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MEMBRANE TRANSPORT IN THE RABBIT ALVEOLAR MACROPHAGE. THE SPECIFICITY AND CHARACTERISTICS OF AMINO ACID TRANSPORT SYSTEMS

MIN-FU TSAN AND RICHARD D. BERLIN
Department of Physiology, Harvard Medical School, Boston, Mass. 02115 (U.S.A.)
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SUMMARY

Amino acid transport in the rabbit alveolar macrophage was studied with a rapid sampling technique which made possible the determination of initial rates of uptake. Lysine was transported by a single saturable system, designated AM-1, with a K_m of 0.1 mM and a v_{max} of 0.44 nmole/45 sec per 10^6 cells. Competition studies revealed that almost all natural amino acids had some affinity for the carrier, though it varied by several orders of magnitude. In general, there is preferential affinity for amino acids with a long lipolytic side chain and a terminal positive charge. Na^+ and K^+ inhibited lysine transport. A kinetic study of the Na^+ inhibition revealed that its effect was due to depression of the v_{max}, K_m remaining constant (noncompetitive kinetics). Leucine was transported by the AM-1 system and at least one other system.

INTRODUCTION

Many recent investigations of the macrophage have focussed on its metabolism, particularly during phagocytosis and pinocytosis[1-3] and on protein synthesis and lysosome formation[4,5]. The high rates of macrophage activities indicated in these studies clearly depend on the availability of exogenous substrates such as the essential amino acids. However, virtually nothing has been reported on the mechanisms by which these compounds enter cells (*i.e.* their membrane transport).

Although detailed studies have been made of amino acid transport in intestine and kidney, the general conception of the membrane transport of amino acids in non-epithelial tissues is derived from its characterization in the Ehrlich ascites tumor cell[6], and, to a lesser extent, the erythrocyte[6,7]. The latter highly specialized cells are unique in several transport systems. It is important to determine to what degree the ascites tumor cell is representative of normal animal cells.

In this paper we report a detailed analysis of amino acid transport (expecially lysine) in the alveolar macrophage, utilizing a rapid sampling technique[8] developed in this laboratory with polymorphonuclear leukocytes. The study indicates important differences as well as similarities in amino acid transport between macrophages,

Ehrlich ascites tumor cells and epithelia. In addition a novel effect of alkali metal ions on a transport system is revealed: depression of the v_{max} for lysine transport.

MATERIALS AND METHODS

Chemicals

[^{3}H]Lysine and [^{3}H]leucine were obtained from Schwarz Biochemical, Inc., [^{3}H]lysine was purified by ascending paper chromatography in *tert.*-butanol–methyl-ethyl ketone–formic acid–water (40:30:15:15, by vol.). The strips of paper containing radioactive lysine were cut out and eluted with modified Hanks solution (see below) in the cold. [^{14}C]Lysine (uniformly labelled) obtained from New England Nuclear, was used in some experiments. [^{14}C]Inulin was also obtained from New England Nuclear and purified by ascending paper chromatography in *n*-butanol–ethanol–water (52:18:30, by vol.).

Animals

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

The preparation of alveolar macrophages

Alveolar macrophages were obtained by the method of MYRVIK *et al.*[9] with some modifications. Briefly, either rabbits which had been injected with glycogen solutions intraperitoneally overnight for other purposes, or uninjected animals were killed by injecting air into the marginal ear vein. The thoracic cavity was opened and the upper part of the trachea was dissected free and clamped shut with a hemostat to avoid entrance of blood into the lung when the trachea was transsected. Transection was then performed above the clamp and the lungs, heart and trachea were freed and placed immediately in ice-chilled modified Hanks solution. The heart was dissected carefully away and the lungs with trachea still clamped were washed with cold modified Hanks solution until they were free of blood. This step was important for avoiding contamination by erythrocytes. The trachea was then opened and canulated with a plastic canula connected to a burette. Modified Hanks solution was introduced into the trachea by gravity until the lungs were fully distended. This required approx. 60–80 ml of the solution for the first washing and 50 ml for subsequent washings. The canula was disconnected from the burette, the lungs inverted and the effluent collected in a pre-cooled flask. Caution was made to avoid contamination of fluid from the external surface of the lungs. The same washing was repeated twice and the fluid was pooled. Further washing did not increase significantly the yield of cells. The cell suspension was centrifuged at 50 × *g* for 10 min at 4° and the supernatant discarded. The cells were resuspended in modified Hanks solution which consists of the following: 139 mM NaCl 20 mM K^+, 10 mM phosphate adjusted to pH 7.4 ± 0.05 with NaOH and 5 mM glucose. Preliminary results showed that modified Hanks solution can maintain alveolar macrophages in a viable condition, as judged by their uptake of neutral red, their impermeability to eosin y and their uptake of amino acids, for at least 4 h. With this method $20 \cdot 10^6$–$100 \cdot 10^6$ macrophages can be procured from one rabbit as compared to $8 \cdot 10^6$–$16 \cdot 10^6$ cells by MYRVICK *et al.*[9] and an average of $20 \cdot 10^6$ by COHN AND WIENER[10]. The preparation was more than 90% homogeneous. The contaminating cells were primarily polymorphonuclear leukocytes.

Cell water content

The cell water content of a cell pellet was determined by drying to a constant weight and correcting for the extracellular fluid contamination by dilution of [^{14}C]-inulin. The measurements gave 1.670 ± 0.112 μl (± S.D.) per 10^6 cells which is about 5 times the volume of rabbit polymorphonuclear leukocytes[8].

Determination of amino acid uptake by a rapid sampling technique

The rapid sampling technique developed by HAWKINS AND BERLIN[8] was readily adapted to the macrophage. Briefly, monolayers were made on 22-mm-diameter coverslips by incubating 0.5-ml aliquots of cell suspension for 30 min at 37°. In the first few experiments $0.5 \cdot 10^6$ macrophages per coverslip were used, but later the use of $0.3 \cdot 10^6$ per coverslip was established. At the lower density, the cells appeared less crowded by phase contrast microscopy. By direct count more than 99 % of the cells adhere to glass throughout the experiment. After the monolayers were formed the coverslips were drained, and approx. 0.4 ml of the incubation media containing the radioactive amino acids to be tested, prewarmed to 38.5°, were placed over the monolayer. When the incubations were completed, the coverslips were drained and rinsed consecutively in four beakers containing cold modified Hanks solution, which removed extracellular contaminating radioactivity. The coverslips were then prepared for counting by liquid scintillation. Samples were done in triplicate and averaged.

In order to examine the possible loss of intracellular label during the washing process, the monolayers were first incubated with 0.1 mM [^{3}H]lysine and [^{14}C]inulin for 2 min. The coverslips were then rinsed in cold modified Hanks solution for periods up to 2 min. The results revealed that the rinsing process was very effective, removing all the [^{14}C]inulin with negligible lossess of intracellular [^{3}H]lysine.

The radioactivity recovered from the monolayers by this technique arises hypothetically from the sum of three processes: (1) carrier-mediated transport, (2) diffusion-mediated transport and (3) extracellular contamination. Corrections for diffusion and extracellular contamination were applied to the total radioactivity in order to obtain the carrier-mediated component. The radioactivity recovered after incubation of monolayers at 37° at supersaturating concentration of substrate was considered to be equal to the sum off diffusion and contamination components. Contamination alone was considered to be the radioactivity adsorbed to the coverslips alone (no cells present) after brief exposure to the radioactive substrate. On this basis it was determined that the diffusion component was negligible. In practice therefore, the carrier-mediated component was determined by substracting from the total radioactivity the value for contamination. This value was equivalent to less than 0.1 μl of radioactive medium per coverslip and in general varied from 0 to < 5 % of the total radioactivity recovered after a 45-sec incubation. Fig. 1 shows the result of the time course uptake of 0.1 mM lysine by alveolar macrophages. It was found that the uptake was linear within a 2-min incubation period and when the results were corrected for extracellular contamination, the line extrapolated through zero uptake at zero time. The results presented in this paper were obtained by incubating monolayers for 45 sec and expressed as the amount of amino acid transported per 45 sec per coverslip ($0.3 \cdot 10^6$ cells) unless otherwise indicated. With this incubation period a final intracellular to extracellular distribution ratio of 2 was obtained when the medium was 0.1 mM lysine. This uptake was considered as the initial rate for the kinetic analysis.

Chromatography

The fate of lysine in alveolar macrophages was analyzed by determining the fraction of radioactivity which was acid insoluble and by analysis of the acid-soluble extract by paper chromatography in *tert.*-butanol–methyl ethyl ketone – formic acid–water (40:30:15:15, by vol.) and *tert.*-butanol–methyl ethyl ketone–water–NH_4OH (40:30:20:10, by vol). After incubating mono-layers with 0.1 mM lysine for 45 sec, the coverslips were drained, washed and broken into beakers containing 0.5 M KOH.

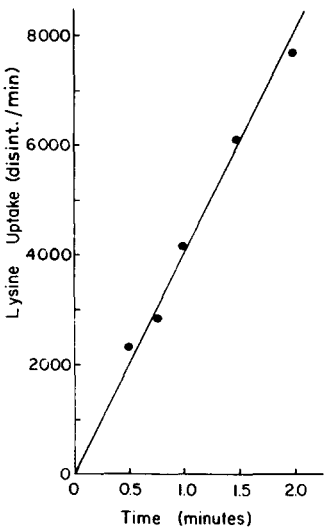


Fig. 1. Time course of lysine uptake by rabbit alveolar macrophages. Cell monolayers were incubated with 0.1 mM [^{3}H]lysine for the times indicated and the radiolabel of the cells was measured as described in MATERIALS AND METHODS.

After shaking at 38.5° for 30 min to extract the soluble material, the pH of the solution was adjusted to less than 2 by adding concentrated $HClO_4$ in the cold for more than 1 h. The precipitate was washed twice with 0.1 M cold $HClO_4$ and dissolved again in 0.5 M KOH. Aliquots of acid-soluble and acid-insoluble fractions were neutralized to phenol red with $HClO_4$ or KOH and were counted in Bray's solution by liquid scintillation. The results showed that no radioactivity was incorporated into the acid-insoluble fraction. Aliquots of the neutralized acid-soluble fraction were placed on paper and developed in the above solvents. The chromatogram was cut into 1 cm × 2 cm strips which were placed in vials and counted by liquid scintillation in a toluene-based solution. It was found that there was no significant metabolism of the radioactive lysine during the 45-sec incubation.

RESULTS

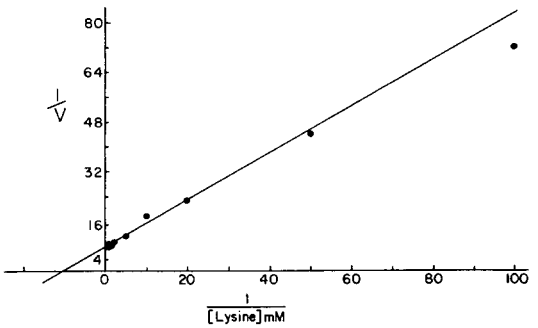
The kinetics of lysine transport

Initial rates of lysine influx were measured at substrate concentrations between 0.01 and 10 mM. The number of disint./min per coverslip was corrected for extracellular contamination and converted to nMoles transported per 10^6 cells. The data were expressed in a double reciprocal plot, from which the K_m and v_{max} were cal-

culated. The derived values of K_m and v_{max} of lysine transport were quite constant. Fig. 2 shows the results of a typical experiment. The K_m was 0.1 mM and the v_{max} was about 0.44 nmole/45 sec per 10^6 cells. Although more than one transport system for lysine has been described in a variety of tissues and cells including rabbit ileum[22], human renal cortex[22] and the Ehrlich ascites tumor cell[13], only one lysine transport system in alveolar macrophages can be resolved from these kinetic data. Although the presence of more than one lysine transport system with similar K_m cannot be excluded from the kinetic analysis alone[14], further results were consistent with the existence of a single system.

Chemical specificity of the lysine transport system

There is both experimental and genetic evidence suggesting that in intestine and kidney cystine and the dibasic amino acids, lysine, arginine and ornithine but not



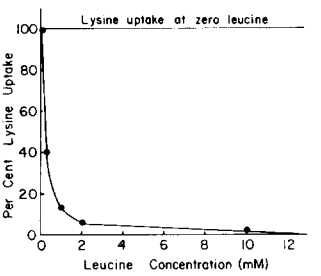


Fig. 2. Kinetics of lysine uptake. Cell monolayers were incubated in the presence of [^{3}H]lysine in concentrations from 0.01 to 10 mM for 45 sec. The monolayers were rinsed and counted as described in MATERIALS AND METHODS. The points represent averages of three determinations. v was expressed as nmoles/45 sec per $0.3 \cdot 10^6$ cells.

Fig. 3. Inhibition of lysine uptake by leucine. Cell monolayers were incubated with 0.1 mM [^{3}H]lysine for 45 sec in the presence of leucine at various concentrations. 100% lysine uptake was the value obtained in the absence of leucine. Each point represents the average of three determinations.

neutral amino acids share the same transport system[14,15]. In alveolar macrophages lysine transport can be completely inhibited by the neutral amino acids leucine and histidine. Thus, we see immediately that the specificity for lysine transport in the macrophage is different from epithelia. These results further confirmed our kinetic study which indicated that there was only one lysine transport system. Fig. 3 illustrates an experiment in which leucine brought about complete inhibition of lysine transport. These striking differences of the specificity of lysine transport system in alveolar macrophages from those previously reported prompted us to carry out an intensive survey of its specificity. It was shown (see below) that leucine is a competitive inhibitor of lysine. Hence we shall assume that the kinetics of inhibition of all other amino acids on lysine transport is competitive in nature. Under this assumption, the K_i's which characterize the affinity of the amino acids to the carrier may be determined simply from their inhibition of transport at a single concentration of lysine. Almost all natural amino acids and some amino acid analogs were tested.

For the survey, 0.1 mM lysine (its K_m) was used. The K_i of the inhibitor can be calculated from a simple formula (Eqn. 4)*.

As shown in Table I, the amino acids tested were classified into two groups: low and high affinity. Low affinity amino acids were defined as those with K_i's more than 10-fold the K_m for lysine. Some of the chemical characteristics of the specificity are immediately apparent from these results. It is obvious that the α-amino carboxylic group is required for the binding of the substrate to the carrier. Thus the affinity of histamine (XI, K_i = 3.84) is one-tenth that of histidine (XX, K_i = 0.35) and that of ε-amino caproic acid (VIII, K_i = 5.5) is significantly lower than norleucine (XIX, 0.36). Some length of side chain is required as suggested by the result with glycine (I, > 10) which has the poorest affinity of the compounds tested. It is also clear that in general the longer the side chain, the higher the affinity (compare glycine (I, > 10 no carbon side chain), alanine (XII, 4.4, one carbon, α-amino-butyric acid (XIV, 3.2, two carbons), with norvaline (XVIII, 0.44, three carbons) and norleucine (XIX, 0.36, four carbons). In addition the α-hydrogen can not be substituted. α-Amino-isobutyric acid (VII, 7.1) and cycloleucine (V, 8.09) have very low affinities. Substitutions at the β-carbon decrease the affinity as can be seen (Fig. 4) by comparison of isoleucine (XVII, 1.1, β-methyl substituted), with leucine (XXIII, 0.081, β-methyl substituted) and norleucine (straight chain); and valine (IV, > 10, β-methyl substituted) with norvaline (straight chain). The low affinity of threonine (VI, 7.9) may be due in part to the β-hydroxyl group. It can also be seen that a carboxylic group at the end of the side chain decreases the affinity [see aspartic acid (IX, 5.1) and glutamic acid (X, 4.5)]. The elimination of the negatively charged carboxylic group by formation of an amide markedly increases the affinity. Thus the affinity of glutamine (XXI, 0.17) was more than 20-fold that of glutamic acid. On the other hand the amino group or guanido group at the end of the side chain (terminal positive charge) increases the affinity [see lysine, arginine (XXIV, 0.05) and ornithine (XXV, 0.045)]. The structural basis of the affinity of the unsaturated cyclic amino acids such as phenylalanine (XIII, 3.9), tryptophan (XVI, 1.4) and histidine (XX, 0.35) is unclear.

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* $$v = \frac{v_{max}[S]}{K_m + [S]} \quad (1)$$

$$v_i = \frac{v_{max}[S]}{K_m\left[1 + \frac{I}{K_i}\right] + [S]} \quad (2)$$

from Eqns. 1 and 2

$$\frac{v_i}{v} = \frac{K_m + [S]}{K_m\left[1 + \frac{[I]}{K_i}\right] + [S]} \quad (3)$$

substituting 0.1 mM for $[S]$ and K_m:

$$K_i = \frac{v_i[I]}{2v - 2v_i} \quad (4)$$

where $[S]$ and $[I]$ are the concentration of substrate and inhibitor respectively, v_i and v are the velocities with and without inhibitor.

TABLE I

INHIBITION OF LYSINE TRANSPORT

Cell monolayers were incubated with 0.1 mM [^{3}H]lysine for 45 sec in the presence of the inhibitor. The amount of inhibition was determined by comparison with a control without inhibitor. All amino acids tested were in the L-form except α-aminoisobutyric acid and nor-leucine in which the DL-form was used. The K_i was calculated from Eqn. 4 (see text) and from the concentration of the L-form (assuming that the D-form has no affinity for the carrier). Each value represents the average of three to five determinations.

Amino acids and analogs with low affinity		*Amino acids and analogs with high affinity*	
Inhibitor	K_i (*mM*)	*Inhibitor*	K_i (*mM*)
I. Glycine	>10	XVIII. Norvaline (α-aminovaleric acid)	0.44
II. Serine	>10	XIX. Nor-leucine (α-aminocaproic acid)	0.36
III. Proline	>10	XX. Histidine	0.35
IV. Valine	>10	XXI. Glutamine	0.17
V. Cycloleucine	8.09	XXII. Methionine	0.15
VI. Threonine	7.9	XXIII. Leucine	0.081
VII. α-Aminoisobutyric acid	7.1	XXIV. Arginine	0.05
VIII. ε-Aminocaproic acid	5.5	XXV. Ornithine	0.045
IX. Aspartic acid	5.1		
X. Glutamic acid	4.5		
XI. Histamine	3.84		
XII. Alanine	4.4		
XIII. Phenylalanine	3.9		
XIV. α-Aminobutyric acid	3.2		
XV. Cystine	1.6		
XVI. Tryptophan	1.4		
XVII. Isoleucine	1.1		

Effect of cations on lysine transport system

There is abundant evidence that alkali metal ions exert marked effects on amino acid transport systems in various tissues and cells[16–24]. Lysine transport in the macrophage is also affected; but the effect of cations is a novel one. Table II summarizes the results of experiments in which Na^+ in the medium was replaced by K^+, choline, sucrose and mannitol. Two striking findings were noted. First, replacement of Na^+ with K^+ had no effect on the rate of lysine transport. Secondly, replacement of Na^+ with equimolar sucrose or mannitol caused a 50 % stimulation of lysine transport. Theoretically this stimulation of lysine transport could have been due to an inhibitory effect of Na^+ or K^+. We varied the ionic concentration of Na^+ and K^+ by varying the proportions of isotonic solutions of mannitol, NaCl, KCl, Na_2SO_4 and $K_4Fe(CN)_6$ in an attempt to study the effects of Na^+ and/or K^+ concentration on lysine transport. It was assumed for these purposes that the dissociation of the electrolytes was complete. In each case 10 mM phosphate buffer was present in the medium so that the pH was equal to 7.4 in all of the incubation media. Thus we were able to keep the medium iso-osmotic yet vary concentration of Na^+ and K^+. The alveolar macrophages tolerated these artificial conditions quite well during 45-sec exposure as judged by their impermeability to eosin Y and by the accumulation of lysine. As can be seen from Fig. 5, there was an inverse linear relationship between Na^+ and/or K^+ concentration and lysine uptake. The effect of K^+ was the same as that of Na^+.

Kinetic studies were carried out to see whether the inhibitory effect of Na^+ was

competitive or non-competitive. Lysine uptake was measured at two different ionic concentrations. The upper curve in Fig. 6 was obtained with lysine diissolved in modified Hanks solution (139 mM Na^+, 20 mM K^+) and the lower curve in 20 mM K^+, Na^+ being replaced by isotonic mannitol. Na^+ exerted a non-competitive inhibitory effect on lysine transport; the K_m for lysine remaining the same, while v_{max} decreased from 0.66 to 0.44 nmole/45 sec per 10^6 cells.

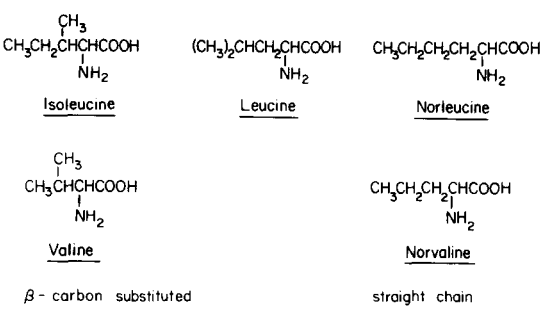
The inhibitory effect of leucine on lysine transport was also investigated in

TABLE II

THE EFFECT OF ALKALI METAL IONS ON LYSINE TRANSPORT

Cell monolayers were incubated with 0.1 mM [^{3}H]lysine for 45 sec in various media including modified Hanks solution, and solutions in which NaCl was replaced by isotonic KCl, choline chloride, sucrose or mannitol. The amount of inhibition or stimulation was determined by comparison with lysine transport in modified Hanks solution. Each result is the average of at least three determinations. —, inhibition; +, stimulation.

Medium	Na^+	K^+	*Choline*$^+$	*Inhibition or stimulation* (%)
Modified Hanks solution	139	20	—	0
Na^+ replaced by K^+	—	159	—	+ 3
Na^+ replaced by choline$^+$	—	20	139	−15
Na^+ replaced by sucrose	—	20	—	−48
Na^+ replaced by mannitol	—	20		−51



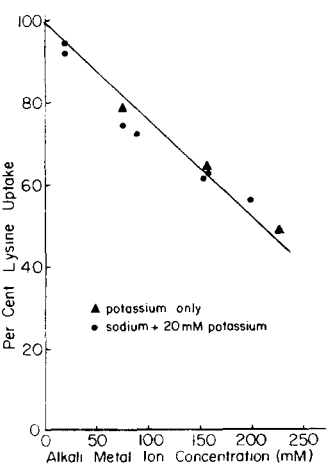


Fig. 4. The structure of leucine and valine and their analogs.

Fig. 5. Effect of Na^+ and K^+ on the rate of lysine uptake. Cell monolayers were incubated for 45 sec with 0.1 mM [^{3}H]lysine in the presence of variable ionic concentrations at constant osmolarity. To maintain isotonicity (300 mosM) solutions of isotonic NaCl, KCl and the multivalent salts, Na_2SO_4 and $K_4Fe(CN)_6$ were diluted with isotonic mannitol. It was assumed that all species were completely ionized. In each case potassium phosphate buffer (10 mM with respect to phosphate) was present; the final pH of the medium was 7.4. 100% lysine uptake was the value obtained on incubation in the total absence of alkali metal ions (mannitol *plus* 16 mM Tris buffer (pH 7.4) or in pure mannitol solutions). The addition of Tris had no effect on uptake. Each point was the average of at least three determinations.

modified Hanks solution as compared with the Na^+ substituted medium. The results are depicted in Fig. 7. Leucine exerted a competitive inhibitory effect on lysine transport in the presence or absence of Na^+. In a double reciprocal plot the lines intersected at the same point of the ordinate at the two different Na^+ concentrations respectively, although the v_{max} of lysine transport had been shifted in the presence of Na^+.

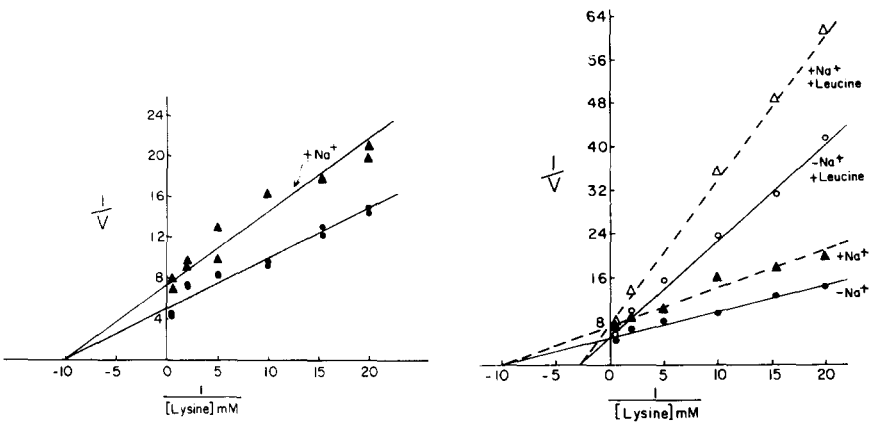


Fig. 6. Reciprocal plots of lysine uptake in the presence and absence of Na^+. Cell monolayers were incubated for 45 sec in the presence of [^{3}H]lysine in concentrations from 0.05 to 2 mM. All points plotted were the mean of three determinations. The media contained either modified Hanks solution (139 mM Na^+, 20 mM K^+) (●—●) or a solution in which the Na^+ was replaced by mannitol (0 mM Na^+, 20 mM K^+) (▲—▲). v was expressed as nmoles/45 sec per $0.3 \cdot 10^6$ cells). See text for interpretation.

Fig. 7. Effect of Na^+ on leucine inhibition of lysine uptake. Double reciprocal plots of uptake at variable lysine concentrations with and without leucine at either 139 or 0 mM Na^+. The experimental conditions were the same as in Fig. 6 except leucine was present as described below. ▲---▲, uptake of lysine in 139 mM Na^+ and 20 mM K^+; ●—●, uptake of lysine in 20 mM K^+ (results are the same as in Fig. 6); △---△, uptake of lysine in the presence of 0.25 mM leucine in 139 mM Na^+ and 20 mM K^+; ○—○, uptake of lysine in the presence of 0.5 mM leucine in 20 mM K^+. Each point was the mean of at least three determinations. v was expressed as nmoles/45 sec per $0.3 \cdot 10^6$ cells). See text for explanation.

However, while the nature of the effect remains competitive, leucine affinity is enhanced by Na^+. In modified Hanks solution the K_i of leucine was 0.097 mM; whereas in 20 mM K^+ solution (Na^+ being replaced by mannitol) the K_i was 0.21 mM, a more than 2-fold decrease of affinity. The effect of K^+ on leucine affinity was also studied. The K_i of leucine was found to be the same (0.21 mM) in media in which Na^+ was totally replaced by either mannitol or K^+. Thus, we have clearly demonstrated two completely different effects of Na^+ on this transport system. It decreases the v_{max} of lysine transport. It also increases the affinity of leucine on the lysine transport system. The former effect is shared by K^+ but the latter effect is Na^+ specific.

The effect of bivalent and polyvalent cations was also determined. In order to eliminate the effects of the monovalent cations, these experiments were done in isotonic mannitol solution with potassium phosphate replaced by Tris buffer. As shown in Table III, except for Th^{4+}, all multivalent cations exerted some inhibition, but the effects of Mg^{2+} and Ca^{2+} at physiologic levels are especially noteworthy. Further experiments are required to explore the nature of this effect.

Leucine transport in rabbit alveolar macrophages

In the previous sections we described lysine transport in some detail. As can be seen from Table I, almost all natural amino acids have some affinity for the carrier, though it varies by several orders of magnitude. It is conceivable that most amino acids can get into the cells through this transport system. One may ask whether there are alternative transport systems for some amino acids in these cells. As an approach to this question, the capacity of lysine to inhibit leucine transport was determined. The results are shown in Fig. 8. Not more than 50 % of leucine transport could be inhibited by lysine. Thus leucine is transported by at least two systems, only one

TABLE III

THE EFFECT OF BIVALENT AND POLYVALENT CATIONS ON LYSINE TRANSPORT

Cell monolayers were incubated with 0.1 mM [^{3}H]lysine for 45 sec in iso-osmotic mannitol solution with Tris buffer in the presence of the multivalent cations. The amount of inhibition or stimulation was determined by comparison with a control without the multivalent cations. Each result is the mean of three determinations. −, inhibition; +, stimulation.

Ionic concn.	*Inhibition or stimulation (%)*
None	0
2 mM Mg^{2+}	− 25
2 mM Ca^{2+}	− 18
1 mM La^{3+}	− 9
1 mM Th^{4+}	− 3

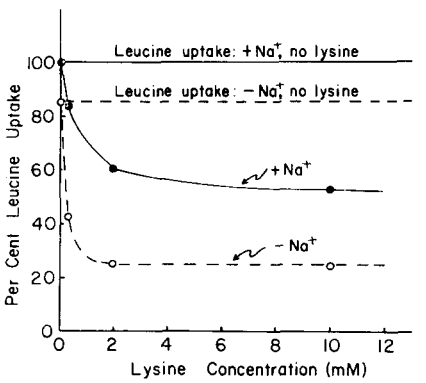


Fig. 8. Inhibition of leucine uptake by lysine in the presence and absence of Na^+. Cell monolayers were incubated with 0.1 mM [^{3}H]leucine for 45 sec in the presence of various lysine concentrations. 100 % leucine uptake was the value obtained in the absence of lysine in modified Hanks solution (139 mM Na^+ and 20 mM K^+). Leucine uptake in 0 mM Na^+, 20 mM K^+ was 85 % (zero lysine concentration). ●—●, and ○---○ denote leucine uptake in the presence of various lysine concentrations in modified Hanks solution and in 0 mM Na^+, 20 mM K^+, respectively. Each point was the mean of three determinations.

shared by lysine. The effect of Na^+ on leucine transport was also studied. Replacement of Na^+ with mannitol from modified Hanks solution decreased leucine transport 15 %. (Compare upper horizontal lines.) In the absence of Na^+, 70 % of the reduced leucine transport can be inhibited by lysine (see Fig. 8). On the other hand, the alternate

pathway for leucine transport represented by the amount of leucine transport not inhibited by lysine, is reduced in the absence of Na^+.

Exchange diffusion

The stimulation of tracer influx by high intracellular substrate concentrations has been repeatedly described in animal cells and designated exchange diffusion[25,26]. The existence of such transconcentration effects in the lysine transport system was readily demonstrated. The initial rate of [^{14}C]lysine influx at 0.1 mM was determined after cells were preloaded by incubation for 10 min with various concentrations of [^{3}H]lysine. After preincubation the monolayers were rinsed with warm modified Hanks solution and the intracellular concentration of lysine before and after the initial rate determination was calculated from the content of ^{3}H and the specific activity of extracellular lysine. Preincubation with modified Hanks solution alone was used as control. In Fig. 9, the per cent of stimulation of lysine uptake was plotted against the intracellular concentration of lysine determined prior to the initial rate measurement. The results when plotted against the intracellular concentration measured at the end of the rate determination did not differ significantly. The maximal stimulation is about 2-fold.

In order to show that the stimulation of lysine transport by the removal of Na^+ from the incubation media as described in the previous section, and exchange diffusion are independent processes, monolayers were preincubated with lysine such that the final intracellular concentration would induce near-maximal exchange diffusion, and initial rates then measured in the presence and absence of Na^+ and/or K^+. The per cent of stimulation of lysine uptake thus obtained was equal to that of cells without lysine preincubation.

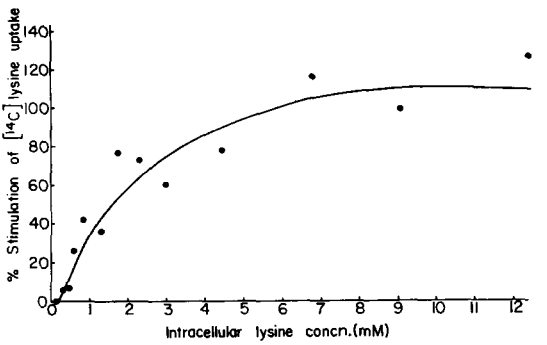


Fig. 9. Effect of preloading with [^{3}H]lysine on [^{14}C]lysine uptake. Monolayers were preloaded by incubation for 10 min with various concentrations of [^{3}H]lysine. After pre-incubation the monolayers were rinsed with warm modified Hanks solution and the intracellular concentration of lysine prior to the initial rate determination with 0.1 mM [^{14}C]lysine was measured. Preincubation with modified Hanks solution alone was used as control. The per cent of stimulation of [^{14}C]lysine uptake was plotted against intracellular lysine concentration.

DISCUSSION

Although attention was focused on lysine transport, none of the amino acid transport systems revealed in these studies can be identified with the lysine or dibasic amino acid transport system described in the intestinal epithelium and proximal renal

tubule. Unlike the epithelial system, the neutral amino acids, leucine and histidine, can bring about complete inhibition of lysine transport, and, in general, the membrane carrier of the macrophage has a high affinity for amino acids with long lipophilic side chains (see Table I). On the other hand the macrophage system has only poor affinity for cystine. The specificity of the macrophage system (which we shall designate AM-1, the first system described in the alveolar macrophage) is relatively broad. At the same time it is not the only amino acid transport system identified in these cells: there are at least two systems for leucine.

Attempts have been made to generalize the transport systems of Ehrlich ascites tumor cells to other mammalian cells[6]. However, several differences in the lysine transport systems of alveolar macrophages and tumor cells are readily apparent.

(A) Lysine enters by a single carrier in the macrophage and by three in the tumor cell[13].

(B) In the macrophage, lysine transport is completely inhibitable by neutral amino acids such as leucine and histidine, but only partially inhibited by these amino acids in tumor cells.

(C) Although phenylalanine has appreciable affinity for the lysine carrier in the macrophage, the affinity of alanine is very poor; in the tumor cell, on the other hand both phenylalanine and alanine are significant inhibitors of lysine transport[13].

The effect of alkali metal ions on the transport of non-electrolytes (*e.g.* amino acid and sugar) has been studied in a variety of animal cells and tissues[11,16–23,27–31] and certain marine bacteria[24]. In most cases the evidence supports the hypothesis that Na^+ functions as a co-substrate for the transport system (or carrier). The ultimate driving force involved in the active accumulation of these non-electrolytes is provided by the Na^+ gradient which is maintained by the Na^+ pump[17,27]. The effects of Na^+ on membrane transport thus far reported in the literature may be divided into four categories depending on whether K_m and/or v_{max} is altered by Na^+. The data are summarized in Table IV. In Type I, Na^+ decreases the K_m but has little or no effect on v_{max}. On the other hand, in Type II, Na^+ increases v_{max} but has little or no effect on K_m. GOLDNER *et al.*[30] ascribed the difference of Na^+ effect on sugar transport found in rabbit ileum (Type II) and hamster small intestine (Type I) to differences of technique. However, it could also be due to the species difference. In this connection it is interesting that in studies of intestinal sucrase, KOLÍNSKÀ AND SEMENZA[32] reported a Type I Na^+ effect in rat and hamster while a Type II effect was found in rabbit and human.

WONG *et al.*[24] reported that in a marine pseudomonad, as the Na^+ concentration in the suspending medium increased from 0 to 50 mM, the K_m for α-aminoisobutyric acid transport decreased, while v_{max} remained essentially constant. Between 50 and 200 mM Na^+, the K_m remained constant while v_{max} continued to increase (Type III effect). The authors attributed the effects of Na^+ at low concentrations to its requirement for the operation of the transport system. At 50–200 mM Na^+ was said to modify the porosity of the cytoplasmic membrane in such a way that it reduced the rate of leakage of α-aminoisobutyric acid from cells. Type IV Na^+ effect is a combination of Type I and Type II, where Na^+ decreases the K_m as well as increases the v_{max}.

We might emphasize at this point that the above effects are relatively specific to Na^+; it cannot be substituted by other cations such as K^+, Li^+ or choline. Na^+ always facilitates the transport of these non-electrolytes by either increasing the

TABLE IV

SUMMARY OF THE EFFECTS OF Na^+ ON MEMBRANE TRANSPORT OF NON-ELECTROLYTES

Type	*Na^+ effect*			*Transport system (substrate)*	*Tissue or cell*	*Ref.*
	K_m	*v_{max}*	*Specificity*			
				6-Deoxyglucose	Hamster small intestine	27, 28
				6-Deoxyglucose	Rat jejunum	29
				Alanine, valine, leucine	Rabbit ileum	16–18
				Alanine	Rabbit jejunum	19
I	↓	o	(+)	Glycine (high K_m system)	Rabbit ileum	20
				Glycine	Pigeon erythrocyte	7
				α-Aminoisobutyric acid	Rabbit lymph node cell	21
				Lysine (high K_m system)	Rabbit ileum	11
II	o	↑	(+)	3-Methyl glucose	Rabbit ileum	30
				α-Methyl-D-glucoside	Rabbit kidney cortex	31
III	↓	o	(+)	α-Aminoisobutyric acid (at Na^+ concn. of 0–50 mM)	Marine pseudomonad	24
	o	↑	(+)	α-Aminoisobutyric acid (at Na^+ concn. of 50–200 mM)		
				Alanine	Mouse ascites tumor cell	22, 23
					Rabbit reticulocyte	
IV	↓	↑	(+)	Glycine	Rabbit reticulocyte	23
				α-Aminoisobutyric acid	Mouse ascites tumor cell	22
					Rabbit reticulocyte	
					Pigeon erythrocyte	
V	o	↓	K^+ shares the effect	Lysine (AM-1 system)	Rabbit alveolar macrophage	

affinity of the substrate to the carrier (*e.g.* decreasing the K_m) or by increasing the rate of the transport, or both. All these effects are consistant with the hypothesis that Na^+ behaves as a co-substrate for the transport system, inducing some allosteric change of the carrier perhaps. The Na^+ gradient appears to provide the driving force for the intracellular accumulation of non-electrolytes.

The effects of Na^+ observed in this study can not be categorized in any of the types enumerated. From Figs. 5 and 6, it was apparent that Na^+ decreased the v_{max} of lysine transport by the AM-1 system but had no effect on the K_m. These effects are categorized as Type V in Table IV. As shown in this table this effect of Na^+ is shared by K^+ and in this sense is non-specific. Inhibition of enzyme activity by Na^+ has also been described. From our own laboratory it was demonstrated that the activity of adenylate pyrophosphorylase (EC 2.4.2.7) purified from extracts of *Bacillus subtilis* was inhibited by Na^+ but not by K^+ (ref. 33).

Reports of the effects of Na^+ on lysine transport in tumor cells are somewhat conflicting. It is apparent from the data of CHRISTENSEN AND LIANG[13] that substitution of sucrose for Na^+ increased the amount of lysine transport which was non-inhibited by neutral amino acids. This was a small fraction of the total lysine transport and the kinetics of the effect were not examined. Recently CHRISTENSEN *et al.*[34] described a Na^+-facilitated reaction of neutral amino acids with a cationic amino acid transport system in ascites tumor cells. They suggested that Na^+ occupied the position

normally taken by the charged terminal amino group of the dibasic amino acid, and facilitated the inhibition of dibasic amino acid transport by neutral amino acids. Theoretically, these two findings of CHRISTENSEN *et al.*[34] suggest that Na^+ should compete with lysine for the carrier.

It is clear in our study that the affinity of leucine for the AM-1 system is also increased specifically by Na^+. On the other hand the effects of Na^+ on lysine transport do not provide evidence for interaction of Na^+ with the carrier site which binds the cationic terminal group of lysine since the kinetics indicate that Na^+ affects v_{max} and not affinity. The dissimilarities of lysine transport in tumor cells and macrophages have already been pointed out, however. The existence of multiple lysine transport systems in the tumor cell makes comparison between the cells difficult at best.

As discussed above the accumulation of non-electrolytes by animal cells has been largely explained by the effect of Na^+ to increase the affinity of non-electrolytes to the carrier or to increase the rate of translocation of these Na^+–substrate–carrier complexes across the membrane or both; and accomplished by the asymmetry of Na^+ distribution across the cytoplasmic membrane under physiological conditions (high Na^+ outside; low Na^+ inside). However, the accumulation of lysine (and probably other basic amino acids) in alveolar macrophages cannot be explained by this mechanism, since the effects of Na^+ and K^+ are equivalent and the total cation concentration is approximately equal intra- and extracellularly. On the other hand there is competition for exit of lysine with the neutral amino acids sharing the AM-1 system which could lead to its accumulation. Thus lysine accumulation in turn also becomes dependent on the asymmetry of Na^+ distribution, which determines the concentration of the competitive neutral amino acids.

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