

## POTASSIUM AND AMINO ACID TRANSPORT IN HUMAN LEUKOCYTES EXPOSED TO PHAGOCYTTIC STIMULI

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### ABSTRACT

Influxes of potassium and amino acids were measured in suspensions of human polymorphonuclear leukocytes (PMNs) under resting conditions and after various phagocytic stimuli. Both ouabain-sensitive (or pump) and ouabain-insensitive (or leak) influxes of K were determined. In 5 mM external K, mean total K influx was  $0.69 \text{ nmol}/10^6 \text{ cells} \times \text{min}$ , of which 52% was ouabain-sensitive. Ouabain binding was irreversible, and, as in erythrocytes, was inhibited by K. At external concentrations of 0.1 mM, influxes of lysine and leucine were entirely carrier-mediated, with means of  $0.021 \text{ nmol}/10^6 \text{ cells} \times \text{min}$ , and  $0.019 \text{ nmol}/10^6 \text{ cells} \times \text{min}$ , respectively. After incubation of PMNs with zymosan or latex particles, the K pump was reduced more than 60%, whereas amino acid influxes were inhibited only by 30%. PMNs were also exposed to cytochalasin B before challenge by particles: the drug prevented phagocytosis but not surface binding of zymosan, nor did it influence transport of K or amino acids. After pretreatment of PMNs with cytochalasin B, interaction of zymosan with their surface resulted in the same degree of inhibition of influxes of K and amino acids as when the cells were permitted to phagocytose the particles. In contrast, exposure of PMN to latex particles, which do not bind to cytochalasin B-treated cells, after pretreatment of cells with cytochalasin B did not result in inhibition of influxes. Treatment of cells with colchicine had no effect on either membrane transport or its inhibition after exposure to various phagocytic stimuli. These results indicate that the surface membranes of PMNs are functionally heterogeneous with respect to the association of transport sites for the different solutes. Moreover, loss of specific membrane functions from phagocytosing cells may result from the surface-attachment phase of particle-cell interactions, since the interactions of zymosan particles with PMNs in the absence of phagocytosis also inhibited transport of solutes.

During the process of particle ingestion, polymorphonuclear leukocytes (PMNs) internalize large portions of their surface membrane, but the relationship between internalization and carrier-mediated membrane transport remains unclear. Tsan and Berlin (1971) demonstrated that although the phagocytosis of polyvinyl toluene latex particles by rabbit PMNs and alveolar macro-

phages resulted in internalization of an estimated one-third to one-half of the surface membrane, the rate of carrier-mediated influx of several organic solutes (lysine and adenosine in macrophages; adenosine and adenine in PMNs) was not appreciably reduced. It was suggested that transport rates remained intact not because internalized transport sites were replaced, but rather because transport sites on the surface membrane were spatially separated from areas of the membrane that had become internalized during phagocytosis.

More recently, Ukena and Berlin (1972) showed that transport rates of adenine and lysine were reduced 40% after phagocytosis of latex particles by rabbit PMNs pretreated with colchicine or vinblastine (which interfere with microtubule function) (Weisenberg et al., 1968; Berry and Shelanski, 1972); whereas transport rates of untreated cells were unaffected. Colchicine had only a small effect on solute transport by resting (nonphagocytic) cells. The authors concluded that the integrity of microtubules was required for the apparent topographic separation of areas of the membrane involved in transport and phagocytosis, or at least for the cells' ability to achieve functional separation.

We have reexamined the relation between transport and phagocytic functions in human PMNs by measuring transport of potassium and two amino acids in resting cells and in cells after ingestion of two different kinds of particles. Our results indicate that there is a complex, functional relationship between transport sites, particle-binding sites, and area of the surface membrane involved in phagocytosis. Particle ingestion resulted in a large inhibition of K transport; amino acid transport was also inhibited but to a lesser extent. In cells treated with cytochalasin B, which inhibits phagocytosis, surface binding of particles had the same effect on transport as internalization of surface membrane. Finally, colchicine was without effect on these aspects of membrane function.

## MATERIALS AND METHODS

### *Preparation of Leukocytes*

Leukocyte suspensions were prepared as previously described from venous blood obtained from healthy young male donors (Weissmann et al., 1971). Blood was drawn into heparinized syringes and mixed with 0.25 vol of a 6% dextran solution (mol wt 190,000–230,000; Pharmachem, Bethlehem, Pa.) in 145 mM NaCl. Sedimentation of the red cells (facilitated by the dextran) was

allowed to proceed for 30 min; the leukocyte-rich supernate was removed and centrifuged at 150 g for 7 min at room temperature. The pellet was suspended in 145 mM NaCl to which 3 vol of distilled water were added to lyse the remaining red cells. After mixing for 20 s, the suspension was restored to isotonicity by addition of an appropriate volume of 615 mM NaCl. The leukocytes were sedimented by centrifugation at 150 g for 7 min, resuspended, and washed by an additional centrifugation in phosphate-buffered saline containing calcium and magnesium (NaCl 137 mM, KCl 2.7 mM, CaCl<sub>2</sub> 0.6 mM, MgCl<sub>2</sub> 1.0 mM, Na<sub>2</sub>HPO<sub>4</sub> 8.1 mM, KH<sub>2</sub>PO<sub>4</sub> 1.5 mM, pH 7.4). The final leukocyte suspensions were more than 85% PMNs, with a platelet-to-leukocyte ratio of approximately 5:1. Suspensions containing 98–99% PMNs were prepared similarly after initial fractionation of venous blood on Hypaque/Ficoll gradients, according to the method of Böyum (1967).

### *Experimental Procedures: Preincubation*

Leukocyte counts were performed and suspensions were adjusted to contain  $2-4 \times 10^6$  cells/ml. Cells were dispensed in 0.5-ml volumes into disposable plastic tubes. Autologous serum was added to a final concentration of 10%. The cells were incubated with various pharmacologic agents and particles at 37°C in a total volume of 1 ml. Cells prepared in this manner were shown previously to be over 90% viable and capable of active phagocytosis (Weissmann et al., 1971).

### *Potassium Influx*

Unidirectional influxes of K were measured with <sup>42</sup>K. The <sup>42</sup>K medium was prepared in buffered saline identical in composition to that given above except for the absence of K. Autologous serum was added to a final concentration of 10%. <sup>42</sup>KCl (New England Nuclear, Boston, Mass.) or <sup>42</sup>K<sub>2</sub>CO<sub>3</sub> (ICN Corp., Chemical & Radioisotopes Div., Irvine, Calif.) was added to give a final <sup>42</sup>K concentration of 5 mM. (For K<sub>2</sub>CO<sub>3</sub>, the pH was restored to 7.4 with HCl). In some experiments, the K medium contained various pharmacologic agents, as described in Results. In experiments designed to measure influx of K and amino acids simultaneously, medium also contained [<sup>14</sup>C]amino acids, as described below.

At the end of the preincubation, cells were sedimented by centrifugation (7 min, 150 g) and the supernate was removed by aspiration. The cells were resuspended with Pasteur pipettes in 1 ml of the buffered saline (at 37°C) containing <sup>42</sup>K. This resuspension procedure also served to separate clumps of cells which may have formed during the preincubation. The tubes were incubated at 37°C, generally for 10 min. The <sup>42</sup>K influx was stopped by addition of 2 ml of cold buffered saline and immersion of the tubes in an ice bath. The cell suspensions were centrifuged in the cold (4°C) at 300 g for 10 min and the supernate removed by aspiration. The cells were washed

free of extracellular radioactivity by resuspension in 2 ml of ice-cold buffered saline and centrifugation for 10 min. This procedure was repeated two more times for a total of three washes in cold saline.

After three washes, the radioactivity of the supernate was negligible. As will be shown below, K and amino acid fluxes are reduced by 90% in the cold. Therefore, the washing procedure removes mainly extracellular isotope, and the radioactivity of the washed pellets of cells gives a good approximation of the influx during the incubation at 37°C.

The washed pellets of cells were extracted in 0.5 ml of 2% Triton X-100. The extract was transferred quantitatively to a glass counting vial (27 × 58 mm) by rinsing the tubes with additional portions of Triton X-100 solution. The radioactivity of the <sup>42</sup>K was determined from the Cerenkov scattering in a Beckman LS-100 liquid scintillation counter (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.). Total counting time never exceeded 45 min, so correction was not made for radioactive decay.

Radioactivity of the flux medium was determined at the same time on a volume of an appropriate dilution equal to the volume of the cell extracts.

The influx of <sup>42</sup>K was a linear function of the number of cells per milliliter (between 2 × 10<sup>6</sup> and 8 × 10<sup>6</sup>), and nearly a linear function of time (between 10 and 30 min), as shown in Table I. Therefore in 10 min no correction was made for back flux of <sup>42</sup>K, and this time was chosen as the standard time for measurement of <sup>42</sup>K influx.

The unidirectional influx of K was calculated from radioactivity in counts per minutes of the medium, K concentration of the medium, the radioactivity of the cells, and the number of cells, by the equation:

$$i_{MK} = R_i \cdot \frac{(K)_o}{R_o} \quad (1)$$

where:  $i_{MK}$  = unidirectional K influx in nmol/10<sup>6</sup> cells × min,  $R_i$  = cpm of cell extract/10<sup>6</sup> cells × min,  $R_o$  =

cpm/liter of the medium, and  $(K)_o$  = K concentration of the medium in nmol/liter.

The cells were assumed to be steady-state with respect to K concentration during the 10-min flux period. In general, this appears to be a good assumption, though some of the pharmacological agents and other treatments may cause slight losses of K.

### Amino Acid Influx

Unidirectional influxes of amino acids were measured like the K influxes. When K and amino acid influxes were measured simultaneously, a [<sup>14</sup>C]amino acid, lysine or leucine (New England Nuclear), usually at a final concentration of 0.1 mM, was added to the medium containing <sup>42</sup>K. The cells were incubated for 10 min at 37°C and the flux was stopped by addition of ice-cold buffered saline, as described above. The cells were centrifuged and washed three times at 4°C. They were then extracted in 2% Triton X-100 and <sup>42</sup>K radioactivity was determined from Cerenkov scattering. Only the emission from <sup>42</sup>K was detected, since the beta emission of <sup>14</sup>C is not detected in the absence of fluor.

After 1 wk (more than 10 half-lives of <sup>42</sup>K), 10 ml of Bray's solution (Bray, 1960) was added to the counting vials containing the cell extracts and dilutions of the medium, and the radioactivity of the [<sup>14</sup>C]amino acid was determined. Each sample was counted to at least 10,000 counts. With the use of an external standard it was determined that there were negligible differences in counting efficiency between samples. Unidirectional influxes of amino acids were calculated exactly like the K influxes.

### Phagocytosis

The following particles were employed: zymosan (ICN Nutritional Biochemicals Div., International Chemical G Nuclear Corp., Cleveland, Ohio) and polystyrene latex, 1.10 μm in diameter (Dow Chemical USA, Midland, Mich.). Cells were exposed to particles for 30 min at the following particle-to-cell ratios: zymosan, 10:1; latex, 100:1.

### Statistical Test

Levels of significance of differences between relative fluxes after various treatments were determined with the randomization test for matched pairs (two-tailed), a nonparametric test with 100% power-efficiency (Siegel, 1956). Differences at the 0.06 level were regarded as significant.

### Compounds

Ouabain and colchicine were purchased from Sigma Chemical Co., St. Louis, Mo. Cytochalasin B was obtained from Imperial Chemical Industries, Cheshire, England, and dissolved at a concentration of 50 μg/ml in phosphate-buffered saline containing 0.1% dimethylsulf-

TABLE I

Unidirectional K Influxes in Human PMNs Related to Numbers of Cells and Times of Exposure to <sup>42</sup>K

Number of cells	Exposure time	K influx
× 10 <sup>6</sup>	min	nmol/min × 10 <sup>6</sup> cells
2	10	0.57
2	30	0.44
4	10	0.57
4	30	0.53
8	10	0.60
8	30	0.53

Fluxes are means of duplicate determinations.

oxide (Matheson Coleman & Bell, East Rutherford, N. J.).

## RESULTS

### Potassium Influx

Table I demonstrates that, under these conditions, K influx is a linear function of cell number up to  $8 \times 10^6$  cells in 1 ml of suspension medium, and is approximately a linear function of time up to 30 min. Fig. 1 shows the time course of  $^{42}\text{K}$  influx, expressed as nanomoles of intracellular K exchanged per  $10^6$  cells. If the exchange is a first-order process involving a single intracellular compartment, the following relation should be obeyed:

$$R_t = R_\infty(1 - e^{-kt}) \quad \text{or} \quad \ln\left(1 - \frac{R_t}{R_\infty}\right) = -kt \quad (2)$$

where  $R_t$  is radioactivity, or amount of K exchanged, in the cells at time  $t$ ,  $R_\infty$  is the radioactivity at infinite time or the total amount of exchangeable K, and  $k$  is the rate constant of the net influx of  $^{42}\text{K}$  (equivalent to the sum of the rate constants for the unidirectional inward and outward fluxes of  $^{42}\text{K}$ ).

With the assumption that total exchangeable K ( $R_\infty$ ) is 45 nmol/ $10^6$  cells, values of  $1 - (R_t/R_\infty)$  were plotted on a logarithmic scale against time. As shown in the inset in Fig. 1, a good fit to a straight line was obtained, indicating that  $^{42}\text{K}$  influx is a first-order process into a single compartment, and that the assumption of 45 nmol of exchangeable K/ $10^6$  cells is reasonable.

With  $346 \mu\text{m}^3$  as the mean cell volume of human PMNs (Hempling, 1973), 45 nmol exchangeable K/ $10^6$  cells gives an intracellular concentration of 130 mmol/liter of cells exchangeable K. This value is essentially the same as published concentrations of K in human leukocytes determined by chemical analysis (Baron and Roberts, 1963: 100 mM/liter cell water; Baron and Ahmed, 1969: 137 mM/liter cell water; Lichtman and Weed, 1969: 118 mM/kg cell water; Cividalli and Nathan, 1974: 119 mM/liter cell water). Therefore, the single exchangeable compartment of K represents essentially all of the intracellular K.

The mean K influx determined on cells from 13 donors was 0.69 nmol/ $10^6$  cells  $\times$  min ( $\pm$  0.095 SEM). Assuming a mean cell volume of  $346 \mu\text{m}^3$ , unidirectional K influx can be expressed as 2.0 mmol/liter cells  $\times$  min. Cividalli and Nathan (1974) reported unidirectional K influxes of 1.3 to 1.6 mmoles/liter cell water  $\times$  min. Their method

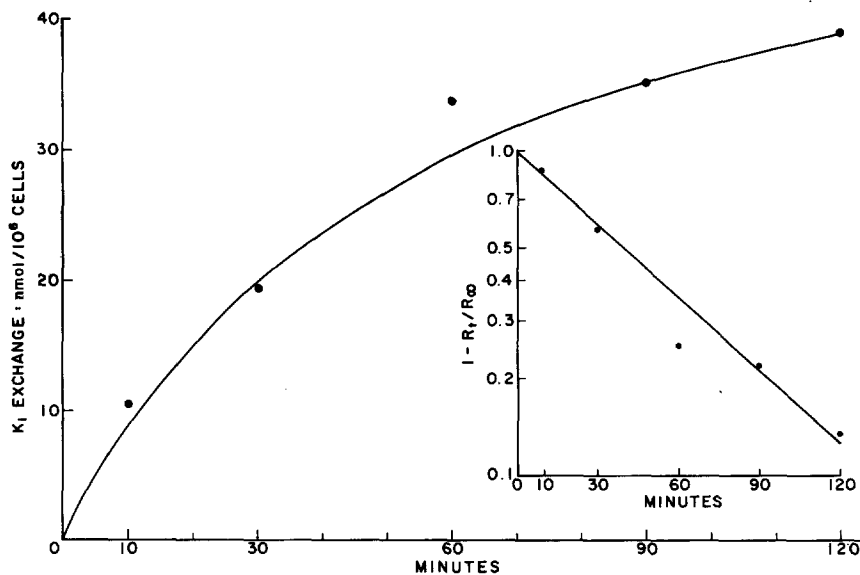


FIGURE 1 Time course of  $^{42}\text{K}$  influx in human PMNs, expressed as nanomoles of K exchanged per  $10^6$  cells. Inset shows data recalculated as  $1 - (R_t/R_\infty)$  where  $R_t$  is the radioactivity of the cells at times  $t$  and  $R_\infty$  is radioactivity at time infinity, assuming that a total of 45 nmol/ $10^6$  cells of intracellular K is available for exchange with  $^{42}\text{K}$ . See text for further explanation. Points represent means of two determinations.

involved washing the cells by filtration rather than centrifugation.

A 10-min preexposure of  $10^{-5}$  M ouabain inhibited K influx by 52% (mean, 13 donors). The range of the ouabain-sensitive component of K influx (38–69% of total influx) was considerably less than the range of absolute total K influx (0.30–1.25 nmol/ $10^6$  cells  $\times$  min). Increasing the ouabain concentration to  $10^{-3}$  M gave no further inhibition of K influx. Exposure to  $10^{-6}$  M ouabain for 10 min caused 77% of the inhibition observed in  $10^{-5}$  or  $10^{-3}$  M ouabain. With the procedures employed, ouabain binding was irreversible: the K influx measured on cells preexposed to ouabain, but washed free of unbound ouabain, was the same as the flux measured in the presence of  $10^{-4}$  M ouabain.

As in red blood cells, K inhibited ouabain binding. Preexposure to  $5 \times 10^{-7}$  M ouabain for 10 min in 1 mM K resulted in complete inhibition of total ouabain-sensitive influx, whereas incubation with  $5 \times 10^{-7}$  M ouabain in 5 mM K resulted in 66% inhibition of the ouabain-sensitive K influx. By analogy with red cells, the ouabain-sensitive K flux will be referred to here as the active or pump flux and the ouabain-insensitive flux as the passive or leak flux. It has not been established that the K gradient in PMNs is maintained by active transport, as indicated for red blood cells (Glynn, 1956; Dunham and Gunn, 1972) and other systems. The designations of pump and leak fluxes are used operationally, though ouabain may well act on PMNs in the same manner as on red cells.

### Amino Acid Influxes

Double reciprocal plots of lysine and leucine influxes vs. external concentration are shown in Figs. 2 *a* and *b*. The correspondence between the experimental points and linearity suggests influx of both amino acids by saturable transport systems. These data enabled approximation of the kinetic constants of the fluxes,  $V_{\max}$  and apparent  $K_m$ . These values, along with mean fluxes at 0.1 mM external amino acid concentration, are given in Table II. All determinations involved 10-min exposures to the labeled amino acids. The influxes of [ $^{14}$ C]lysine and leucine had not reached saturation in 15 min, but the net rate of entry had declined. Thus after incubation for 10 min in the tracer, unidirectional influx would be underestimated from net tracer accumulation. Nevertheless, as will be shown below, fluxes based on incubation of 10

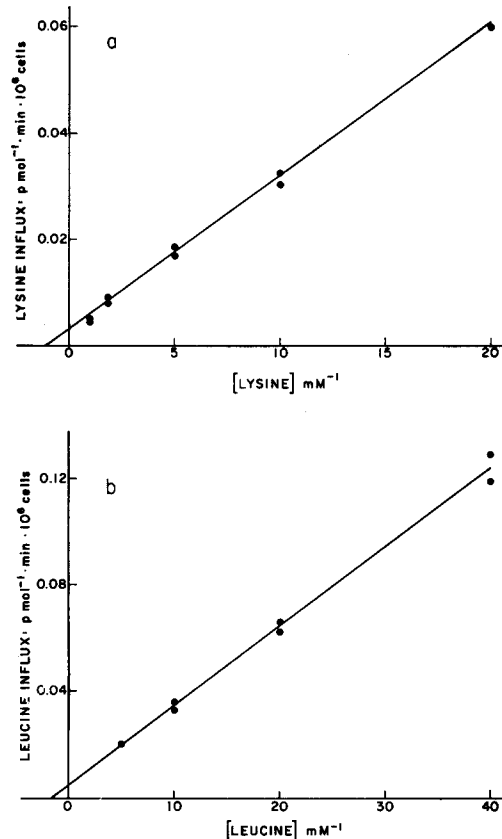


FIGURE 2 Lineweaver-Burke plots of amino acid influxes in human PMNs. Cells were incubated in media with various amino acid concentrations, lysine (2*a*), or leucine (2*b*) for 10 min. Results are given as reciprocal picomoles/ $10^6$  cells  $\times$  min vs. reciprocal millimoles/liter. Points represent single determinations.

min give valid estimations of relative flux rates. The  $V_{\max}$  and apparent  $K_m$  found by Ukena and Berlin (1972) for lysine influx in rabbit PMNs were 0.025 nmol/ $10^6$  cells  $\times$  min and 0.25 mM, respectively. The  $V_{\max}$  was considerably lower than that found here.

Preliminary data indicate that lysine and leucine influxes are by separate mechanisms: 2 mM leucine had no effect on lysine influx cells in 0.1 mM lysine. In contrast, Tsan and Berlin (1971) found a marked inhibition of lysine influx by leucine in rabbit alveolar macrophages.

The method for measuring influxes involved washing the cells free of extracellular label by centrifugation and resuspension in ice-cold buffered saline. To obtain an indication of the extent to

which intracellular label is removed during washing, influxes of K and lysine were measured at 4°C and compared with fluxes measured at the same time at 37°C. Both total K influx and lysine influx were reduced by 90% at 4°C. Also, as expected, most of the K influx at 4°C was ouabain insensitive. Therefore, during washing in the cold, there is some loss of intracellular tracer, but only a small fraction of that which entered during the incubation at 37°C.

### Potassium Influxes and Phagocytosis

Potassium influxes were compared in control cells and cells exposed to zymosan or latex parti-

TABLE II  
Amino Acid Influxes in Human PMNs Based upon 10-min Exposures to [<sup>14</sup>C]Amino Acid

	Fluxes at 0.1 mM*		Kinetic constants†	
	nmol/10 <sup>6</sup> cells × min	mmol/liter cells × min	nmol/10 <sup>6</sup> cells × min	K <sub>m</sub> (apparent) mmol/liter
Lysine	0.021 (n = 6)	0.061	0.33	0.9
Leucine	0.019 (n = 3)	0.055	0.22	0.7

\* External amino acid concentration.

† Taken from appropriate extrapolations of the data in Fig. 2.

cles for 30 min before measurement of the flux. The results of several experiments are summarized in Fig. 3. The ouabain-sensitive or pump flux and ouabain-insensitive or leak flux are given as the percents of these fluxes in control cells. In cells exposed to zymosan, pump influx was reduced by more than 60% compared to that in the control cells. The reduction in the pump was significant at the 0.016 level. The leak influx was reduced about 20% (*P* = 0.03). Similar results were obtained in cells exposed to latex; the pump was inhibited by more than 50% and the K leak was reduced by 20%.

The effects of exposure to zymosan were observed only if fresh autologous serum was present in the reaction mixtures. If serum pretreated by heating to 56°C for 30 min was employed, only a slight inhibition of K influx was observed. K influx was unaffected in cells exposed to zymosan-treated serum (1 mg/ml) in the absence of particles.

To determine if inhibition of K influx was due simply to membrane internalization as a consequence of phagocytosis, cells were treated with cytochalasin B (5 μg/ml) for 10 min before exposure to particles. As shown in Fig. 3, cytochalasin alone may have reduced both ouabain-sensitive and -insensitive K fluxes slightly (*P* = 0.09 for both). In cells exposed to zymosan after cytochalasin B treatment, the K pump flux was again reduced, but to an extent not significantly different from that observed after exposure to zymosan alone (*P* = 0.375). In contrast, there was little or no inhibition of the K pump in cytochalasin B-treated cells exposed to latex particles.

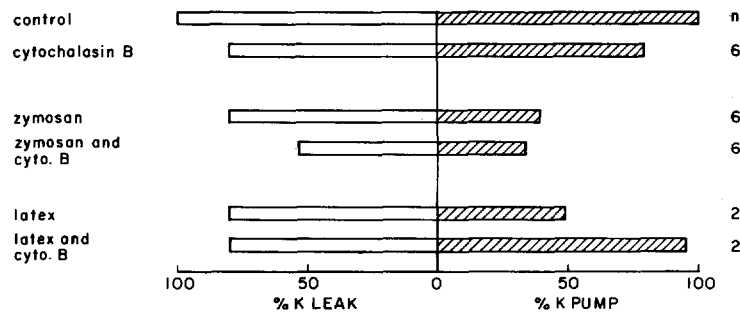


FIGURE 3 K influxes in human PMNs after various treatments as percent of K influxes, both leak (ouabain-insensitive) or pump (ouabain-sensitive), in untreated or control cells. Aliquots of cells were exposed to zymosan particles (10 particles/cell) or latex particles (100 particles/cell) for 30 min before measurement of the fluxes. Some of these aliquots were preexposed to cytochalasin B (5 μg/ml) for 15 min before addition of the particles; total exposure to cytochalasin B was 45 min. Total K influxes and % pump flux were close to the mean given in the text for all experiments. *n* = number of replicated experiments.

### Amino Acid Influxes and Phagocytosis

In Table III are the results of experiments in which lysine influx, from an external concentration of 0.1 mM, was measured along with K influx in cells exposed to particles. Lysine influx was reduced in cells that had ingested zymosan or latex, but far less than the reduction in the K pump. In a total of seven experiments, in which K and amino acid influxes were measured simultaneously after exposure to particles, the inhibition of K influx was significantly greater than the inhibition of amino acid influx ( $P = 0.03$ ). Table III also shows that treatment with cytochalasin B had no effect on lysine influx and only slightly reduced K influx. In cells exposed to zymosan after treatment with cytochalasin B, both the lysine and K pump influxes were nearly the same as in cells exposed to zymosan without prior cytochalasin treatment.

Since the methods employed results in an underestimation of unidirectional amino acid influx, it is possible that the extent of inhibition after phagocytosis is also underestimated. Accordingly, unidirectional influxes of amino acids were determined in resting cells and in cells after exposure to zymosan, based upon incubation times in the tracers of 0.5 and 10 min. The results are shown in Table IV. The fluxes calculated from the 10-min incubation

are underestimated four- to fivefold relative to those based on the 0.5-min incubation; shorter incubation times might reveal an even greater underestimation. Nevertheless, the extent of inhibition of amino acid influx after exposure to zymosan was not reduced. The levels of inhibition based upon 10-min incubations were actually greater than those from 0.5-min incubations. The important point to be derived from these results is that the difference in inhibition of K and amino acid influxes after endocytosis cannot be ascribed to an underestimation of the inhibition of amino acid influx.

### Purified PMNs

To exclude the possibility that the results observed in the experiments described above were due to the presence of mononuclear cells in the leukocyte suspensions, PMNs were purified with a Hypaque-Ficoll gradient. The cell suspensions obtained contained 98% PMNs. The effects of exposure of these cells to zymosan on K and lysine

TABLE III

*K and Lysine Influxes, Measured Simultaneously, in Control Cells, and in Cells after Exposure to Zymosan or Latex for 30 min*

	Influxes		
	Lysine	K pump	K leak
	% of control		
Zymosan			
Zymosan	66	30	37
Cytochalasin B*	102	71	95
Cyto B + zymosan*	63	22	34
Total control influxes: lysine, 0.022 nmol/10 <sup>6</sup> × min; K, 1.17 nmol/10 <sup>6</sup> × min. (K pump, 60%).			
Latex			
Latex	67	48	81
Cytochalasin B*	89	98	90
Cyto B + latex*	86	95	80
Total control influxes: lysine, 0.014 nmol/10 <sup>6</sup> × min; K, 0.37 nmol/10 <sup>6</sup> × min (K pump, 66%).			

\* PMNs were preincubated with cytochalasin B (5 mg/ml) at 37°C for 15 min. Cytochalasin was also present during exposure to particles.

TABLE IV

*Amino Acid Influxes in Human PMNs: Effect of Time of Incubation in [<sup>14</sup>C]Amino Acid on Apparent Unidirectional Influx and on Apparent Percent Inhibition of Influx by Preexposure to Zymosan Particles for 30 min.*

	Incubation time in tracer <i>min</i>	Control influx	Inhibition after zymosan
		nmol/10 <sup>6</sup> cells × min	%
Lysine	0.5	0.091	14
	10	0.022	24
Leucine	0.5	0.089	20
	10	0.018	34

TABLE V

*K and Lysine Influxes in a Purified Preparation of PMNs*

	Influxes		
	Lysine	K pump	K leak
	% of control		
Zymosan*	80	16	75
Total control influxes: lysine, 0.018 nmol/10 <sup>6</sup> × min; K, 0.92 nmol/10 <sup>6</sup> × min (K pump, 59%).			

Differential counts; 98% PMNs, 2% eosinophils.

\* PMN's were exposed to zymosan for 30 min.

influxes are shown in Table V, along with the absolute total influxes in the control cells. Both the absolute fluxes and the inhibition observed in cells exposed to zymosan were generally the same as those observed with suspensions containing 85% PMNs, and, indeed, the K pump was inhibited much more than in less purified preparations.

### Effect of Colchicine

K and amino acid influxes were measured in resting cells, cells exposed to zymosan or latex particles, and cells preincubated with  $5 \times 10^{-6}$  M colchicine for 1 h before exposure to particles. The results are shown in Table VI. After ingestion of zymosan or latex the amino acid influx was inhibited slightly. The K pump was inhibited somewhat more than usual with both zymosan and latex, whereas the K leak was uncharacteristically not inhibited with zymosan.

Colchicine treatment alone had little or no effect on amino acid or K influxes. The important conclusion to be drawn from these results is that colchicine did not cause an increase in inhibition of K pump influx or amino acid influx after particle ingestion. The percent inhibitions of the K pump and lysine influx were the same in cells exposed to zymosan in the presence or absence of colchicine.

TABLE VI  
*Effects of Colchicine on Inhibition of K and Amino Acid Influxes by Particle Ingestion*

	Influxes		
	Leucine	K pump	K leak
	% of control		
Zymosan			
Zymosan	75	20	96
Colchicine	104	92	97
Colchicine + zymosan	76	20	79
Total control influxes: leucine, 0.013 nmol/ $10^6 \times$ min; K, 0.69 nmol/ $10^6 \times$ min (K pump: 54%).			
Latex			
Latex	76	36	82
Colchicine	96	75	109
Colchicine + latex	72	66	57
Total control influxes: leucine, 0.015 nmol/ $10^6 \times$ min; K, 0.44 nmol/ $10^6 \times$ min (K pump: 69%).			

PMNs were preincubated with colchicine ( $5 \times 10^{-6}$  M) at 37°C for 1 h; Colchicine was also present during exposure to particles.

With latex the inhibition of the K pump was markedly less with colchicine than without. Both zymosan and latex produced a slightly greater inhibition of the K leak after colchicine pretreatment.

### DISCUSSION

The experiments cited in this report were designed to determine the relationship between two functions of the PMN plasma membrane: solute transport and phagocytosis. Using methods that have been utilized for the measurement of K and amino acid fluxes in other cell types, we were able to measure the transport of these solutes in human PMNs under resting conditions and after exposure to various phagocytic stimuli.

The mean unidirectional K influx in resting PMNs measured in the present study, 2.0 mmol/liter cells  $\times$  min, is similar to the K influxes recently reported by Cividalli and Nathan (1974) (1.3–1.6 mmol/liter cell water  $\times$  min; three determinations). No other determinations of K influx in PMNs have come to our attention. The similarity between our calculated value for exchangeable intracellular K and previously reported chemical determinations of K concentration in PMNs is a basis for confidence not only in the value for exchangeable K but in the values for unidirectional influx as well.

There was a large range in absolute K influxes in different preparations of cells in the present work. This may have been due to actual differences in the cells from the various donors, since no such variability was found between duplicate determinations within an experiment. Furthermore, despite variability in absolute fluxes between experiments, the relative effects to which major significance is attached were consistent.

There might be concern that the K fluxes are underestimated due to loss of  $^{42}\text{K}$  from the cells during washing. Cividalli and Nathan (1974) presented evidence for K loss from human leukocytes in the cold. Though Berlin and Wood (1964) found that exposure to 4°C for 2 h had no significant effect on K concentration in leukocytes, the procedures employed here for the preparation of the leukocyte suspensions used in the experiments might also be expected to cause loss of K from the cells. Lichtman and Weed (1972) have shown conclusively that any such losses are restored by subsequent incubation for 1 h at less at 37°C in physiological concentrations of K. All of the



experiments in the present study involved incubations at 37°C for 30–60 min before measurement of K influx.

A large fraction of K influx, about one half, was insensitive to ouabain. This was not due to a low affinity for ouabain, since  $10^{-5}$  M and  $10^{-3}$  M ouabain gave the same levels of inhibition. If one makes the common assumption that the ouabain-sensitive K influx is active and the ouabain-insensitive flux is passive, one can in simple thermodynamic terms approach the question: is it likely that there is a passive K influx of 1.0 mmol/liter  $\times$  min? If the cells are in steady-state with respect to K and all of K efflux is passive, K efflux will equal total K influx, or 2.0 mM/liter  $\times$  min. From the fluxes and the K concentrations, 130 mM inside and 5 mM outside, one can calculate the electrical potential difference across the membrane with the flux ratio equation (Ussing, 1949):

$$\frac{{}^iM}{{}^oM} = \frac{C_o}{C_i} e^{EF/RT} \quad \text{or} \quad E = \frac{RT}{F} \ln \frac{C_i}{C_o} \cdot \frac{{}^iM}{{}^oM} \quad (3)$$

where  ${}^iM$  and  ${}^oM$  are the unidirectional influx and efflux,  $C_o$  and  $C_i$  are the outside and inside concentrations,  $E$  is the electrical potential difference, and  $F$ ,  $R$ , and  $T$  have their usual meanings. With the values just given and 37°C,  $E$  is predicted to be 68 mV. If  $E$  were determined entirely by the K concentration difference, it would be 87 mV calculated by the Nernst equation. Therefore  $E$  predicted by flux ratio analysis is less than the K equilibrium potential. If Na permeability were relatively high, and the Na gradient were a major determinant of  $E$ , then  $E$  might not be as high as 68 mV. Nevertheless, the membrane potential predicted by a passive K influx, 50% of total influx, is not unreasonable.

Finally the ouabain-insensitive K influx might consist in part of a ouabain-insensitive active K influx analogous to the ouabain-insensitive Na efflux in red cells (Hoffman and Kregenow, 1966; Sachs, 1971). Cividalli and Nathan (1974) found no effect of ethacrynic acid on K concentration in human leukocytes; this drug inhibits the ouabain-insensitive Na pump in red cells.

The results of our experiments provide new descriptions of the functional topography of the surface membrane of human PMNs. The topography appears to have two dimensions: (a) a distribution with respect to one another of transport sites for different solutes; and (b) a functional relation-

ship between transport sites, particle binding sites, and areas of the membrane involved in endocytosis.

Evidence bearing on the first aspect of cell surface topography is provided by the large difference between inhibition of amino acid influx and inhibition of K influx after exposure to phagocytic stimuli. After ingestion of zymosan or latex particles or after surface binding of zymosan in cytochalasin-treated cells (see below), the K pump was inhibited 50–75%, while simultaneously measured lysine or leucine influxes were inhibited 30% or less. Assuming that the observed inhibition of transport was due primarily to internalization or inactivation of the surface membrane as a consequence of particle ingestion, these results indicate a topographical separation between the two kinds of transport sites. K transport sites appear to be more closely associated with areas of the membrane involved in the binding and phagocytosis of certain particles than the transport sites of amino acids. This functional topography need not reflect static spatial heterogeneity, but may indicate a mobilization of K sites toward (or amino acid sites away from) areas of the surface involved in particle contact and/or ingestion.

The data summarized in Table IV demonstrate that the differences between levels of inhibition of K and amino acid influxes were not due to an underestimation of the inhibition of amino acid influxes, though the absolute unidirectional influx of amino acids was indeed underestimated.

After ingestion of zymosan, ouabain-insensitive K influx was always reduced, though there was considerably more variability than in the reduction of ouabain-sensitive K influx. In some experiments, the K leak was reduced little or not at all after particle ingestion, even though the usual inhibition of the K pump was observed (e.g., Table VI). Therefore, little significance can be attached to effects of phagocytic stimuli on the K leak. The reduction usually observed may reflect a decrease of the total surface area of the cell. Conversely, there may be a spatial heterogeneity in areas of the membrane permitting passive K flux. It has been suggested that in red cells a component of passive cation fluxes, like active fluxes, takes place through specific, selective sites (Dunham and Hoffman, 1971). The inhibition of passive K flux in PMNs by membrane internalization is consistent with a mediation of the passive fluxes by such heterogeneously distributed specific sites.

Requirements for optimal phagocytosis of zymosan and latex particles differ in the systems employed in this study. Fresh serum must be present in the case of zymosan, since opsonization is a prerequisite for ingestion of this particle. There is considerable evidence that a portion of the third component of serum complement, C3b, is the factor that promotes adherence of these particles to the PMN surface membrane via specific membrane receptors (Winkelstein et al., 1972; Johnston et al., 1973; Lay and Nussenzweig, 1968). The lack of inhibition of K influx observed in PMNs exposed to zymosan in the presence of heated (complement-inactivated) serum is therefore expected. Fresh serum is not an absolute requirement for the ingestion of polystyrene latex particles, and, though opsonization by immunoglobulins has been suggested, specific binding of latex to PMNs has not been demonstrated (Van Oss and Stinson, 1970; Skeel et al., 1969).

The second aspect of the functional topography of human PMNs indicated by our results is a complex relationship between transport sites, particle-binding sites, and areas of the membrane involved in phagocytosis.

Support for this relationship comes from the results of experiments in which cytochalasin B-treated PMNs were exposed to zymosan or latex particles before measurement of the solute influx. Cytochalasin B, at the concentration used in these experiments, inhibits the ingestion of most particles by human PMNs (Davies et al., 1973; Zurier et al., 1973). Nevertheless, particles (particularly zymosan) can adhere to cells treated with this compound (Zurier et al., 1973). Thus the effects on solute transport of particle binding per se can be distinguished from effects of ingestion and membrane internalization.

Cytochalasin B-treatment had little effect on lysine and K influx in resting cells. Zigmond and Hirsch (1972) similarly reported a lack of effect of cytochalasin B on leucine influx in horse leukocytes, though deoxyglucose transport was strongly inhibited. The inhibition of both lysine and K pump influx observed when untreated cells were exposed to zymosan was also seen with cytochalasin B-treated cells exposed to this particle. As in the former case, inhibition of K influx was again greater than inhibition of lysine influx. In contrast, when cytochalasin B-treated PMNs were exposed to polystyrene latex, no inhibition of solute influx was observed. Our interpretation of these results is as follows: In normal cells (without cytochalasin B

treatment) both zymosan and latex are ingested irrespective of differences in surface binding, and the internalization of the surface membrane that accompanies ingestion results in a reduction of solute transport. In the nonphagocytic system (cytochalasin B-treated PMNs) surface binding of zymosan but not latex occurred, and this was sufficient to inhibit K and lysine influx. In both systems, inhibition of K influx exceeded inhibition of lysine influx. Thus there appears to be a spatial separation of sites responsible for lysine and K transport as well as an association of K pump sites with areas of the PMN surface membrane involved in particle ingestion, and specifically with zymosan-binding, perhaps C3b receptor sites.

There may be a static association of K pump sites and C3b receptors in restricted areas of the membrane; or, conversely, the K pump sites may be mobilized toward the receptor sites when these latter sites are occupied. The inhibition of K transport by occupation of zymosan-binding sites may be steric: the zymosan particles may simply mask the K sites. It might also be allosteric, resulting from a conformational change in the K pump sites mediated through the zymosan-binding sites. In this instance, the transport sites may not be inhibited, but rather may have a reduced affinity for the substrate, thus reducing transport at subsaturating concentrations of substrate. A related possibility is that binding of particles to the surface may change the fluidity of the cell membrane, which may in turn affect transport sites.

While the interpretation of the results in terms of membrane topography is consistent with the results, and appears to us the most likely context for explaining the results, another possibility should be considered. Phagocytic stimuli alter the metabolism of PMNs, most strikingly by stimulating C1 oxidation of glucose (Skeel et al., 1969). Possibly the reduction of active transport accompanying particle ingestion is due to a redirection of metabolic energy away from transport mechanisms. Though this possibility cannot be ruled out, it must be considered in light of a large overall increase in metabolism in PMNs associated with phagocytosis (Sbarra and Karnovsky, 1959).

There are two important differences between the present results and those obtained by Berlin's group on transport and phagocytosis. First, in the present study, transport was inhibited after phagocytosis of particles. Tsan and Berlin (1971) observed no inhibition after phagocytosis in otherwise untreated cells. Second, in the present system,

disruption of microtubules had no effect on the inhibition of transport association with particle ingestion, whereas Ukena and Berlin (1972) found that after colchicine treatment, phagocytosis did result in inhibition of transport.

These differences may be attributable to one or more factors. First, cells from different species of mammal were used: human cells in the present study and cells from rabbits in the studies of Berlin's group. Second, in the present study, PMNs were obtained from peripheral blood. Berlin's group used alveolar macrophages and peritoneal PMNs. The experiments involving treatment with colchicine were performed only with PMNs. Finally, in the present work, the cells were in suspension, whereas Berlin's group used cells that had been allowed to adhere to glass. Attachment to glass may be associated with changes in the functional state of PMNs. For example, it has been suggested that microtubules are involved in attachment and spreading of macrophages (Reaven and Axline, 1973). Attached cells might therefore respond to colchicine differently from cells in suspension.

Recently Berlin's group reported further evidence on regulation of membrane topography that is in part complementary to that given here (Oliver et al., 1974). It was found that lectin-binding sites in rabbit PMNs were internalized selectively during phagocytosis of droplets of an oil emulsion. The evidence for selective internalization was a reduction of lectin-binding sites per milligram of membrane protein after phagocytosis. Thus, these results correspond to the selective internalization of K transport sites reported here. Pretreatment with colchicine for 30 min at  $5 \times 10^{-6}$  M did not inhibit phagocytosis, but did eliminate the selective internalization of lectin-binding sites. In contrast, colchicine did not have a corresponding effect on the selective internalization of K transport sites in the present study. It is important to point out that this last study from Berlin's group used PMNs in suspension rather than attached to glass, as in their previous work. The interpretation of the results on lectin binding may be complicated by the fact that at 37°C, lectins induce their own internalization by a process that remains unclear (Oliver et al., 1974), whereas PMNs do not otherwise appear to internalize membrane in the absence of specific phagocytic stimuli (Henson, 1971).

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