EFFECT OF PHAGOCYTOSIS ON MEMBRANE TRANSPORT OF NONELECTROLYTES*

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Phagocytosis and carrier-mediated membrane transport are two distinct membrane functions by which substances are translocated across the plasma membrane. Yet, little is known about the distribution of these functions over the cell surface. During phagocytosis, a part of the plasma membrane is internalized (1, 2). This internalized membrane may include transport sites (carriers). On the other hand, transport sites may be distributed in such a way that different parts of the plasma membrane are involved in transport and phagocytosis.

In this study, the activities of specific transport systems were determined before and after large portions of the surface membrane had been interiorized by phagocytosis of inert particles. Five separate transport systems characterized in this laboratory, whose activity can be measured with great sensitivity, have been examined: adenosine[1] and two adenine (3) transport systems in rabbit polymorphonuclear leukocytes; adenosine[2] and lysine (4) transport systems in alveolar macrophages. The results show that the rates of transport were unaffected in all systems, even after an estimated 35–50% of the membrane had been internalized. It was also shown that the constancy of transport did not depend on the introduction into the surface of new transport sites during phagocytosis. The results indicate that the membrane is mosaic in character with geographically separate transport and phagocytic sites.

Materials and Methods

Animals.—New Zealand white rabbits of either sex, weighing between 2 and 4 kg each, were used.

Chemicals.—Adenine-8-^{14}C, adenosine-^{3}H, and lysine-^{3}H were obtained from Schwarz BioResearch, Inc., Orangeburg, N.Y. Lysine-^{14}C was obtained from New England Nuclear Corp., Boston, Mass. *p*-Chloromercuribenzene sulfonic acid (PCMBSA)[3]-^{203}Hg was obtained

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[3] *Abbreviations used in this paper:* PCMBSA, *p*-chloromercuribenzene sulfonic acid; PVT, polyvinyl toluene; -SH, sulfhydryl group.

from Amersham-Searle Corp., Arlington Heights, Ill., and unlabeled PCMBSA was obtained from Sigma Chemical Company, St. Louis, Mo. Adenosine-^{3}H and lysine-^{3}H were purified by ascending paper chromatography as described previously (4).[1]

Polyvinyl toluene latex (2.02 μ in diameter) and polystyrene latex (0.1 μ in diameter) were obtained from the Dow Chemical Company, Midland, Mich.

The Preparation of Leukocytes and Alveolar Macrophages.—Polymorphonuclear neutrophilic leukocytes were obtained from sterile peritoneal exudates by the method of Kaiser and Wood (5). Alveolar macrophages were obtained by the method of Myrvik, Leake, and Fariss (6), as modified by Tsan and Berlin (4). The cells were harvested with modified Hanks' solution as described previously (3, 4). Cell monolayers used in all experiments were formed as previously described on round glass cover slips at a cell density of 2 million polymorphonuclear leukocytes, and 0.3 million alveolar macrophages, per cover slip (3, 4).

Quantitation of Phagocytosis.—Quantitative measurement of phagocytosis was based on the method of Roberts and Quastel (7). Briefly, after the formation of monolayers, the cover slips were drained and aliquots of approximately 0.4 ml of the incubation media containing 0.5% by weight of polyvinyl toluene latex particles (2.02 μ in diameter), 2 mM Mg^{++}, and 2 mM glucose in modified Hanks' solution, prewarmed to 38.5°C, were placed over the monolayers. After incubation for 30 min, the cover slips were drained and rinsed consecutively in four beakers containing ice cold modified Hanks' solution. The cover slips were sealed with Parafilm in 20 ml beakers containing 2 ml of dioxane, and extracted overnight. The extracted polyvinyl toluene was measured with a Beckman spectrophotometer (Beckman Instruments, Inc., Fullerton, Calif.) at 274 mμ. The controls were dioxane extracts of monolayers incubated with the same media at 0°C for 30 min. The results expressed as the number of particles taken up were calculated from the extinction coefficient (2.4×10^{-3} OD units/μg per ml at 274 mμ) and the density (1.03 g/ml at 20°C) of polyvinyl toluene (8). Phase-contrast microscopy revealed that after 30 min incubation at 38.5°C, polymorphonuclear leukocytes or alveolar macrophages were heavily loaded with particles which made accurate direct counting impossible. On the other hand, when incubated at 0°C some particles were found adherent to the glass and to the cell periphery. Few can be identified intracellularly by phase-contrast microscopy and consequently the 0°C value was subtracted from that obtained at 38.5°C to obtain the approximate number of particles actually taken up by the cells.

Determination of Membrane Transport of Nonelectrolytes by a Rapid-Sampling Technique.—The rapid-sampling technique, developed by Hawkins and Berlin (3), was used in this study. The 45 sec uptake of the radio-labeled substrates, which we have shown to represent the initial rate of transport before and after phagocytosis, was determined in each transport system. Five separate transport systems (3, 4), [1, 2] which have been characterized in this laboratory, were examined. Table I summarizes the kinetic characteristics of these transport systems.

For the determination of membrane transport after phagocytosis, monolayers were first incubated with media containing particles. At the end of the incubation, the cover slips were drained and washed with modified Hanks' solution at room temperature, then the rate of transport was determined. Controls were done with the media containing no particles and the rate of transport determined in the same way. All the experiments were done with quadruplicate monolayers and averaged. The results are expressed as the amount of substrate transported per 45 sec/cover slip, unless otherwise indicated.

Sensitivity of the Rapid-Sampling Technique.—The maximal transport rate (V_{max}) is proportional to the number of carriers of a transport system and hence, the initial rate of transport is often used as an assay of the number of membrane carriers (9, 10). The results depicted in Fig. 1 are the rates of lysine transport measured with different amounts of alveolar macrophages per cover slip. It is apparent that the rate of lysine transport is proportional to the amount of alveolar macrophages and hence, to the number of carriers. The sensitivity of the

TABLE I

Summary of the Kinetics of Membrane Transport Systems of Nonelectrolytes in Rabbit Alveolar Macrophages and Polymorphonuclear Leukocytes

Transport systems	K_m	V_{max}*	Reference or footnote
	mM		
Alveolar macrophages			
Lysine transport system	0.1	0.44×10^{-3}	(4)
Adenosine transport system	0.04	0.32×10^{-3}	FN2
Polymorphonuclear leukocytes			
Adenosine transport system	0.01	1×10^{-5}	FN1
Adenine transport system			
High K_m system	100	1.37×10^{-2}	(3)
Low K_m system	0.007	0.57×10^{-5}	(3)

* Unit, μmoles/45 sec per million cells.

‡ FN, footnote.

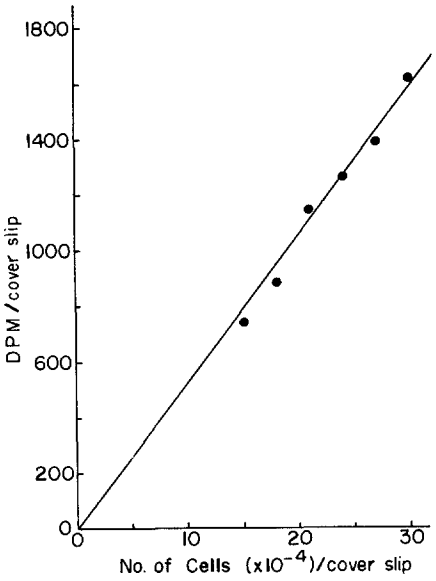

FIG. 1. Rate of lysine transport as a function of the number of macrophages per monolayer. Each point was the mean of four determinations.

assay is such that a decrease of greater than 7% in number of carriers is associated with a statistically significant ($P < 0.05$) decrease in transport rate.[4]

Uptake of Radioactive p-Chloromercuribenzene Sulfonic Acid by Alveolar Macrophages.— Monolayers were incubated with 0.005 mM PCMBSA-^{203}Hg in modified Hanks' solution

[4] This result is based on an analysis of the variance within groups of quadruplicate monolayers as routinely employed in the transport assay.

containing 5 mM glucose at 38.5°C for 20 min. After incubation, the cover slips were drained and washed and prepared for counting by liquid scintillation as described previously (3, 4). Because of the instability of this compound in solution (11), fresh solutions were used in each experiment. When cover slips alone (no cells) were incubated with PCMBSA-^{203}Hg for 20 min and washed in the usual manner, no radioactivity was found adsorbed to the glass.

RESULTS

Quantitative Determination of Phagocytosis.—Table II shows the number of polyvinyl toluene (PVT) particles (2.02 μ in diameter) phagocytosed by polymorphonuclear leukocytes and alveolar macrophages after 30 min incubations as determined by the spectrophotometric method. Averages of 14 and 51 particles were ingested by each leukocyte and macrophage, respectively. Electron microscopic evidence indicates that particles of this size are ingested individually (1, 12). The internalized particles appear to be tightly enveloped

TABLE II

Phagocytosis of Polyvinyl Toluene Latex Particles (2 μ in Diameter) by Rabbit Alveolar Macrophages and Polymorphonuclear Leukocytes

Cell types	No. of particles ingested/cell	Total surface area of particles ingested	Estimated cell surface	% Plasma membrane internalized during phagocytosis
Polymorphonuclear leukocytes	14 ± 2 (9)*	175 ± 30 μ^2/cell	500 μ^2	~35
Alveolar macrophages	51 ± 15 (16)	650 ± 190 μ^2/cell	1400 μ^2	~50

*Number in parenthesis: number of determinations.

by the plasma membrane. Hence, the total surface area of the particles should approximately represent the surface membrane interiorized by phagocytosis. The results of this calculation are included in Table II.

An accurate estimation of the total plasma membrane is impossible at this moment, especially of cells spreading over the cover slip. However, based on the previous measurements of the cell water in these two cell types (1.67 μl/million alveolar macrophages and 0.35 μl/million polymorphonuclear leukocytes) (3, 4) and assuming that the cells are spherical, a rough estimation of the cell surface can be made. It is readily calculated that alveolar macrophages have an area of 700 μ^2/cell (15 μ in diameter) and polymorphonuclear leukocytes have an area of 250 μ^2/cell (9 μ in diameter). Certainly these figures are underestimated. Scanning electron microscopic evidence (13, 14) suggests that after surface contact, leukocytes and macrophages are rather flat with increased diameters and a variety of projections. Even if we suppose that the actual surface area (Table II) is twice the above calculated value, the portion of the surface membrane interiorized during phagocytosis under the conditions of our experiment

is from 35% (polymorphonuclear leukocytes) to 50% (alveolar macrophages) of the entire cell surface membrane.

None of the experimental arguments to follow depend on the precise area of membrane internalized or of the cell itself. The approximate calculations indicate only that if transport sites were evenly distributed over the membrane, the per cent of the cell membrane lost to the surface by phagocytosis would undoubtedly include a fraction of the transport carriers sufficient to be readily detectable by our analysis (>7%).

TABLE III

Effect of Phagocytosis on the Membrane Transport in the Rabbit Alveolar Macrophage and Polymorphonuclear Leukocyte as Determined by the Rate of Transport at a Single Concentration

Transport systems	Conc.	Rate of transport *(±SD)		P‡
		Control	Phagocytosis	
	mM			
Alveolar macrophages				
Lysine	0.1	1.33 ± 0.27 (6)§	1.26 ± 0.21 (6)	>0.20
Adenosine	0.04	0.35 ± 0.14 (4)	0.34 ± 0.14 (4)	>0.25
Polymorphonuclear leukocytes				
Adenosine	0.01	11.39 ± 2.13 (3)	10.49 ± 0.14 (3)	>0.20
Adenine				
High K_m system	5	1560 ± 287 (7)	1580 ± 375 (7)	>0.80
Low K_m system	0.008	5.97 ± 1.51 (7)	6.29 ± 1.30 (7)	>0.10

* The rate of transport was expressed as (10^{-4} μmoles/45 sec per 0.3 million cells) for alveolar macrophages and (pmoles/45 sec per 2 million cells) for polymorphonuclear leukocytes.

‡ P values were determined from paired differences.

§ Number in parenthesis: number of experiments. Each experiment was done with quadruplicate monolayers and averaged.

Effect of Phagocytosis on Membrane Transport.—The rate of transport in five systems was measured after the cells were induced to phagocytize PVT particles (2.02 μ in diameter) for 30 min. The results were as shown in Table III. It is obvious that the rate of transport was not significantly different after phagocytosis. The same determinations were also done with polystyrene particles (0.1 μ in diameter) for the lysine and adenosine systems in alveolar macrophages with identical results (not shown in Table III).

The kinetics of carrier-mediated membrane transport follows enzyme kinetics and the initial reaction is often represented by the following model (15–17),

$$(a) \qquad C + Se \underset{k_3}{\overset{k_1}{\rightleftharpoons}} CS \xrightarrow{k_2} C + Si,$$

where C represents carrier, and Se and Si represent extracellular and intracellular substrate concentration, respectively. CS represents the concentration of the carrier-substrate complex. The k (k_1, k_2, and k_3) represent the rate constants.

If Ct represents the total number of carriers, then the maximal transport rate can be expressed as:

$$(b) \qquad V_{max} = k_2 [CtS].$$

It is obvious that V_{max} is proportional to the total number of carriers under each experimental condition. The initial rate (V) of transport at a single con-

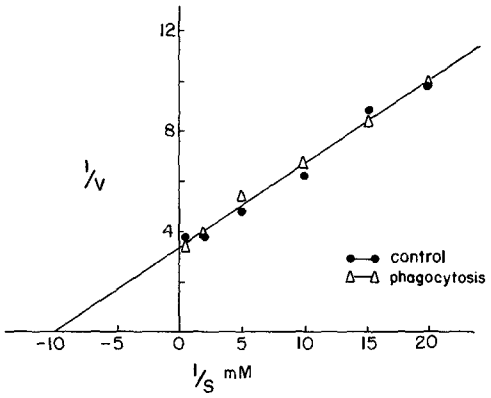

FIG. 2. Kinetics of lysine transport in alveolar macrophages with and without phagocytosis. Double reciprocal plots of lysine transport in macrophages with and without phagocytosis. Cell monolayers were first incubated with 0.5% PVT latex for 30 min, rinsed, and then 45 sec uptake of lysine-^{3}H in concentrations from 0.05 mM to 2 mM was measured. Control was incubated in the absence of particles. Each point was the mean of four determinations. V was expressed as 10^{-3} μmoles/45 sec per 0.3 million cells.

centration S is determined by the following equation:

$$(c) \qquad V = V_{max}\left(\frac{[S]}{[S] + K_m}\right) = k_2[CtS]\left(\frac{[S]}{[S] + K_m}\right)$$

From equation c, it is apparent that three factors can influence the initial rate, namely, k_2, $[CtS]$, and K_m. The number of carriers are reflected by the initial rates under various experimental conditions only when k_2 and K_m remain remain constant. Therefore it is of prime importance to find out whether these factors are changed by phagocytosis. The possibility that K_m is affected by phagocytosis was determined by kinetic studies. It is assumed that in these experiments k_2 can be affected only by exchange diffusion, a phenomenon characteristic of the lysine transport system. The following experiments reveal that

K_m remains constant after phagocytosis and that lysine transport is identical in control and phagocytic cells under conditions of maximal exchange diffusion.

Effect of Phagocytosis on the Kinetics of Membrane Transport.—Kinetic studies of lysine and adenosine transport in alveolar macrophages with and without phagocytosis were carried out first and the results as expressed by double reciprocal plots are shown in Figs. 2 and 3. The K_m and V_{max} of both transport systems remained the same after phagocytosis. Therefore the rates of transport shown in Table III, as determined at a single substrate concentration, are satisfactory indices of the V_{max} of the transport systems.

Effect of Exchange Diffusion on Lysine Transport of Alveolar Macrophages with and without Phagocytosis.—It has been shown in our previous study (4) that the

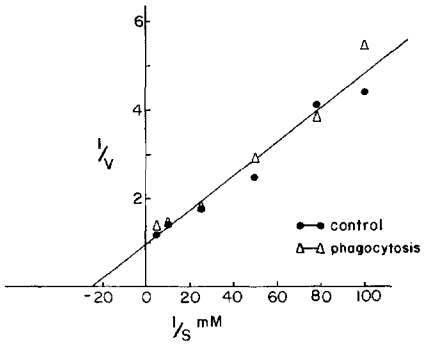

FIG. 3. Kinetics of adenosine transport in alveolar macrophages with and without phagocytosis. Phagocytosis was induced as in Fig. 2, 45 sec uptake of adenosine-^{3}H in concentrations from 0.01 mM to 0.2 mM was measured. Each point was the mean of four determinations. V was expressed as 10^{-4} μmoles/45 sec per 0.3 million cells.

rate of lysine transport as measured by radioactive tracer is influenced by the intracellular lysine concentration. The stimulation of isotope flux by transmembrane concentration (exchange diffusion) is presumably because of an increase of k_2 in equation c and attains a maximum at high intracellular lysine concentrations (4). For this one transport system the existence of exchange diffusion does not permit us to infer the constancy of carrier number from the isotope flux data. It is possible that the intracellular concentration of lysine changed after phagocytosis, so that although the affinity of lysine for the carrier remains unchanged, the number of carriers is reduced but compensated by an increased rate because of exchange diffusion (i.e., k_2 increased, see equation c). In order to rule out this possibility, alveolar macrophages were loaded with 5 mM lysine-^{14}C during phagocytosis. The final intracellular concentration attained was sufficient to induce near maximal exchange diffusion. After phagocytosis the rate of lysine transport was then measured with lysine-^{3}H. From

Table IV it is shown that alveolar macrophages accumulated lysine to the same concentration with and without phagocytosis. Moreover, the rate of transport was stimulated more than twofold in comparison with cells not preloaded with lysine. There was no difference between macrophages with and without phagocytosis. From this observation and the above kinetic study it is concluded that the lysine transport system remains unchanged (both K_m and V_{max}) and the number of carriers remains the same after phagocytosis.

The Specificity of Adenine Transport in Polymorphonuclear Leukocytes after Phagocytosis.—At low concentration adenine is converted to adenosine monophosphate (AMP) by the enzyme adenine phosphoribosyltransferase (EC 2.4.2.7) immediately after it is transported into polymorphonuclear leukocytes (18). Transport rather than enzymatic conversion is the rate-limiting step of adenine uptake (3). After phagocytosis the rate of adenine entry remains the same

TABLE IV

Effect of Lysine Loading on Lysine Transport in Alveolar Macrophages with and without Phagocytosis

	Rate,* no lysine loading	Conc. of intracellular lysine after loading‡	Rate,* after lysine preloading
		mM	
Control	1.24	9.44	2.82
Phagocytosis	1.20	8.67	2.84

* Unit, 10^{-4} μmoles/45 sec per 0.3 million cells.

‡ Calculated from the cell content of the radioactive C^{14} and the specific activity of the medium lysine.

(Table IV). It is important to insure that transport is still the rate-limiting step. As an approach to this question, adenine analogs with various affinities for transport carrier as compared to the enzyme were used to inhibit the 45 sec uptake of adenine (18). It is clear from Table V that the specificity of adenine transport remains the same after phagocytosis (i.e., has not shifted to the specificity of the enzyme. Compounds A and C which are good inhibitors of the extracted enzyme do not inhibit uptake.) This indicates that transport is still the rate-limiting step of adenine uptake even after phagocytosis.

Hypotheses to Explain the Constancy of Transport after Phagocytosis.—It is clear from the data of Table III that the number of transport carriers (i.e. the rate of transport) remains constant even after a large fraction of the surface membrane is interiorized during phagocytosis. Two interpretations seem most likely as illustrated in Fig. 4. The circle represents the hypothetical cell and L represents a lysine carrier. Our experimental findings indicate that after phagocytosis the number of lysine carriers remains the same. The first possibility is that some carriers are internalized when the membrane is internalized with the

ingested particle, but they are replaced immediately during phagocytosis, as represented by the appearance of new lysine carriers (NL). The second possibility is that phagocytosis and membrane transport involve different parts of the plasma membrane, so that no transport carriers are internalized during phagocytosis.

As an approach to distinguish these two possibilities, lysine carriers were first inactivated and then phagocytosis was induced (Fig. 5). If new transport carriers appeared during phagocytosis, some restoration of lysine transport should occur. On the other hand, if the constancy of the number of carriers is because of preservation of the lysine carriers, then there will be no restoration of lysine transport after phagocytosis.

In the following experiments it is demonstrated that the potent sulfhydryl

TABLE V

*Effect of Phagocytosis on the Specificity of Adenine Transport (Low K_m System) in Polymorphonuclear Leukocytes**

Compound	Conc.	% Inhibition		% Inhibition (or *Ki*)‡	
		Control	Phagocytosis	Transport	Enzyme
	mM				
A 5-amino-4-imidazole-carboxamide	1	5	7	5	56
B 4-aminopyrazole [3,4-d]pyrimidine	0.5	47	54	*Ki*(0.23 mM)	*Ki*(0.27 mM)
C 7-methyladenine	1	12	13	12	48

* Concentration of adenine tested was 0.008 mM.

‡ Figures taken from ref. 18 for comparison.

inhibitor PCMBSA at low concentrations (*a*) does not penetrate the cytoplasmic membrane, (*b*) selectively inactivates the lysine transport system, and (*c*) exerts minimal inhibition of phagocytosis.

Uptake of Radioactive PCMBSA by Alveolar Macrophages.—The uptake of PCMBSA-^{203}Hg by alveolar macrophages was carried out by incubating monolayers with 0.005 mM PCMBSA-^{203}Hg for various intervals. The results are shown in Fig. 6. The uptake is nearly constant after 20 min incubation. If monolayers were fixed in absolute ethanol for 10 min in order to destroy the permeability barrier, rinsed, and then exposed to the same concentration of PCMBSA for 20 min, the uptake of radioactivity was increased over 35-fold. This indicates that in unfixed cells maximal uptake of mercury is attained when fewer than 3% of the cell's sulfhydryl groups have reacted, i.e., the PCMBSA is normally prevented from access to the intracellular compartment. This result is in accord with studies on PCMBSA and erythrocytes in which it was also demonstrated that the agent did not penetrate the membrane (11).

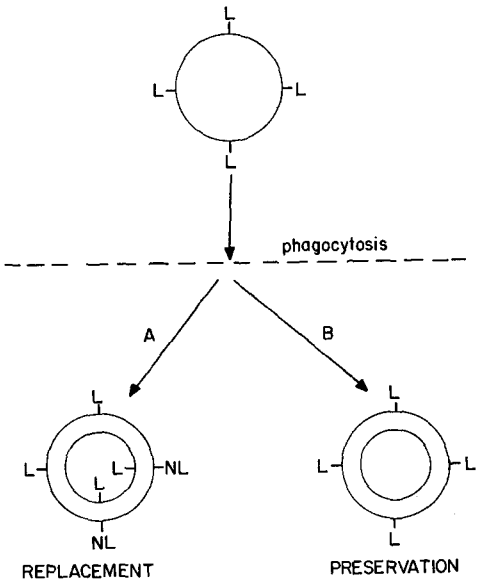

FIG. 4. Two hypotheses to explain the constancy of transport after phagocytosis.

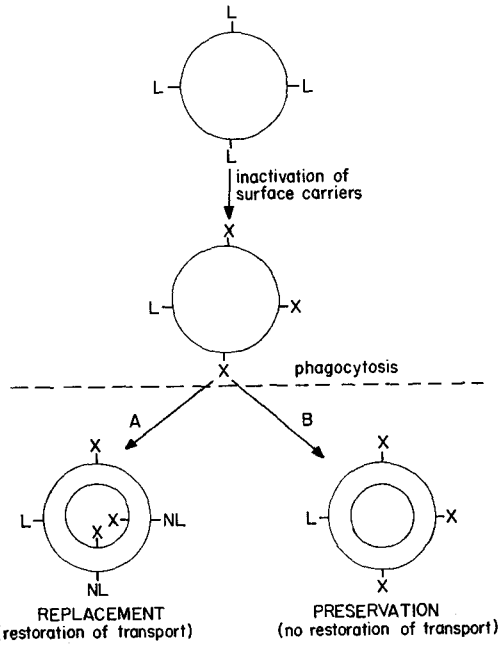

FIG. 5. Experimental approach to distinguish replacement from preservation.

Effect of PCMBSA on Phagocytosis and Membrane Transport in Alveolar Macrophages.—Fig. 7 shows the effects of various concentrations of PCMBSA on lysine and adenosine transport in alveolar macrophages. Macrophages tolerate the compound quite well over the concentrations tested as judged by

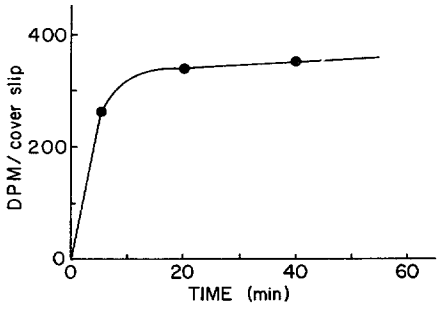

FIG. 6. Time-course uptake of radioactive PCMBSA by alveolar macrophages. Monolayers were incubated with 0.005 mM PCMBSA-^{203}Hg (30,000 dpm/ml) for the time interval indicated. dpm, drops per minute.

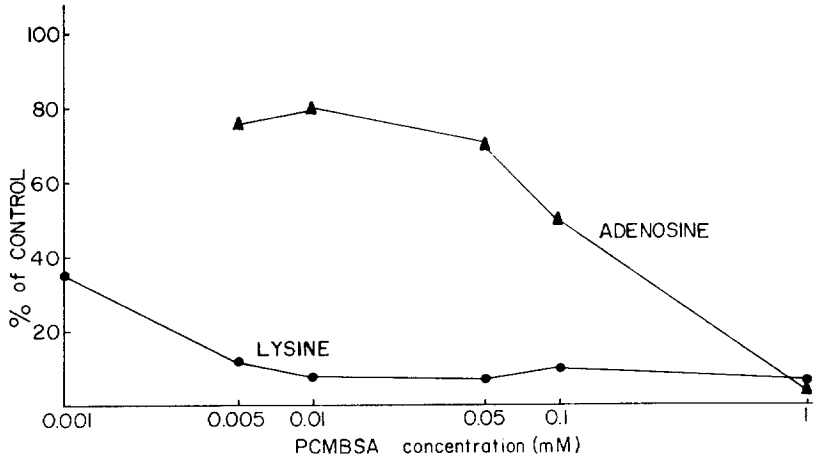

FIG. 7. Effect of PCMBSA on membrane transport in alveolar macrophages. Cell monolayers were preincubated with various concentrations of PCMBSA for 20 min, rinsed, and then the rate of lysine and adenosine transport determined. The results were expressed as the per cent uptake of control preincubated in the absence of PCMBSA.

the impermeability of the cell membrane to eosin Y. Lysine transport is extremely sensitive to PCMBSA inhibition. At 0.005 mM 90% of lysine carriers were inactivated while adenosine transport was only inhibited by 20%. The effect of PCMBSA on the phagocytosis of alveolar macrophages was also examined. After preincubation of 0.005 mM PCMBSA for 20 min, the phago-

cytosis of alveolar macrophages was inhibited by 30% as quantitated by the spectrophotometric method.

Effect of Lysine on the PCMBSA Inhibition of Lysine Transport in Alveolar Macrophages.—If the inhibition of lysine transport is because of the covalent binding of PCMBSA to the sulfhydryl (-SH) group of the lysine carrier at the active site, it may be possible to use a high concentration of lysine to protect the carrier from PCMBSA inhibition (analogous to substrate protection of enzymes). Alveolar macrophages were first incubated in 20 mM lysine-^{14}C in the modified Hanks' solution (with 5 mM glucose) with or without 0.005 mM PCMBSA for 20 min. At the end of the incubation, the monolayers were drained and washed with modified Hanks' solution at room temperature to remove all of the free PCMBSA and extracellular lysine-^{14}C. The rate of lysine transport was then measured with lysine-^{3}H. The results are shown in Table VI. Without

TABLE VI

Effect of Lysine on the PCMBSA Inhibition of Lysine Transport in Alveolar Macrophages

	Rate,* no lysine loading	Conc. of intracellular lysine after loading§	Rate,* after lysine loading
		mM	
Control	1.25	15.3	2.42
PCMBSA	0.22	18.2	2.26
% Inhibition	82		7

* Unit, 10^{-4} μmoles/45 sec per 0.3 million cells.

§ Calculated from the ^{14}C content and the specific activity of the 20 mM lysine.

lysine protection, 82% of lysine transport was inhibited by 0.005 mM PCMBSA, whereas the simultaneous presence of 20 mM lysine with the PCMBSA blocked its effect almost completely. There is no direct reaction of PCMBSA with lysine as shown below. The rates of lysine transport after lysine loading are maximal with or without PCMBSA because of the high intracellular lysine concentration which induces the maximal rate of exchange diffusion.

Uptake of Radioactive PCMBSA in the Presence and Absence of Lysine by Alveolar Macrophages.—The uptake of PCMBSA-^{203}Hg by alveolar macrophages was carried out by incubating monolayers with 0.005 mM PCMBSA-^{203}Hg for 20 min in the presence and absence of 20 mM lysine. As shown in Table VII, the difference of PCMBSA-^{203}Hg uptake, which is statistically highly significant by the method of paired differences, is presumably because of the protection of lysine carriers by the high concentration of lysine, and corresponds to the lysine protection of lysine transport in Table VI. However, it is important to rule out the possibility that this difference is because of the direct interaction of lysine with PCMBSA-^{203}Hg. Chromatographic analyses of PCMBSA-^{203}Hg, PCMBSA-^{203}Hg + lysine, and PCMBSA-^{203}Hg + cysteine

incubation media were carried out in four different solvent systems: (*a*) *tert*-butanol:methyl ethyl ketone:formic acid:water (40:30:15:15), (*b*) 5-butanol:methyl ethyl ketone:water:ammonium hydroxide (40:30:20:10), (*c*) *n*-butanol:glacial acetic acid:water (50:25:25), and (*d*) isobutyric acid:ammonium hydroxide:water (66:1:33). PCMBSA-^{203}Hg + cysteine could be separated from PCMBSA-^{203}Hg by solvents *c* and *d*. The R_f values of the end product of PCMBSA-^{203}Hg + cysteine were 0.43 for solvent *c* and 0.46 for solvent *d*. In the case of PCMBSA-^{203}Hg + lysine the radioactivity was recovered in a single spot at the same R_f value as PCMBSA-^{203}Hg alone in all four solvents. The addition of 10 mM of cysteine reduces ^{203}Hg uptake by more than 95%, indicating that PCMBSA binding to monolayers or cysteine depends on the same functional group (i.e., Hg).

On the basis of the lysine protection of PCMBSA inhibition of lysine transport (see Table VI) and the assumptions that (*a*) the decrease in ^{203}Hg bound in the presence of lysine is exclusively because of protection at the lysine carrier and (*b*) that one molecule of PCMBSA reacts with one lysine carrier, the difference of PCMBSA-^{203}Hg uptake by alveolar macrophages in the presence and absence of 20 mM lysine can be used to calculate the number of lysine carriers per cell. Under the conditions of these experiments, the difference in ^{203}Hg uptake corresponds to approximately 90% of the lysine carriers. From the specific activity of the PCMBSA-^{203}Hg (see Table VII legend) and the number of cells per cover slip, it is readily calculated that there are about 20 ± 7.7 (±SD) million lysine carriers per alveolar macrophage.

TABLE VII

*Uptake of PCMBSA-^{203}Hg by Alveolar Macrophages**

Addition	Conc.	Uptake (dpm/20 min/cover slip)	*P*‡
	mM		
None	—	396 ± 92 (7)§	
Lysine	20	342 ± 82 (7)	<0.001

* Monolayers were incubated with 0.005 mM PCMBSA-^{203}HG (30,000 dpm/ml) for 20 min in the presence or absence of lysine.

‡ The statistical analysis was based on the paired differences of the seven experiments.

§ Number in parenthesis: number of experiments; each experiment was done with quadruplicate monolayers.

Effect of Phagocytosis on the PCMBSA Inhibition of the Membrane Transport in Alveolar Macrophages.—From the above experiments PCMBSA would appear a suitable agent for use in the experimental approach outlined in Fig. 5. Lysine transport in alveolar macrophages was first inactivated by 0.005 mM PCMBSA and the effect of phagocytosis on the reappearance of transport activity examined. As noted above, phagocytosis is inhibited only 30% by

PCMBSA at this concentration. If new transport carriers appeared during phagocytosis, some restoration of lysine transport should occur. On the other hand, if the constancy of the number of carriers is because of preservation of the lysine carriers, then there will be no restoration of lysine transport after phagocytosis. As shown in Table VIII no restoration of lysine transport after phagocytosis was observed. The constancy of a second independent transport system (adenosine) with and without phagocytosis indicates that the failure of reversal of lysine transport after phagocytosis could not be because of the inhibition of a hypothetical renewal process of the membrane by PCMBSA.

TABLE VIII

Effect of Phagocytosis on the PCMBSA Inhibition of the Membrane Transport in Alveolar Macrophages

	Rate of transport (10^{-4} μmoles/45 sec per 0.3 million cells)				% Recovery
	Control	+ PCMBSA	After subsequent incubation		
			No phagocytosis	Phagocytosis	
Lysine transport	1.33	0.16	0.20	0.19	−5
Adenosine transport	0.43	0.35	0.39	0.37	−5

TABLE IX

Effect of Lysine Protection on the PCMBSA-Inhibited Transport of Lysine in Alveolar Macrophages with and without Phagocytosis

	Rate,* no lysine loading	Conc. of intracellular lysine after loading§	Rate,* after lysine loading
		mM	
Control	0.19	16.6	0.42
Phagocytosis	0.21	16.0	0.40
% Control	110		95

* Unit, 10^{-4} μmoles/45 sec per 0.3 million cells (after PCMBSA treatment).

§ Calculated from the ^{14}C content and the specific activity of the 20 mM lysine.

Although the washing procedure is extremely effective (see Materials and Methods) and removes any contaminating PCMBSA, it is possible that after incubation and washing some of the reversibly bound PCMBSA migrates to and inactivates newly formed transport sites. To rule out this possibility, lysine transport was first inactivated with 0.005 mM PCMBSA. Phagocytosis was then induced in the presence of 20 mM lysine-^{14}C to prevent any inactivation of newly formed carriers. The rate of lysine transport was then measured after phagocytosis with 0.1 mM lysine-^{3}H. The results are shown in Table IX. Again no difference of lysine transport was found between macrophages with and without phagocytosis, ruling out the possibility that new carriers were formed and immediately inactivated by "migratory" PCMBSA.

DISCUSSION

Polymorphonuclear leukocytes and alveolar macrophages have an enormous capacity to ingest inert particles. As estimated in this study on the average, each leukocyte ingested 14 PVT particles of 2.02 μ in diameter and each macrophage ingested 51 PVT particles. The results are consistent with the estimate of Roberts and Quastel (7) for human leukocytes. Although monolayers incubated at 0°C were used to correct for nonspecific adsorption of particles to monolayers, these figures might be still overestimated, because it is possible that at 38.5°C more particles stick to the glass and to the surface of the cells. On the other hand, some particles might be only partially "internalized." Nevertheless, these figures give us some basis for determining how much membrane could be internalized during phagocytosis. In terms of the volume, the ingested particles are equivalent to less than 20% of the cell water. However, in terms of the surface membrane interiorized, if we assume that the cells are spherical and the surface is smooth, then almost 100% of the original surface membrane is internalized in these experiments during phagocytosis. In fact, a similar figure has often been used by other workers (12, 19) to infer a requirement for extensive membrane synthesis during phagocytosis. Since after surface contact the phagocytes are rather flat with increased diameters and a variety of processes (13, 14), such an approximation is admittedly too low. To make some allowance for this we have arbitrarily doubled the area of the smooth spherical cell. Even so, the membrane internalized still amounts to 30–50% of this hypothetical surface. It is difficult to imagine how 14 or 50 PVT particles (2 μ in diameter) could be individually wrapped in cytoplasmic membrane without involving an appreciable amount of the surface membrane. For these experiments it is necessary only that the per cent of surface internalized is detectable by the transport assay. The rapid-sampling technique for the measurement of the rate of transport, employed in this study, is exceedingly sensitive. A change of only 7% in the number of carriers can be easily detected by this technique and the part of the plasma membrane involved in phagocytosis surely exceeds this figure. No change in the transport rates of five separate transport systems were observed after phagocytosis. Moreover, on the basis of kinetic studies of lysine and adenosine transport (Figs. 2 and 3) and the exchange diffusion of lysine transport (Table IV) in alveolar macrophages, and the specificities of adenine transport in polymorphonuclear leukocytes (Table V), it is clear that in contrast to metabolism which is altered by phagocytosis (20), membrane transport is unchanged. Therefore the observation that the rates of the membrane transport of five separate transport systems remain constant after a large portion of the surface membrane is internalized during phagocytosis, indicates that the number of carriers is maintained constant. Two interpretations seemed most likely (Fig. 5). Firstly, the surface membrane could be replaced immediately during phagocytosis, so that the number of carriers remained constant, even though

some carriers are internalized during membrane internalization. Secondly, phagocytosis and membrane transport could involve different parts of the plasma membrane, so that no transport sites are internalized during phagocytosis. In this experiment after PCMBSA treatment 90% of the lysine carriers were inactivated. An appearance of new carriers, even if only 10% of the total carrier number, would have caused a 100% increase in this reduced activity. No recovery was observed indicating that no new carriers appear during phagocytosis. Since carrier number remains constant, it follows that no carriers are internalized during this process. The possibility that new lysine carriers are inactivated by PCMBSA before their appearance on the surface membrane need not be considered since it was shown that PCMBSA does not penetrate the plasma membrane.

Thus different parts of the surface membrane are involved in phagocytosis and membrane transport. From the functional point of view, therefore, the plasma membrane is not homogeneous. These observations complement those of several other workers who have demonstrated inhomogeneity of the anatomical distribution of antigens over the cell surface. Thus Cerottini and Brunner (21), using an immunofluorescent technique, showed that mouse H-2 isoantigens are located in discrete areas on the cell surface, and recently, this has been confirmed by Stackpole et al. by electron microscopy of visually labeled antibody to H-2 isoantigens (22). Smith and Hollers (23) demonstrated that fluorescein-labeled concanavalin A bound only to the posterior region of the plasma membrane of the motile lymphocyte, which was functionally distinct from the anterior region of the plasma membrane.

The impermeability of the plasma membrane to PCMBSA makes this compound an ideal tool to quantitate surface sulfhydryl groups (11). Shapiro, Kollman, and Martin (24) have recently advanced evidence that there may be at least five classes of sulfhydryl groups involving different membrane functions on the human erythrocyte membrane. The lysine transport system in alveolar macrophages is extremely sensitive to PCMBSA (Fig. 7). Moreover, this PCMBSA inhibition of lysine transport can be prevented by high concentrations of lysine. This makes it probable that the reaction between the -SH group of the lysine carrier and PCMBSA is at the active site of the carrier. Fox and Kennedy (25) have used this "substrate protection" for the protection and identification of the lactose transport carrier in *Escherichia coli*. By using PCMBSA-^{203}Hg, the membrane -SH groups of alveolar macrophages reacting with PCMBSA were measured in the presence and absence of 20 mM lysine (Table VII). Under these conditions the -SH groups responsible for lysine transport are 13% of the total -SH groups of membrane. Under the assumption that 1 mole of PCMBSA reacts with 1 mole of lysine carrier, it is readily calculated that there are approximately $(20 \pm 7.7) \times 10^6$ ($\pm$SD) lysine carriers/alveolar macrophage. If we further assume that the total surface area of an alveolar

macrophage is approximately 1.400 μ^2 (see Table II), then there is an average surface density of $(14 \pm 5) \times 10^3$ carriers/μ^2 plasma membrane. If the lysine carriers are evenly distributed over the cell surface with a density of $(14 \pm 5) \times 10^3/\mu^2$, it would be impossible for carriers not to be internalized during phagocytosis of PVT particles of 12 μ^2 surface area (2 μ in diameter). The following hypotheses may then be considered to explain the preservation of transport after phagocytosis: (*a*) the carriers cluster into groups; (*b*) the membrane involved in phagocytosis arises intracellularly; or (*c*) during phagocytosis the carriers are pushed in the liquid membrane away from the part of membrane to be internalized.

From the previously measured V_{max} of lysine transport in alveolar macrophages (4) it is also calculated that the turnover-number of the lysine carrier is 20 moles lysine/mole carrier per min. The number of glucose carriers has been estimated in a variety of tissues (11, 26–28). These estimates are undoubtedly too high since the experiments on which they are based did not employ substrate protection or other means of providing evidence for specificity. The minimal number of lactose transport carriers was estimated to be about 9000/*E. coli* by the isolation of M protein (29). If we assume that *E. coli* are cylindrical, with a diameter of 0.2 μ and a height of 1 μ, it may be calculated that *E. coli* has a density of 14×10^3 lactose carriers/μ^2 plasma membrane which fortuitously agrees exactly with the surface density of the lysine carriers in alveolar macrophages. It is further calculated from the V_{max} of lactose transport (30) that the turnover-number of lactose carriers is 5000 moles lactose/mole carrier per min.

Quantitative determination of lysine carriers by PCMBSA-^{203}Hg binding as a function of phagocytosis was not carried out in this study. Although it is possible to obtain an actual figure of lysine carriers by this method, yet it is not so sensitive as the indirect approach based on the measurements of the initial rate of lysine transport, since only a small fraction (13%) of the membrane sulfhydryl groups reacting with PCMBSA corresponds to lysine carriers. Furthermore, washing of the monolayers, a step necessary for the phagocytosis experiment, increases the uptake of PCMBSA-^{203}Hg two- to threefold. It is currently not known why the washing process increases PCMBSA-^{203}Hg binding. Perhaps some surface sulfhydryl groups are uncovered during washing or some cells are damaged. Since there are many more sulfhydryl groups located inside the diffusion barrier only a very small fraction, for example 1% or less, of cells damaged during washing may account for this increased binding of PCMBSA-^{203}Hg.

A variety of membrane markers, such as membrane adenosine triphosphatase (31), surface antigens (21, 22), and concanavalin A binding sites (23), has been used to study the organization of the cell surface. Transport carriers are integral components of the plasma membrane and can be followed quantitatively. Phagocytosis is also a membrane function and involves internalization of parts

of the surface membrane. In this study, the distribution of transport and phagocytic functions over the cell surface has been analyzed. The use of these separate membrane functions clearly provides a useful approach to the study of the organization of the cell surface.

SUMMARY

The activities of specific transport systems were determined before and after large portions of the surface membrane had been interiorized by phagocytosis of inert particles. In five separate transport systems in rabbit polymorphonuclear leukocytes (adenosine and two adenine transport systems) and alveolar macrophages (adenosine and lysine transport systems), the rate of transport was unaffected even after an estimated 35–50% of the membrane had been internalized. Studies of the kinetics of lysine and adenosine transport, exchange diffusion of lysine transport in alveolar macrophages, and the specificities of adenine transport in polymorphonuclear leukocytes indicate that the nature of the membrane transport systems is not altered by phagocytosis. Therefore the constancy of transport indicates that the number of carriers remains the same before and after phagocytosis. It was also shown that this constancy of transport did not depend on the introduction into the surface of new transport sites during phagocytosis. Therefore transport sites are preserved on the surface during the internalization of membrane which accompanies phagocytosis. The results are best explained by the concept that the membrane is mosaic in character with geographically separate transport and phagocytic sites.

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