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Unexpected additional mode of energization of amino-acid transport into Ehrlich cells

(ATP/alkali-ion gradients/NADH/ouabain/quinacrine)

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ABSTRACT **Ehrlich cells treated with dinitrophenol and iodoacetate rapidly recover their 30-sec uptake of 2-(methylamino)-isobutyrate on treatment with 0.1 mM phenazine methosulfate + 20 mM sodium ascorbate before they begin to recover from the severely depressed ATP levels and alkali-ion gradients. Addition of 10 mM pyruvate also restores uptake of methylaminoisobutyrate before the alkali-ion gradients rise. This restoration is prevented by rotenone, but rotenone does not handicap restoration by phenazine methosulfate/ascorbate. Na^+-independent uptake of 2-aminonorbornane-2-carboxylate by Ehrlich cells is affected the same way. Quinacrine almost completely suppresses uptake of methylaminoisobutyrate within the 30-sec uptake test, even when ATP levels are sustained by pyruvate and alkali-ion gradients are not depressed. Ouabain prevents restoration of both Na^+-dependent and Na^+-independent amino-acid transport by phenazine methosulfate/ascorbate or pyruvate.**

We interpret these results to indicate that amino-acid transport can be energized not only by known means, but also by reducing equivalents, which presumably reach the plasma membrane in the form of NADH from the mitochondria when the source of energy is pyruvate. In support of this hypothesis, the distribution of methylaminoisobutyrate between plasma membrane vesicles and their supporting media was influenced in the predictable way by NADH, quinacrine, and an uncoupling agent, proceeding on the assumption that more of the vesicles had the everted rather than the natural orientation.

We seek here to challenge the conventional picture of the energization of the transport of amino acids against a gradient into animal cells. The most generally accepted explanation is that active transport is driven by cotransport with H^+ or Na^+, with these inorganic ions migrating down gradients of their electrochemical potential. These movements are held to arise ultimately in microorganisms from primary active extrusion of H^+ and in cells of higher organisms from primary active extrusion of Na^+. An apparent inadequacy in the driving force available from the Na^+ gradient in the latter case (1) might be corrected if the present estimates of the transmembrane potentials for such cells were to prove much too low (2, 3). Under some experimental conditions, however, even Na^+-dependent amino-acid transport against a gradient can occur with negligible cotransport of Na^+ (4). Furthermore, one of the systems able to drive the transport of neutral amino acids in Ehrlich ascites tumor cells (4) is totally Na^+ independent. When Na^+ does not move, its gradient appears irrelevant to any question of the adequacy of energization by cotransport with it; hence underestimates of the transmembrane potential would not modify the above conclusion. Various investigators have observed, furthermore, stronger transport by respiring or glycolyzing cells than by those for which these metabolic processes, or energy conservation from them, have been abolished (5). This additional energy production has often been considered to be transferred to transport by way of ATP formation and cleavage, although the evidence is ambiguous (6, 7). The properties of ATPase activity that is stimulated by amino acids seem inappropriate to the known amino-acid transport systems (8), and the uptake of 2-aminoisobutyric acid has been reported to be completely independent of the rate of ATP hydrolysis in Ehrlich cells (9). Schafer and Williams (10) have emphasized that uptake of 2-aminoisobutyric acid, which at neutrality is Na^+-dependent in Ehrlich cells, continues after ATP is largely depleted, even with unfavorable alkali-ion gradients.

The available alternatives to ATP as energizing agent appear severely restricted if we accept the ordinary assumption that all the oxidoreductase activity of the plasma membrane has been transferred to mitochondria in the transition from the bacterial to the animal cell. Recent studies of presumed marker enzymes in membranous fractions of various cells have fortuitously shown, however, the presence of NADH dehydrogenase activity in plasma membrane preparations (11–13). Löw and Crane have noted hormone stimulation of this activity associated with an inhibition by NADH of the activity of membrane adenylate cyclase (13). We propose that an oxidoreduction system in the plasma membrane can participate in energization of both Na^+-dependent and Na^+-independent amino-acid transport, allowing the utilization of reducing equivalents which can be made available by shuttle from the mitochondria and presumably also arise in the cytoplasm.

EXPERIMENTAL PROCEDURES

Methods for handling the cells and for measuring the uptake of amino acids have been described (14). A preliminary incubation of the cells for 15 min in hypoosmotic medium (30 mM NaCl/35 mM KCl/1.2 mM KH_2PO_4/1.2 mM $MgSO_4$/0.5 mM $CaCl_2$/12.5mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (Hepes)-NaOH, pH 7.35), followed by a 15-min incubation in standard medium, was systematically used to lower the content of endogenous amino acids to about 40% of basal values without appreciable loss of the subsequent transport capacity. Standard incubation medium was similar to Krebs-Ringer solution with 25 mM Hepes-NaOH, pH 7.35, as buffer. Occasionally a "high-K^+ medium", containing 25 mM KCl, was used in an effort to preserve cellular content of K^+ within reasonable values during treatment with inhibitors. ATP was determined fluorometrically by measuring the reduction of $NADP^+$ in the presence of glucose, hexokinase, and glucose-6-phosphate dehydrogenase; Na^+ and K^+ were determined by flame photometry.

RESULTS AND DISCUSSION

The 30-min uptake of the model amino acids, 2-(methylamino)-isobutyric acid (MeAIB) (fully Na^+-dependent), the norbornane amino acid (Na^+-independent), and 1-aminocy-

Abbreviations: Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; MeAIB, 2-(methylamino)-isobutyric acid.

Table 1. Restoration of 30-sec uptake of 20 μM of the norbornane amino acid (BCH) after 30-min treatment with rotenone (100 ng/ml)

Circumstance	Uptake of MeAIB	Uptake of BCH	Cell [ATP]	Cell [Na^+]	Cell [K^+]	Na^+-K^+ gradient*
Before rotenone	2.56	7.29	2.26	32	81	47.8
After 5-min restoration with no substrate	0.65	3.52	0.09	68	50	13.9
Ascorbate/phenazine methosulfate	2.33	7.05	0.12	63	66	19.6
Ascorbate/phenazine methosulfate + 2 mM ouabain	0.74	4.43	0.22	74	48	12.2

See legends of Figs. 1 and 2 for details. The cation levels are in mEq/kg of cell water.
* $([Na^+]_{out} \times [K^+]_{in})/([Na^+]_{in} \times [K^+]_{out})$.

clopentane carboxylic acid (partly Na^+-dependent, partly Na^+-independent), bore a linear relation with the cellular ATP level and with the logarithm of the quantity $([Na^+]_{out} \times [K^+]_{in})/([Na^+]_{in} \times [K^+]_{out})$ in cells treated with graded doses of dinitrophenol or rotenone. These experiments show that unless conditions are carefully selected, the responses of the several indications of cellular energization will be closely associated, so that cause-and-effect relations will not be revealed. For example, the relation found between the Na^+-independent uptake of the norbornane amino acid and the alkali-ion gradient has no obvious direct basis. On the other hand, we could double the cellular levels of ATP by a 60-min incubation with 10 mM glucose and 0.25 mM adenine without measurable effect on amino-acid transport, despite the observed linearity. Under these conditions the levels of ADP and P_i may also have been modified.

A 30-min treatment of the Ehrlich cells with 0.1 mM dinitrophenol plus 1 mM iodoacetate halved the subsequent 30-sec uptake of 20 μM MeAIB. Washing these cells and placing them in fresh medium containing 10 mM pyruvate led to recovery of MeAIB influx and of ATP levels much faster than recovery of the alkali-ion gradient. Rotenone prevented this recovery and pyruvate failed to produce recovery with rotenone-treated cells. In contrast, glucose restored the recovery in rotenone-treated cells, but not in cells treated with dinitrophenol and iodoacetate.

Transport of MeAIB after treatment with either dinitrophenol plus iodoacetate or rotenone was also restored with the artificial donor system for electrons, phenazine methosulfate (0.1 mM) plus sodium ascorbate (20 mM). This restoration was not accompanied by a significant increase of cellular ATP levels and there was only a slow increase in the alkali-ion gradients (Fig. 1). The failure of ATP to rise suggests that the artificial donor system, in contrast to pyruvate, produces its effect without a mitochondrial contribution. The same behavior applies to the uptake of 2-aminonorbornane-2-carboxylate (Table 1), which we find fully Na^+-independent in the Ehrlich cell (4). A hypothetical explanation of the restoration as arising from an increase of electrochemical Na^+ gradient produced by an increase of transmembrane potential due to the activity of sodium pump (2, 3) would not fit in the case of the norbornane amino acid.

The restoration was prevented in all cases by 2 mM ouabain (Table 1), a remarkable finding because the alkali-ion gradient and amino-acid transport were only sluggishly diminished by ouabain in cells not treated with these metabolic inhibitors. The action of ouabain on the plasma membrane gains interest from the observation that it applies as well to the Na^+-independent uptake of the norbornane amino acid (Fig. 2). Apparently the scope of its action on membrane energetics is much wider than

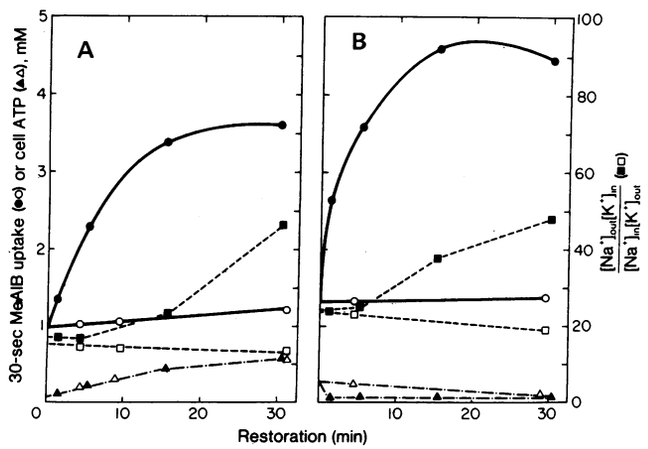

FIG. 1. Time course of restoration by phenazine methosulfate (0.1 mM) plus sodium ascorbate (20 mM) of the 30-sec uptake of MeAIB, after 30-min treatment of the Ehrlich cells with (*A*) 0.1 mM dinitrophenol + 1 mM iodoacetate or (*B*) rotenone (100 ng/ml). MeAIB uptake is expressed as the distribution ratio reached in 30 sec. MeAIB was 20 μM in the suspending fluid. Solid symbols, in the presence of phenazinemethosulfate/ascorbate; open symbols, no phenazinemethosulfate/ascorbate.

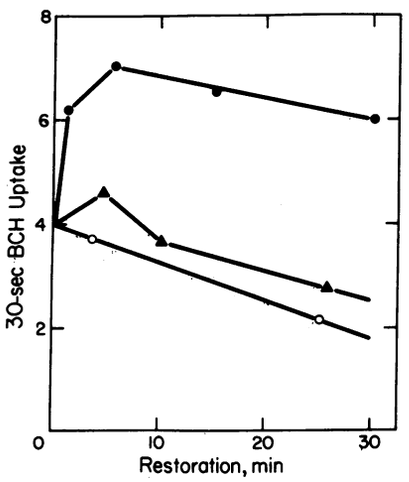

FIG. 2. Recovery of uptake of 2-aminonorbornane-2-carboxylate (BCH) after 30-min treatment of Ehrlich cells with rotenone (100 ng/ml). The norbornane amino acid was 20 μM in the suspending fluid. Its uptake is expressed as the distribution ratio reached in 30 sec. Restoration of the alkali-ion gradient was the same as in Fig. 1*B*. (●) In the presence of phenazinemethosulfate/ascorbate; (▲) phenazinemethosulfate/ascorbate plus 2 mM ouabain; (○) no phenazinemethosulfate/ascorbate.

Table 2. Comparison of inhibition produced by quinacrine on the 30-sec uptake of 1 mM MeAIB with that produced by other inhibitors

Previous treatment with inhibitor	Inhibitor	MeAIB uptake	Cell [ATP]	Cell [Na^+]	Cell [K^+]	Na^+-K^+ gradient
None	None	1.09	2.37	30	71	46
	1.8 mM quinacrine	0.34	1.18	22	58	52
	100 ng/ml rotenone	1.02	1.96	30	72	47
	10 mM NaCN	1.04	1.33	30	72	47
	10 mM NaN_3	1.01	1.47	29	72	48
30 min	None	0.87	2.51	24	83	70
	1.8 mM quinacrine	0.20	0.15	80	12	3
	100 ng/ml rotenone	0.64	0.18	39	40	21
	10 mM NaCN	0.68	0.08	42	37	17
	10 mM NaN_3	0.71	0.35	36	48	26

For other details, see Table 1.

has often been supposed, perhaps even wider than the proposal of Kimmich that it acts on an ATPase serving for the transport both of the organic metabolite and the alkali ions (15).

The inhibition by quinacrine (atebrin) of the plasma membrane NADH reductase has been considered characteristic by Crane and Löw (13). Table 2 shows that quinacrine proved a potent inhibitor of MeAIB uptake; within the 30 sec of contact used for the uptake test it suppressed MeAIB uptake more than any other agent tested. We can argue that in this 30 sec the dehydrogenase activity of the plasma membrane would be much more likely to be reached and blocked than that of the mitochondrion. This argument is also supported by the observations that the inhibition by quinacrine does not follow the relation with ATP levels obtained with other inhibitors (Table 2) and that the addition of pyruvate to the incubation medium largely prevents the decrease of ATP levels produced by quinacrine, without preventing the inhibition of MeAIB accumulation (data not presented).

Preliminary results with plasma membrane vesicles prepared from Ehrlich cells (8) showed (Table 3) a 52% decrease of MeAIB entry by 1.8 mM NADH applied externally. Quinacrine (0.4 mM) and trifluoromethoxycarbonylcyanide phenylhydrazone (0.33 mg/ml) increased MeAIB uptake by 51 and 56%, respectively. These effects correspond to acceptance by the plasma membrane of NADH to energize amino-acid transport, if we suppose that more of our vesicles were everted than were right side out.

On the basis of the above results, we provisionally propose that amino-acid transport can be energized by the alkali-ion gradient, by the cellular ATP, or by reducing equivalents, which may reach the plasma membrane from the mitochondrion by a shuttle. The natural electron acceptor that presumably accounts for these effects remains unidentified. The malate-aspartate shuttle involves two transaminations. Hence, the widely observed action of analogs of vitamin B_6 to produce an early inhibition of amino-acid transport may be pertinent. A failure of 0.2 mM aminoxyacetate to block the restorative effect of pyruvate did not, however, support a major role of that shuttle.

Table 3. Levels of MeAIB reached in plasma membrane vesicles of Ehrlich cells incubated 30 sec in 0.2 mM [^{14}C]MeAIB

	nmol/mg of protein
Control	0.148
0.4 mM quinacrine	0.231
0.33 mg/ml trifluoromethoxycarbonyl cyanide phenylhydrazone	0.224
1.8 mM NADH	0.071

Krebs-Ringer phosphate medium (pH 7.4) at 37°. MeAIB appeared to have reached a steady state of distribution in this time.

Note that the ability of the phenazinemethosulfate/ascorbate combination or of NADH to energize transport by the plasma membrane has different and more extended implications in the animal cell than it does in the vesicles of *Escherichia coli* (16) or *Bacillus subtilis* (17), because for these organisms the plasma membrane is known to contain the respiratory chain. The close interaction among the modes of energization is illustrated by ouabain, which prevents energization of transport under conditions in which the alkali-ion gradient cannot be implicated, even though this agent is usually considered to react specifically with the Na^+-K^+ transport ATPase. The continuing influence of the transmembrane potential was also seen in the cells treated with dinitrophenol and iodoacetate; the presence of valinomycin or thiocyanate produced a 40–60% enhancement of MeAIB uptake without any increase in the ATP level and despite the low gradients of the alkali ions.

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