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COMPLEXITY IN VALINOMYCIN EFFECTS ON AMINO ACID TRANSPORT

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SUMMARY

^{3}H-labeled dibenzyldimethylammonium ion, applied as an indicator for the potential differences across the plasma membrane, enters the Ehrlich ascites-tumor cell during 2 h to an apparent steady-state concentration about twice that of the suspending medium. In the presence of valinomycin, the distribution ratio reached was increased to about four, the cells meanwhile undergoing a substantial loss of K^+ and a corresponding gain of Na^+. Valinomycin decreased the extent to which four model amino acids, selected to represent the uphill operation of the three transport system, were accumulated by the respiring cell. Simultaneously, the cellular level of ATP was sharply decreased.

These observations indicate as expected that the ATP is not a direct factor in the accumulation of the dibenzyldimethylammonium ion. They further indicate that the diminution of cellular ATP by valinomycin handicaps uphill transport more than the accompanying increase in the transmembrane potential may possibly stimulate it.

INTRODUCTION

Gibb and Eddy [1] have observed that valinomycin treatment of a Na^+-enriched mouse ascites tumor cell, previously depleted of ATP by treatment with cyanide and 2-deoxyglucose, is able to stimulate the Na^+-dependent uptake of certain amino acids. This effect they attribute to an enhancement of the potential difference across the plasma membrane, at the expense of a normally directed potassium gradient. Since the linked flux of an amino acid with Na^+ is taken to be electrogenic, this response to an intensification of the potential difference (inside negative) might be expected. Similar results were said to be obtained with metabolizing cells.

This demonstration acquires physiological significance if the Na^+–K^+ pump is taken to be electrogenic in its normal operation, through an excess of the extrusion of Na^+ over the introduction of K^+. The results of Gibb and Eddy suggest accordingly

DDA^+, dibenzyldimethylammonium ion.

that the unidentified portion of the energy needed to account for Na^+-dependent amino acid accumulation may arise to some degree and under selected conditions from the electrogenicity of the sodium pump.

The smallness of the potential difference observed across the plasma membrane of the Ehrlich ascites tumor cell has concerned us in another connection [2]. Furthermore, previous observations in this laboratory [2] seemed to indicate that the transmembrane potential may not exert an important influence on amino acid accumulation, since isosmotic replacement of chloride ion in the incubation medium with nonpenetrant anions such as sulfate had only small effects on the uptake of several selected amino acids. The validity of this demonstration depends of course on whether the diffusion potential of the chloride ion makes an important contribution to the transmembrane potential, i.e., whether an appreciable part of the migration of the ion is electrogenic.

In the present study, we explored further the effect of induced membrane potentials on amino acid transport, by observing the sodium-dependent and the sodium-independent uptake of several amino acid analogs, in the presence and absence of valinomycin. In an effort to measure the potential difference, we used the lipid-soluble cation dibenzyldimethylammonium (DDA^+), which has already been used, along with other lipid-soluble cations and anions, to monitor the potential difference across other membrane systems such as those of mitochondria and submitochondrial particles [3, 4], also of cells of *Streptococcus faecalis* [5, 6], and of vesicles formed from *Escherichia coli* [7].

The present results apparently confirm for the Ehrlich cell the prediction that valinomycin should increase the transmembrane potential difference by increasing the contribution that the K^+ diffusion potential makes to it. At the same time, however, in our hands and with the cell line used, the ionophore inhibited rather than stimulated both the Na^+-dependent and the Na^+-independent components of the accumulative uptake of the amino acids tested.

These results indicate that the transmembrane potential of the metabolizing Ehrlich cell may at most be only slightly larger than the values observed by microelectrodes, or calculated from the Cl^- distribution. Furthermore the results do not support a major contribution of the transmembrane potential difference to the energization of amino acid transport in the Ehrlich cell under the conditions of our study.

MATERIALS AND METHODS

Dibenzyldimethylammonium (DDA^+) chloride, was purchased from Aldrich Chemical Company, tetraphenylboron sodium salt, from K and K Laboratories, valinomycin from Calbiochem, and a mixture of hexokinase and glucose-6-phosphate dehydrogenase from Sigma Chemical Company. Tritiated DDA^+, was obtained as the chloride from Dr. Frank Harold, National Jewish Hospital and Research Center, Denver, Colo.

The Ehrlich cells were of a hypotetraploid strain maintained for hundreds of generations by Dr John Jacquez of this University. The cells were propagated as usual in Swiss albino mice. The methods of collecting the cells and studying transport by them have been described previously [8, 9].

Cellular Na^+ and K^+ levels were determined with digital flame photometer from Instrumentation Laboratory, using an internal lithium standard.

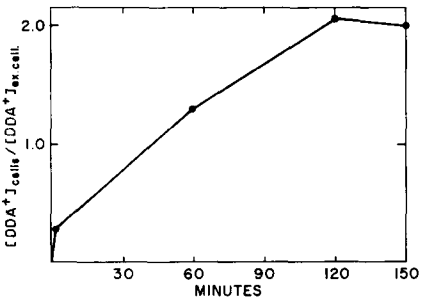
ATP was measured fluorimetrically by the formation of $NADPH+H^+$ from the coupled reaction of hexokinase and glucose-6-phosphate dehydrogenase [10].

RESULTS

^{3}H-labeled DDA^+ uptake by the Ehrlich cell in the presence of valinomycin

The time course of DDA^+ uptake is shown in Fig. 1. The results show that in 2 h DDA^+ at 0.1 mM is already at a steady state of distribution. At this low concentration of DDA^+ the distribution ratio appears to be nearly concentration independent. Table I compares the distribution ratio reached by DDA^+ at the steady state in the absence and in the presence of valinomycin. Potential differences were calculated by inserting the figure for the distribution ratio into the Nernst equation, assuming a simple electrophoretic response of the cation to the potential difference,

$$E = \frac{RT}{F} \ln \frac{DDA^+_i}{DDA^+_0}$$



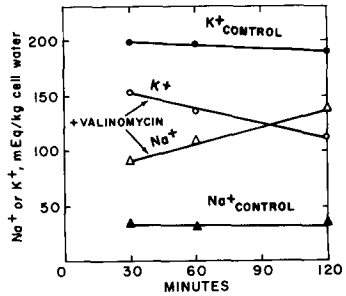


Fig. 1. Time course of the uptake of DDA^+ by the Ehrlich cell. Cells were incubated at 37 °C in ordinary Krebs–Ringer bicarbonate medium. DDA^+ was present at a concentration of 0.1 mM. Typical result.

Fig. 2. Apparent levels of intracellular Na^+ and K^+ during DDA^+ uptake in the absence and in the presence of valinomycin.

TABLE I

Effect of valinomycin on the distribution of DDA^+ in the Ehrlich cell. Cells were incubated for 2 h at 37 °C in Krebs–Ringer bicarbonate medium. DDA^+ was present at a concentration of 0.1 mM, valinomycin at a final concentration of $5 \cdot 10^{-7}$ M, added in 5 μl of ethanolic solution/ml medium. The same amount of ethanol was added to the controls. Each line describes a different experiment.

Control		Valinomycin	
Apparent distribution ratio	ΔE (mV)	Apparent distribution ratio	ΔE (mV)
1.89	−16.9	3.54	−33.8
2.32	−22.5	3.19	−31.0
2.05, 2.14	−19.2, −20.3	4.54, 3.75	−40.4, −35.3

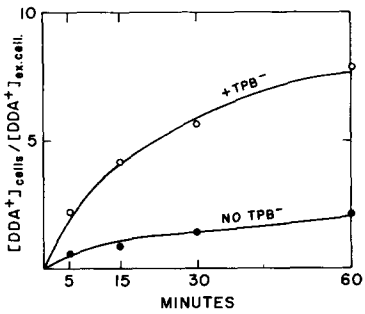
The distribution ratio for the cation between the inside and the outside of the cell was always higher than 1, corresponding to a negative potential inside, relative to that outside. The values calculated for the potential difference show a range from -17 to -23 mV in the absence of valinomycin and from -31 to -40, in its presence. Attempts to change the distribution of DDA^+ by means of isosmotic substitution of the external chloride in the medium by sulfate were unsuccessful. These results raise a question as to the electrogenic character of the passage either of Cl^- or DDA^+ across the membrane. Essentially no change in the distribution of Na^+ and K^+ occurred with the uptake of DDA^+ from a 0.1 mM solution during 2 h. In the presence of valinomycin the cellular K^+ decreased and the cellular Na^+ increased (Fig. 2). At high concentrations of DDA^+, for example 20 mM, its distribution ratio was about one after 60 min of incubation, although intracellular K^+ was lowered to values less than 10 mequiv./kg cell water whether valinomycin was present or not.

The values for the untreated cells in Table I are consistent with those obtained with microelectrodes by Lassen et al. [11], namely -24 ± 7 mV, a value which is considered to represent a Donnan potential. Lassen and his associates concluded that this value is the actual membrane potential across the plasma membrane of the Ehrlich cell, and that the lower figure of around -11 mV reported by other authors represents a junction potential between damaged cells and their environment.

The higher figure for the potential difference in the presence of valinomycin is likely to represent an approach to a potassium diffusion potential induced by the ionophore.

Effect of tetraphenylboron on DDA^+ uptake

Low concentrations of the lipid-soluble anion tetraphenylboron are known to stimulate the uptake of DDA^+ in other systems [4–7]. Fig. 3 shows that tetraphenylboron at a concentration of $5 \cdot 10^{-7}$ M enhanced both the rate and net uptake of DDA^+.



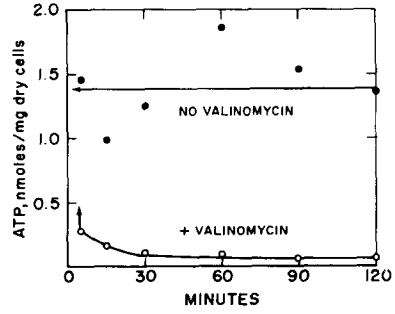


Fig. 3. Effect of tetraphenylboron on the uptake of DDA^+ in the Ehrlich cell. Cells were incubated at 37 °C in Krebs–Ringer bicarbonate buffer. Potassium was omitted from the incubation medium in order to avoid precipitation of the tetraphenylboron. The concentration of DDA^+ and tetraphenylboron were 0.1 mM and $5 \cdot 10^{-7}$ M, respectively.

Fig. 4. ATP levels in the Ehrlich cell in the presence of valinomycin. Cells were incubated at 37 °C in ordinary Krebs–Ringer bicarbonate medium. Valinomycin was added as in Table I. The ATP levels of the two aliquots of cells undoubtedly were identical at the beginning, as suggested by the arrowheads at the left.

The maximal effect was reached in approximately one hour, no further increase in the uptake being seen with longer incubation intervals (data not shown). The maximal distribution ratio of DDA^+ attained with tetraphenylboron present was about 8, corresponding to a potential difference of about -55 mV, if DDA^+ is indeed serving as an indicator under these conditions.

Effect of valinomycin on amino acid uptake

The effect of valinomycin on the uptake of several amino acids, substrates of three transport systems of the Ehrlich cell [2], is shown in Table II. The amino acids tested were homoarginine, representing the cationic system Ly^+, 2-(methylamino)-isobutyric acid, a model substrate for Na^+-dependent System A, 4-amino-1-methylpiperidine-4-carboxylic acid, a substrate principally of Na^+-independent System L selected because it shows unambiguous uphill transport into the Ehrlich cell [2] and *S*-2-aminoethyl-L-cysteine (thialysine) concentrated mainly by System A but also by System L. The results show that valinomycin not only fails to stimulate the uptake of these amino acids, but instead leads to a decrease in their uptake.

TABLE II

Effect of valinomycin on the accumulation of [^{14}C]homoarginine, 4-amino-1-methylpiperidine-4-[^{14}C] carboxylic acid, [*carboxy*-^{14}C]-2-(methylamino)-isobutyric acid and [^{3}H]thialysine by the Ehrlich cell. Uptake from ordinary Krebs–Ringer bicarbonate medium was observed during 90 min for the first two amino acids, and during 60 min, for the last two. The amino acids were added at 0.8 to 1 mM. Valinomycin was added as for Table I.

Amino acid	Cellular uptake (mmoles/kg cell water)	
	Without valinomycin	With valinomycin
Homoarginine	5.8	4.0
4-Amino-1-methylpiperidine-4-carboxylic acid	14.1	7.4
2-(Methylamino)-isobutyric acid	20.1	7.4
Thialysine	22.4	12.8

DISCUSSION

The Ehrlich cell shows an oxygen consumption during ordinary incubation corresponding to a considerable rate of catabolism of endogenous metabolites, probably lipids [12]. The values for the potential difference observed in Table I, which correspond rather well to those estimated by the chloride distribution (see for example, Hempling [13]), as well as those obtained by a microelectrode [11], appear therefore to apply for metabolizing cells. The failure of sulfate replacement for chloride to modify significantly the distribution of DDA^+ may mean that little electrogenic migration of Cl^- occurs across the plasma membrane of this cell.

Whether the effect of tetraphenylboron on DDA^+ distribution implies the presence of a somewhat higher membrane potential is by no means clear, since the action of this anion is not understood. Bakeeva et al. [4] proposed that it can serve as a

carrier for the organic cation and hence that it is not the free DDA^+ but the neutral anion-cation complex that moves through the substance of the membrane. A dissociation at the inner membrane surface to release the cation would nevertheless permit its migration to be electrogenic. These authors offer the alternative explanation that tetraphenylboron may neutralize some positive charges in the membrane (e.g. cationic choline groups on phospholipids) which may otherwise hinder the migration of DDA^+. As illustrated in Fig. 1 DDA^+ at 0.1 mM was already at a steady state of distribution in 2 h. Accordingly tetraphenylboron does not appear to have merely accelerated the approach to equilibrium, and the higher distribution ratio of DDA^+ provoked by tetraphenylboron probably represents an induced potential, rather than the physiological transmembrane potential. Tetraphenylboron is well known to be reactive with K^+, although it is not likely to act simply as a K^+ ionophore, since incubation at $5 \cdot 10^{-7}$ M tetraphenylboron did not significantly modify the K^+ level of the Ehrlich cell (data not shown).

The observed valinomycin inhibition of the uptake of the test amino acids may very well arise from a concurrent decrease in the cellular ATP level (Fig. 4). Levinson [14] observed that the increase in intracellular Na^+ and the decrease in intracellular K^+ that occurs in K^+-depleted Ehrlich cells treated with valinomycin paralleled a fall in the cellular ATP level. This decrease presumably limits the activity of the (Na^+, K^+)-dependent ATPase. Addition of glucose to the medium largely reversed this effect of valinomycin. Poole et al. [15] also noted that glucose addition reversed the valinomycin-induced decrease of intracellular K^+, seen in that case in Ehrlich cells not previously depleted in K^+. The interpretation of both these papers was that valinomycin lowered the ATP levels by uncoupling oxidative phosphorylation in the mitochondria.

In isolated mitochondria, valinomycin can under some conditions act as an uncoupler of oxidative phosphorylation [16, 17]. Furthermore we noted that valinomycin is able under the conditions of our experiments to release the inhibition of O_2 consumption produced by the previous treatment with oligomycin, an effect also observed by Levinson [14] in K^+-depleted cells. We believe therefore that valinomycin under the conditions of our study was acting simultaneously to uncouple oxidative phosphorylation in the mitochondrion and to allow K^+ to diffuse down its concentration gradient across the plasma membrane.

We have previously observed that uncouplers of oxidative phosphorylation inhibit uptake of amino acids studied here, in association with the lowering of the cellular ATP level [2]. Conceivably, therefore, a stimulating effect of the intensification of the potential gradient in the present experiments could have been hidden by the simultaneously lowered ATP level. Because ATP had first been largely depleted in the experiments of Gibb and Eddy, a further diminution in the ATP level was less likely to have contributed to the effects they observed for valinomycin. These authors note, however, that similar effects of valinomycin were observed in metabolizing cells in the presence of 10 mM glucose.

Our experiments, although not primarily designed to determine how much the potential gradient contributes to energization of the uphill transport of amino acids, appear to indicate that under the given conditions a diminution in the cellular ATP level exerts a deterent effect on both transport systems larger than any stimulating effect of an increase in the transmembrane potential.

The observation that the distribution ratio that can be observed for DDA^+ is increased in the presence of valinomycin, despite a major depletion of ATP, supports the assumption that this cation is not actively transported by the plasma membrane of the Ehrlich cell.

ACKNOWLEDGEMENTS

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