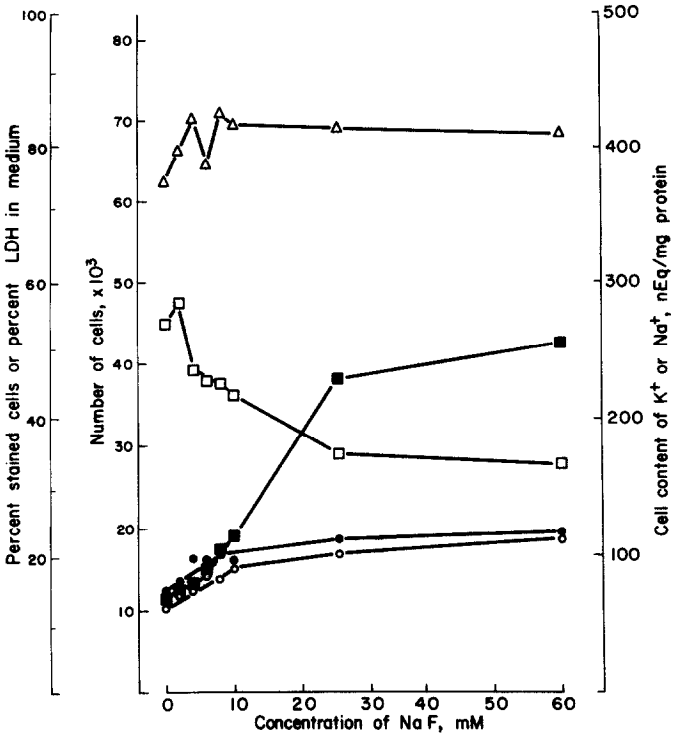

FIG. 2. Effect of NaF on permeability of spleen cell. Isolated rat spleen cells, suspended in the incubation medium described in the text, were treated and subsequently analyzed as described in the legend to Fig. 1; (△) cell count; (○) stained cells; (●) extracellular LDH; (□) cellular K^+; (■) cellular Na^+.

The number of cells unable to exclude trypan blue after exposure to rising concentrations of F^-, increased twofold in suspensions of leukocytes, was less than 1.5-fold when spleen cells were used and was virtually constant in experiments with Ehrlich cells (Figs. 1–3). The above changes occurred as a consequence of increasing the concentration of F^- up to 10 mM. Higher F^- content in the incubation medium had no further effect on cellular uptake of trypan blue. Differentiation of stained cells on the basis of their optical properties is illustrated in Fig. 4 by the oscilloscopic display, obtained after incubation of a control and a pretreated cell suspension with trypan blue. Uptake of the dye was reflected in altered coordinates of the stained cells on the oscilloscopic screen, leading to an accumulation of the dots representing these cells along the abscissa.

The pattern by which cellular LDH was released into the external medium, as a result of incubation with the metabolic inhibitor, was markedly similar to observed changes in the uptake of trypan blue. With

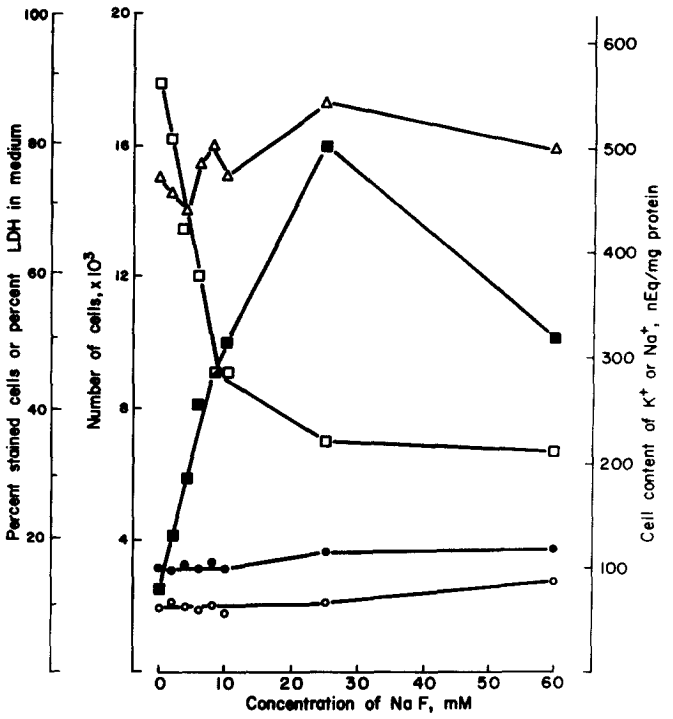

FIG. 3. Effect of NaF on permeability of Ehrlich cells. Harvested mouse Ehrlich cells, suspended in the incubation medium described in the text, were treated and subsequently analyzed as described in the legend to Fig. 1: (△) cell count; (○) stained cells; (●) extracellular LDH; (□) cellular K^+; (■) cellular Na^+.

concentrations of NaF increasing to 10 mM, the activity of LDH in the medium rose 2.5-fold with leukocytes, somewhat less than 1.5-fold in suspensions of spleen cells, and was unchanged in experiments using Ehrlich cells (Figs. 1–3). Again, higher concentrations of the metabolic inhibitor induced no additional changes.

Whereas NaF caused minor changes in dye uptake or in leakage of cytoplasmic enzyme, it markedly affected concentrations of cellular K^+ and Na^+. After exposure of the cells to 60 mM F^- for 2 hours, the ratio K^+/Na^+ in leukocytes, spleen cells, and Ehrlich cells decreased from 4.8 to 1.1, from 4.0 to 0.66, and from 7.6 to 0.7, respectively. As investigated with leukocytes, the effect of NaF could not be reversed by thorough washing of the cells. It is of interest to note the considerable changes in ion content of Ehrlich cells, in which this pretreatment had no effect on either the extent of cell staining or release of cellular LDH.

Cell permeability after exposure to hypertonic conditions. In general, the changes in the plasma membrane of cells induced by increased tonicity were more pronounced than those caused by NaF. Whereas the

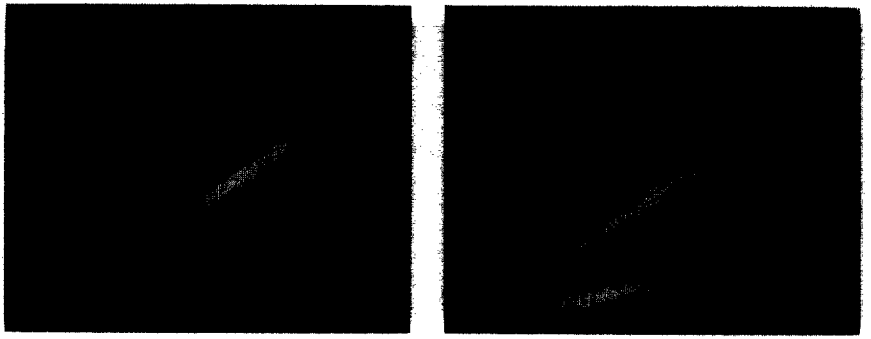

FIG. 4. Cytographic analysis of perturbed and control cells. Abscissa: amplitude of A pulse; ordinate: amplitude of S pulse. Displayed is the oscilloscopic dot pattern of a suspension of rat spleen cells incubated for 15 minutes at 37° with 0.2% trypan blue after initial incubation at 37° for 2 hours without (left) and with (right) 60 mM NaF. Whereas sensor A was insensitive to the presence of absorbing material in the cells, sensor S was affected by light passing through the cells. Thus lightabsorbing material in the cells (i.e., trypan blue) reduced the pulse generated by sensor S while not appreciably affecting the amplitude of the pulse generated by sensor A. The population of stained cells which accumulated along the abscissa (right) corresponded to 25% of the total number of cells present. The settings of the Cytograf, model 6301, were as outlined in Materials and Methods.

cell count after incubation with the latter compound remained essentially constant, a decreasing number of cells were counted in suspensions of all three cell types subsequent to hypertonic exposure. Following incubation for 15 minutes in a solution with a tonicity fourfold that of the isotonic medium, the cell count of both leukocytes and spleen cells decreased by 35% (Figs. 5 and 6). The corresponding change in Ehrlich cells was smaller; only 9% fewer cells were counted after the hypertonic treatment (Fig. 7).

As a consequence of a tonicity of up to fourfold that of the isotonic concentration, uptake of trypan blue occurred in relatively few cells (Figs. 5–7). After exposure to the highest tonicity, the percentage of stained leukocytes rose from 8 to 15 (Fig. 5). The change in uptake of trypan blue by cells from the spleen was even less pronounced, increasing from 8 to 11% (Fig. 6), whereas the number of stained Ehrlich cells remained virtually unchanged (Fig. 7).

The pattern by which cellular LDH was released after exposure to hypertonicity was similar to that obtained for the uptake of trypan blue. However, the extent of changes in release of cellular LDH as a consequence of hypertonicity were in general somewhat greater relative to those observed in uptake of the dye. The percentage of LDH in the incubation media of both leukocytes and spleen cells increased from 5.5 to 22 (Figs. 5 and 6). Corresponding to the limited decrease in cell count, retention of LDH by Ehrlich cells was greater; increase of this en-

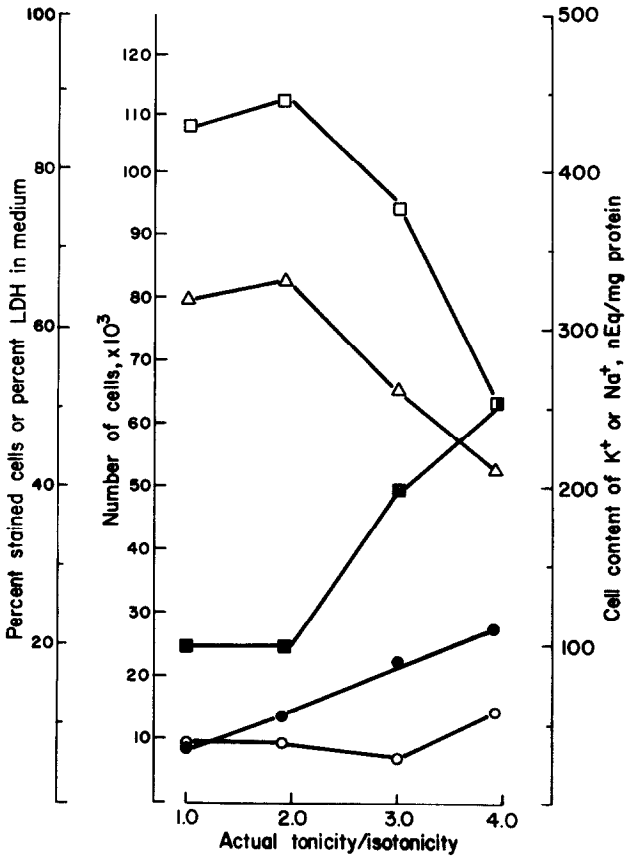

FIG. 5. Effect of hypertonicity on permeability of leukocytes. Isolated rat leukocytes were incubated at 37° for 15 minutes in media of increasing tonicity. The latter solutions were prepared by proportionally increasing the concentration of components in the standard buffer medium described in the text. The pH of the media was adjusted to 7.4. Subsequently, the cell count (△), extent of cell staining by trypan blue (○), activity of LDH (●), and the cellular contents of K^+ (□) and Na^+ (■) were determined as described in the text. Enzyme activity was expressed as outlined in the legend to Fig. 1. Presented are mean values of four experiments using different preparations of cells. The standard deviation of the mean was less than ± 10%.

zymatic activity in the external medium of pretreated cells was only between 10 and 12% (Fig. 7).

High tonicity caused rapid and extensive changes in the K^+ and Na^+ contents of all three cell types (Figs. 5–7). In leukocytes and spleen cells the steep decline of cellular K^+ began as the concentration in the medium increased more than twofold above isotonicity (Figs. 5 and 6). Moreover, in Erhlich cells a significant drop in concentration of K^+ occurred at even slight hypertonicity (Fig. 7). Depletion of K^+ was accom-

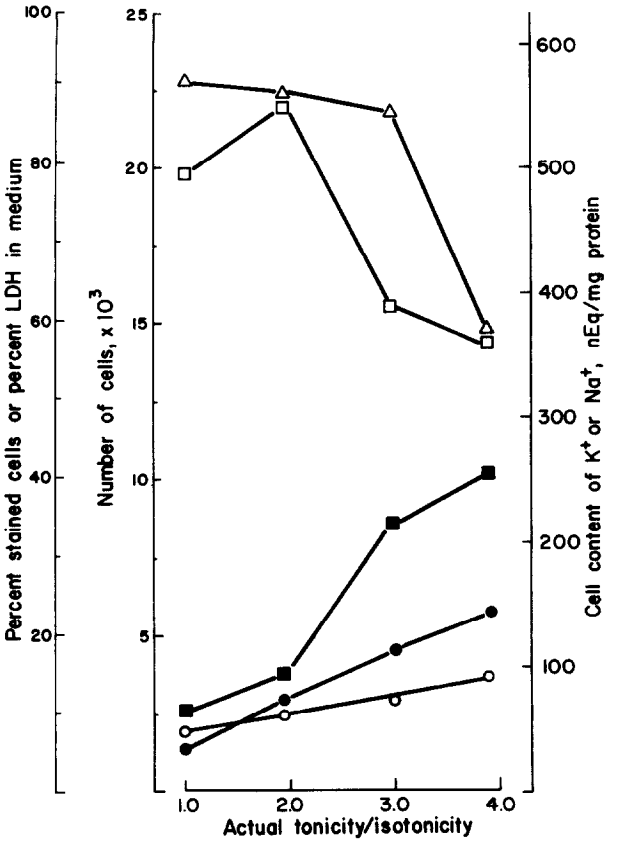

FIG. 6. Effect of hypertonicity on permeability of spleen cells. Isolated rat spleen cells were incubated and subsequently analyzed as described in the legend to Fig. 5. (△) Cell count; (○) stained cells; (●) extracellular LDH; (□) cellular K^+; (■) cellular Na^+.

panied by an increase in cellular Na^+. With increasing tonicity, the ratio K^+/Na^+ in leukocytes, spleen cells, and Ehrlich cells declined from 7.6 to 1.4, from 4.4 to 1.0, and from 9.0 to 0.6, respectively.

Effect of heat treatment. In order to evaluate the effect of drastic conditions on some of the here investigated cellular parameters, suspensions of leukocytes were heated for 8 minutes at 80°. Whereas a drop of only 13% in the cell count was found to occur, more than 95% of the cellular LDH was released into the incubation mixture.

DISCUSSION

Assessment of the ability to carry out specific biologic functions is an essential step in characterizing isolated cells, and various methods have been used to ascertain this property of isolated cells. The applied criteria

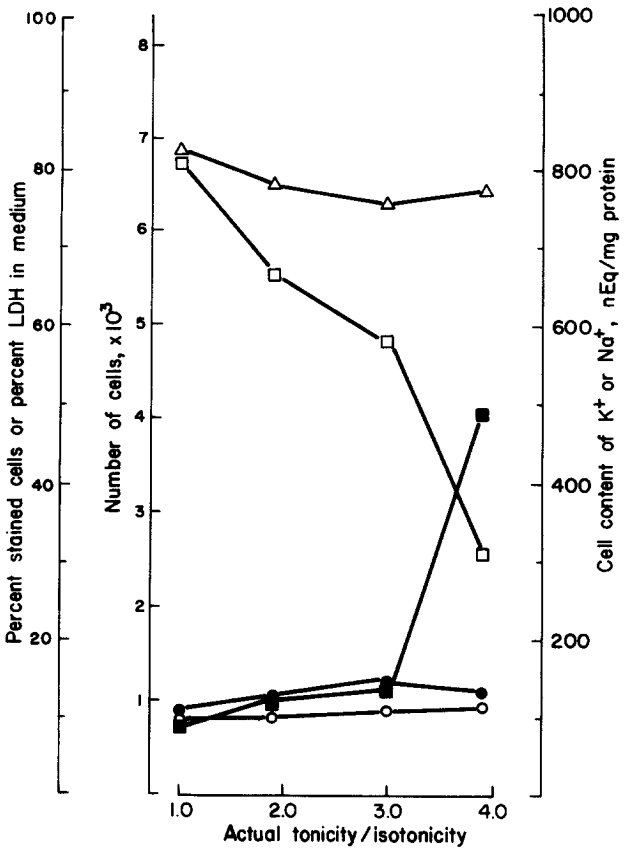

FIG. 7. Effect of hypertonicity on permeability of Ehrlich cells. Harvested mouse Ehrlich cells were incubated and subsequently analyzed as described in the legend to Fig. 5. (△) Cell count; (○) stained cells; (●) extracellular LDH; (□) cellular K^+; (■) cellular Na^+.

include investigation of cell motility in phagocytosis (13,14), light or electronmicroscopic examination (14–17), measurement of oxygen uptake (13–20), estimation of various enzyme activities (14–17, 19, 21), incorporation of radiolabeled amino acids into cell protein (18,20,22), and estimation of levels of NADH during aerobic and anaerobic metabolism (18). However, interpreting viability as cell integrity, the most frequently applied parameter to ascertain presence of live cells is the exclusion by the latter of dyes, such as trypan blue. Observations during a previous study in which we investigated the viability of isolated blood cells by several criteria, including the cellular uptake of trypan blue (7,8), led us to conclude that the latter test might not accurately reflect the permeability of the cell membrane toward a large number of biologically important, low molecular weight compounds. In the present study

the efficiency of the trypan blue test was compared with other parameters of cell permeability in isolated leukocytes, spleen cells, or Ehrlich cells in response to adverse conditions.

As a result of pretreatment with NaF, the cell count of either of the cells changed little. Exposure of the cells to hypertonic conditions caused a more pronounced decline in cell count, except for Ehrlich cells, in which the decrease after exposure to maximal tonicity was only 9%, as opposed to a drop of up to 35% in the number of leukocytes and spleen cells. It is of interest to note the limited change in cell count of leukocytes after their exposure to heat. On the other hand, under identical conditions most of the cellular LDH was released into the incubation medium. These findings underline the ambiguous value of cell counts in assessing cell integrity.

Incubation of the cells with NaF resulted in rather restricted increases in the extent of cellular uptake of trypan blue. Similarly, only limited responses were observed in release of the cytoplasmic enzyme LDH, the assay of which in the external medium was used to indicate a leaking plasma membrane. Even after exposure of the cells to a tonicity of up to fourfold that of normal, increases in the number of stained cells as well as in released LDH in suspensions of all three cell types were only moderate. The highest uptake of the dye, obtained in leukocytes and spleen cells at concentrations of the external medium corresponding to four times that of the isotonic composition, was 22%. Interestingly, Ehrlich cells were again, similar to the findings for cell count, more resistant to adverse conditions; maximum responses in both cell staining and LDH release were limited to about 12%. Reported percentages of various freshly isolated cells stained by trypan blue range from 1 to 5% (15,22), to 20 to 30% (20).

In contrast to the rather limited changes of the cellular parameters which indicate passage across the plasma membrane of compounds with high molecular weights, contents of both K^+ and Na^+ in all three investigated cell types responded markedly to the adversity of external conditions. Incubation of either of the cells with 10 mM NaF, a treatment which induced little change of the other two investigated parameters, strongly altered the cellular concentrations of these two ions, leading to considerable decrease in the value of the ratio, cellular K^+/Na^+. This decrease was primarily due to a decline in the potassium content of the cells, which continued decreasing as a function of increasing concentrations of the metabolic inhibitor in the suspending medium. Similar responses were found after exposure of the cells to hypertonicity. It is of significance that in Ehrlich cells, which were quite resistant to the latter pretreatment as determined by the moderate changes in cell count, cell staining, and release of LDH, the ratio K^+/Na^+ decreased substantially In addition to early onset of the responses to cellular ion content as com-

pared to dye uptake or LDH release (e.g., Fig 7), the difference in the extent of changes should be emphasized. Whereas the number of stained leukocytes increased from 8 to 15% after exposure to a medium with a tonicity fourfold that of normal, the ration K^+/Na^+ under these conditions fell from 7.6 to 0.9 and the cellular K^+ content declined from 500 to 85 nEq/mg protein (Fig. 5).

On the basis of the data accumulated in this study, the ratio of cellular K^+ to Na^+ or, with increased efficiency, the concentration of cellular K^+, proved to be the optimal indicator of alterations in plasma membrane integrity. On the other hand, dye uptake, the often applied criterion of cell viability, and a test frequently used to indicate the presence of "intact" cells, was a rather poor index of changes in cell permeability. Various other measures of viability of isolated cells, including cell staining and retention of cellular LDH, have been proposed and applied (23). There has been no comprehensive study relating all these cellular parameters. In characterizing isolated human and rat blood cells, we previously estimated cell count, trypan blue exclusion, and oxygen uptake, as well as cell contents of ATP, K^+, and Na^+ (7). These parameters remained essentially constant during a prolonged incubation of the isolated cells; however, no attempt was made to induce changes. Tullis (24) compared several methods for evaluating the viability of isolated leukocytes. Results of that study indicated that oxygen consumption was the most sensitive indicator of cell viability, followed in descending order by phagocytic ability, ameboid motility, brownian motion, resistance to impermeable dyes and finally, morphological integrity. However, if viability is interpreted as existing cell integrity, then respiration, which can continue in disrupted cellular preparations, cannot be a measure of the aforesaid cellular property. Likewise, it seems unreasonable to expect that exclusion or retention of compounds with molecular weights of about 900 (trypan blue) and 135×10^3 (LDH) should necessarily be representative indicators of possible leakage of smaller cellular components.

In addition to the sensitive and marked response of cellular K^+ to adverse conditions, its importance in growing cells is well recognized (25,26). Also well documented is the major role of this ion in maintaining the potential difference across the plasma membrane, typical for most living cells and closely related to their function (25,27). Thus the cellular concentration of K^+, in addition to reflecting membrane integrity, can serve as an index of metabolic conditions within the cell.

SUMMARY

Various methods were compared for estimation of cell viability. Cell count, uptake of trypan blue, release of cellular LDH, as well as cell contents of K^+ and Na^+ were determined in leukocytes, spleen cells, and Ehrlich cells exposed to adverse conditions. After incubation of these

cells with NaF, the cell count remained essentially constant; the extent of cell staining and release of LDH increased to a limited level in suspensions of leukocytes and spleen cells and was virtually unchanged in experiments with Ehrlich cells. On the other hand, pretreatment with NaF induced rapid and marked changes in contents of K^+ and Na^+ in all three cell types. Exposure of the cells to hypertonic conditions caused more pronounced, but similar overall effects. As a consequence of increased tonicity, the cell count decreased and considerable leakage of LDH occurred in all the cells investigated. Whereas the contents of K^+ and Na^+ in all pretreated cells changed markedly, the cellular uptake of trypan blue was again the least responsive index of cell permeability. The results showed cell staining by trypan blue to be a relatively poor measure of cell viability and suggest use of the ratio cellular K^+/Na^+ or, even more efficiently, the cell content of K^+ as sensitive indicators of plasma membrane integrity.

ACKNOWLEDGMENT

This work was supported in part by the United States Public Health Service Grants 5P11 GM15559 and R01-DA-00254.

REFERENCES

1. Pappenheimer, A. M., *J. Exp. Med.* **25,** 633 (1917).
2. Hanks, J. H., and Wallace, J. H., *Proc. Soc. Exp. Biol. Med.* **98,** 188 (1958).
3. Medzihradsky, F., Marks, M. J., and Carr, E. A., Jr., *Biochem. Pharmacol.* **21,** 1625 (1972).
4. Medzihradsky, F., Marks, M. J., and Metcalfe, J. I., *Advan. Biochem. Psychopharmacol.* **8,** 537 (1973).
5. Marks, M. J., and Medzihradsky, F., *Mol. Pharmacol.* **10,** 837 (1974).
6. Marks, M. J., and Medzihradsky, F., *Biochem. Pharmacol.* **23,** 2951 (1974).
7. Medzihradsky, F., Marks, M. J., and Metcalfe, J. I., *Biochem. Med.* **10,** 153 (1974).
8. Medzihradsky, F., and Metcalfe, J. I., *J. Lab. Clin. Med.* **85,** 342 (1975).
9. Inui, Y., and Christensen, H. N., *J. Gen. Physiol.* **50,** 203 (1966).
10. Melamed, M. R., Kamentsky, L. A., and Boyse, E. A., *Science* **163,** 285 (1969).
11. Bio/Physics Systems, Inc., "Cytographic Analysis," Mahopac, New York, 1971.
12. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J., *J. Biol. Chem.* **193,** 265 (1951).
13. Rabinowitz, Y., *Blood* **23,** 811 (1964).
14. Lentz, P. E., and DiLuzio, N. R., *Exp. Cell Res.* **67,** 17 (1971).
15. Fallon, H. J., Frei, E., III, Davidson, J. D., Trier, J. S., and Bark, D., *J. Lab. Clin. Med.* **59,** 779 (1962).
16. Rose, S. P. R., *Biochem. J.* **102,** 33 (1967).
17. Suzanger, M., and Dickson, J. A., *Exp. Cell. Res.* **63,** 353 (1970).
18. Hommes, F. H., Draisma, M. I., and Molenaar, I., *Biochem. Biophys. Acta* **222,** 361 (1970).
19. Rose, S. P. R., and Sinha, A., *Life Sci.* **9,** 907 (1970).
20. East, A. G., Louis, L. N., and Hoffenberg, R., *Exp. Cell Res.* **76,** 41 (1973).
21. Leise, E. M., Morita, T. N., Gray, I., and LeSane, F., *Biochem. Med.* **4,** 327 (1970).
22. Pertoft, H., Back, O., and Kiessling-Lindahl, K., *Exp. Cell Res.* **50,** 355 (1968).

23. Rose, S. P. R., *in* "Applied Neurochemistry" (A. N. Davison and J. Dobbing, Eds.), pp. 332–355. F. A. Davis, Philadelphia, 1968.
24. Tullis, J. L., *Blood* **8,** 563 (1953).
25. Steinbach, H. B., *Perspect. Biol. Med.* **5,** 338 (1962).
26. Lubin, M., *in* "The Cellular Functions of Membrane Transport' (J. F. Hoffman, Ed.), pp. 193–211. Prentice-Hall, Englewood Cliffs, 1964.
27. Schoffeniels, E., "Cellular Aspects of Membrane Permeability." Pergamon Press, Oxford, 1967.