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Potasssium transport in human blood lymphocytes treated with phytohemagglutinin.

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Research Article

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Potassium Transport in Human Blood Lymphocytes Treated with Phytohemagglutinin

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ABSTRACT We have confirmed that phytohemagglutinin (PHA) rapidly enhances the uptake of potassium (K^+) by human blood lymphocytes. PHA, however, did not produce an increase in lymphocyte K^+ concentration. The apparent steady-state of cell K^+ concentration despite the marked increase in uptake of $^{42}K^+$ could be explained by either an increase in K^+-K^+ exchange or an increase in concentrative (active) K^+ accumulation in association with an increase in the leak of K^+ from the cell.

We compared, therefore, the uptake of $^{42}K^+$ with the decrement in cellular K^+ content when active transport was inhibited by ouabain. These studies established that K^+-K^+ exchange was negligible in human blood lymphocytes and that the increase in $^{42}K^+$ uptake after PHA treatment represented concentrative transport. Our studies did indicate that $^{42}K^+$ exodus from PHA treated lymphocytes increased markedly from 19 to 38 mmol · l cell water^{-1} · h^{-1}. Within the same time period K^+ influx into PHA-treated lymphocytes increased from 20 to 38 mmol · l cell water^{-1} · h^{-1}. Thus, PHA produces a marked increase in the permeability of the lymphocyte membrane to K^+, and the increase in active K^+ influx in PHA-treated lymphocytes may represent a homeostatic response by the membrane K^+ transport system to the increase in K^+ efflux.

Increased K^+ turnover was observed at the lowest concentrations of PHA which produced an observable increase in [^{3}H]thymidine incorporation into DNA. Thus, PHA produces an increase in K^+ permeability that closely parallels its mitogenic effect. The rapid increase in K^+ influx preceding blastogenesis and mitogenesis is required, therefore, to maintain normal intracellular K^+ concentration. An adequate intracellular K^+ concentration is essential for the synthetic processes required for cell transformation or division.

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INTRODUCTION

The addition of plant lectins to lymphocyte cultures results in a marked alteration in the transport function of the plasma membrane. The rates of accumulation of sugars (1), nucleosides, (2) and amino acids (3, 4) increase, perhaps as an adaptive response to the enhanced requirements for the synthetic processes soon to follow. Preceding the increased rates of substrate transport is a marked enhancement of potassium (K^+) uptake (5–7). It was hypothesized that the increased rate of K^+ uptake, which is one of the earliest physiological changes in lectin-treated lymphocytes, may lead to an increased concentration of cell K^+ (5). The latter event could be an essential requirement, perhaps a trigger for subsequent events in lymphocyte transformation. Several lines of evidence made this an intriguing hypothesis. First, cardiac glycosides which specifically inhibit the transport of sodium (Na^+) and K^+ are potent inhibitors of lectin-induced (8) and spontaneous lymphoid cell proliferation (9, 10). Second, internal K^+ concentration has been shown to be correlated with the rate of protein synthesis in bacteria (11) and animal cells (12, 13). Third, complex changes in K^+ movement are seen during the mitotic cycle of lymphoblasts and an apparent fall and rapid rise in cellular K^+ occurs during the DNA synthetic phase of mitosis (14), the same stage of the cell cycle in which cardiac glycosides have their major inhibitory effect on cell division (10). Fourth, the activity of plasma membrane Na^+, K^+ ATPase is higher in proliferating than nonproliferating hematopoietic cells (15) and its activity increases in the membranes of lymphoid cells treated with lectins (16).

The increased rate of $^{42}K^+$ accumulation after phytohemagglutinin (PHA)[1] treatment of human lymphocytes could result in an increase in cellular K^+. Alternatively, the increase in K^+ uptake could be in response to an increased leak of K^+ from the cell and act, thereby, to restore and maintain cellular K^+ at normal levels. Since there is considerable evidence that an adequate level of internal K^+ is required for protein synthesis (11–13) and cell division (8–10), the compensatory increase in K^+ uptake may be no less important in allowing the cell to sustain its synthetic rates.

In this report we have examined the quantitative relationship of $^{42}K^+$ exodus to $^{42}K^+$ uptake in untreated and PHA-treated human lymphocytes. Since the turnover of $^{42}K^+$ could represent either K^+-K^+ exchange (17–19) or active (concentrative) K^+ accumulation in response to the leak of K^+ from lymphocytes, we have compared the uptake of $^{42}K^+$ to the reduction in cell K^+ when active transport is inhibited by ouabain in untreated and PHA-treated lymphocytes. The initial rate of fall in cell K^+ in the presence of ouabain can be considered equal to the active transport of K^+ against its gradient. Thus, K^+-K^+ exchange represents the difference between the rate of uptake of $^{42}K^+$ and the rate of ouabain-induced K^+ loss (18, 19).

The experimental results are compatible with the following sequence of events. PHA leads to an increase in membrane K^+ permeability. This results in an enhanced leak of K^+ from the cell. Active transport of K^+ is accelerated to maintain the cell K^+ content at normal levels. In so doing, the possible deleterious effect of reduced cellular K^+ on macromolecule synthesis is avoided.

GLOSSARY OF SYMBOLS

α	Time constant of K^+ diffusion, min^{-1}
α_e	Time constant of K^+ diffusion as measured in a $^{42}K^+$ efflux experiment, min^{-1}
α_e'	Time constant of K^+ diffusion as measured by the rate of change of K^+ concentration when K^+ transport is inhibited, min^{-1}
α_i	Time constant of K^+ diffusion as measured in a $^{42}K^+$ influx experiment, min^{-1}
C_{int}	Internal K^+ concentration, femtomole/liter
C_{ext}	External K^+ concentration, femtomole/liter
K^+	Potassium
k	Ratio of count rate to quantity of isotope present, $counts \cdot minute^{-1} \cdot femtomole^{-1}$
N	Cell count, number of cells
Φ_{in}	Influx, $femtomole \cdot cell^{-1} \cdot h^{-1}$
Φ_{out}	Efflux, $femtomole \cdot cell^{-1} \cdot h^{-1}$
Q	Quantity of K^+, femtomole
$\rho(t)$	Count rate as function of time
$\rho_{ext}(t)$	Count rate of supernate as function of time.
$\rho_{int}(t)$	Count rate of cells as a function of time.
ρ_{trap}	Count rate due to extracellular trapped isotope
t	Time, minute.
V_{cw}	Volume of cell water per cell, liter/cell
V_{sup}	Volume of supernate, liter
V_{tc}	Total cell volume, liter/cell sample
V_{tcw}	Total cell water volume $\simeq 0.78\ V_{tc}$, liter/cell sample

[1] *Abbreviation used in this paper*: PHA, phytohemagglutinin.

METHODS

Lymphocyte preparation. To acquire the number of lymphocytes necessary for our studies, the mononuclear cell-rich residues (approximately 20 ml) from the plateletpheresis of healthy donors were obtained (20). The mononuclear cell-rich residue was diluted to 300 ml with tissue culture medium 199 containing Earles salts (TC 199) and heparin (5 U/ml). The cell suspension was sedimented at 200 *g* for 15 min and the platelet-rich supernatant fraction discarded. The sedimented cells were diluted with TC 199 and placed on a step-gradient consisting of 32.8% (wt/vol) sodium metrizoate:9% (wt/vol) Ficoll (1:2.4 vol/vol) (21). The cell layer at the interface of gradient and buffer was removed, diluted in TC 199, and washed twice with the same medium at 4°C. The cells were resuspended in glass bottles containing 250 ml of TC 199 with 20% (vol/vol) fetal calf serum, plus penicillin (100 U/ml), and streptomycin (100 μg/ml) at a concentration of 5×10^6 cells/ml. The cell suspension was incubated at 37°C under air and 7% CO_2 in a shaking water bath (20 oscillations/min) overnight (approximately 16 h) during which time the cells equilibrated and adherence of monocytes to the glass bottle occurred. The suspension was then gently stirred, decanted into 50-ml plastic tubes, and washed once with TC 199 by centrifugation at 200 *g*. The cell suspensions consisted of more than 85% lymphocytes, as judged by morphologic appearance and the cell size distribution. The proportion of cells excluding trypan blue dye was greater than 95%. Prior studies indicated that the lymphocytes obtained in the aforementioned manner were similar in their responsiveness to PHA and K^+ content to lymphocytes obtained directly from venous blood (20).

Cell incubations. The K^+ content and $^{42}K^+$ flux measurements were determined in lymphocytes suspended in TC 199 with 20% (vol/vol) fetal calf serum containing penicillin-G (100 U/ml) and streptomycin (100 μg/ml). These suspensions were incubated in air with 7% CO_2 at 37°C in a shaking water bath (100 oscillations/min). The pH of the cell suspension was maintained at 7.4 ± 0.1. PHA (Burroughs Wellcome and Co., Greenville, N. C.) was dissolved in TC 199 and 20 μl/ml added to cell incubations to achieve the final concentrations required by individual experiments. A stock solution of 10 mM ouabain octahydrate (Sigma Chemical Company, St. Louis, Mo.) was prepared in TC 199 at 37°C. For the study of the effect of varying doses of ouabain, dilutions of the stock solution were added in amounts required by individual experiments. All other experiments were performed at 2 μM ouabain which was achieved by the addition of 20 μl/ml of a 1:100 dilution of the stock solution.

Cell K^+ content. Cell content was measured in either of two ways which yielded similar results. In earlier experiments, 10 ml of a lymphocyte suspension containing 2×10^6 cells/ml was incubated in culture tubes and 10 μl (5 μCi) of ^{131}I-albumin (sp act = 0.044 mCi/mg) added. The cells were sedimented, and the supernate removed so that the residual volume was less than 0.05 ml. 2.8 ml of 15 mM LiCl was added to each vial and the cell suspen-

sion was dispersed by repeated aspiration through an 18 gauge needle and transferred to vials for measurement of radioactivity. Subsequently, the contents were sonicated at 25 W for 30 s and the K^+ content measured by means of a flame photometer with a LiCl blank (22). The cell K^+ content was calculated from the formula:

$$\text{cell } K^+ \text{ (femtomole/cell)} = \left[(K^+ \text{ in cell pellet}) - \left(\frac{\text{cpm } ^{131}\text{I cell pellet}}{\text{cpm } ^{131}\text{I/ml supernate}} \right) \times (K^+/\text{ml supernate}) \right] \cdot [\text{cell count}]^{-1}$$

—where cell K^+ is expressed as femtomole/cell, cell pellet K^+ as femtomole/pellet and supernate K^+ as femtomole/milliliter.

The cell K^+ content in later experiments was determined by sedimenting 10 ml of lymphocyte suspension, removing the supernate by aspiration so that the residual volume was less than 0.05 ml, and washing the undisturbed cell pellet with 15 ml of 20 mM HEPES buffered 142 mM choline chloride, pH 7.3 (HEPES-ChCl). Extensive study indicated that the medium trapped between cells rather than atop cells and on the sides of the tube was trivial and could be ignored. The tubes were recentrifuged to insure sedimentation of any disturbed lymphocytes; the wash solution was removed, and the cell pellet was resuspended in 2.8 ml of 15 mM LiCl. The cells were disrupted by sonication at 25 W for 30 s, and the K^+ content measured directly. The K^+ content of the wash solution was immeasureable, and hence no subtraction of trapped K^+ was necessary. The measurements of cell K^+ content after washing the undisturbed pellet were indistinguishable from those made with the ^{131}I-albumin technique when the results were compared in the same lymphocyte population.

Cell K^+ concentration. To convert K^+ content (femtomole/cell) to K^+ concentration (millimole/liter cell water) we measured cell volume in each experiment and used the following formula to convert cell volume to cell water:

$$\text{cell water} = 0.78\ V_c \cdot 10^{-15}$$

— where cell water is expressed as liter/cell, V_c represents the volume of the cell in the μm^3/cell and 10^{-15} represents the liters per μm^3.

The percentage of cell water by weight was determined by methods previously described (23) and was 78±0.4 g H_2O/100 g cells. The specific volume of the cell solids was considered to be 1.0. The K^+ content was converted to K^+ concentration with the formula:

$$\text{cell } K^+ \text{ concentration} = \frac{\text{cell } K^+ \text{ content}}{\text{cell } H_2O \text{ content}} \times 10^{-12}$$

—where K^+ concentration is expressed as millimole/liter, cell K^+ content as femtomole/cell, cell water as liter/cell, and 10^{-12} represents the mmol per fmol.

$^{42}K^+$ transport—efflux. To determine the rate of $^{42}K^+$ efflux, ^{42}KCl, 4 μCi/ml (sp act 0.18 mCi/mg) was added to the lymphocyte suspension before incubating the cells overnight (~16 h). The cell suspension was sedimented at 200 *g* for 10 min, the supernate discarded, and the cells washed thrice at 4°C with TC 199 before resuspension at 3±1 × 10^6 cells/ml in 120 ml of TC 199 plus fetal calf serum at 37°C. 30-ml-portions of the cell suspensions were incubated in air with 7% CO_2 in a shaking water bath at a pH of 7.4±0.1. PHA (0–8 μg/ml) was added and 1-ml samples were removed immediately and at 20-min intervals thereafter. The samples were sedimented at 8,000 *g* for 1.5 min in an Eppendorf Micro Centrifuge (Brinkmann Instruments, Inc., Westbury, N. Y.) at 4°C and the supernates removed. The radioactivity in 0.8 ml of each supernatant fraction and in the total cell suspension was measured. The movement of label during the efflux process is represented by simple diffusion in which the rate of movement is dependent on the label's concentration inside the cell. The following formula may be used for determining the time constant, α_e, in terms of the appearance of label in the supernate:

$$e^{-\alpha_e t} = 1 - \frac{\rho_{ext}(t)}{\rho_T} \text{ (see Equations 5 and 6 Appendix)}$$

—where $\rho_{ext}(t)$ is the count rate of a sample of the supernate and ρ_T is equal to the count rate of the label contained in a given volume of suspension. α_e is equal to the slope of the line formed when the quantity $\left(1 - \frac{\rho_{ext}(t)}{\rho_T}\right)$ is plotted semi-logarithmically against time. The derivation of these calculations is presented in the Appendix.

The exodus rate, Φ_{out}, in femtomoles · $cell^{-1}$ · h^{-1} is equal to $\alpha \cdot V_{cw} \cdot (C_{int}) \times 60$ min/h, where V_{cw} is the cell water expressed as liter/cell, and C_{int} is the cellular concentration of K^+ in femtomole/liter.

$^{42}K^+$ transport—influx. To determine the rate of K^+ influx, $^{42}K^+$ (2 μCi/ml) was added to flasks containing 30 ml of a suspension of 4 × 10^6 lymphocytes/ml. PHA had been added to the experimental flasks 30 min previously. Ouabain was added at time zero. At 15-min intervals for a total of 90 min, 1.5-ml samples were removed and transferred to 15-ml plastic tubes and sedimented at 1,000 *g* for 2.5 min in a Sorvall RC-3 centrifuge (DuPont Instruments, Sorvall Division, Newtown, Conn.). The supernates were removed and the cells washed twice with 15 ml of HEPES-ChCl at 4°C without disturbing the cell pellets. Cell pellets were resuspended in 1.4 ml of HEPES-ChCl and transferred to vials for measurement of their radioactivity. The radioactivity of 1.5 ml of the total cell suspension was similarly determined.

Prior studies from our laboratory have shown that PHA-treated lymphocytes lose over 20% of their cellular K^+ content when washed once (24). However, we have found that this loss does not occur when the cell pellet is not dispersed during washing. Lymphocytes previously labeled with ^{42}KCl lost 1.5±0.7% of their ^{42}K during a single HEPES-ChCl wash. PHA-treated cells that were dispersed during washing lost 20.1±6.9% of ^{42}K, whereas PHA-treated cells that were not dispersed lost 2.2±0.9% of ^{42}K. We were able, therefore, to measure the accumulation of ^{42}K in PHA-treated cells by avoiding cell dispersion during washing. The residual volume of the original supernate after two 15-ml HEPES-ChCl washes was 9 nl/pellet as measured with ^{14}C-sucrose. This represented 0.7% of the total pellet water which was 1.3 μl/pellet. The residual volume of supernate was, therefore, an insignificant component in the K^+ influx measurements since it accounted for less than 0.2% of the total radioactivity which accumulated during a 90-min experiment.

The data from the influx experiment allows calculation of (*1*) the $^{42}K^+$ influx, Φ_{in}, from the accumulation rate of isotope and (*2*) the $^{42}K^+$ efflux constant α_i from the exponential decrease of the accumulation rate which results from the $^{42}K^+$ back flux to the medium. The later is dependent on K^+ concentration being in a steady state. Influx is calculated from the equation.

$$\Phi_{in} = \frac{C_{ext} V_{sup}}{N \rho_{ext}} \left. \frac{d\rho_{int}}{dt} \right|_{t=0} \text{ (See Equation 22 Appendix)}$$

—where ρ_{int} and ρ_{ext} are the count rates of the cells and of the supernate, C_{ext} is the external K^+ concentration in femtomole/liter, V_{sup} is the volume of supernate in liters and N the number of cells. The efflux constant α_i is calculated from the equation:

$$e^{-\alpha_i t} = 1 - \frac{\rho_m(t)}{\rho_{int}(\infty)} \text{ (See Equation 24 Appendix)}$$

The derivations of these formulae are shown in the appendix.

[^3H]thymidine incorporation into DNA. Lymphocytes were suspended in TC 199 with 20% heat inactivated isologous human or fetal calf serum from a single lot. Prior studies showed that the lymphocyte response to PHA was similar when either serum was used. PHA (20 μl) was added to 1 ml of a suspension of 10^6 lymphocytes to provide the concentration of lectin required by individual experiments. Cell suspensions were incubated under air and 7% CO_2 at 37°C in closed plastic culture tubes. 2 μCi of [^{3}H]thymidine (sp act 2 Ci/mM) was added at 48 h and the tubes agitated and regassed with air and 7% CO_2. At 72 h, the cells were dispersed and 100 μl samples placed onto duplicate paper filter discs suspended on straight pins. Nucleic acids were precipitated with 10% trichloracetic acid at 4°C and washed with solvent as previously described (24, 25). Each filter disc was placed in a liquid scintillation vial with 5 ml of Bray's solution and the radioactivity determined.

RESULTS

Lymphocyte K^+ concentration in response to PHA. Because accelerated K^+ accumulation which has been observed after PHA stimulation of lymphocytes might result in increased K^+ concentration, we attempted to measure K^+ concentration with a standard technique that required cell washing. PHA-stimulated lymphocytes exchanged cellular K^+ for external cations during the washing procedure, which suggested an altered plasma membrane permeability to K^+ (24). We adopted, therefore, a technique to measure intracellular K^+ so as to avoid cell washing and used ^{131}I-albumin to measure trapped supernatant K^+ (22). Lymphocytes were exposed to 0–8 μg/ml PHA, a range which encompassed the concentration which produces maximal stimulation of [^{3}H]thymidine (0.5 μg PHA/ml) into DNA. As shown in Fig. 1, the lymphocyte K^+ concentration (125–136 mmol/l cell water) was unchanged when measured 15, 120, and 300 min after exposure of lymphocytes to 1, 2, 4, or 8 μg/ml PHA. When samples were taken immediately after addition of PHA (≈0.5–2.0 min) a slight but not statistically

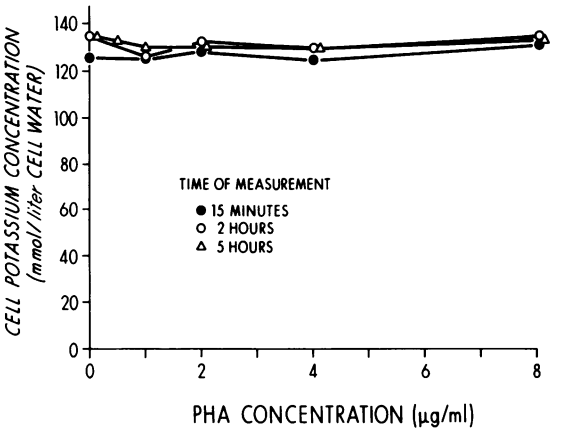

FIGURE 1 The cellular K^+ concentration (mmol/liter cell water) of human blood lymphocytes after exposure to increasing concentrations of PHA (0–8 μg/ml) is shown. K^+ concentration was measured in unwashed cells (see Methods) avoiding the loss of intracellular K^+ that occurs in lectin-treated lymphocytes. K^+ concentration was measured at 15 min, 2, and 5 h after addition of PHA.

TABLE I
$^{42}K^+$ Efflux in Untreated and PHA-Treated Human Lymphocytes

	α_e	Control Efflux		α_e	PHA Efflux	
Exp. no.	min^{-1}	$fmol \cdot cell^{-1} \cdot h^{-1}$	$mmol \cdot l cell\ water^{-1} \cdot h^{-1}$	min^{-1}	$fmol \cdot cell^{-1} \cdot h^{-1}$	$mmol \cdot l cell\ water^{-1} \cdot h^{-1}$
1	0.0031	4.3	21.0	0.0062	8.3	40.6
2	0.0030	4.1	22.3	0.0057	7.7	41.8
3	0.0027	3.7	19.0	0.0061	8.2	42.0
4	0.0026	3.5	17.8	0.0056	7.5	38.1
5	0.0025	3.4	18.2	0.0055	7.4	39.6
6	0.0025	3.4	17.4	0.0057	7.6	38.9
7	0.0025	3.2	16.7	0.0066	8.5	44.2
Mean	0.0027	3.7	18.9	0.0059	7.9	40.7
SD	±0.0003	±0.4	±2.0	±0.0004	±0.4	±2.1

PHA was present at 8 μg/ml. Efflux values were calculated from α_e, a diffusion constant, determined from the rate of $^{42}K^+$ exodus from previously labeled lymphocytes (see methods).

TABLE II

Comparison of Influx and Efflux in Untreated and PHA Treated Human Lymphocytes

Exp. no.	α_t	Efflux		Influx	
	min^{-1}	$fmol \cdot cell^{-1} \cdot h^{-1}$	$mmol \cdot l cell\ water^{-1} \cdot h^{-1}$	$fmol \cdot cell^{-1} \cdot h^{-1}$	$mmol \cdot l cell\ water^{-1} \cdot h^{-1}$
Control					
1	0.0029	3.9	20.0	3.7	19.0
2	0.0025	3.4	17.4	3.8	19.5
3	0.0026	3.5	18.0	3.9	20.0
4	0.0028	3.6	18.5	4.2	21.5
5	0.0031	4.2	21.5	3.4	17.4
6	0.0032	3.9	20.0	4.2	21.5
7	0.0030	4.0	20.5	4.7	24.1
Mean	0.0029	3.8	19.4	4.0	20.4
SD	±0.0003	±0.3	±1.5	±0.4	±2.2
PHA					
1	0.0054	7.2	36.9	7.5	38.5
2	0.0051	6.8	34.9	7.2	36.9
3	0.0056	7.6	39.0	9.4	48.2
4	0.0065	8.5	43.6	7.5	38.5
5	0.0050	6.9	35.4	4.7	24.1
6	0.0073	9.0	46.2	7.9	40.5
7	0.0047	6.1	31.3	7.6	40.0
Mean	0.0057	7.4	38.2	7.4	38.1
SD	±0.0009	±1.0	±5.2	±1.4	±7.2

PHA was present at 8 μg/ml. Influx values were derived from the rate of $^{42}K^+$ accumulation by the lymphocytes. Efflux values were calculated from the same experiments by determining α_t (see methods).

significant decrease in the cell K^+ in PHA-treated (133 ±9 SD) as compared to untreated (141±9 mmol/l cell water) cells was observed. In this current series of studies 69 measurements were made in eight lymphocyte populations during 5 h exposure to 8 μg/ml PHA. K^+ concentration in PHA treated cells (124 ±19 SD) was not different from untreated cells (128 ±23 mmol/l cell water).

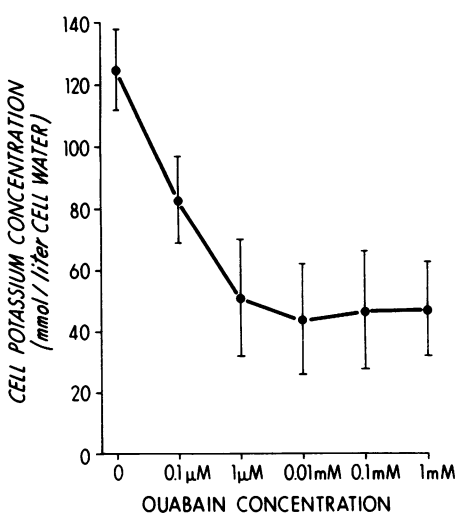

FIGURE 2 Lymphocyte cell K^+ concentration after 5 h of incubation in the presence of 0–1 mM ouabain. Each point represents the mean±2 SE of triplicate determinations in four lymphocyte populations.

Lymphocyte K^+ exodus in response to PHA. Since K^+ uptake has been observed to be accelerated after PHA stimulation, K^+ content can remain constant only if exodus of K^+ is accelerated by exposure to PHA. In prior experiments, we observed the ^{86}rubidium exodus from rat and human lymphocytes was increased after treatment with PHA (26). Table I shows the time constant, α_e, for efflux of K^+ from human lymphocytes after treatment with 8 μg/ml of PHA. The mean ±SD of the seven efflux constants for untreated lymphocytes was 0.0027±0.0003 min^{-1}. The efflux constant increased with PHA treatment to 0.0059±0.0004 min^{-1} ($P < 0.01$). These time constants correspond to absolute K^+ exodus rates of 3.7 and 7.9 fmol · $cell^{-1}$ · h^{-1} or 18.9 and 40.7 mmol · 1 cell $water^{-1}$ · h^{-1} in control and PHA-treated lymphocytes, respectively. In view of the large PHA-induced increase in K^+ efflux and the absence of a change in K^+ content, we tested the possibility that the increment in efflux was quantitatively equal to the increment in influx.

Lymphocyte $^{42}K^+$ turnover in response to PHA. To minimize the effect of variations among individual

cell donors, $^{42}K^+$ influx and efflux were measured in the same lymphocyte populations. In Table II, the efflux and influx in seven populations of unstimulated human blood lymphocytes are shown. Both influx and efflux were calculated from each influx experiment. The mean efflux calculated from α_i was 3.8 fmol · cell^{-1} · h^{-1} (19.4 mmol · 1 cell water^{-1} · h^{-1}). This was not significantly different from the efflux measured independently from α_e or from the mean influx of 4.0 fmol · cell^{-1} · h^{-1} (20.4 mmol · 1 cell water^{-1} · h^{-1}). This is the expected relationship of efflux to influx, since K^+ content in untreated cells remains unchanged during the 90-min incubation required for flux determinations (i.e. apparent steady-state).

The influx and efflux in the same lymphocytes exposed to 8 μg/ml PHA are also shown in Table II. The mean efflux of 7.4 fmol · cell^{-1} · h^{-1} (38.2 mmol · 1 cell water^{-1} · h^{-1}) calculated from α_i was not significantly different from the efflux measured independently from α_e and was identical to the measured influx. Influx and efflux were also measured in two lymphocyte populations at 0.8 μg/ml PHA. Potassium efflux (5.8 and 5.9) and influx (5.6 and 6.2 fmol · cell^{-1} · h^{-1}) were increased when compared to control values, but remained similar to each other.

Dose-response curve for the effect of ouabain on cell K^+ concentration. To determine the concentration of ouabain that maximally inhibited K^+ accumulation, we treated human blood lymphocytes with ouabain (1 mM–0.1 μM) for 5 h and measured cell K^+ concentration (Fig. 2). The cell K^+ concentration of untreated lymphocytes was not altered significantly during this time. When lymphocytes were studied in the presence of 1 μM or greater ouabain, K^+ concentration fell from a mean of 125 mmol/l cell water to about 50 mmol/l cell water in 5 h.

The concentration of K^+ in cells after 5 h exposure to ouabain was very similar to the value predicted if our $^{42}K^+$ uptake studies represented concentrative (active) transport. The predicted cellular K^+ when ouabain-sensitive K^+ uptake is inhibited can be calculated from the equation:

$$C_{int}(t) = e^{-\alpha t}(C_{int}(o) - C_{ext}) + C_{ext}$$

An exact treatment should take into account that the membrane potential can effect the diffusion rate. In this case the membrane potential is too small (27, 28) to influence the results, and its omission simplified the mathematical treatment of the data. For this calculation we used the mean values determined experimentally for (*1*) the time constant for K^+ exodus (α_e) (0.0027 min^{-1}), (*2*) $C_{int}(o)$ (125 mmol/l cell water) and (*3*) C_{ext} (5 mmol/l). When the equation was solved for $C_{int}(t)$ the predicted cell K^+ concentration after 5 hours of inhibition of K^+ transport was 58 mmol/l cell water. This value was within the 95% confidence limits of the mean of the observed K^+ measurements in the ouabain-treated lymphocytes (Fig. 2).

The fall in lymphocyte K^+ after 5 h incubation in 1 μM ouabain was 96% of that induced by 1 mM ouabain. Subsequently, therefore, the effects of ouabain on K^+ content and transport were studied at a concentration of 2 μM. We established first that this concentration provided maximal inhibition of K^+ transport as judged by inhibition of $^{42}K^+$ uptake (see later), and second that it did not alter the plasma membrane permeability to K^+ as judged by studies of $^{42}K^+$ efflux (data not shown).

Ouabain-sensitive $^{42}K^+$ influx in untreated and PHA-treated human blood lymphocytes. Previous studies have shown that the PHA-induced increase in $^{42}K^+$ influx is ouabain sensitive (7). Ouabain is a specific inhibitor of the Na^+,K^+ ATPase, and of active K^+

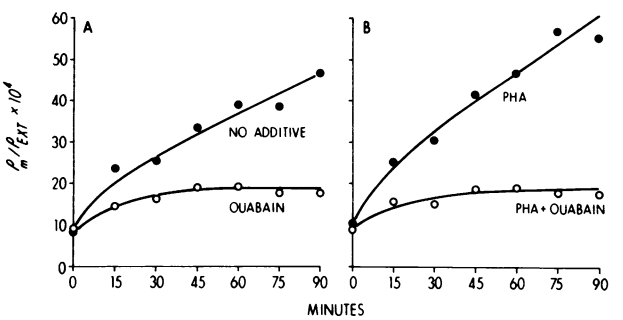

FIGURE 3 The accumulation of $^{42}K^+$ by A) untreated and B) PHA-treated (8.0 μg/ml) lymphocytes in the presence and absence of ouabain. The closed circles represent the relative count rates (ρ_{int}/ρ_{ext}) during 90 min of study in the absence of ouabain. The open circles represent the relative count rates during 90 min of study in the presence of 2 μM ouabain.

TABLE III
The Effect of Ouabain on $^{42}K^+$ Influx in Human Lymphocytes

Exp. no.	Control		PHA	
	fmol · cell^{-1} · h^{-1}	*mmol · l cell water^{-1} · h^{-1}*	*fmol · cell^{-1} · h^{-1}*	*mmol · l cell water^{-1} · h^{-1}*
No ouabain				
1	3.4	17.4	4.7	24.1
2	4.2	21.5	7.9	40.5
3	4.7	24.1	7.6	40.0
Ouabain				
1	0	0	0	0
2	0.2	1.0	0.4	2.1
3	0	0	0	0

PHA was present 8 μg/ml. Ouabain was present at 2 μM.

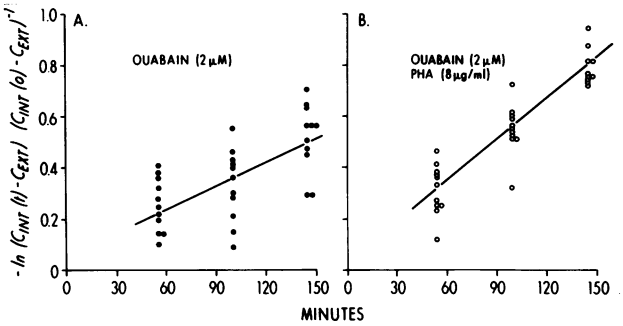

FIGURE 4 Active K^+ transport by (*A*) untreated and (*B*) PHA-treated (8.0 μg/ml) lymphocytes. The ordinate is a negative logarithmic function of the cell K^+ content at each time shown on the abscissa. All measurements were made in the presence of 2 μM ouabain, a concentration we found to inhibit concentrative K^+ transport. The slopes of the lines represent the time constant for efflux α'_e (min^{-1}). In these studies the α'_e for untreated cells was 0.0028 min^{-1} and for PHA-treated cells was 0.0053 min^{-1}.

influx (29). Thus, these previous reports suggest but do not prove that lectin stimulation results in an increase in the rate of concentrative uptake of K^+ into the cell. This uncertainty exists because ouabain-sensitive $^{42}K^+$ influx could represent either net K^+ transport or ouabain-sensitive K^+-K^+ exchange (17–19). Therefore, we measured ouabain-sensitive $^{42}K^+$ influx in untreated and PHA-treated lymphocytes and compared it to the ouabain-induced net change in K^+ content.

The closed circles in Fig. 3A show the accumulation rate of $^{42}K^+$ in untreated human lymphocytes. The addition of ouabain markedly reduced the $^{42}K^+$ uptake as shown by the open circles. The closed circles in Fig. 3B show the accumulation of $^{42}K^+$ in PHA-treated human lymphocytes. PHA caused an increase in the rate of accumulation of $^{42}K^+$. Ouabain prevented $^{42}K^+$ uptake in the presence of PHA as shown by the open circles. The effect of ouabain on the influx of $^{42}K^+$ in three lymphocyte populations is shown in Table III. More than 95% of the $^{42}K^+$ uptake was ouabain-sensitive; K^+ influx being near zero in the ouabain-treated cells.

Active K^+ transport in untreated and PHA-treated lymphocytes. Since active transport of K^+ may be quantified by measuring the rate of decrement in cell K^+ when the K^+ pump is inhibited maximally by ouabain, lymphocyte K^+ content was measured in the presence of 2 μM ouabain. The exodus in K^+ was calculated from a plot of the function:

$$-\ln(C_{int}(t) - C_{ext})(C_{int}(o) - C_{ext})^{-1} \text{ versus time.}$$

The graphic representation of the results of 11 measurements is shown in Fig. 4A. The slope of the regression line represents a time constant for K^+ efflux, α'_e. In the steady-state, influx equals efflux; therefore, the rate of K^+ influx in resting lymphocytes based on the reduction in cell K^+ content produced by ouabain can be calculated. α'_e was 0.0028 min^{-1} in untreated lymphocytes. This corresponds to a net influx of 3.9 fmol · $cell^{-1}$ · h^{-1} or 19.0 mmol · 1 cell $water^{-1}$ · h^{-1}.

Similar measurements were made of K^+ content in the same populations of human blood lymphocytes treated with 8 μg/ml PHA (Fig. 4B). The slope of the regression line, α'_e, was 0.0053 min^{-1} and corresponded to a net K^+ influx rate of 7.4 fmol·$cell^{-1}$·h^{-1} or 38.0 mmol · 1 cell $water^{-1}$ · h^{-1}.

K^+-K^+ exchange in untreated and PHA treated lymphocytes. To examine the possible role of K^+-K^+ exchange in PHA stimulated K^+ turnover, we compared isotopic $^{42}K^+$ influx and K^+ influx calculated from the net change in cell K^+ in the presence of ouabain. The measurements of K^+ influx which are derived from the rate of decrement of cell K^+ when K^+ transport is inhibited with ouabain and the $^{42}K^+$ uptake in untreated and lectin-treated cells are shown in Table IV. The K^+ influx rates derived by the former method were 3.9 and 7.4 fmol·$cell^{-1}$·h^{-1} (19 and 38 mmol·1 cell $water^{-1}$·h^{-1}) for untreated and PHA-treated lymphocytes. $^{42}K^+$ influx was 4.0 and 7.4 fmol·$cell^{-1}$·h^{-1} (20 and 38 mmol·1 cell $water^{-1}$·h^{-1}) for untreated and PHA-treated lymphocytes. Since exchange is defined as the difference between the isotopic ($^{42}K^+$) flux and the ouabain-induced cellular K^+ loss, little $^{42}K^+$ uptake could be related to K^+-K^+ exchange in these studies.

Nearly all of the isotopic K^+ influx was ouabain-sensitive (Table III). The ouabain-sensitive $^{42}K^+$ influx must at least equal the net K^+ influx in both

TABLE IV
Comparison of $^{42}K^+$ Influx with Ouabain-Induced K^+ Loss in Human Lymphocytes

	$^{42}K^+$ Influx	Ouabain-Induced Cell K^+ Loss	$K^+ - K^+$ Exchange
Control			
fmol·$cell^{-1}$·h^{-1}	4.0	3.9	0.1
mmol·lcell $water^{-1}$·h^{-1}	20	19	1
PHA 8 μg/ml			
fmol·$cell^{-1}$·h^{-1}	7.4	7.4	0
mmol·lcell $water^{-1}$·h^{-1}	38	38	0

The data for $^{42}K^+$ uptake are the mean of determinations in seven lymphocyte populations. The data for ouabain-induced cell K^+ loss are derived from the regression lines in Fig. 4. $K^+ - K^+$ Exchange is the difference between these two values.

untreated and PHA-stimulated cells. These observations, in fact, were consistent with the isotopic data, in which $^{42}K^+$ influx fell to zero in both untreated and PHA-treated lymphocytes exposed to maximally inhibitory concentrations of ouabain.

Relationship of increased K^+ turnover to [^3H]-thymidine incorporation. Since K^+ content remains constant and K^+ efflux equals K^+ influx after PHA stimulation, measurement of either efflux or influx serves as an indication of the rate of K^+ turnover. To consider the relationship of increased K^+ turnover to PHA stimulation of blastogenesis, we measured the PHA dose response of [^{3}H]thymidine incorporation into DNA and compared it to the PHA effect on K^+ efflux. The data in Fig. 5 show that [^{3}H]thymidine incorporation progressively increased as the PHA concentration was increased from 0 to 0.5 μg/ml and fell thereafter. K^+ exodus likewise increased as the PHA concentration was raised to the optimal PHA concentration, 0.5 μg/ml. Importantly, even at the lowest PHA concentration tested, 0.05 μg/ml, an increase in K^+ exodus was associated with the stimulation of [^{3}H]thymidine incorporation. At higher concentrations of PHA, K^+ turnover continued to increase, although thymidine incorporation into DNA fell.

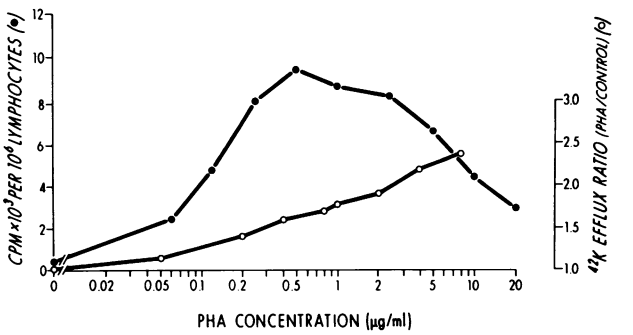

FIGURE 5 The incorporation of [^{3}H]thymidine into DNA is compared to K^+ turnover at increasing concentrations of PHA. The [^{3}H]thymidine incorporation into DNA is shown on the left ordinate as radioactivity recovered in TCA precipitated material from 10^6 lymphocytes. The closed circles represent the mean of duplicate determinations from six different lymphocyte populations. The ratio of ^{42}K efflux in PHA-treated to untreated lymphocytes is shown on the right ordinate. The open circles represent the mean of duplicate determinations from two different lymphocyte populations.

DISCUSSION

The cellular Na^+ and K^+ concentrations in human lymphocytes are maintained by a system of leaks and pumps common to all nucleated cells in which plasma membrane Na^+,K^+ activated ATPase transduces the energy required for uphill ion movements against their respective gradients. It has been suggested that maintenance of a high K^+, low Na^+ environment within the cell provides (*1*) a mechanism for cell volume regulation (30), (2) for the transport of substrates dependent on an ionic gradient for accumulation (31) and (3) an appropriate ionic environment for protein synthesis (11–13). The role of transport in lymphocyte mitosis has been studied in several laboratories. Quastel and co-workers have shown that K^+ transport is altered in lymphocytes exposed to PHA. In their studies, $^{42}K^+$ uptake was accelerated after PHA stimulation while $^{42}K^+$ exodus was unchanged or slightly decreased (5). Thus, they suggested that a rise in cellular K^+ content may occur. However, measurements of K^+ content in stimulated lymphocytes were not made, and therefore, this inference was not verified directly.

For this reason, we attempted to measure lymphocyte K^+ content with a standard technique which included washing the cells free of external K^+. K^+ content of lymphocytes treated with PHA was markedly reduced, rather than increased; however, this proved to be a factitious change due to an increase in membrane permeability whereby K^+ exchanged for external cations during cell washing (24). An alternate technique which avoids washing before cellular K^+ measurement established that no change in K^+ content could be demonstrated between 10 min and 24 h after PHA treatment (22). Since Quastel et al. found an increment in K^+ influx of about 150% of the control rate (6 vs. 4 fmol · cell^{-1} · h^{-1}) when lymphocytes were exposed to PHA (5), a measureable increase in content should have occurred (2 fmol/h) within the time period of our observations if efflux did not increase. The maintenance of a steady state in cellular K^+ concentration strongly suggested that an increment in K^+ influx must be balanced by a proportionate increase in K^+ efflux.

Furthermore, the observation that PHA treatment altered membrane K^+ permeability such that K^+ was lost to washing solutions suggested that K^+ exodus might be increased during incubation in a physiologic medium. Indeed, studies of rat thymic and human blood lymphocytes showed a significant PHA-induced increment in the efflux of $^{86}Rb^+$ as did preliminary studies with $^{42}K^+$ (26). The present measurements substantiate that PHA induces a significant increase in K^+ efflux from cells studied in a physiologic medium. To test whether the increase in influx was equivalent to the increase in efflux, efflux and influx were measured in the same lymphocyte populations to avoid the effect of variations among individual cell donors. The presence of equivalent rates of $^{42}K^+$ efflux and $^{42}K^+$ influx in untreated and PHA-stimulated cells was consistent with the inability to detect a change in K^+ content over 24 h of study.

The increase in K^+ turnover in PHA-stimulated lymphocytes could represent K^+-K^+ exchange diffusion

or a PHA induced K^+ leak from the cell with compensatory active K^+ influx to maintain cell K^+ content. Since we could demonstrate little K^+-K^+ exchange diffusion in untreated or lectin-treated lymphocytes, the $^{42}K^+$ exodus measurements represent K^+ leak and the $^{42}K^+$ uptake represents K^+ pump activity.

The increase in influx is closely related to the increase in efflux. Thus, it is likely that the rate of concentrative uptake of K^+ is adjusted to the rate at which K^+ leaks from the cell. The inward K^+ pump may respond to a transient, slight reduction in cellular K^+ or an increase in Na^+ or both. Despite measurements as early as 2 min after PHA treatment, reduction in cellular K^+ was not detectable. Since the exodus rate of K^+ from PHA-treated lymphocytes was about 19 mmol · 1 cell water^{-1} · h^{-1} greater than untreated cells and steady-state content was about 128 mmol/l cell water, it would require at least 1 h for K^+ to fall below the lower range of normal (i.e. <108 mmol/l cell water). The latter calculation assumes no compensatory increase in K^+ uptake during this period. Since K^+ uptake does increase rapidly after PHA treatment, the failure to be able to identify a fall in cellular K^+ is not unexpected. Moreover, it suggests that the rate of transport of K^+ into the lymphocyte is closely controlled to maintain cellular concentration when K^+ permeability increases.

Since K^+ efflux is equal to K^+ influx in both untreated and PHA-treated cells, measurement of either influx or efflux represents a measurement of the K^+ turnover rate. With $^{42}K^+$ efflux, we compared K^+ turnover to [^{3}H]thymidine incorporation into DNA at increasing concentrations of PHA. K^+ turnover and DNA synthesis appeared to be correlated in that both increased as the PHA concentration was increased from 0 to 0.5 μg/ml. At PHA concentrations above 2 μg/ml, DNA synthesis was inhibited, but K^+ turnover continued to increase. This suggests that the increased K^+ permeability and the ability of K^+ transport to respond to maintain K^+ content was not affected by concentrations of PHA which impaired [^{3}H]thymidine incorporation. This apparent dissociation of K^+ turnover and [^{3}H]thymidine incorporation does not invalidate a hypothesis which links these two phenomena, causally. It is likely that the membrane transport ATPase is more resistant to toxic effects of PHA than are internal metabolic pathways, like DNA synthesis. The inability to dissociate increased K^+ turnover and [^{3}H]thymidine incorporation at low concentrations of PHA is compatible with a causal link between these two events.

It is well established that the proliferative capacity of both lectin-stimulated and spontaneously dividing lymphocytes is absolutely dependent upon Na^+,K^+ transport activity. Cardiac glycosides, specific inhibitors of plasma membrane Na^+,K^+ ATPase activity, are potent inhibitors of lymphocyte mitosis (8–10). Our studies suggest that the increase in K^+ transport seen soon after lectin treatment of lymphocytes does not contribute to mitogenesis by increasing cell K^+ content as previously suggested, rather its importance may be to maintain the level of cellular K^+ required for macromolecular synthetic processes.

MATHEMATICAL APPENDIX

For the purpose of analysis, we consider K^+ movement across the membrane divided into a uni-directional influx component, Φ_{in} and a uni-directional efflux component, Φ_{out}.

In these experiments we have followed the movement of radioactive K^+ which is proportional to the ratio of its concentration to the total K^+ concentration. Thus, the rate of change of the quantity (Q^*_{int}) of radioactive K^+ within a cell is given by

$$\frac{dQ^*_{int}}{dt} = \Phi_{in}\frac{C^*_{ext}}{C_{ext}} - \Phi_{out}\frac{C^*_{int}}{C_{int}} \qquad (1)$$

where C_{int} and C_{ext} are the internal and external K^+ concentrations and asterisks refer to quantities and concentrations of radioactive isotopes.

Efflux. In the efflux measurements, cells were preloaded with radioactive K^+ to a concentration C^*_{int} (0). At time zero the cells were transferred to a medium free of radioactive isotope. As the radioactive K^+ diffuses out of the cell, it becomes sufficiently diluted so that we can neglect C^*_{ext} in the above equation. We have, therefore,

$$\frac{dQ^*_{int}}{dt} = -\Phi_{out}\frac{C^*_{int}}{C_{int}} = -\Phi_{out}\frac{Q^*_{int}}{Q_{int}} \qquad (2)$$

for which we have the solution

$$Q^*_{int}(t) = Q^*_{int}(0)\, e^{-\alpha_e t} \qquad (3)$$

where

$$\alpha_e = \frac{\Phi_{out}}{Q_{int}} \qquad (4)$$

and the subscript e indicates α as measured by an efflux experiment. In the case under discussion in this paper, it is difficult to determine Q^*_{int} directly, because the incubation time with optimal conditions is relatively short, and only a small fraction of Q^*_{int} diffuses out during that time.

To avoid this difficulty, we measured instead the radioactive K^+ appearing in the supernate. In the process of transferring the labeled cells to the nonradioactive solution in which the efflux was measured, some radioactive K^+ was trapped, apparently on the cell surfaces, since the intercellular space is very small. We designate the count rate due to trapped isotope, ρ_{trap}. In terms of the count rate due to the aggregate of the cells and the trapped isotope the supernatant count rate is

$$\rho_{ext}(t) = \rho_{int}(0)(1 - e^{-\alpha_e t}) + \rho_{trap} \qquad (5)$$

The quantity

$$\rho_T = \rho_{int}(0) + \rho_{trap} \qquad (6)$$

is the total count rate from the cells plus that due to ectocellular trapped isotope so that

$$\rho_{ext}(t) = \rho_T - \rho_{int}(0)\, e^{-\alpha_e t} \qquad (7)$$

$$e^{-\alpha_e t} = \frac{\rho_T - \rho_{ext}(t)}{\rho_{int}(0)} = \frac{\rho_T}{\rho_{int}(0)}\left(1 - \frac{\rho_{ext}(t)}{\rho_T}\right) \quad (8)$$

We have found it convenient to express this in the form

$$-\alpha_e t = \ln\left(1 - \frac{\rho_{ext}(t)}{\rho_T}\right) + \ln\left(\frac{\rho_T}{\rho_{int}(0)}\right) \quad (9)$$

and have plotted

$$1 - \frac{\rho_{ext}(t)}{\rho_T} \quad (10)$$

semilogarithmically against t, in Fig. 6A for untreated and PHA-treated lymphocytes. The slope of the resulting line is α_e. The quantity $\ln \frac{\rho_T}{\rho_{int}(0)}$ does not affect the slope and was neglected for the calculation of α_e.

From Equation 4

$$\Phi_{out} = \alpha_e Q_{int} \quad (11)$$

Influx. The influx studies were performed by putting unlabeled cells into a medium containing radioactive $^{42}K^+$ at a concentration, C^*_{ext}, which remains essentially unchanged throughout the experiment. From Equation (*1*) we find that the influx is given by

$$\Phi_{in} = \frac{C_{ext}}{C^*_{ext}} \frac{dQ^*_{int}}{dt}\bigg|_{t=0} \quad (12)$$

since at time zero C^*_{int} is negligibly small. It is not possible to measure the rate of accumulation dQ^*_{int}/dt exactly at time zero. We therefore have measured it as soon after time zero as possible, and make use of a solution to Equation (1) given by

$$Q^*_{int}(t) = Q^*_{int}(\infty)(1 - e^{-\alpha_i t}) \quad (13)$$

and

$$\alpha_i = \frac{\Phi_{out}}{Q_{int}} \quad (14)$$

to extrapolate the measured value of dQ^*_{int}/dt from a time greater than zero back to the zero time value. The definition of α_i is identical to that of α_e in Equation 4. The distinction of subscript is made only to indicate whether the numerical value obtained is calculated from an efflux or influx experiment. This solution, Equation 13, is based on a steady state approximation which may not be rigorously true for chemically perturbed cells. In the following analysis, however, its use is limited to providing a small correction factor which will be sufficiently accurate since $Q^*_{int}(\infty)$ does not change radically.

The extrapolation of dQ^*_{int}/dt is done as follows: we express the count rate $\rho_{int}(t)$ of the cells in terms of its calculated value at infinite time. We measure, however, $\rho_m(t)$ which includes a trapped component.

$$\rho_m(t) = \rho_{int}(\infty)(1 - e^{-\alpha_i t}) + \rho_{trap} \quad (15)$$

We calculate $\rho_{int}(\infty)$ from steady-state values as follows:

$$\rho_{int}(\infty) = \rho_{ext}(0)\frac{V_{tcw}C_{int}}{V_{sup}C_{ext}} \quad (16)$$

We chose a convenient time, t_r, for measurement of $d\rho_m/dt$ from the observed rate of accumulation of Q^*_{int}. Let the count rate at this time $\rho_m(t_r)$ be divided by $\rho_{int}(\infty)$ to yield the fraction, r.

$$r = \frac{\rho_m(t_r)}{\rho_{int}(\infty)} = (1 - e^{-\alpha_i t_r}) + \frac{\rho_{trap}}{\rho_{int}(\infty)} \simeq 1 - e^{-\alpha_i t_r} \quad (17)$$

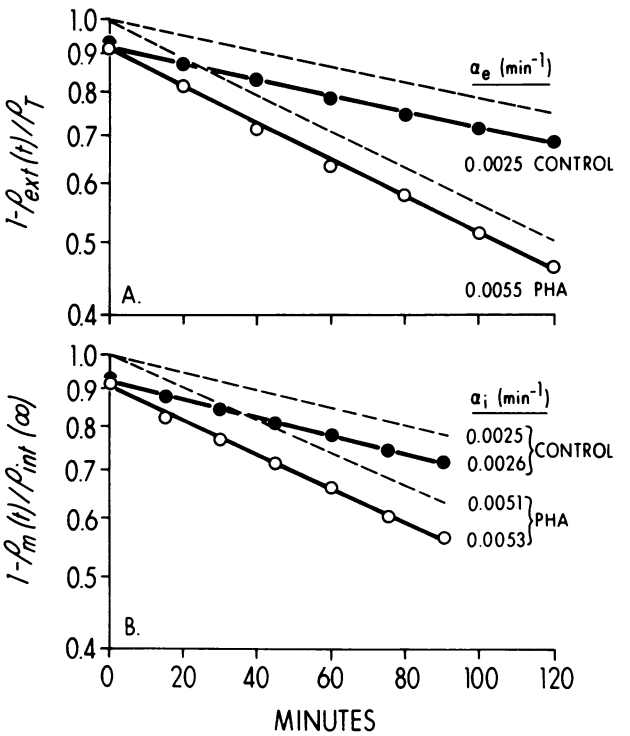

FIGURE 6 Graphic determination of the quantities α_e and α_i is shown. In Fig. 6A the ordinate is a logarithmic plot of 1 minus the ratio of the observed count rate to ρ_T as defined by equation 6 in the Appendix. In Fig. 6B the ordinate is a logarithmic plot of 1 minus the ratio of the observed count rate to its rate extrapolated to infinite time, and the abscissae represent the time of measurement. In both Fig. 6A and 6B the observed points are linear as represented by the solid lines. These lines, however, do not intersect unity at time zero. The offset of the zero-time intercept is accounted for by ectocellular radioactivity, as discussed in the text. The interrupted lines represent the same data points corrected for ectocellular radioactivity. In the correction for α_e, Fig. 6A. interrupted lines are exactly parallel to the solid lines. The mathematical correction does not alter the observed time constants for efflux. In the correction for α_i, Fig. 6B, the interrupted lines are displaced from the solid lines by a constant quantity at each point. In the case of influx this decreases the observed time constant for influx by 5%.

The derivative of ρ_{int} at time t_r is then related to its zero time derivative by

$$\frac{d\rho_{int}}{dt} = \frac{d\rho_m}{dt} = \rho_{int}(\infty)\alpha_i e^{-\alpha_i t} \quad (18)$$

$$\frac{d\rho_{int}}{dt}\bigg|_0 = \rho_{int}(\infty)\alpha_i$$

$$= \frac{1}{e^{-\alpha_i t_r}} \frac{d\rho_{int}}{dt}\bigg|_{t_r} = \frac{1}{1-r} \frac{d\rho_{int}}{dt}\bigg|_{t_r} \quad (19)$$

We define a counting factor k which relates the counting rates to the quantities of label so that

$$\rho_{ext} = kC^*_{ext}V_{sup} \quad (20)$$

and

$$\left.\frac{d\rho_{int}}{dt}\right|_0 = kN \left.\frac{dQ^*_{int}}{dt}\right|_0 \quad (21)$$

where N is the number of cells. Thus, from Equations 12, 20, and 21.

$$\Phi_{in} = \frac{C_{ext}V_{sup}}{N\rho_{ext}} \left.\frac{d\rho_{int}}{dt}\right|_0 \quad (22)$$

where the zero time derivative is obtained from Equation 19.

To establish confidence in the validity of the above extrapolation and allow comparison of α_e the efflux constant measured directly, to α_i, the efflux constant derived from the influx measurements, we determined α_i from Equation 15 as follows:

Recalling Equations 15 and 16

$$\rho_m(t) = \rho_{int}(\infty)(1 - e^{-\alpha_i t}) + \rho_{trap} \quad (15)$$

$$\rho_{int}(\infty) = \rho_{ext}(0)\frac{V_{tcw}C_{int}}{V_{sup}C_{ext}} \quad (16)$$

Equation 15 is then solved for α_i as follows:

$$\alpha_i t = -\ln\left[1 - \frac{\rho_m(t) - \rho_{trap}}{\rho_{int}(\infty)}\right] \quad (23)$$

As yet, we do not know the value of ρ_{trap}. We estimate it by plotting the quantity

$$-\ln\left[1 - \frac{\rho_m(t)}{\rho_{int}(\infty)}\right] \quad (24)$$

against time, as in Fig. 6B, then

$$\rho_{trap} = \rho_m \ (t \text{ extrapolated to zero}) \quad (25)$$

We can now plot

$$1 - \frac{\rho_m(t) - \rho_{trap}}{\rho_{int}(\infty)} \quad (26)$$

semilogrithmically against time from which α_i can be determined.

Empirically, we find that α_i determined from the above equation agrees very closely with the α_e.

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