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ACCUMULATION OF ARGININE BY DOG KIDNEY CORTEX MITOCHONDRIA

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SUMMARY

From experiments on mitochondria isolated from dog renal cortex it has been shown that:

1. The volume of distribution of arginine is initially similar to that for sucrose (approx. 70% of mitochondrial pellet water) but slowly increases toward 100% of the pellet water at 0°. Incubation at 30° permits mitochondrial accumulation of arginine and the apparent volume of distribution quickly exceeds the pellet water; cooling to 0° does not cause loss of the arginine but removal of arginine from the medium permits rapid reversal of uptake.

2. The uptake of arginine obeys Michaelis–Menten kinetics with $K_m = 8.9$ μM and has an energy of activation of 10 kcal/mole.

3. Arginine uptake is inhibited by ornithine, lysine, glycine and cystine and not by several other amino acids and metabolic inhibitors.

4. Arginine uptake at 30° approaches a maximum value which obeys adsorption theory.

It is suggested that arginine uptake into the sucrose-inaccessible space of mitochondria is carrier mediated and that excess uptake is on the basis of adsorption. The possible relationship to arginine reabsorption by the kidney is discussed.

INTRODUCTION

It is generally accepted that most amino acids are freely filtered from plasma at the renal glomeruli[1,2] and that the epithelium of the proximal segment of the nephron reabsorbs 90 to more than 99% of these filtered acids[3]. The mechanism by which this transport occurs remains a fundamental problem.

Administration of sufficient amounts of one amino acid parenterally to experimental animals led to decline in the number of mitochondria in the cells of the proximal tubule and to simultaneous appearance of droplets with staining properties similar to mitochondria[4] and which contained the injected amino acid in high concentration[4,5]. As a result of these and other findings, OLIVER[6] suggested (as part of a more general theory) that mitochondrial substance binds amino acids as a step in

Abbreviations: PPO, 2,5-diphenyloxazole; POPOP, 1,4-bis-2-(5-phenyloxazolyl)-benzene.

the process of renal reabsorption. A proposal similarly invoking mitochondria as agents in cellular transport has been suggested by others[7].

Mitochondria have already been shown to take up a variety of substances from media when incubated *in vitro*: electrolytes[8,9], substrates of the Krebs cycle[10], adenine nucleotides[10], pyridine nucleotides[11]. Protein synthesis[12,13,14] and hippurate synthesis[15,16] by mitochondria *in vitro* have also been demonstrated. The present studies were to test whether dog renal cortex mitochondria could capture arginine in non-protein form *in vitro*, to characterize the uptake particularly with regard to its adsorptive nature, and to compare mitochondrial uptake of arginine to reabsorption of arginine by the kidney. A preliminary report has been given[17].

METHODS

Tissue preparation

Mitochondria were prepared from canine renal cortex essentially according to the method of SCHNEIDER AND HOGEBOOM[18]. The kidney was obtained under pentobarbital anesthesia and promptly chilled in 150 mM KCl solution. The nuclear fraction sedimenting during the first centrifugation was discarded, and the mitochondria that were contained in the supernatant were sedimented at 5000 $\times$ *g* for 10 min. The mitochondria were then washed 6 times by resuspending the pellet in 0.25 M sucrose of twice the volume of original tissue followed by centrifuging at 5000 $\times$ *g* for 10 min. The final pellet was resuspended in 0.25 M sucrose of volume equal to the original tissue. The sucrose was boiled and cooled immediately prior to beginning the isolation.

Incubation

4 ml of the final mitochondrial suspension were usually added to 1.6 ml of stock medium at 0°. In the case of zero-time controls the 5.6 ml were then added to 0.4 ml of 154 mM NaCl at 0° containing arginine (and inhibitor) at the desired concentration; mixed; divided equally between two preweighed, prechilled, glass centrifuge tubes; centrifuged at 0° at 25000 $\times$ *g* for 1.5 min; decanted; and the inner walls of the tubes wiped dry with filter paper. (The 1.5-min centrifugation time does not include the 2.5 min required to reach 25000 $\times$ *g* nor the 3 min to decelerate.) For mitochondria which were to undergo incubation, the 5.6 ml were equilibrated for 3 min in a Dubnoff metabolic shaker (generally at 30°); then (zero time) the suspension was added to the 0.4 ml of NaCl + arginine $\pm$ inhibitor solution which had also been thermally equilibrated. The arginine uptake was terminated (generally after 2 min) by dumping the 6 ml of incubation mixture into a steel tube in an ice bath and shaking for 3 min to accomplish rapid cooling to 0°. As with the zero-time control, the suspension was divided between 2 centrifuge tubes; centrifuged; decanted; and the walls of the tubes wiped dry. The wet weight of pellets was obtained by weighing promptly. When sucrose as well as water content and arginine was to be determined, the volumes were increased 50%, and the final mixture was divided equally among 3 tubes. All of the above steps were carried out without delay. Centrifugation was accomplished in an automatic, refrigerated Servall RC-2.

The final incubation medium was one previously found suitable for oxidative phosphorylation[19] except that no phosphate acceptor system was present. The rate of oxidation was thus limited by the activity of adenosine triphosphatase in the system.

The final concentrations (mM) were: α-ketoglutarate, 6.67; cytochrome *c*, 0.012; adenosine triphosphate, 2.0; $MgSO_4$, 7.5; phosphate buffer (pH 7.3), 13.3; Na^+, 30; Cl^-, 26; K^+, 42; sucrose, 167.

Analyses

The pellet water content was obtained by difference after drying 1 pellet of each pair overnight at 100–105°. The percentage of pellet water thus determined was applied to the duplicate pellet.

The other pellet of each pair was disrupted thoroughly with the aid of a stirring rod in 2 ml of Hydroxide of Hyamine-10X, and dissolved by shaking in a water bath at 80° for 15 to 30 min (see ref. 20). Disruption of the pellet was more complete, the time required at 80° was much shorter, and the color developed was less if disruption was accomplished immediately upon Hyamine addition. After dissolving the pellet, sufficient 20 % H_2SO_4 in absolute ethanol was added to make the Hyamine neutral to litmus. These solutions were transferred to counting vials using 13 ml of toluene counting fluid containing 0.4 % 2,5-diphenyloxazole (PPO) and 0.01 % 1,4-bis-2-(5-phenyloxazolyl)-benzene (POPOP). An appropriate aliquot of each supernatant was treated similarly except that the treatment at 80° was omitted. ^{14}C determination was accomplished with a Packard Tri-Carb model 3214 liquid scintillation counter using [^{14}C]toluene as internal standard.

In some experiments sucrose was determined[21] on a $CdSO_4$ filtrate of a triplicate pellet and on appropriately diluted supernatant.

Paper chromatograms were prepared in triplicate of standard [^{14}C]arginine solution, medium, and aqueous extract of pellet, each having carrier arginine added. Phenol saturated with water was allowed to ascend for 3 h and the paper was dried at room temperature. One of each triplicate set was sprayed with ninhydrin to locate the arginine spot, and the other strips were cut up (limiting the arginine to one square) and placed in individual counting vials. 1 ml of Hydroxide of Hyamine-10X was added to each followed by incubation[22] at 80° for 20 min, and then neutralized with the 20 % H_2SO_4 in ethanol. 14 ml of toluene counting fluid were added to each vial and counted as described above.

Chemicals

Chemicals were obtained as follows: unlabeled amino acids from Nutritional Biochemical, Cleveland; α-ketoglutaric acid, disodium adenosine triphosphate, type II cytochrome *c* from Sigma, St. Louis; L-[^{14}C]arginine (uniformly labeled) with specific activity of 246 mC/mM, [^{14}C]toluene from New England Nuclear, Boston; PPO, POPOP from Pilot Chemicals, Watertown, Mass.; Hydroxide of Hyamine 10-X from Packard Instrument, Downers Grove, Ill.; antimycin A, actinomycin D, puromycin dihydrochloride by kindness of Mr. M. J. Weinstein, Schering Corporation, Bloomfield, N.J. Other chemicals were reagent grade. Water was triple glass distilled.

Calculations

'Arginine space' is defined as the ratio of the disint./min per ml of mitochondrial pellet water to the disint./min per ml of supernatant. Because this space generally exceeded 1, (*i.e.*, exceeded the water content of the pellet), it must be considered a virtual space. 'Sucrose space' is defined in an analogous way. 'Arginine uptake' by

mitochondria is the arginine content (calculated from pellet activity and the initial specific activity of the arginine in the medium) per g of wet pellet sedimented after a period of incubation *minus* the arginine content per g of wet pellet sedimented at zero time (immediately after mixing and while maintained continuously at 0°).

'Arginine excess-uptake' is the amount of arginine in a pellet in excess of an amount calculated as the product of pellet water and supernatant concentration, expressed per g of wet pellet.

RESULTS

Arginine space

Immediately after the mitochondrial suspension was mixed with arginine at 0° (time = 0), the arginine space was only 0.7 of the pellet water (Fig. 1). In 23 experiments the initial arginine space had a mean value of 0.723 ± 0.022 (S.E.) and

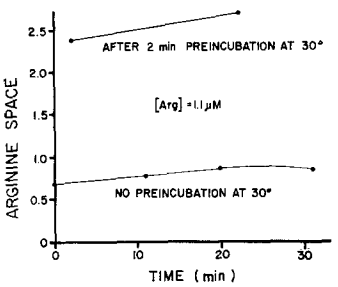

Fig. 1. Arginine space of mitochondrial pellet at 0° with and without preincubation at 30°. The mitochondrial suspension was incubated in 1.1 μM arginine in complete medium (see METHODS).

was statistically significantly larger than the sucrose space which had a mean of 0.666 ± 0.017 (S.E.) ($0.025 < P < 0.05$ calculated by analysis of paired samples) (Table I). With continued incubation at 0°, the arginine space approached a value of 1, indicating that arginine might be able to penetrate into all regions of the mitochondrial water but that penetration into approx. 30 % of the pellet water is very slow at 0°. Arginine might have partially penetrated into the sucrose-inaccessible space during the few min required to mix and centrifuge the 'zero-time' sample, thus accounting for the slightly larger arginine than sucrose space at zero time.

After a brief 2-min exposure of mitochondria to arginine at 30°, arginine space exceeded mitochondrial water by 2.4-fold (Fig. 1). In 23 experiments measuring

TABLE I

MITOCHONDRIAL PELLET COMPOSITION

	Zero time, 0°	*2 min, 30°*	*P (n = 23)*
Water fraction of pellet	0.713 ± 0.0053	0.730 ± 0.0030	<0.001
Pellet sucrose space	0.666 ± 0.017	0.714 ± 0.017	<0.001
Pellet arginine space	0.723* ± 0.022	1.953 ± 0.133	<0.001

* Significantly larger than pellet sucrose space at zero time ($P < 0.05$)

arginine space after 2 min at 30° (Table I), the space had a mean value of 1.953 ± 0.133 (S.E.). In contrast, the sucrose space after 2 min at 30°—0.714 ± 0.017 (S.E.)—was only slightly larger ($P < 0.001$) than at zero time.

Upon cooling the suspension to 0°, the arginine which had been taken up did not leak out (Fig. 1). On the contrary, uptake continued but at a very slow rate—about 2 % of the rate obtained at 30°. The relatively low uptake rate at 0° permitted the uptake reaction to be essentially stopped by cooling followed by prompt centrifugation. The slow uptake at 0° just prior to and during separation by centrifugation would cause overestimation of uptake during the initial 2 min at 30° by approx. 5 %.

Because radioactivity was being used as a measure of arginine concentration, it was necessary to demonstrate that the activity present in the pellet and medium following incubation was still mainly associated with arginine. To accomplish this, chromatograms of the following were prepared: (a) unincubated [^{14}C]arginine medium, (b) [^{14}C]arginine medium after use in an 8-min incubation, and (c) boiled aqueous extracts of a mitochondrial pellet following 8 min of incubation with [^{14}C]arginine at 30°. (Within experimental error, the extract contained 100 % of the pellet radioactivity.) The radioactivity was largely associated with the arginine spot in all three instances, the spots containing 95, 91, and 81 % of the recovered activity, respectively. When the activities present in the supernatant and in the pellet are corrected by the factors 0.91 and 0.81, respectively, the pellet in this experiment still contained 2.3 times as much arginine per ml of pellet water as the medium contained. Because approx. one-fourth of the pellet water is extramitochondrial[8], the intramitochondrial concentration would be correspondingly higher. Considering the mild condition used to extract the pellet, arginine must have existed somewhere in the mitochondrial pellet either free in solution at greater than twice the concentration in the medium or it must have existed in a form which easily reverts to free arginine.

Arginine uptake as a function of time

Fig. 2 indicates that arginine uptake rate over the first 2 min of incubation approximates initial uptake rate with arginine concentrations between 0.091 and 10.1 μM. In subsequent experiments, uptake over this interval was used as a measure of initial uptake rate.

If the data are interpreted as indicating that there is no barrier to arginine movement into all parts of the sucrose space, the component of pellet arginine that is present in the sucrose space would be taken up coincident with mixing. Interest would then center on arginine taken up in excess of that calculated to be in the sucrose space (the product of sucrose space and arginine concentration in the medium). Such an uptake could logically be expressed per unit dry mitochondrial weight. Because of the changing pellet water, it would not be possible, in general, to compare quantitatively the uptake calculated in this way with the arginine uptake defined under METHODS. As an example, however, by using the mean values of Table I, it can be shown that on the average, a 2-fold increase in the rate of entry of arginine 'into' the sucrose-inaccessible space would be calculated as a 1.92-fold increase in arginine uptake as defined in METHODS. Therefore, if the above model of arginine distribution in the pellet is correct, the present method of calculating arginine uptake would systematically underestimate a change in rate by about 8 %.

Effect of temperature on uptake

Fig. 3 shows the relationship of initial uptake velocity to temperature between 22° and 37°. The data indicate an energy of activation of 10 kcal/mole of arginine uptake.

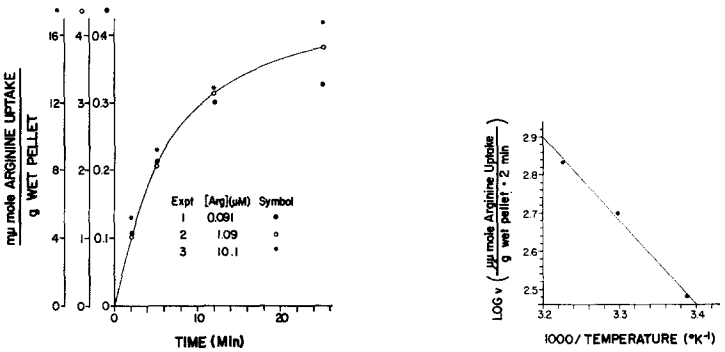

Fig. 2. The effect of time and arginine concentration on arginine uptake by mitochondria. The 3 experiments used 3 separate mitochondrial preparations.

Fig. 3. Influence of temperature on velocity of arginine uptake by mitochondria. Arginine concentration was 0.98 μM.

Reversibility of arginine uptake

Because arginine taken up by mitochondria at 30° does not leak out at 0° (Fig. 1) and because of the possibility that the arginine taken up might exist in a loosely bound form, it was of interest to see whether the uptake reaction could be reversed by removing the arginine from the medium. Fig. 4 shows the usual rapid uptake of arginine at 30° during the first 2 min of incubation. Following this uptake mitochondria were rapidly cooled to 0°, separated by centrifugation, resuspended in 0.25 M sucrose, and added to arginine-free medium. Subsequent incubation at 30° revealed a rapid loss of arginine from the mitochondria into the arginine-free medium indicating reversibility of the uptake reaction.

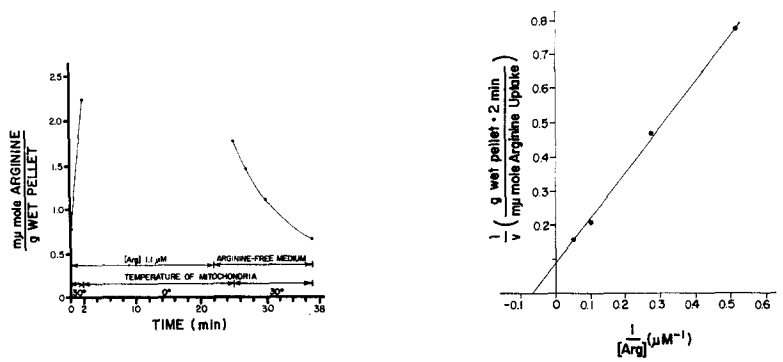

Fig. 4 Uptake of arginine by mitochondria followed by rapid release of arginine into a medium free of arginine. Arginine concentration initially was 1.1 μM.

Fig. 5. A double reciprocal plot showing the effect of arginine concentration on initial velocity of arginine uptake by mitochondria.

Kinetics of uptake reaction

Fig. 5 is a double reciprocal plot of the Lineweaver–Burk type relating arginine concentration to initial uptake velocity. 5 such experiments yield a mean value for K_m of 8.9 ± 1.8 (S.E.) μM and a mean value for V of 7.4 ± 1.7 (S.E.) mμM arginine uptake per g wet pellet per 2 min.

Inhibition of uptake

Table II lists data from a series of experiments testing whether other natural amino acids could inhibit arginine uptake by mitochondria. The greater inhibition shown by ornithine, lysine, and cystine is consistent with their strong competition with arginine for reabsorption *in vivo*[1,23–26]. The interaction of glycine with arginine reabsorption *in vivo* is not well defined; variously, glycine has been reported to inhibit[27], or to enhance arginine reabsorption[28], or to have no effect[23]. Also glycine reabsorption can be depressed by arginine[1]. Histidine given *in vivo* may decrease the maximum rate at which arginine is reabsorbed[23], increase arginine excretion[29], or show little effect[1].

TABLE II

INHIBITION OF MITOCHONDRIAL ARGININE UPTAKE

2 min incubation at 30°; arginine at 1.1 μM, inhibitor at 25 μM. Each datum is the average from 2 or more experiments except for tryptophane and aspartate.

L-Amino acid inhibitor	*% of control uptake*
Ornithine	43
Lysine	66
Glycine	75
Cystine	77
Leucine	88
Glutamate	91
Alanine	94
Tryptophane	100
Aspartate	101
Histidine	102

Omission from the incubation medium of either exogenous substrate, or ATP, or Mg^{2+} does not alter the initial rate of arginine uptake. Maleic acid (5 mM), an amino aciduric agent[30] and an inhibitor of α-aminoisobutyric acid uptake by kidney slices[31], shows no inhibition of arginine uptake by mitochondria. Similarly actinomycin D (17 μg/ml), an inhibitor of protein synthesis by beef-heart mitochondria[32], and puromycin (20 μM), an inhibitor of protein synthesis by microsomes[33], do not inhibit arginine uptake under these conditions. Antimycin A (4 μg/ml), cyanide (1 mM), and 2,4-dinitrophenol (0.5 mM)—agents inhibiting oxidative phosphorylation in various ways and all capable of inhibiting protein synthesis by beef-heart and rat-liver mitochondria[34]—each fail to inhibit arginine uptake. Therefore, the uptake of arginine by mitochondria is not particularly sensitive to alterations in oxidative metabolism nor to inhibitors of protein synthesis.

Adsorption of arginine

The 4 experiments of Fig. 6 demonstrate that the amount of arginine excess-uptake as affected by arginine concentration is consistent with the equation derived by KLOTZ[35] for ion binding to protein: $r/(A) = kn - kr$. As applied to these experiments, r is the arginine excess-uptake (presumably bound) after 12 min incubation at 30°; (A) is the arginine concentration of the media; k is the equilibrium constant; and n is the number of binding sites per unit of mitochondrial pellet. The 4 separate experiments yield a mean $k = 0.17 \pm 0.017$ (S.E.)l/μmole. The mean of $1/k$ for the 4 experiments is 6.0 ± 0.62 (S.E.) μM indicating that 50% of the sites would be bound when arginine is at that concentration.

The mean value of n is 2.4 ± 0.86 (S.E.) mμmoles per g wet pellet. The large S.E. indicates that the number of binding sites in the final preparations is variable.

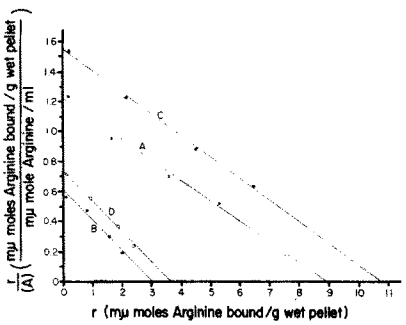

Fig. 6. A test of the applicability of the adsorption equation of KLOTZ relating ion binding to protein to ion concentration. The arginine excess-uptake (see METHODS) is plotted along the abscissa as bound arginine (r); (A) is arginine concentration. Temperature was 30°; incubation time was 12 min. A–D represent 4 experiments using 4 different mitochondrial preparations.

DISCUSSION

It has previously been demonstrated that mitochondria from a variety of tissues can synthesize protein[12,13,14,32]. The ability of mitochondria to capture arginine may serve *in vivo* to compartmentalize intracellular arginine at a site of essential protein synthesis. The minimum arginine concentration permitting protein synthesis in tissue culture[36,37] approximates K_m demonstrated for this mitochondrial uptake.

One can view the arginine taken up in either of two ways[38]. It might be accumulated as arginine free in solution within some part of the pellet water. Or it might be adsorbed onto a component of the mitochondria. The later view would seem the more likely considering the lack of inhibition by metabolic inhibitors and the good agreement with theory that is demonstrated in Fig. 6.

However, if the accumulated arginine were free in solution in a subcompartment of the pellet, it would be of interest to calculate the concentration ratio that was apparently effected by the uptake reaction: 2 min of incubation with arginine at 1 μM and at 30° caused the pellet to contain approximately twice as much arginine as would have occurred on the basis of uniform distribution between mitochondrial water and medium. Longer incubation easily increased this to 4 times as much (Fig. 2). Because the pellet extramitochondrial space, which amounts to about a fourth of the pellet would not contain any of the extra arginine, it must be concentrated within the 3/4 of

the pellet water which is 'intramitochondrial', thus making the concentration ratio 5. If all of the extra arginine were to exist in the pellet water which is initially free of arginine (28 %) or in the water inaccessible to sucrose (33 %), the ratio would be about 10. It thus appears that if the arginine taken up remains free in solution in an intramitochondrial compartment, the uptake mechanism can achieve a 5- to 10-fold build up in the concentration over that in the medium.

The alternate explanation of arginine capture—argine binding loosely to an element of mitochondria—leads to other interesting considerations: The kinetic data are consistent with the formation, as one step in the uptake phenomenon, of an arginine complex of the enzyme–substrate type. The straight-line relationship of Fig. 6 depends on the assumption that arginine achieves a uniform concentration throughout the pellet water equal to the concentration in the supernatant. (If one were to calculate the excess-uptake, *i.e.* the bound arginine, as the difference between total pellet arginine and the product of sucrose space and supernatant arginine concentration, the loci of points in Fig. 6 would be concave upward and the equation of KLOTZ[35] would not be satisfied.) There is a positive correlation between the initial rate of uptake and the arginine excess-uptake suggesting that both functions may depend on the same mitochondrial element. Also the difference between the mean values for K_m and $1/k$ is not significant. It might, therefore, be that a carrier molecule in the inner mitochondrial membrane accounts for both entrance of arginine into the sucrose-free space and for adsorption of arginine.

The concentration range of arginine used in this study is appropriate to the study of the reabsorptive mechanism as it exists in the nephron. It is reported that about 99.4 % of the filtered arginine undergoes reabsorption[3]. Evidence indicates that this occurs within the proximal segment of the nephron[24,25] where four-fifths to seven-eighths[40] of the filtered water is reabsorbed. From this it is easily calculated that arginine concentration in the lumen must be finally reduced to 3 to 5 % of the concentration in the glomerular filtrate. Because arginine concentration in the plasma is about 70 μM and because it is not bound to plasma protein, the final concentration in the lumen of the proximal segments must be on the order of 3 μM. This is about one-third of the K_m value determined for the mitochondrial uptake reaction. The higher concentrations present in the earlier regions of the segment would keep the mechanism operating near maximum velocity.

Both arginine uptake by canine renal mitochondria and arginine transport in the intact dog kidney display maximum rates (V and T_m, respectively). However, there is a large discrepancy between the two values. T_m is approx. 0.65 μmole arginine per g kidney·min (see ref. 41); whereas, V is only 0.0037 μmole arginine per g wet mitochondria·min. Even if kidney were composed entirely of mitochondria, V would be 175 times too small to account for T_m.

Some possible explanations for the deficiency in V can be listed: (1) The mitochondrial uptake sites might display a larger V *in vivo* in the presence of activators and a natural environment. (2) A large fraction of the mitochondrial uptake sites present *in vivo* may be detached and removed or destroyed during the isolation of the mitochondria. (3) The uptake sites may be generally damaged during isolation so that they function at a lower maximum rate. (4) The uptake sites studied in these experiments may only be responsible for producing a low urinary arginine concentration by virtue of their low K_m, other more rapidly acting sites but with high K_m

being responsible for the bulk of renal arginine reabsorption. (5) The mitochondrial uptake of arginine may be only remotely related to renal reabsorption *in vivo*.

In order for mitochondrial uptake of arginine to be directly involved in arginine transfer across the epithelial cells, a mechanism must exist for 'unloading' mitochondrial arginine into interstitial fluid. The nature of such a mechanism is, of course, unknown. RASMUSSEN[9] has commented that '... the close association of the mitochondrial and plasma membranes in various cells may lead to a functional unit of parallel membranes having considerably different properties than those of the individual membranes studied in isolation. Such a relationship could confer a vectorial component to mitochondrial ion exchanges, and to a direct participation of these mitochondria in transcellular cation transport'.

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