Proc. Natl. Acad. Sci. USA
Vol. 78, No. 1, pp. 363–367, January 1981
Cell Biology

Hoechst 33342 dye uptake as a probe of membrane permeability changes in mammalian cells

(flow cytometry/membrane transport/colchicine resistance/murine lymphocytes)

MARC E. LALANDE, VICTOR LING, AND RICHARD G. MILLER

The Ontario Cancer Institute and the Department of Medical Biophysics, University of Toronto, 500 Sherbourne Street, Toronto, Ontario, Canada M4X 1K9

Communicated by J. Tuzo Wilson, October 6, 1980

ABSTRACT **Flow cytometric analysis of the uptake of the DNA-specific and fluorescent probe Hoechst 33342 (HO342) offers a simple and rapid method for measuring membrane transport rates in mammalian cells and identifying cellular subpopulations that differ in their membrane transport rates. In a Chinese hamster ovary wild-type cell line and in three colchicine-resistant lines derived from it, the rate of uptake of HO342 dye decreases as the stepwise resistance to colchicine increases. A colchicine-sensitive revertant cell line shows an increased rate of dye uptake. Drug resistance in these lines has previously been shown to be related to changes in transport rate. In a manner similar to colchicine, the rate of uptake of HO342 dye shows nonsaturation kinetics. The effect of KCN, a metabolic inhibitor, on HO342 dye uptake, both in the presence and in the absence of glucose, is similar to that previously observed for colchicine uptake. When murine spleen cells are stained with HO342 under appropriate conditions, one sees two populations of lymphocytes differing in HO342 fluorescence intensity, a difference not related to DNA content. The two subpopulations show the same relative difference in both colchicine and HO342 uptake. HO342 dye appears, therefore, to enter mammalian cells by the same mechanism as colchicine—i.e., by unmediated diffusion—and can be used as a probe of cytoplasmic membrane permeability. Potential applications of the dye in studies of drug resistance, detection of activated T cells, and recognition of lymphocyte subpopulations are discussed.**

We describe a sensitive and rapid method for assessing membrane permeability by using a flow cytometer (1) and the bisbenzimidazole dye Hoechst 33342 (HO342) (2). This dye binds both specifically and quantitatively to DNA and, once bound, becomes strongly fluorescent (3). Unlike other DNA-specific fluorescent dyes (4), HO342 is readily taken up by living cells and is nontoxic (5). Although HO342 can be used for flow cytometric (FCM) analysis of DNA content of viable cells (5, 6), HO342 stained murine lymphocyte populations show differences in fluorescence intensity not related to DNA content (7). An attempt to understand these differences led to the present study, in which we conclude that they are due to differences in the rate of HO342 transport across the cytoplasmic membrane. Ling and Thompson (8) have isolated a series of Chinese hamster ovary (CHO) cell lines showing various resistances to the drug colchicine and have shown that the extent of resistance correlates directly with a reduction in drug permeability. Subsequent studies (9) showed that the kinetics of colchicine uptake are characteristic of an unmediated diffusion process. We show here that viable CHO cells acquire HO342 fluorescence at a rate inversely related to their degree of colchicine resistance. Thus HO342 probably enters CHO cells via the same mechanism as colchicine; moreover, we show that colchicine enters murine lymphocytes at a rate proportional to the rate of HO342 fluorescence increase.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U. S. C. §1734 solely to indicate this fact.

MATERIAL AND METHODS

Cell Lines and Culture Conditions. Stock cultures of the wild-type CHO cell line Aux B1, the colchicine-resistant mutant lines CH^RA3, CH^RB3, and CH^RC5 (8), and the revertant line I10-1 isolated from CH^RC5 (10) were maintained in monolayer in plastic tissue culture flasks (Falcon Plastics, Oxnard, CA) containing α medium (11) supplemented with 10% fetal calf serum and antibiotics (streptomycin and penicillin). One day prior to FCM analysis, the cells were treated with trypsin, transferred to glass roller tubes, and grown in suspension at 37°C.

Spleen Cell Suspensions. Cell suspensions from 8- to 12-week-old female RNC mice were prepared by pressing the organs through a wire mesh (60 gauge).

Cell Staining Procedures. Cells (10^6 per ml) were incubated at 37°C in α medium, prewarmed to 37°C, and containing HO342 dye. Dye concentrations and incubation times varied as indicated in the text. After incubation, cells were collected by centrifugation, resuspended in cold (4°C) phosphate-buffered saline and placed on ice for subsequent FCM analysis. The presence of serum in dye-containing medium was found to decrease the rate of uptake of HO342 dye and, hence, all incubations were performed in serum-free medium.

Instrumental Procedures. The flow cytometer–cell sorter used in these studies was designed and built at the Ontario Cancer Institute (12). Our instrumentation and procedures are basically similar to those in widespread use elsewhere (13). Cells were illuminated with a Spectra Physics 164-05 argon ion laser adjusted to emit 50 mW at the combined 351- and 364-nm lines. Fluorescence emission from HO342-stained cells was passed through a Schott KV-418-nm shortwave-cutoff filter. Dead cells can be detected on the basis of their decreased forward light scattering ($\pm 12°$ half angle) and were excluded by setting the window of the light scatter single-channel analyzer to accept only pulses above the dead cell region of the scatter spectrum. In this way, all fluorescence spectra included in this report, except those of Fig. 2, represent the fluorescence from viable cells only. Changes in instrument gain can be accounted for within individual experiments by analyzing a sample at the different gain settings. Fluorescence intensities between experiments cannot, however, be directly compared because no appropriate fluorescence reference standard exists at present. The fluorescence spectra obtained by FCM analysis were stored in a Digital PDP-11/10 computer and the modal fluorescence intensities of the observed peaks were estimated by Gaussian fit. Bidirectional cell sorting was performed using a recently built set of sorting logic circuitry (unpublished).

Abbreviations: HO342, Hoechst 33342 dye; FCM, flow cytometric; CHO, Chinese hamster ovary; LI and HI, low-intensity and high-intensity fluorescence populations.

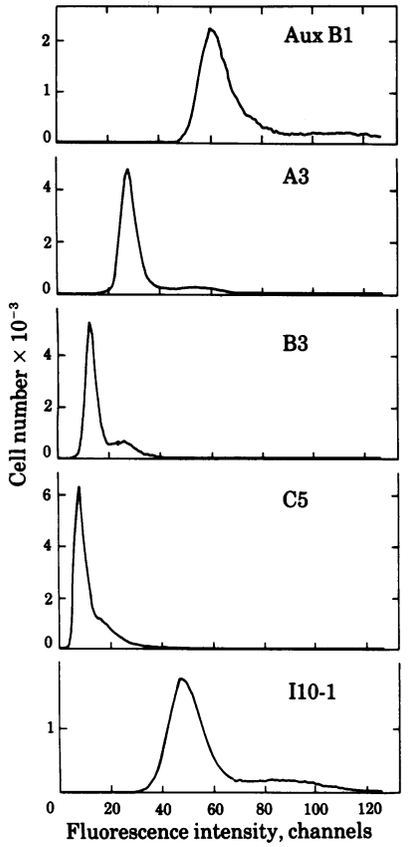

FIG. 1. FCM analysis of HO342-stained unfixed CHO cells. Cells from the different CHO cell lines were incubated in α medium containing 10 μM HO342 for 30 min at 37°C. The modal fluorescence intensities of the G_1 peaks, as estimated by Gaussian fit, are 61.6 (Aux B1), 38.1 (A3), 13.4 (B3), 8.3 (C5), and 49.4 (I10-1). The mean standard error of the estimates of the modal intensities for all experiments (Figs. 1 to 8) is 1.0 ± 0.2%. The abscissa is a linear (128-channel) scale of fluorescence intensity.

Drugs and Chemicals. HO342 dye was a kind gift from H. Loewe, Hoechst, Frankfurt, Federal Republic of Germany. Radioactive colchicine (*ring C methoxy*-^{3}H) in benzene/ethanol with a specific activity of 5 Ci/mmol (1 Ci = 3.7×10^{10} becquerels) was obtained from New England Nuclear. Potassium cyanide was purchased from Fisher and D-glucose was obtained from British Drug House, Toronto, ON, Canada.

RESULTS

Flow Cytometry of HO342-Stained CHO Cells. Fig. 1 shows FCM spectra for five CHO cell lines viably stained with HO342: wild type, three colchicine-resistant lines, and a revertant line. The three colchicine-resistant mutant lines—CH^RA3 (A3), CH^RB3 (B3), and CH^RC5 (C5)—were derived from the wild type, Aux B1, by a step-wise selection procedure (8) and display increasing resistance to colchicine (see Table 1). The revertant line, I10-1, was isolated from the CH^RC5 line (10) and has recovered nearly full drug sensitivity. Each spectrum of Fig. 1 is a DNA histogram whose dominant feature is the G_1 peak. The modal fluorescence intensity of this peak decreases in step with increasing resistance to colchicine (Fig. 1 and Table 1). In Fig. 2, the results of staining fixed cells of the Aux B1, C5, and I10-1 lineages with HO342 are shown. In this case, the modal fluorescence intensity of the G_1 peak is essentially the same in all cases, indicating that the three cell lines have the same G_1 DNA content. Therefore, the reduction in HO342 fluorescence staining intensity of the mutants compared to the wild-type cells does not reflect a difference in DNA content but may be associated with the mechanism of resistance to colchicine—i.e., reduced permeability.

Table 1. Resistance to colchicine and decrease in HO342 fluorescence intensity

Cell line	Relative resistance to colchicine*	Relative decrease in HO342 fluorescence†
Aux B1	1.0	1.0
CH^RA3	6.0	2.1
CH^RB3	21.0	4.6
CH^RC5	184.0	7.4
I10-1	3.0	1.3

* See ref. 10.

† Ratio of modal fluorescence intensities (Fig. 1) of the G_1 peak for Aux B1 to the modal intensity of the cell line being examined.

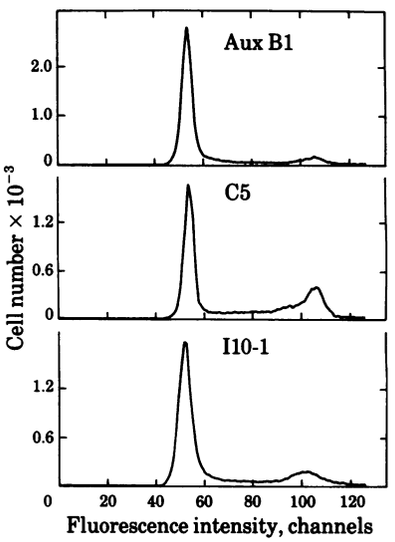

FIG. 2. FCM analysis of HO342-stained fixed CHO cells. After fixation in 70% (vol/vol) ethanol for 30 min, CHO cells were collected by centrifugation, resuspended in phosphate-buffered saline containing 10 μM HO342, and placed on ice for 30 min. The modal intensities of the G_1 peaks, as determined by Gaussian fit, are 53.8, 54.3, and 52.2 for the Aux B1, C5, and I10-1 cells, respectively.

Uptake Kinetics of HO342 by CHO Cells. We first measured the uptake of HO342 by Aux B1 cells as a function of time. Cells were incubated for various periods of time at 37°C in me-

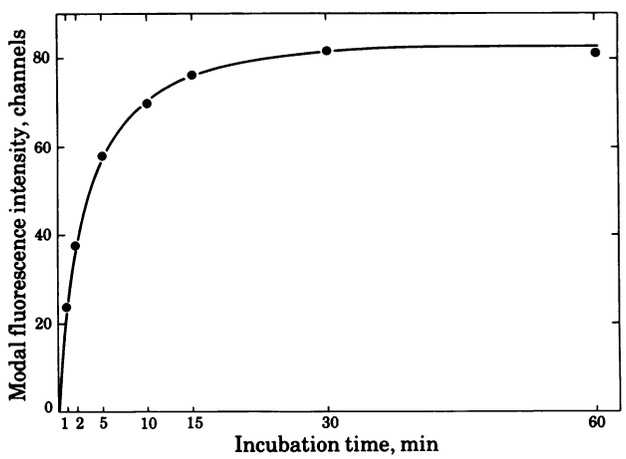

FIG. 3. Time course of uptake of HO342 by Aux B1 cells. Aux B1 cells were suspended in α medium containing 5 μM HO342 at 37°C and aliquots were removed after various periods of time (1–90 min). The modal intensities of the G_1 peaks were determined by FCM analysis.

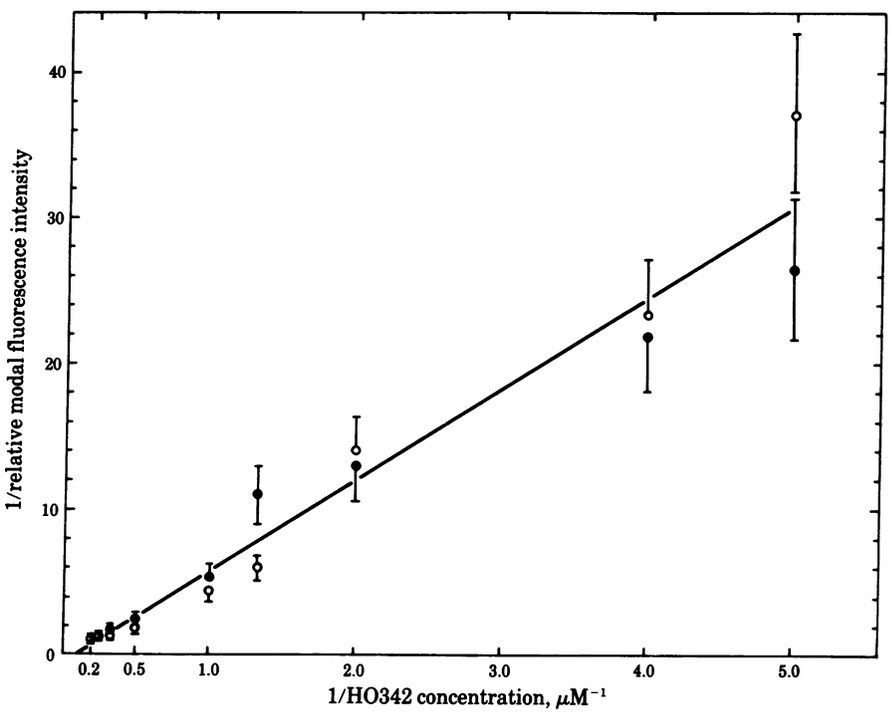

FIG. 4. Lineweaver–Burk plot of HO342 uptake in Aux B1 cells. Samples of Aux B1 cells were suspended for 1 min at 37°C in α medium containing increasing concentrations of HO342 (0.2–5 μM). The modal intensity of the G_1 peak measured at the 5 μM dye concentration was assigned a value of 1 and measurements obtained at the lower dye concentrations were plotted relative to this value. The results from two different experiments are shown. Error bars are 1 SD. The straight line is a result of linear regression analysis ($r^2 = 0.96$) and has a y axis intercept of -0.504 ± 0.781.

dium containing 5 μM dye and the fluorescence intensity of the G_1 peak was measured (Fig. 3). This fluorescence intensity increases rapidly, reaching a plateau within 30 min of incubation. Similar results were obtained for the mutant A3 but with the plateau being reached in 90 min rather than 30 min (data not shown). The rate of increase appears to be nearly linear up to 2 min, 1 min being the shortest time for which accurate data could be obtained.

The effect of concentration on the rate of uptake of HO342 was measured by incubating Aux B1 cells for 1 min in medium containing HO342 dye in various concentrations from 0.2 to 5 μM and then measuring the fluorescence intensity of the G_1 peak. The data, when analyzed on a Lineweaver–Burk plot (Fig. 4), fit a straight line whose intercept is not significantly different from zero. Thus, no evidence of saturation is seen, consistent with an unmediated diffusion process. Carlsen *et al.* (9) obtained similar results for colchicine.

Effect of Cyanide on Uptake of HO342. As another approach to determine whether the uptake of HO342 into viable cells was mediated by the same mechanism as that previously observed for colchicine, we examined the effect of a metabolic inhibitor, KCN, on the kinetics of dye uptake both in the presence and in the absence of glucose. Metabolic inhibitors such as KCN stimulate the colchicine uptake of CHO cells by increasing membrane permeability to the drug, an increase that can be prevented by the addition of glucose (15). These effects are more marked for the membrane mutant lines, indicating that resistance to colchicine in CHO cells involves an energy-dependent barrier (15). The uptake of HO342 is affected in the same way (Fig. 5). The fluorescence intensity of the G_1 peak of C5 cells incubated for 10 min in 2 μM HO342 is 20-fold more intense when 2 mM KCN is included in the incubation mixture (compare Fig. 5 *A* and *B*) but is little changed when glucose is also included (Fig. 5*C*). In Fig. 5*D*, the cells were incubated for 20 min in 2 mM KCN and 2 μM HO342,—i.e., for twice as long as in Fig. 5*B*. The fluorescence increased markedly. When, however, cells were incubated as above for 10 min in KCN plus HO342 but then 15 mM glucose was added before the incubation was continued for a further 10 min, the results of Fig. 5*E* were obtained. The addition of glucose not only prevented a further cyanide-dependent increase in fluorescence intensity (Fig. 5*D*) but also resulted in a loss of fluorescence intensity relative to the value obtained at the end of the 10-min incubation in the presence of cyanide (Fig. 5*B*). The C5 cells, therefore, seem to possess an energy-dependent mechanism for exporting the dye.

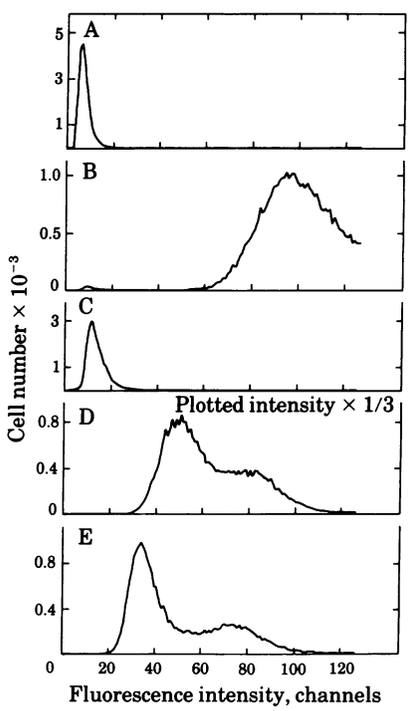

FIG. 5. Effect of cyanide on uptake of HO342 by CH^RC5 cells. CH^RC5 cells were suspended for 10 min at 37°C in phosphate-buffered saline containing 2 μM HO342 (*A*) and either 2 mM KCN (*B*) or 2 mM KCN plus 15 mM glucose (*C*). After the 10-min incubation in dye and KCN, either the incubation was prolonged for 10 min in 2 mM KCN (*D*) (analyzed at 1/3 the instrumental gain) or a sample was removed and analyzed 10 min after glucose was added to a final concentration of 15 mM (*E*).

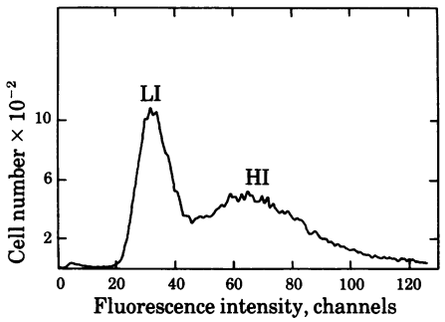

FIG. 6. FCM analysis of HO342-stained murine lymphocytes. Spleen cells were suspended in α medium containing 2.5 μM HO342 for 10 min at 37°C. LI and HI refer to the low- and high-intensity fluorescence peaks, respectively.

Uptake of HO342 by Murine Lymphocytes. Fig. 6 shows that viable murine spleen cells stained with HO342 dye contain a low-intensity (LI) and high-intensity (HI) fluorescence population. We have shown (7) that this is not related to a difference in DNA content. It may, however, be a result of a difference in permeability to the Hoechst dye.

The fluorescence intensity of the LI and HI peaks with increasing incubation time in 2.5 μM HO342 dye is shown in Fig. 7. Both peaks reach the same plateau of fluorescence intensity, the HI peak within 60 min and the LI within 90 min. Fluorescense intensity after 2 min of staining as a function of dye concentration is shown in Fig. 8, plotted as a Lineweaver–Burk plot. The curves are linear, as was also seen for CHO cells (Fig. 4). These results suggest that the uptake of HO342 in murine lymphocytes is mediated by a mechanism similar to that of colchicine or HO342 in CHO cells. To investigate further this similarity, murine spleen cells were incubated in medium containing 5 μM HO342 and radioactive colchicine. The cells of the LI and HI fluorescence peaks were sorted simultaneously by using the cell sorting capabilities of our instrument. Radioactive counts incorporated by the cells from each of the sorted fractions were then determined by standard methods (8). The results are presented in Table 2. The cells of the HI peak incorporated 2.5 times more radioactive colchicine during the incubation than did the cells of the LI peak. The ratio of the HO342 fluorescence intensity of the HI to the LI peak was 1.9, as estimated by taking the ratio of the slopes of the uptake

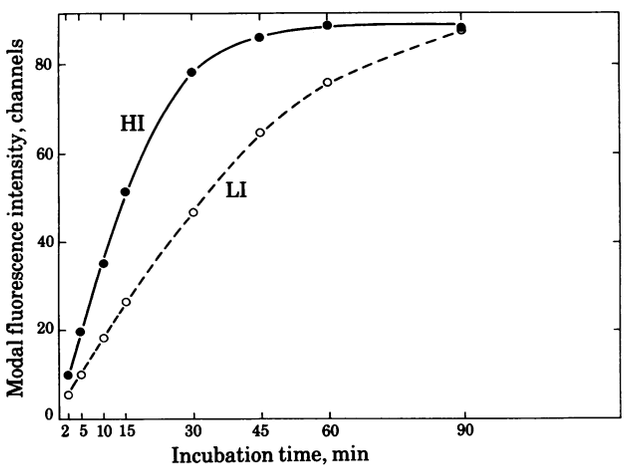

FIG. 7. Time course of HO342 uptake by murine lymphocytes. Spleen cells were suspended in α medium containing 2.5 μM HO342 at 37°C and samples were removed after various periods (2–90 min) of incubation. The modal intensities of the HI (●) and LI (○) peaks were measured by FCM analysis.

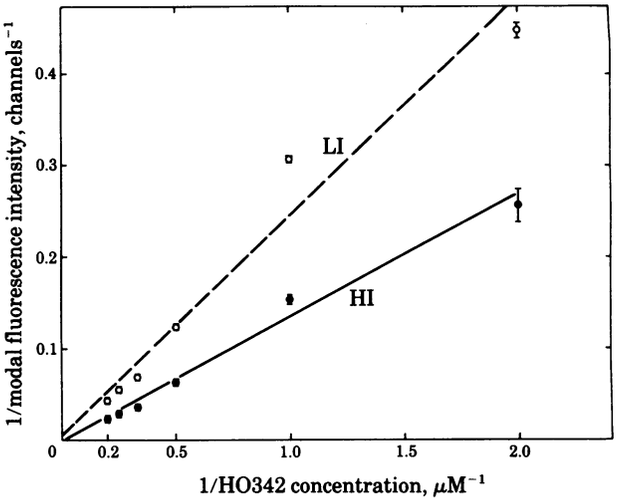

FIG. 8. Lineweaver–Burk plot of HO342 uptake by murine lymphocytes. Samples of spleen cells were suspended in α medium containing increasing concentrations (0.5–5 μM) of HO342 for 2 min at 37°C. The modal intensities of the HI (●) and LI (○) peaks were determined by FCM analysis. Error bars are 1 SD. Linear regression analysis of the reciprocal of the HI and LI modal intensities yields the solid (r^2 = 0.99) and broken (r^2 = 0.96) lines, which have y axis intercepts of −0.003 ± 0.008 and 0.006 ± 0.022, respectively.

curves shown in Fig. 8. Therefore, the LI and HI populations show about the same relative difference in colchicine uptake and HO342 fluorescence intensity.

DISCUSSION

In this report, we have studied the membrane transport of the DNA-specific fluorescent dye H0342, and we conclude that the mechanism of uptake in CHO cells and in murine lymphocytes appears to be identical to that of colchicine. The data summarized in Table 1 indicate that the degree of resistance to colchicine in CHO cells is directly related to a decrease in fluorescence intensity upon staining with HO342. The uptake kinetics presented in Fig. 4 are consistent with an unmediated diffusion process, previously shown to be the transport mode of colchicine (9). As well, both the cyanide-dependent stimulation of colchicine uptake by the CH^RC5 cells and its abrogation by the addition of glucose (15) can also be observed for HO342 dye (Fig. 5).

The colchicine-resistant CHO mutants studied here show extensive cross-resistance to a number of unrelated compounds. Although, when different mutant CHO lines are compared, the numerical extent of cross-resistance may vary from compound to compound, the rank order of resistance amongst the different lines is invariant or nearly so (14). This is also what is seen in Table 1; the cell lines tested are equivalently ordered by either their resistance to colchicine or their decreased uptake of

Table 2. Colchicine uptake of LI and HI lymphocyte fluorescence populations

Sorted fraction	cpm/10^6 cells	
	Exp. 1	Exp. 2
HI	68.4 ± 5.6	82.5 ± 6.2
LI	29.6 ± 4.9	29.6 ± 4.9

Murine lymphocytes were suspended in α medium containing 5 μM HO342 and radioactive colchicine at 2 μCi/ml for 15 min at 37°C. The HI and LI fractions were sorted simultaneously as described in the text and radioactive counts were measured by standard methods (8). Values are corrected for background counts, which were 27.8 ± 2.8 (mean ± SD) for 10^6 unlabeled cells.

HO342, although the numerical extent of the changes is not the same. The mechanism that accounts for variations in the degree of cross-resistance is not known. In Fig. 5, the addition of glucose to CH^RC5 cells in which uptake has been stimulated by cyanide enables the colchicine-resistant cells to export HO342 dye. The energy dependence of this phenomenon implies that an active mediated process may be involved in export of the dye. We do not understand why the fluorescence distributions of cells treated with KCN (Fig. 5*D* and *E*) show some structure at fluorescence intensities higher than that of the G_1 peak. It is not due to the presence of S and G_2+M cells, because these are not observed in the control (Fig. 5*A*). The existence of this structure does not affect our interpretation of the spectra.

The HO342 technique would appear to be generally useful for studies of membrane transport and appears to offer advantages over conventional methods involving the incorporation of radiolabeled compounds in that it is both more rapid (5 min per measurement) and more sensitive. In addition, one can sort cells on the basis of their rate of dye uptake. It may be that the study of membrane transport by using a fluorescent probe that complexes to DNA is not suitable in all instances. Transport of the dye at the level of the nuclear membrane, saturation of DNA binding sites, and differences in chromatin conformation affecting the availability of dye–DNA binding sites must also be considered, although none of these factors appears to have been a problem in the studies reported here.

In the case of murine lymphocytes, two subpopulations differing in their rate of dye uptake can be identified (see LI and HI peaks of Fig. 6). The LI and HI fluorescence populations show nonsaturation uptake kinetics (Fig. 8) and an approximately 2-fold difference in both HO342 fluorescence intensity and colchicine uptake (Table 2). This argues strongly that the two unrelated compounds, HO342 and colchicine, enter the cell by the same mechanism. The presence in unstimulated spleen cells of two subpopulations that differ in their transport properties is apparently attributable to differences in membrane transport between T and B lymphocytes, because these have been found to be present mainly in the LI and HI peaks, respectively (16).

A major limitation in many *in vitro* tests of cellular function is that one can only indirectly study interactions between the precursor cells of interest through measuring their differentiated progeny obtained after several days of culture. In studies of T lymphocyte activation, the HO342 technique may provide a partial solution to this problem. After stimulation with the mitogen concanavalin A, most T cells rapidly undergo a transition from the LI to the HI peak (7). After stimulation in a mixed lymphocyte reaction, a much smaller number of T cells move from the LI to the HI peak (17). These include cytotoxic T lymphocyte precursor cells specific for the stimulator cells of the mixed lymphocyte reaction, all other cytotoxic lymphocyte precursor cells remaining in the LI peak (17). We conclude from the results reported here that the LI to HI transition is a result of changes in membrane transport after stimulation of the T cells. It is not known whether similar changes take place after activation of other kinds of cells.

There are several previous reports of changes in T cell membrane properties after activation. Differences in calcium transport (18) and in electrophoretic mobility (19) have been noted within minutes of stimulating T lymphocytes with the mitogen concanavalin A. Although both phenomena are thought to be due to membrane changes, they do not appear to be directly related to the membrane changes detected with our HO342 technique. There is no significant movement of T cells from the LI to the HI peak until 3 hr after concanavalin A stimulation (ref. 7; unpublished data). Several studies indicate that different lymphocyte subpopulations undergo rapid changes in membrane potential after activation with mitogens (20–22). Judging from the structure of HO342 (3), it should be neutral at the pH we have used for labeling, so it is unlikely that the transport changes we see are a direct effect of changes in membrane potential. One study (23) shows that membrane microviscosity of T lymphocytes changes shortly after stimulation with a lymphokine, a phenomenon possibly related to the changes we have seen (7, 17).

In conclusion, the HO342 technique described here would appear to be a simple and rapid technique for measuring membrane transport rates or changes in membrane transport rate that, as discussed above, have potential application in such diverse areas as fundamental studies of membrane transport, clinical studies of drug resistance, and studies of lymphocyte activation.

The authors gratefully acknowledge the skillful assistance of Alice Chase and Michael Naik in maintaining the cell lines and of Mark McCutcheon in performing the cell sorting. This work was supported by the National Cancer Institute of Canada.

1. Melamed, M. R., Mullaney, P. F. & Mendelsohn, M. L., eds. (1979) *Flow Cytometry and Sorting* (Wiley, New York).
2. Loewe, H. & Urbanietz, J. (1974) *Arzneim. Forsch.* **124**, 1927–1933.
3. Latt, S. A. & Stetten, G. (1976) *J. Histochem. Cytochem.* **24**, 24–33.
4. Crissman, H. A., Stevenson, A. P., Kissane, R. J. & Tobey, R. A. (1979) in *Flow Cytometry and Sorting*, eds. Melamed, M. R., Mullaney, P. F. & Mendelsohn, M. L. (Wiley, New York), pp. 243–261.
5. Arndt-Jovin, D. J. & Jovin, T. M. (1977) *J. Histochem. Cytochem.* **25**, 585–589.
6. Pallavicini, M. G., Lalande, M. E., Miller, R. G. & Hill, R. P. (1979) *Cancer Res.* **39**, 1891–1897.
7. Lalande, M. E. & Miller, R. G. (1979) *J. Histochem. Cytochem.* **27**, 394–397.
8. Ling, V. & Thompson, L. H. (1974) *J. Cell. Physiol.* **83**, 1116.
9. Carlsen, S. A., Till, J. E. & Ling, V. (1976) *Biochim. Biophys. Acta* **455**, 900–912.
10. Ling, V. (1975) *Can. J. Genet. Cytol.* **17**, 503–515.
11. Stanners, C. P., Elicieri, G. & Green, H. (1971) *Nature (London) New Biol.* **230**, 52–54.
12. Price, G. B., McCutcheon, M. J., Taylor, W. B. & Miller, R. G. (1977) *J. Histochem. Cytochem.* **25**, 597–600.
13. Loken, M. R. & Herzenberg, L. A. (1975) *Ann. N.Y. Acad. Sci.* **254**, 163–171.
14. Bech-Hansen, N. T., Till, J. E. & Ling, V. (1976) *J. Cell. Physiol.* **88**, 23–31.
15. See, Y. P., Carlsen, S. A., Till, J. E. & Ling, V. (1974) *Biochim. Biophys. Acta* **373**, 242–252.
16. Loken, M. R. (1980) *J. Histochem. Cytochem.* **28**, 36–39.
17. Lalande, M. E., McCutcheon, M. J. & Miller, R. G. (1980) *J. Exp. Med.* **151**, 12–19.
18. Freedman, M. H., Raff, M. C. & Gomperts, B. (1975) *Nature (London)* **255**, 378–382.
19. Blume, P., Malley, A., Knox, R. J. & Seaman, G. V. F. (1978) *Nature (London)* **271**, 378–380.
20. Taki, M. (1970) *Mie Med. J.* **19**, 245–262.
21. Kiefer, H., Blume, A. J. & Kaback, H. R. (1980) *Proc. Natl. Acad. Sci. USA* **77**, 2200–2204.
22. Shapiro, H. M., Natale, P. J. & Kamentsky, L. A. (1979) *Proc. Natl. Acad. Sci. USA* **76**, 5728–5730.
23. Puri, J., Shinitzky, M. & Lonai, P. (1980) *J. Immunol.* **124**, 1937–1942.