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THE INVOLVEMENT OF THE MEMBRANE OXIDOREDUCTION SYSTEM IN STIMULATING AMINO ACID UPTAKE IN EHRLICH ASCITES TUMOR CELLS

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The addition of 5 mM ascorbate plus 0.09 mM phenazine methosulfate stimulated 2- to 3-fold the initial rate of 2-aminoisobutyric acid transport into Ehrlich cells. This was observed under the conditions in which glycolysis and mitochondrial electron transport were blocked by iodoacetate and KCN, and the cellular ATP level was maintained below 0.1 mM. Proton conductors, carbonylcyanide *m*-chlorophenylhydrazone and SF6847 did not affect the stimulation of 2-aminoisobutyric acid uptake caused by ascorbate plus phenazine methosulfate. Ascorbate was replaced by NADH but not by NADPH, and phenazine methosulfate was the only effective acceptor in stimulating 2-aminoisobutyric acid uptake. The stimulating effect of ascorbate plus phenazine methosulfate was due to an increase in the V value for 2-aminoisobutyric acid but not in the K_m value. This effect required the presence of an Na^+ gradient and was accompanied by an increase in $^{22}Na^+$ influx. The molar ratio of 2-aminoisobutyric acid to Na^+ uptake enhanced by ascorbate plus phenazine methosulfate was calculated to be 1 : 1. Quinacrine, an inhibitor of NADH oxidoreductase in the plasma membrane, inhibited both the enhanced rate of 2-aminoisobutyric acid and Na^+ transport without affecting the basal transport activity. The stimulatory effect of ascorbate plus phenazine methosulfate was also observed with other amino acids, alanine, glycine, proline and cycloleucine which are known to be transported via an Na^+-dependent system but not with leucine and threonine. These results suggest that a redox system in the plasma membrane participates in energy coupling for amino acid transport by increasing the rate of cotransport with Na^+.

Introduction

Active transport by System A of neutral amino acids is known to be Na^+-dependent in Ehrlich cells [1,2], in which amino acids are cotransported with Na^+ [2–4], and the direct driving force for the concentrative transport is an electrochemical potential caused by alkali-ion gradients [5–9].

In addition to energization of transport due to alkali-ion gradients, Garcia-Sancho et al. [10] and Christensen [11] have recently reported that the addition of ascorbate plus phenazine methosulfate increases not only Na^+-dependent uptake of 2-methylaminoisobutyric acid but also Na^+-independent uptake of norbornane amino acid in Ehrlich cells. This effect was then shown to be inhibited by quinacrine, an inhibitor of the cell membrane oxidoreductase system [12,14]. Plasma membrane preparation of animal cells was found to contain NADH oxidoreductase and its activity was also specifically inhibited by quinacrine, which did not inhibit the same enzyme activity in mitochondrial and microsomal membranes [12–15].

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Abbreviations: Norbornane amino acid, 2-aminobicycloheptane-2-carboxylic acid (2-aminonorbornane-2-carboxylic acid); Hepes, *N*-2-hydroxyethylpiperazine-*N'*-ethanesulfonic acid; CCCP, carbonylcyanide *m*-chlorophenylhydrazone.

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These studies suggest that a redox system in the plasma membrane can participate in energization of both Na^+-dependent and Na^+-independent amino acid transports [10]. However, the mechanism by which the transport is energized by reducing equivalents has not been elucidated. We extended these studies in an attempt to explain the mechanism stimulating the transport of amino acids in Ehrlich cells by exogenous hydrogen donors and electron acceptors and found that the addition of ascorbate or NADH plus phenazine methosulfate stimulated the cotransport of 2-aminoisobutyric acid with Na^+ at an one-to-one ratio.

Materials and Methods

Preparation of cell suspension. Ehrlich cells were grown for one week in the peritoneal cavity of ICR Swiss female mice. Cells collected from the ascites were washed four times with 150 mM KCl containing 10 mM Hepes (pH 7.4) at room temperature for 2 min at $100 \times g$. The washed cells were suspended in an incubation medium composed of 140 mM Na^+, 10 mM K^+, 2 mM Mg^{2+}, 20 μM Ca^{2+}, 1 mM SO_4^{2-}, 0.5 mM PO_4^{3-}, 146 mM Cl^- and 10 mM Hepes (pH 7.4). Na^+-free medium was prepared by replacing 140 mM NaCl with 140 mM choline chloride in the incubation medium.

Transport measurement. Cell suspension of 1 ml (0.5 mg protein/ml) was preincubated for 30 min at 37°C with 1 mM iodoacetate and 0.5 mM KCN to block the mitochondrial electron transfer system and to maintain a low cellular ATP level. Ascorbate and phenazine methosulfate at 5 mM and 0.09 mM, respectively, were added immediately prior to the start of uptake. The uptake was started by adding the labeled amino acid at a given concentration and incubation was continued for an appropriate period. The reaction was stopped by diluting the incubation medium 9-fold with ice-cold 0.9% NaCl solution and by centrifugation for 10 s at $800 \times g$. After the supernatant was discarded and the inner surface of the centrifuge tube was wiped carefully two times with paper, the pellet was dissolved in 0.2 ml of NCS tissue solubilizer. After mixing with 15 ml of Bray solution [16], radioactivity was counted using a Packard Tri-Carb liquid scintillation spectrometer (model 3320). The rate of 2-aminoisobutyric acid uptake at 37°C in the absence of Na^+ increased linearly with increase in extracellular 2-aminoisobutyric acid concentrations, indicating the uptake at 37°C by non-saturable components. The uptake rate was therefore expressed by subtracting the uptake at 37°C in the absence of Na^+ throughout the experiments.

Radioactive Na^+ uptake was assayed by the same procedure as amino acid transport, except that $^{22}Na^+$ uptake after incubation for a given period was stopped by centrifugation for 10 s at $800 \times g$ at room temperature. This method minimized the influence of $^{22}Na^+$ efflux and successfully permitted quantitative assay.

Protein was estimated by the method of Lowry et al. [17] using bovine serum albumin as standard. The rate of amino acid and $^{22}Na^+$ uptake was expressed as nmol per mg protein for an appropriate short period.

Assay of cellular ATP, Na^+ and K^+. Cellular ATP was extracted with 0.6 M perchloric acid containing 10% ethanol at below 0°C. After centrifugation, the supernatant was neutralized with 3 M K_2CO_3, and centrifuged and the supernatant was used for assay of ATP. ATP was determined by a coupled enzymatic method for hexokinase and glucose-6-phosphate dehydrogenase measuring the reduction of $NADP^+$ fluorometrically [18]. Intracellular Na^+ and K^+ were determined by flame photometry.

Chemicals. 2-[1-^{14}C]aminoisobutyric acid (51.6 mCi/mmol), L-[1-^{14}C]alanine (50 mCi/mmol), [U-^{14}C]glycine (118 mCi/mmol), L-[U-^{14}C]proline (270 mCi/mmol), L-[U-^{14}C]threonine (236 mCi/mmol), L-[U-^{14}C]leucine (59 mCi/mmol), 1-aminocyclopentan-1-[^{14}C]carboxylic acid (cycloleucine) (59 mCi/mmol) and ^{22}NaCl (3.54 Ci/mmol) were purchased from New England Nuclear and NCS from Sigma. SF6847 was provided through the courtesy of Dr. Hiroshi Terada, Tokushima University Faculty of Pharmaceutical Sciences, and carbonylcyanide *m*-chlorophenyl hydrazone (CCCP) was obtained from Sigma. All other chemicals were of the highest purity commercially available.

Results

Effect of ascorbate/phenazine methosulfate on transport of 2-aminoisobutyric acid

The time course of 2-aminoisobutyric acid uptake

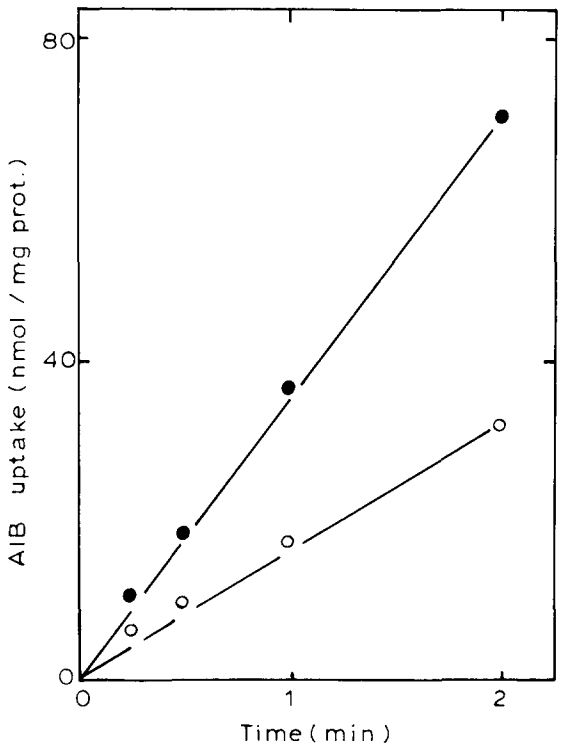

Fig. 1. Effect of ascorbate/phenazine methosulfate on the uptake of 2-aminoisobutyric acid (AIB). Ehrlich cells were preincubated for 30 min at 37°C in the presence of 1 mM iodoacetate and 0.5 mM KCN. The uptake of 2-aminoisobutyric acid at 3 mM was started in the presence of either 5 mM ascorbate alone (○) as control or 5 mM ascorbate plus 0.09 mM phenazine methosulfate (●) and determined as described in Materials and Methods.

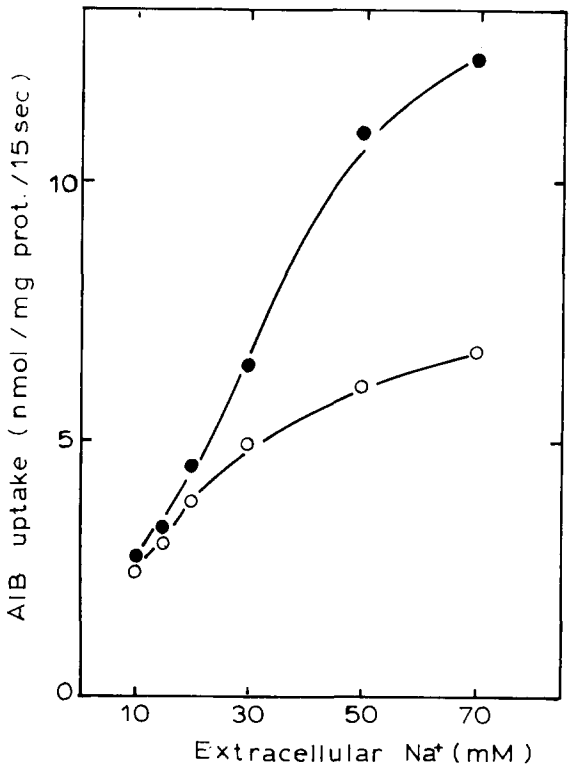

Fig. 2. Effect of extracellular Na^+ concentrations. Ehrlich cells were pretreated with iodoacetate and KCN as described and then the initial rate of uptake of 3 mM 2-aminoisobutyric acid (AIB) for 15 s at 37°C was followed in the presence of Na^+ gradient under the conditions in which the medium contained either ascorbate alone (○) or ascorbate/phenazine methosulfate (●). The Na^+ gradient (10–70 mM) in the medium was prepared by replacing NaCl with choline chloride to a total concentration of 70 mM.

in the presence and absence of ascorbate/phenazine methosulfate is shown in Fig. 1. The addition of 5 mM ascorbate plus 0.09 mM phenazine methosulfate enhanced 2-fold the initial uptake rate. Since the cell suspension was preincubated for 30 min with 1 mM iodoacetate and 0.5 mM KCN in the absence of glucose, cellular ATP decreased to 0.07–0.1 mM. This low ATP level remained at 0.07–0.1 mM during the incubation period with ascorbate/phenazine methosulfate, which indicates that this stimulation of the uptake of 2-aminoisobutyric acid was independent of the cellular ATP level.

These results partly confirm the results reported by Garcia-Sancho et al. [10]. However, as described later in this paper, the effect of ascorbate/phenazine methosulfate on the uptake of 2-aminoisobutyric acid requires the presence of an Na^+ gradient (see Fig. 2), which is different from the result [10] showing that Na^+-independent uptake of norbornane amino acid is also enhanced by the addition of ascorbate/phenazine methosulfate.

Effect of extracellular Na^+ concentration

Since 2-aminoisobutyric acid is actively taken up by Ehrlich cells through the mechanism of Na^+ cotransport [2], it is important to test whether or not an Na^+ gradient is required for the increase in 2-aminoisobutyric acid uptake induced by ascorbate/phenazine methosulfate. Effect of extracellular Na^+ concentrations on the initial uptake rate revealed

that the presence of an Na^+ gradient was required to manifest the stimulating effect of ascorbate/phenazine methosulfate (Fig. 2).

Since intracellular Na^+ was at a level of 25 to 35 mM during the uptake period (see Table II), extracellular Na^+ concentration above 30 mM enhanced the uptake rate by increasing the Na^+ gradient. This was further supported by pretreatment of Ehrlich cells with 4 μM gramicidin for 30 min at 37°C, which resulted in a complete abolishment of the increase in the uptake due to ascorbate/phenazine methosulfate. After treatment, the uptake of 2-aminoisobutyric acid (nmol/mg of protein/min) was 4.92 and 4.70, respectively, in the presence and absence of ascorbate/phenazine methosulfate.

Effect of proton conductors

As described above, the effect of ascorbate/phenazine methosulfate to stimulate Na^+-dependent transport of 2-aminoisobutyric acid was demonstrated in the presence of iodoacetate and KCN. Among proton conductors tested, a nearly identical stimulation of the uptake, 2.1- to 2.9-fold, was found in the presence of CCCP (0.5 and 1.0 μM) or SF6847 (0.01 and 0.1 μM) when ascorbate/phenazine methosulfate were added. These two proton conductors did not change the rate of the basal transport. 2,4-Dinitrophenol (0.1 mM), however, caused a 40% decrease in the basal transport, although a stimulation of 2.3-fold in the 2-aminoisobutyric acid uptake was noted by ascorbate/phenazine methosulfate.

Iodoacetate and KCN were therefore present in the incubation medium throughout the experiments to block glycolytic ATP synthesis and electron transfer which couples to oxidative phosphorylation, respectively. The results obtained also indicate that a proton gradient is not involved in energization of the transport of 2-aminoisobutyric acid enhanced by the addition of ascorbate/phenazine methosulfate.

Effect of quinacrine

Quinacrine which is an inhibitor of plasma membrane oxidoreductase inhibited 2-aminoisobutyric acid uptake enhanced by the addition of ascorbate/phenazine methosulfate depending on its concentration. Complete inhibition of the increase in the uptake resulted from the addition of 0.4 mM quinacrine without affecting the basal transport (Table I). This is consistent with the result reported on the uptake of 2-methylaminoisobutyric acid [10].

TABLE I

EFFECT OF QUINACRINE ON THE ENHANCED UPTAKE OF 2-AMINOISOBUTYRUC ACID

Ehrlich cells were preincubated with iodoacetate and KCN and then with quinacrine at indicated concentrations for 1 min. The rate of 2-aminoisobutyric acid uptake was then determined in the presence of either ascorbate alone or ascorbate/phenazine methosulfate (PMS).

Addition	Concn. (mM)	Uptake (nmol/mg protein/15 s)	
		Ascorbate	Ascorbate + PMS
None		4.8	10.6
Quinacrine	0.02	4.9	9.7
	0.1	4.8	7.3
	0.4	4.7	5.0

Effect of ouabain

Ouabain, which has been shown to prevent the restoration of 2-methylaminoisobutyric acid uptake [10], was practically ineffective in preventing an increase in the uptake rate of 2-aminoisobutyric acid in our system (Table II). Although treatment of Ehrlich cells for 30 min with iodoacetate and KCN increased the cellular Na^+ concentration from approx. 20 mM to 30 mM, the cellular Na^+ concentration was not further altered during the transport for 1 min in the presence of ascorbate/phenazine methosulfate. This suggests no apparent alteration in the Na^+ gradient during the transport period. The fact that ouabain hardly altered alkali-ion gradients as shown in Table II was compatible with the inability of ouabain to prevent the restoration of the uptake.

Effect of ascorbate/phenazine methosulfate on kinetics of the uptake

The increase in the uptake of 2-aminoisobutyric acid by the addition of ascorbate/phenazine methosulfate was found to be due to an increase in the V value (Fig. 3), that is, 18.3 to 43.8 nmol/mg of protein per min in the presence of iodoacetate

TABLE II

EFFECT OF OUABAIN ON THE UPTAKE OF 2-AMINOISOBUTYRIC ACID AND INTRACELLULAR CONCENTRATIONS OF Na^+ AND K^+

Ehrlich cells were preincubated with 1 mM iodoacetate and 0.5 mM KCN for 30 min at 37°C, 2 mM ouabain was added to the medium 15 min after incubation and incubation was continued for 15 min. The uptake of 2-aminoisobutyric acid was then measured for 1 min at 37°C under the standard conditions described. Intracellular Na^+ and K^+ before and after the uptake were determined by flame photometry and their concentrations were calculated by taking cellular water space of the cell as 6.05 μl per mg of protein. PMS, phenazine methosulfate.

Treatment	Ascorbate + PMS	Uptake (nmol/mg protein/min)	Intracellular concn. (mM)		Alkali-ion gradients *
			Na^+	K^+	
None	–	16.2	20.1	83.6	58.2
	+	29.3	20.5	81.6	55.7
Iodoacetate	–	11.8	28.6	70.2	34.4
+ KCN	+	28.3	28.8	68.7	33.4
Iodoacetate	–	9.9	34.0	56.0	23.1
+ KCN + ouabain	+	23.4	34.6	55.0	22.3

* $[Na^+]_{out} \cdot [K^+]_{in}/[Na^+]_{in} \cdot [K^+]_{out}$ [10].

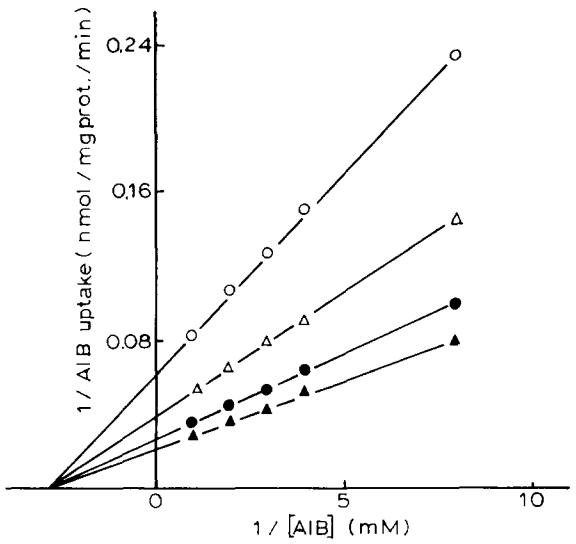

Fig. 3. Effect of ascorbate/phenazine methosulfate on the kinetics of the uptake. Ehrlich cells were preincubated for 30 min at 37°C in the presence (circles) or absence (triangles) of 1 mM iodoacetate and 0.5 mM KCN. Then, 5 mM ascorbate (open symbols) as control and 5 mM ascorbate plus 0.09 mM phenazine methosulfate (solid symbols) were added and the rate of 2-aminoisobutyric acid uptake was determined under the conditions in which extracellular Na^+ was present at 140 mM. Lineweaver-Burk plots of the uptake rate are illustrated. AIB, 2-aminoisobutyric acid.

and KCN, and 26.7 to 50.6 in the absence of the inhibitors. The K_m value (0.37 mM) for the substrate remained unchanged.

These results indicate that the increase in the

TABLE III

HYDROGEN DONORS AND ELECTRON ACCEPTORS INVOLVED IN THE STIMULATION OF THE UPTAKE RATE OF 2-AMINOISOBUTYRIC ACID

Ehrlich cells were preincubated with iodoacetate and KCN as described and then hydrogen donors and electron acceptors at indicated concentrations were added. The uptake rate was determined under the standard assay conditions. PMS, phenazine methosulfate.

Donors	(mM)	Acceptors	(mM)	Uptake (nmol/mg protein/30 s)
None		None		7.0
Ascorbate	5	PMS	0.09	17.1
NADH	1	PMS	0.09	14.0
	2	Glyoxylate	0.1	7.4
	2	Cytochrome *c*	0.1	7.4
	2	Ferricyanide	0.1	7.0
	2	Juglone	0.05	7.3
	2	Indophenol	0.05	7.1
NADPH	2	PMS	0.09	6.8

uptake rate by ascorbate/phenazine methosulfate is not caused by increasing affinity of the transport to the substrate.

Hydrogen donors and electron acceptors effective in stimulating the uptake

Physiological and artificial hydrogen donors and electron acceptors were tested to determine whether these were effective or not in stimulating the uptake of 2-aminoisobutyric acid. As shown in Table III, NADH was an active hydrogen donor being equally as effective as ascorbate when added with phenazine methosulfate, while NADPH was ineffective. Electron acceptors except for phenazine methosulfate were ineffective in enhancing the uptake rate. Quinacrine at 0.4 mM also completely inhibited the enhanced uptake rate by NADH/phenazine methosulfate.

These results obtained with hydrogen donors and electron acceptors and with inhibitors of the plasma membrane redox system suggest that an oxidoreductase in the plasma membrane participates in stimulating the transport of 2-aminoisobutyric acid by Ehrlich cells.

TABLE IV

MOLAR RATIO OF Δ $^{22}Na^+$ TO Δ 2-AMINOISOBUTYRIC ACID TAKEN UP BY EHRLICH CELLS

Ehrlich cells were preincubated with iodoacetate and KCN as described. Labeled Na^+ uptake at 100 mM in the presence of unlabeled 2-aminoisobutyric acid at indicated concentrations was determined for 15 s at 37°C and increase in $^{22}Na^+$ uptake (Δ $^{22}Na^+$) due to the presence of 2-aminoisobutyric acid (AIB) was calculated at each concentration. On the other hand, the uptake of 2-amino[^{14}C]isobutyric acid at indicated concentrations was measured for 15 s at 37°C in the presence of nonradioactive 100 mM NaCl and Δ 2-aminoisobutyric acid uptake was calculated by subtracting the uptake in an Na^+-free medium. The molar ratio was then calculated.

AIB (mM)	Δ Uptake (nmol/mg protein/15 s)		Δ $^{22}Na^+$/ Δ AIB
	$^{22}Na^+$	AIB	
0.25	2.8	3.1	0.90
0.50	4.0	4.9	0.82
0.75	6.1	6.4	0.95
1.0	7.1	7.3	0.97

TABLE V

MOLAR RATIO OF Δ $^{22}Na^+$ TO Δ 2-AMINOISOBUTYRIC ACID UPTAKE STIMULATED BY ASCORBATE/PHENAZINE METHOSULFATE

Labeled $^{22}Na^+$ uptake at 100 mM in the presence of unlabeled 1 mM 2-aminoisobutyric acid, and 2-amino[^{14}C]isobutyric acid uptake at 1 mM in the presence of non-radioactive 100 mM NaCl were carried out with Ehrlich cells treated with iodoacetate and KCN. The uptake rate (15 s) of both $^{22}Na^+$ and 2-aminoisobutyric acid (AIB) was measured in the presence and absence of ascorbate/phenazine methosulfate as described. Increases in the rate of both uptakes (Δ $^{22}Na^+$ and Δ 2-aminoisobutyric acid) due to the addition of ascobrate/phenazine methosulfate were then calculated. Since Δ $^{22}Na^+$ and Δ 2-aminoisobutyric acid were lowered by quinacrine depending on its concentrations, which was added to the medium before starting the uptake, the molar ratio of Δ $^{22}Na^+$ to Δ 2-aminoisobutyric acid uptake was calculated at each concentration of quinacrine.

Quinacrine (mM)	Δ Uptake /nmol/mg protein/15 s)		Δ $^{22}Na^+$/ Δ AIB
	$^{22}Na^+$	AIB	
None	6.1	5.8	1.05
0.02	4.9	5.0	0.98
0.1	2.2	2.1	1.05
0.4	0.3	0.4	0.75

Molar ratio of $^{22}Na^+$ to 2-aminoisobutyric acid taken up in the presence and absence of ascorbate/phenazine methosulfate

The transport of 2-aminoisobutyric acid in Ehrlich cells was accompanied by Na^+ influx, although the molar ratio of the substrate to $^{22}Na^+$ taken up together was not consistent [2,3,19,20]. In order to clarify whether or not the addition of ascorbate/phenazine methosulfate affects the cotransport of 2-aminoisobutyric acid with Na^+ in our system, the uptake rate in the presence of 100 mM Na^+ was measured at various substrate concentrations together with the rate of $^{22}Na^+$ uptake under the same conditions. The molar ratio of 2-aminoisobutyric acid to $^{22}Na^+$ taken up was calculated to be 1 : 1 (Table IV). The addition of ascorbate/phenazine methosulfate stimulated the uptake of both 2-aminoisobutyric acid and Na^+ at 1 : 1 molar ratio (Table V).

These results, together with the fact showing the requirement of Na^+ gradient (Fig. 2), indicate that

TABLE VI

TRANSPORT OF AMINO ACIDS AFFECTED BY ASCORBATE/PHENAZINE METHOSULFATE

Assay of the uptake was carried out under the standard conditions as described. As hydrogen donors, 5 mM ascorbate and 2 mM NADH were added, respectively, with 0.09 mM phenazine methosulfate. Labeled amino acids were used at 1 mM each. PMS, phenazine methosulfate.

Amino acid	Uptake (nmol/mg protein/30 s)			
	Ascorbate	Ascorbate + PMS	NADH	NADH + PMS
2-Aminoisobutyrate	7.0	13.5	6.5	12.3
Alanine	9.7	25.0	10.3	22.9
Glycine	6.7	15.9	7.1	12.5
Proline	8.2	18.8	7.6	15.1
Cycloleucine *	12.0	19.8	11.1	16.9
Threonine	4.7	4.8	4.7	4.6
Leucine	5.1	5.0	4.4	4.4

* Cycloleucine, 1-aminocyclopentan-1-carboxylic acid.

ascorbate/phenazine methosulfate added to the cell suspension stimulate Na^+-substrate cotransport.

The uptake of other amino acids

Active transport of amino acids into Ehrlich cells were carried out mostly by two systems [2]: an Na^+-dependent system A and an Na^+-independent system L. 2-Aminoisobutyric acid is known to be transported by system A in Ehrlich cells [2], although in rat hepatocyte systems ASC and L also participate in the uptake of 2-aminoisobutyric acid [21]. The effect of addition of ascorbate or NADH/phenazine methosulfate on the uptake rate of amino acids was therefore tested (Table VI). Alanine, glycine and proline which are substrate for the Na^+-dependent system [2,22] were stimulated more than 2-fold and cycloleucine that is transported via both systems A and L [23] was also stimulated less than 1.5-fold. On the other hand, the uptake of leucine and threonine was not affected. Although the reason for this phenomenon is obscure, it might be due to transport of leucine and threonine through systems L and ASC, respectively. The simulatory effect of ascorbate or NADH/phenazine methosulfate on the uptake of alanine, glycine, proline and cycloleucine was also abolished by 0.4 mM quinacrine.

Discussion

Garcia-Sancho et al. [10] have first reported that Ehrlich cells treated with 2,4-dinitrophenol and iodoacetate or rotenone rapidly recover the 30-s uptake of 2-methylaminoisobutyric acid as well as norbornane amino acid on treatment with ascorbate/phenazine methosulfate. These results were confirmed in part by the experiments reported in this paper, but there are several important discrepancies.

The stimulatory effect of ascorbate/phenazine methosulfate on the transport of amino acids required the presence of an Na^+ gradient in our experiments (Fig. 2) and probably enhanced the cotransport of the substrate with Na^+ (Table V). On the other hand, the uptake of norbornane amino acid, a model substrate of Na^+-independent system L [2] was also stimulated by ascorbate/phenazine methosulfate [10]. This stimulation of norbornane amino acid uptake should be, therefore, explained by a mechanism different from that proposed in our substrate-Na^+ cotransport system.

Though ouabain completely prevented the restoration of the stimulated uptake [10], similar treatment with ouabain showed only a minor preventive effect on the stimulated uptake in our system (Table II). Ouabain also caused a minor change in alkali-ion gradients in the presence of iodoacetate and KCN, when calculated from the data shown in Table II, on the contrary lowered the gradients slightly. Alkali-ion gradients in the presence of ouabain remained nearly constant during the uptake period

when ascorbate and phenazine methosulfate were present. These results obtained with ouabain indicate that an Na^+/K^+ pump catalyzed by $(Na^+ + K^+)$-ATPase (EC 3.6.1.3) may not be involved in energization of 2-aminoisobutyric acid uptake by Ehrlich cells in the presence of the reducing reagents, and suggest that ouabain does not inhibit the redox system in the plasma membrane. The effect of ouabain of completely preventing the restoration of the uptake of 2-methylaminoisobutyric acid by ascorbate/phenazine methosulfate [10] should be, therefore, explained by the action of ouabain other than that which inhibits $(Na^+ + K^+)$-ATPase as suggested by these authors [24].

The results presented in this paper clearly demonstrated that the presence of ascorbate/phenazine methosulfate stimulate one-to-one molar uptake of $^{22}Na^+$ and 2-aminoisobutyric acid (Table V). Christensen et al. [2] have reported on cotransport of Na^+ and 2-aminoisobutyric acid by Ehrlich cells at a two-to-one ratio in the absence of the reducing reagents.

The driving force which enhances the cotransport of amino acid with Na^+ in the presence of ascorbate/phenazine methosulfate could not be clarified, but appears to be provided through an oxidoreduction reaction in the plasma membrane. This is supported by the facts that (1) NADH but not NADPH acts as hydrogen donor (Table III) and the plasma membrane of Ehrlich cells contains NADH dehydrogenase (acceptor: ferricyanide or cytochrome *c*) but not NADPH-cytochrome *c* reductase [15]. (2) NADH oxidizing activity could be detected using membrane vesicles of Ehrlich cells when phenazine methosulfate was added as an electron acceptor (unpublished observation). (3) Quinacrine, an inhibitor of the plasma membrane NADH dehydrogenase [14], inhibited the enhanced transport of not only 2-aminoisobutyric acid but also of alanine, glycine, proline and cycloleucine due to the presence of ascorbate/phenazine methosulfate (Table I).

Transmembrane potential due to a H^+ gradient may not be involved in the additional energization of 2-aminoisobutyric acid uptake, since the stimulating effect of ascorbate/phenazine methosulfate was observed in the presence of proton conductors such as CCCP and SF6847.

The role of the membrane redox system of Ehrlich cells in energizing the additional active transport of amino acids is obscure at the present time. One approach toward elucidating this role in the transport is to characterize the redox system in the plasma membrane, since ferricyanide and cytochrome *c* were shown to be very active electron acceptors in the plasma membrane preparation of Ehrlich cells [15]. However, these acceptors were inert to support the increase in 2-aminoisobutyric acid uptake by intact Ehrlich cells when added with NADH (Table III). In our recent preliminary experiments, NADH oxidizing activity could be detected with membrane vesicles of Ehrlich cells when phenazine methosulfate was an electron acceptor under the condition in which 2-aminoisobutyric acid uptake was stimulated in the presence of iodoacetate and KCN (unpublished observation).

After submitting our manuscript for publication, we learned of the work of Ohsawa et al. [24] who primarily studied the uptake of 2-aminonorbornane-2-carboxylic acid in energy-depleted Ehrlich cells and reached conclusions different in part to a previous paper [10]. That is, ascorbate/phenazine methosulfate energizes amino acid transport by a pathway linked to mitochondrial ATP production as well as by action at the plasma membrane.

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