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DIBASIC AMINO ACID TRANSPORT IN RAT-KIDNEY CORTEX SLICES

STANTON SEGAL*, LEONARD SCHWARTZMAN**, ALBERTA BLAIR AND DOLORES BERTOLI*

Clinical Endocrinology Branch, National Institute of Arthritis and Metabolic Diseases, National Institutes of Health, Bethesda, Md. (U.S.A.)

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SUMMARY

The effect of anaerobiosis, Na^+ deprivation and pH alteration on the accumulation of L-lysine, L-arginine, L-diaminobutyric acid and L-cystine was studied at physiological levels of substrate in rat-kidney cortex slices. Lysine, arginine and diaminobutyric acid were capable of being actively transported despite oxygen and Na^+ lack in the incubation medium. Initial rates of accumulation of lysine and diaminobutyric acid appeared to be normal under the latter conditions although the steady-state concentration gradients were reduced. Cystine and arginine transport was inhibited initially and there was impairment of the ability to form a concentration gradient, most marked for cystine in Na^+-free medium. The variation of accumulation with pH differed for each of the amino acids, the curve of cystine being more like that of glycine, than the dibasic amino acids. The results lend further support to the concept that cystine transport in kidney slices occurs by a system separate from that of dibasic amino acids and indicate that for the other dibasic amino acids, variation of intragroup transport characteristics is present.

INTRODUCTION

Interest in the transport of dibasic amino acids by mammalian kidney has been stimulated by the observation that L-lysine, L-arginine, L-ornithine and L-cystine occur in large quantities in the urine of human cystinurics[1]. The additional finding that infusion of lysine into both normal humans[2] and dogs[3] caused cystinuria led to the proposal that there is a common transport system in kidney tubules for cystine and the dibasic amino acids[4].

Employing mutual inhibition experiments in rat-kidney cortex slices *in vitro*, ROSENBERG, DOWNING AND SEGAL[5] reported that L-lysine, L-arginine and L-ornithine competitively inhibited the accumulation of each other but that these dibasic amino

* Present address: Dept. of Pediatrics, University of Pennsylvania Medical School, Children's Hospital of Philadelphia, Philadelphia, Pa., U.S.A.

** Present address: Dept. of Medicine, Los Angeles County Hospital, Los Angeles, Calif., U.S.A.

acids did not inhibit L-cystine transport nor did L-cystine inhibit transport of the dibasic amino acids. Subsequently, the same results were observed in studies with human-kidney slices[6]. Concomitantly, although dibasic amino acid transport was found to be defective in kindey slices of human cystinurics, that of cystine was within normal limits.

The purpose of the present investigation has been to delineate the characteristics of dibasic amino acid and cystine transport in rat-kidney cortex slices by criteria other than interaction of the amino acids for transport sites. Transport of lysine, arginine, diaminobutyric acid and cystine has been studied with respect to dependence on oxidative metabolism, Na^+ and pH and the results form the basis of this report.

METHODS

The technique for the study of amino acid transport in kidney cortex slices of the 150–180 g Sprague–Dawley rats has been described in detail previously in several communications[5,7]. Three cortex slices weighing between 50 and 80 mg are incubated in 2 ml of buffer, usually Krebs–Ringer bicarbonate (pH 7.4), with radioactive amino acid. At the end of the incubation the tissue pool of amino acid is extracted with boiling water. With measurement of the radioactivity of the media and water extract and estimation of the total tissue water (80.2±1% wet tissue weight[8]) and inulin space (25.7±1.1% of wet tissue weight[8]) the distribution ratio or ratio of counts/min per ml intracellular fluid to that in the media may be calculated where

$$\text{counts/min in intracellular fluid} = \frac{\text{counts/min in tissue water} - (\text{volume inulin space} \times \text{counts/min in media})}{\text{total tissue water} - \text{inulin space}}$$

Previous experiments with lysine and arginine demonstrated that the radioactivity in the tissue fluid is virtually all in the form of the amino acid added to the media[5]. The case for cystine is somewhat different since CRAWHALL AND SEGAL[9] have recently reported that the intracellular radioactivity is almost entirely cysteine after incubation of slices with cystine. Therefore, unlike the other amino acids, the cystine distribution ratio does not represent a concentration ratio but rather a radioactivity ratio. Since L-diaminobutyric acid was used here with kidney slices for the first time, the hot-water tissue extracts were subjected to paper-chromatographic analysis in butanol–ethanol–water (4:1:2, by vol.) with subsequent assay of radioactivity by cutting the paper into 2-cm strips which were counted by liquid-scintillation techniques. 98% of the radioactivity corresponded with the location of diaminobutyric acid. Experiments were also performed to measure the oxidation of diaminobutyric acid to $^{14}CO_2$ and the incorporation of diaminobutyric acid into protein. With 470 000 counts/min of substrate in 2 ml Krebs–Ringer bicarbonate buffer 100 mg of tissue converted 16 000 counts/min to $^{14}CO_2$ in 60 min and incorporated 50 counts/min per mg of tissue protein in the same period of time. This is about equal to the rate of lysine oxidation and 1/20 the rate of lysine incorporation into protein reported previously employing identical experimental procedures[10].

Anaerobiosis experiments were carried out in Krebs–Ringer bicarbonate buffer (pH 7.4) in which the buffer and flasks were gassed with 95% N_2–5% CO_2. The slices were preincubated for 30 min at 37° before the radioactive amino acid was added in order to exhaust tissue oxygen supplies. Experiments in which no preincubation was carried out revealed slightly higher amino acid uptake at early time points.

In experiments with Na^+-free media, the Na^+ was replaced by Tris in equimolar amounts as described previously[11]. Changes of total tissue water and inulin space known to occur in this medium were applied to the calculation of the distribution ratio[11]. All slices were preincubated for 30 min in Na^+-free buffer at 23° before incubation.

In studies of pH effects, no one buffer sufficed over the entire pH range employed. At pH above 7.4, the $NaHCO_3$ of Krebs–Ringer buffer was replaced by 0.1 M Tris buffer at the appropriate pH. At pH 7.0, 3.5 and 6.0 Krebs–Ringer phosphate buffer and at pH 5.0 and 5.5, 0.1 M sodium acetate replacement of bicarbonate was employed. Concentration of Na^+ was kept constant and equivalent to that of pH 7.4 Krebs bicarbonate buffer by adjustment of the amount of NaCl added. Phosphate itself did not affect transport since at pH 7.4 Krebs–Ringer bicarbonate and phosphate buffer gave identical results for amino acid uptake. Likewise, the amounts of acetate necessary for buffers at the lower pH did not affect transport when added to Krebs–Ringer bicarbonate at pH 7.4. The buffer pH was taken at the start and completion of the 30-min incubations. The acetate buffers were found to increase 0.2 – 0.4 pH units and the Tris buffer to decrease 0.2 pH units. The total tissue water and inulin spaces at pH 5.0 and 5.5 were found to be similar to those found in Na^+-free medium[11] and appropriate changes were made in the calculation of distribution ratios at these pH.

All the radioactive ^{14}C-labeled amino acids used (uniformly-labeled L-lysine, [1-^{14}C]arginine, [2-^{14}C]glycine, [1-^{14}C]cycloleucine and [1-^{14}C]valine with the exception of L-[^{14}C]diaminobutyric acid, which was purchased from CalBiochem Co., were high specific activity compounds purchased from New England Nuclear Corp. and diluted with unlabeled amino acid to make 13 mM solutions of 40 μC/ml: 10 μl of these solutions were added to each 2-ml incubation. L-[^{35}S]Cystine was obtained from Schwarz Biochemical at 24 mC/mmole which was dissolved in dilute alkali to make a solution of 0.2 mM, 48 μC/ml. 10 μl of this solution *plus* 10 μl of 12 mM unlabeled cystine solution was added to each 2 ml of medium. All of the ^{14}C-labeled amino acids were found to be chromatographically pure as was the [^{35}S]cystine by high-voltage paper electrophoresis. In experiments where the substrate concentration was increased to 15 mM or above, unlabeled amino acid commercially obtained was added to the buffer.

RESULTS

Accumulation of dibasic amino acids at low concentrations

The present experiments have been carried out at 0.065 mM, a level similar to that observed in human plasma. At this concentration lysine and arginine are known to be transported by a system with a K_m of about 2.5 mM and $v_{max.}$ of 2.5 mmoles/l per 30 min (ref. 5).

Transport in Na^+-free medium

The distribution ratio of L-lysine, L-arginine, L-diaminobutyric acid and L-cystine under various incubation conditions is shown in Fig. 1. Each of the amino acids demonstrated its own characteristic uptake curve in Krebs–Ringer bicarbonate medium. Since kinetically the slice system may be analyzed as a two-compartment closed system[10], the distribution ratio at steady state is an indication of the ratio of

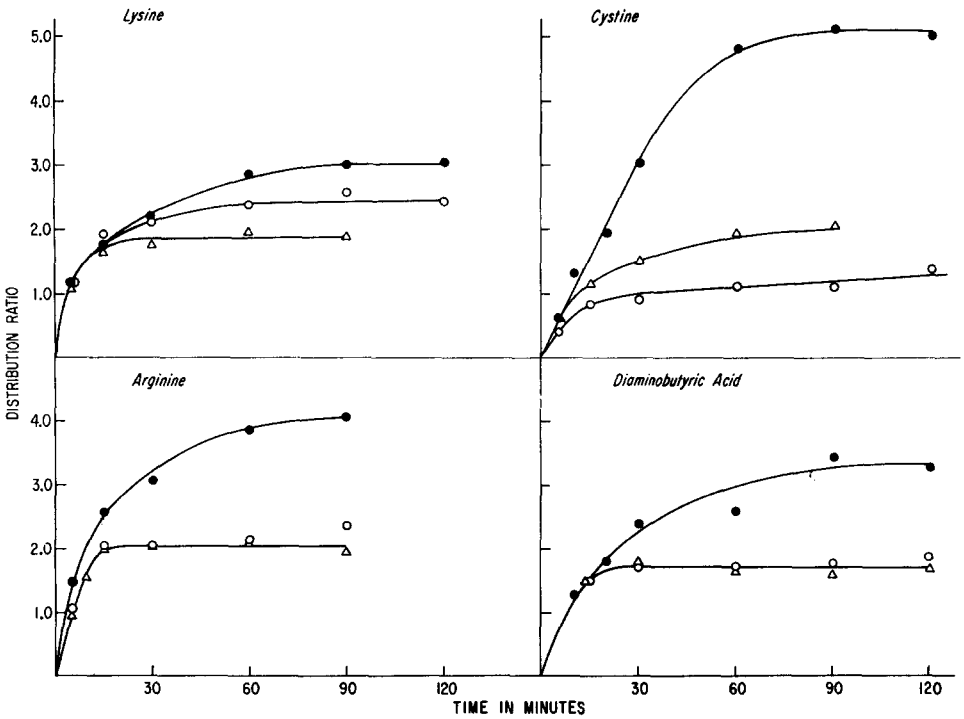

Fig. 1. The accumulation of L-lysine, L-arginine, L-diaminobutyric acid and L-cystine by rat-kidney cortex slices with time. Amino acid concentrations were cystine 0.07 mM, lysine, arginine and diaminobutyric acid 0.065 mM, 2-ml incubation, 0.2 μC/ml. ●—●, Krebs–Ringer bicarbonate buffer (pH 7.4) gassed with 95% O_2–5% CO_2; △—△, Krebs–Ringer bicarbonate buffer gassed with 95% N_2–5% CO_2; ○—○, Krebs–Ringer buffer with all Na^+ replaced by Tris (pH 7.4). Each point is an average of triplicate determinations and paired slices were used for determination of each curve, except for 5-min points as noted in the text.

the amino acid influx and efflux rate constants. The highest steady-state distribution ratio is found for cystine whose uptake curve with its relatively long linear uptake is quite dissimilar to those of lysine, arginine and diaminobutyric acid. The cystine curve is not unlike that observed for α-aminoisobutyric acid and glycine[7].

In Na^+-free medium initial distribution ratios of lysine and diaminobutyric acid are not affected. The 5-min control distribution ratio of lysine was 1.17 (14 determinations) while in Na^+-free medium it was 1.16 (9 determinations) with a P value tested for paired data of >0.9. Subsequent steady-state concentration gradients, though below the controls (Fig. 1), indicate the cells are able to maintain a considerable gradient under this condition. The differences between the control and Na^+-free values of lysine in the steady state are significantly different, $P<0.1$ for 6 additional determinations at 20 min. Arginine on the other hand, has both an impaired early uptake without Na^+, control distribution ratio 1.48 (12 determinations), Na^+-free 1.08 (8 determinations), $P<0.001$ by test for paired data, and a lower steady-state gradient. Cystine reveals a marked inhibition of early influx with a marked inability to achieve a significant concentration gradient. In this regard, cystine resembles α-aminoisobutyric acid and glycine[11,*].

* S. O. THIER, M. FOX AND S. SEGAL, unpublished observations.

Effect of anaerobiosis and various inhibitors

Fig. 1 reveals that all four of the amino acids are able to form concentration gradients under anaerobic conditions. As in Na^+-free medium, diaminobutyric acid and lysine show no impairment of initial uptake (lysine 5-min control distribution ratio 1.17, anaerobic 1.04, 6 determinations, $P>0.3$ for paired data), while arginine and cystine do show impairment (arginine 5-min distribution ratios are control 1.48, anaerobic 0.94, $P<0.001$ by paired data analysis, 4 determinations). The fact that these amino acids show active transport under anaerobiosis is in contrast to the findings with neutral amino acids which are unable to be concentrated intracellularly without oxygen[7].

Table I demonstrates the effect of various inhibitors on dibasic amino acid uptake. Dinitrophenol at 10^{-4} M does not significantly inhibit lysine accumulation at early or late time intervals but quite readily inhibits the uptake of both cystine and arginine. *N*-Ethylmaleimide, *p*-chloromercuriphenylsulfonate and iodoacetate, all sulfhydryl inhibitors, slow the early uptake of cystine to a considerable extent. Iodoacetate and *N*-ethylmaleimide inhibit lysine uptake but *p*-chloromercuriphenylsulfonate is not effective. Ouabain, which inhibits the Na^+ pump mechanism, causes substantially the same effect on cystine and lysine transport as does deprivation of Na^+ from the medium.

Transport dependence on pH

Fig. 2 shows the transport dependence of the dibasic amino acids as well as cystine and glycine on the pH of the medium. It is evident that each of the dibasic amino acids, lysine, arginine, and diaminobutyric acid, has its own characteristic curve of pH dependence, with the only resemblance one to another being the linear curve of lysine and diaminobutyric acid, between pH 5.2 and 8.0. Only arginine showed a peak at 7.4. Cystine uptake was more markedly altered by changes in pH than the dibasic compounds. Perhaps the most pertinent observation is the marked inability

TABLE I

EFFECT OF METABOLIC INHIBITORS ON CYSTINE, LYSINE AND ARGININE ACCUMULATION BY KIDNEY CORTEX SLICES

Concentration of amino acids was cystine, 0.070 mM; lysine and arginine, 0.065 mM in Krebs–Ringer bicarbonate buffer (pH 7.4). 0.2 μC/ml, 2-ml incubation volume.

Inhibition	*Concn. (M)*	*Distribution ratio*					
		Cystine		*Lysine*		*Arginine*	
		30 min	*90 min*	*30 min*	*90 min*	*30 min*	*90 min*
None		3.37 (6)	3.40 (6)	2.35 (6)	2.94 (6)	2.67 (6)	2.98 (6)
Dinitrophenol	10^{-4}	1.46 (6)*	2.42 (6)*	2.72 (6)	2.68 (6)	2.00 (6)*	1.99 (6)*
N-Ethylmaleimide	$2\cdot10^{-4}$	2.34 (3)*		1.77 (3)*			
Iodoacetate	10^{-3}	1.38 (3)*		2.10 (3)			
p-Chloromercuriphenyl-sulphonate	10^{-4}	2.32 (3)*		2.39 (3)			
Ouabain	$8\cdot10^{-4}$		2.40 (6)*		2.40 (6)*	1.66 (6)*	

* Significantly different from controls by paired data analysis. $P < 0.01$.

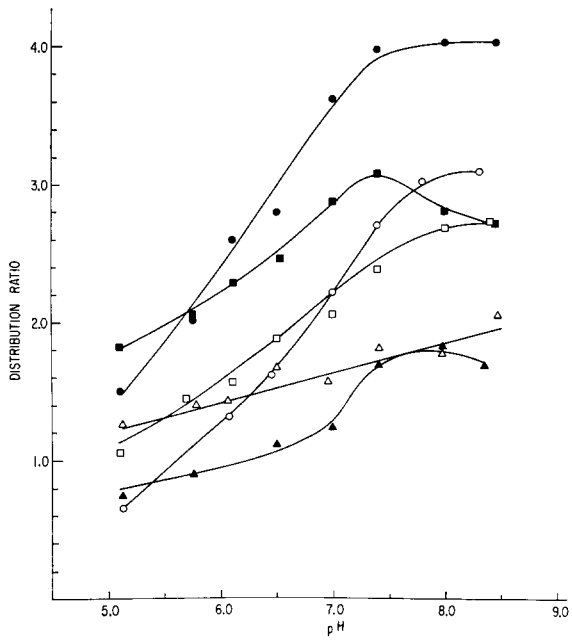

Fig. 2. The change in amino acid accumulation with pH. Buffers used are described under METHODS. Each point is an average of triplicate determinations and the points on each curve represent paired data. Concentration of all amino acids was 0.065 mM except cystine which was 0.01 mM. Lysine at high concentration was 15 mM. Incubation time 30 min. ●—●, glycine; ○—○, cystine; ■—■, arginine; △—△, lysine; □—□, diaminobutyric acid; ▲—▲, lysine, 15 mM.

of all of these to form concentration gradients at pH 5 to 6. Glycine establishes higher concentration gradients at high pH and shows the same general increase in uptake with increasing pH as do the dibasic amino acids. The shapes of the glycine and cystine curves are very similar.

Accumulation at high concentrations

During the course of counterflow experiments reported separately[12] in which the kidney slices were incubated with 30 mM lysine, it was noted that an intracellular concentration gradient developed. This appeared to be inconsistent with previous observations of a K_m of 2.0 and $v_{max.}$ of 2.5 mmoles/l per 30 min for lysine[5]. The possibility that this was an apparent gradient based on lysine displacement of intracellular cations was ruled out by determination of intracellular K^+ and Na^+. That it was due to distortion of tissue water or the extracellular fluid compartment was also eliminated by experimental observations of normal values for these parameters[12]. The characteristics of the transport of lysine were therefore investigated at high concentrations.

Accumulation of 30 mM lysine and the effect of Na^+ deprivation and anaerobiosis

Fig. 3 shows the uptake of lysine at 30 mM under various conditions. In Krebs–Ringer buffer (pH 7.4) there is active transport of lysine with establishment of

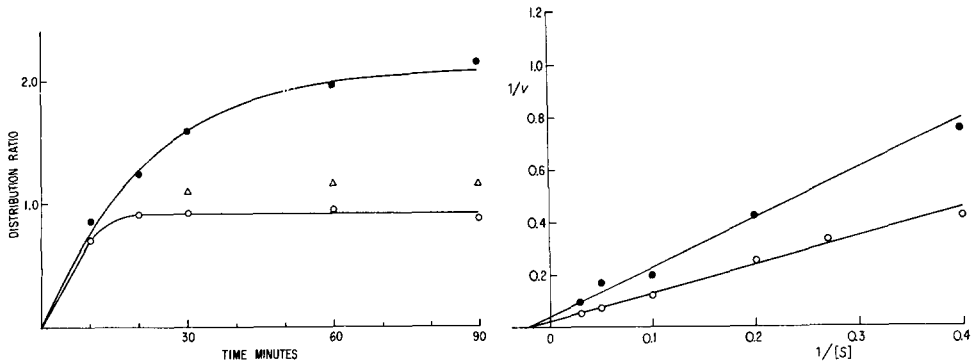

Fig. 3. Accumulation of 30 mM lysine by kidney cortex slices. 0.2 μC/ml. ●—●, Krebs–Ringer bicarbonate buffer, gassed with 95% O_2–5% CO_2; △—△, Krebs–Ringer bicarbonate with 95% N_2–5% CO_2; ○—○, Na^+-free medium.

Fig. 4. Lineweaver–Burk plot of reciprocal of velocity *vs.* reciprocal of substrate concentration. *S* is mM, *v* is mmoles/l per 20 min. ●—●, lysine; ○—○, glycine.

concentration gradients of 2. In the absence of medium Na^+ and under anaerobic conditions there is an inability to form a concentration gradient.

Table II shows the steady-state distribution ratios of several amino acids at medium levels of 15 mM. It appears that all of the amino acids studied were able to form gradients, with glycine being the most effectively concentrated in the intracellular fluid. Thus, other amino acids besides the dibasic ones possess a mechanism for transport besides simple diffusion at high concentration. K_m values and $v_{max.}$ values for α-aminoisobutyric acid, glycine and arginine previously reported[7] did not predict active transport at 15 mM.

Saturability of transport at high concentration

The effect of increasing concentration on lysine and glycine uptake is shown as a Lineweaver–Burk plot in Fig. 4. The amount of amino acid actively accumulated was estimated by subtracting the initial medium concentration according to previous approximations for passive diffusion[5]. The K_m for both amino acids was 50 mM with a $v_{max.}$ of 50 and 100 mmoles/l per 20 min for lysine and glycine, respectively.

TABLE II

CONCENTRATIVE UPTAKE OF AMINO ACIDS AT 15 mM

Conditions in text; averages of triplicate determinations. All values are significantly different from 1.00, the ratio expected from diffusion alone.

Amino acid	*Distribution ratio*	
	60 min	*90 min*
α-Aminoisobutyric acid	1.71	1.67
Glycine	2.33	2.33
Valine	1.50	1.36
Cycloleucine	1.46	1.50
Arginine	1.34	1.51

TABLE III

EFFECT OF AMINO ACIDS ON L-LYSINE AND GLYCINE UPTAKE

Conditions in text. 30-min incubation values are averages of triplicate determinations.

Substrate (10 mM)	*Inhibitor (50 mM)*	*Distribution ratio*
L-Lysine	—	1.51
	α-Aminoisobutyric acid	0.89*
	Glycine	1.55
Glycine	—	2.41
	α-Aminoisobutyric acid	1.70*
	L-Lysine	2.03

* Significantly different from controls by analysis of paired data.

The similar K_m for glycine and lysine under these conditions suggests the possibility of a common system for neutral and dibasic amino acids at high concentrations. The effect of glycine and α-aminoisobutyric acid on lysine uptake, and the effect of lysine and α-aminoisobutyric acid on glycine uptake were studied using 10 mM substrate concentration with inhibitors at 50 mM. The results are shown in Table III. α-Aminoisobutyric acid markedly inhibited lysine uptake but glycine did not. On the other hand, α-aminoisobutyric acid markedly inhibited glycine uptake while lysine had only a slight effect. These findings support the possibility of a common transport mechanism for amino acids at these very elevated levels.

Effect of pH

Fig. 2 shows the changes of lysine accumulation with pH at 15 mM. At low pH the curve is similar to the linear dependence shown at 0.065 mM, but unlike the latter curve, the one for 15 mM records a distinct maximum uptake at pH 8.0.

DISCUSSION

The evidence that lysine, arginine and diaminobutyric acid participate in heteroexchange diffusion[12] as well as competitive inhibition[5] supports the concept that they share a common transport system in rat-kidney cortex slices. The studies reported here at low substrate concentration reveal that these amino acids have similar transport characteristics under conditions of anaerobiosis and Na^+ deprivation, a finding which also identifies then as a distinct group. Under anaerobic conditions, the ability of lysine, arginine and diaminobutyric acid at physiological concentrations to form a concentration gradient is impaired but not to the same extent as α-aminoisobutyric acid, glycine, phenylalanine and tryptophan which cannot achieve any gradient under the same conditions[7,10]. Likewise, the maintainance of ratios of 2 with Na^+ deprivation is in contrast to α-aminoisobutyric acid and glycine which do not form gradients in Na^+-free medium[11].

Certain differences do emerge, however, within the dibasic group in transport properties at lower concentrations. The pH dependence of arginine transport differs from that of lysine and diaminobutyric acid. Initial influx of arginine is partially dependent on the presence of Na^+, whereas lysine influx is not. Arginine uptake is also

more dependent on oxidative metabolism than lysine as shown by both uptake in anaerobic medium and inhibition by dinitrophenol. It seems apparent that the presence of the guanidino group in arginine imparts distinctive transport characteristics different from ordinary diamino acids, although by competitive inhibition and exchange diffusion they share a common transport mechanism.

The cystine transport mechanism at low concentration apears to have characteristics more analogous to the neutral amino acid system than the dibasic system. The normal uptake curve of cystine resembles that of glycine, as does the uptake curve in Na^+-free medium. Moreover, the pH dependence of cystine is like that of glycine rather than like lysine and arginine. These data along with the previously observed lack of inhibition of cystine transport of lysine[5] and the non-participation of cystine in heteroexchange diffusion with dibasic amino acids, strongly indicate that cystine is transported into cells by a system separate from that of dibasic amino acids. However, the interpretation of cystine transport data based on ^{35}S distribution ratios requires further evaluation since the intracellular form after [^{35}S]cystine transport has been found to be largely cystine[9].

At high amino acid concentrations (15 mM and higher) the transport system operative for lysine differs from that at lower concentration. The K_m and $v_{max.}$ for the latter are 2.5 mM ans 2.0 mmoles/l per 30 min, respectively, while that of the former is K_m, 50 mM and $v_{max.}$, 50 mmoles/l per 20 min. Moreover, the high-K_m system does not appear specific for dibasic amino acids, since the several amino acids tested established concentration gradients at 15 mM. Glycine has a similar 50 mM K_m and α-aminoisobutyric acid has been found to markedly inhibit both lysine and glycine uptake. In addition, the high-K_m system is Na^+ and oxygen-dependent and has a different pH dependence from the low-K_m system. A similar non-specific high-K_m transport system for lysine has been found in Ehrlich cells[13].

L-Diaminobutyric acid was employed in these studies since [^{14}C]ornithine was not available and CHRISTENSEN[13] had shown it to be a good model for dibasic amino acid transport in Ehrlich cells. In the kidney-slice system diaminobutyric acid appears to have many of the transport characteristics of L-lysine. However, it is not a non-metabolizable model dibasic amino acid for there is significant oxidation to CO_2 although there is little incorporation into protein.

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