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Energy Sources for Amino Acid Transport in Animal Cells

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The existence of an electrogenic Na^+ pump in Ehrlich cells which substantially contributes to the membrane potential, previously derived from the distribution of the lipid soluble cation tetraphenylphosphonium (TPP^+), could be confirmed by an independent method based on the quenching of fluorescence of a cyanine dye derivative, after the mitochondrial respiration had been suppressed by appropriate inhibitors. The mitochondrial membrane potential, by adding to the overall potential as measured in this way is likely to cause an overestimation of the membrane potential difference (p.d.). But since this error tends to diminish with increasing pump activity, the true p.d. of the plasma membrane should easily account for the driving force to drive the active accumulation of amino acids in the absence of an adequate Na^+ concentration gradient. Accordingly, the F_2-aminoisobutyric acid (AIB) uptake rises linearly with the distribution of TPP^+ at constant Na^+ concentrations, suggesting that each responds directly to membrane potential. There is evidence that the electrogenic (free) movement of Cl^- is slow, at least at normal p.d., whereas a major part of the Cl^- movement across the cellular membrane appears to occur by an electrically silent Cl^--base exchange mechanism. By such a mode Cl^-, together with an almost stoichiometric amount of K^+, may under certain conditions move into the cell against a high adverse electrical potential difference. This "paradoxical" movement of K^+Cl^- contributing to the deviation of the Cl^- distribution from the electrochemical equilibrium distribution, is not completely understood. It is insensitive towards ouabain but can almost specifically be inhibited by furosemide. As a likely explanation a H^+–K^+ exchange pump was previously offered, even though unequivocal evidence of such a pump is so far lacking. According to available evidence the electrogenic movement of free Cl^- is too small, at least at normal orientation of the p.d., to significantly shunt the electrogenic pump potential so that the establishment of such a potential is plausible. The evidence presented is considered strong in favor of the gradient hypothesis since even in the absence of an adequate Na^+ concentration gradient, the electrogenic Na^+ pump will contribute sufficient extra driving force to actively transport amino acid into the cells.

Key words: amino acid transport, gradient hypothesis, electrogenic cation pump, electrolyte movements, ouabain, furosemide

The active uptake of neutral amino acids by Ehrlich cells, as in other animal cells and tissues, is according to many investigators secondary active transport, i.e., driven by the electrochemical potential gradient of Na^+ via cotransport (gradient hypothesis),

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rather than directly by the affinity of a chemical reaction, as in primary active transport. There are other investigators, however, who reject this gradient hypothesis, and, indeed, the evidence available, though strongly in its favor seems not to have been sufficient to fully exclude some contribution of primary active transport to the overall process. The controversy is illustrated by the following 2 findings. It has been shown by Eddy and others that active uptake of neutral amino acids may take place during complete inhibition of metabolism, as long as appropriate gradients of Na^+ and K^+ can be maintained (1). This observation appears to indicate that only the ion gradients are required to drive the transport, but not directly the metabolic energy supply. On the other hand, it has been shown that metabolically fully active cells may also actively accumulate amino acids while gradients of both Na^+ and K^+ are absent or even moderately inverted (2). This observation in contrast to the first one seems to indicate that only metabolic activity, but not the ion gradients, is required for transport. To reconcile these 2 seemingly contradictory observations some researchers have assumed that there are 2 separate pathways for active amino acid transport, one coupled to the Na^+ flow and the other directly coupled to a metabolic reaction. It has never been possible, however, to separate 2 such pathways, even though neither of the 2 mentioned alternatives alone gives optimal transport, which is obtained only in the presence of both Na^+ gradient and respiration. The main arguments raised against the gradient hypothesis nowadays are energetic ones, i.e., the question whether the electrochemical potential gradient of Na^+ is adequate under all conditions (3). The energetic adequacy of any driving device depends on 2 conditions. Firstly, that enough energy is available, and secondly, that the coupling between the driver process and the transport process is tight enough to warrant sufficient transfer of this energy. As to the latter condition, it could be shown by the methods of irreversible thermodynamics that coupling between amino acid influx and Na^+ influx is indeed fairly tight (4) whereas a direct coupling between the same amino acid influx and the hydrolysis of ATP is barely detectable (5). Hence the second condition appears to be fulfilled. The actual amount of energy available from the Na^+ electrochemical potential gradient is still a matter of controversy, especially in the above mentioned case of inverted Na^+ and K^+ gradients (2). More recently, however, we have been questioning whether electrochemical potential difference of Na^+ as it is derived from separate estimates of concentrations and electrical potential differences, may not be greatly underestimated so that its energetic inadequacy, whenever it is observed, may not be real. In particular, the electrical component of this potential difference (p.d.) may be much higher than has hitherto been assumed, especially in view of the evidence that an electrogenic Na^+ transport system (pump) appears to operate in these cells (6). If the p.d. were exclusively due to a disequilibrium of ion distribution, the electrochemical potential difference of Na^+ could not exceed a ceiling value

$$\frac{RT}{F} \ln \frac{[Na^+]' \cdot [K^+]''}{[Na^+]'' \cdot [K^+]'} \; *.$$

During inversion of both Na^+ and K^+ distribution the electrochemical p.d. of Na^+ should then clearly be in the wrong direction. An electrogenic pump, however, has a contribution to the overall electrical potential of its own, which in the nonsteady state may raise the electrochemical p.d. of Na^+ well above the ceiling value so that in spite of inverted gradients the electrochemical p.d. of Na^+ would be in the right direction to drive the amino acid into the cell. There is experimental evidence that the uptake of the neutral amino acid AIB is accelerated by raising the electrical p.d., even in the absence of a Na^+

*The superscripts ′ and ″ refer to extracellular and intracellular space, respectively.

concentration gradient (Fig. 1). It can also be shown that the cells are depolarized by neutral amino acids, which, acting as Na^+ ionophores, tend to shunt the electrical p.d. (7–9).

Somewhat uncertain still, however, is the actual magnitude of the contribution to the overall potential by this pump. All methods applied to estimate the electrical p.d. appear to involve some error: The p.d. estimated on the basis of the Cl^- distribution is by far too small. As will be discussed below, the distribution of this ion is unsuitable for a p.d. estimate. The p.d. measured by microelectrodes (10) is higher but probably not high enough. Apart from the fact that this method has not yet been applied under the condition of inverted alkali ion distribution, the puncture of the cell by microelectrodes has been found to damage the cellular membrane so that owing to a rapid decay, the values obtained by this method are likely to be underestimated. On the other hand, p.d. estimates based on the distribution of passively permeant ions, such as lipophilic tetraphenylphosphonium ions (TPP^+) or fluorescent dyes, give much higher values, which would easily suffice to explain active amino acid transport entirely on the basis of the gradient hypothesis. They are, however, likely to give overestimated values because these presumably also contain the electrical p.d. of the mitochondrial membrane. With some such cations, the interference by mitochondrial p.d. may be so strong that the contribution

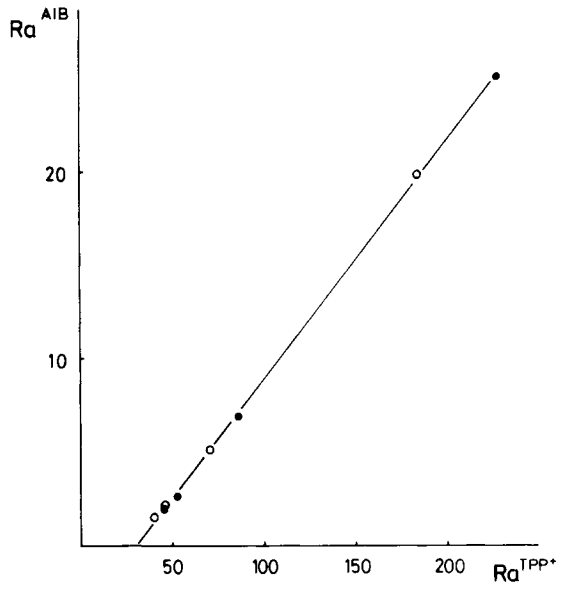


Fig. 1. Effect of electrical potential on the uptake of AIB. The ordinate shows the AIB distribution ratio (R_a) after 4 (○) and 6 (●) min incubation of K^+-depleted Ehrlich cells in the presence of various ouabain concentrations in the extracellular medium. The abscissa shows the corresponding ratios of the distribution of tetraphenylphosphonium (TPP^+) ions, also after 4 and 6 min incubation. The K^+-depleted cells were preincubated with TPP^+ (10 μM) and K^+-free Krebs-Ringer-phosphate buffer, pH 7.4, for 10 min at 37°C before buffers containing AIB (0.1 mM), K^+ (18 mM), and ouabain (0–0.75 mM) were added. It is seen that beyond a certain value of TPP^+ distribution the transient accumulation ration of AIB linearly rises with the ration of TPP^+. The Na^+ concentrations inside and outside the cell are approximately the same in all experiments. To the extent that the TPP^+ distribution ratio indicates the electrical potential, the experiment shows that the transient accumulation ratio of AIB is strongly influenced by the electrical potential or by the difference in electrochemical activity of Na^+.

to the p.d. by the electrogenic pump can be measured only after selectively blocking the mitochondrial respiration and supplying energy to the Na^+ pump from glycolysis. Figure 2 shows the measurements of the p.d. using a method based on the quenching of the fluorescence of a cyanine derivative. This method seems to depend more strongly on mitochondrial interference than does the TPP^+ method. An increase in electrical potential after stimulating the electrogenic Na^+ pump by extracellular K^+ can with this method be observed only after mitochondrial metabolism has been selectively blocked by some respiratory inhibitor whereas the energy source of the electrogenic Na^+ pump is glycolysis. The difference in behavior between lipophilic cations and the fluorescent dyes in this respect might be attributed to a different permeability of the mitochondria membrane to these substances. A p.d. estimate obtained under such precautions appears to provide an independent confirmation of our previous estimates with TPP^+ (Fig. 3). It can be predicted that the effect of extracellular K^+ on the mitochondrial p.d. is opposite to that on the

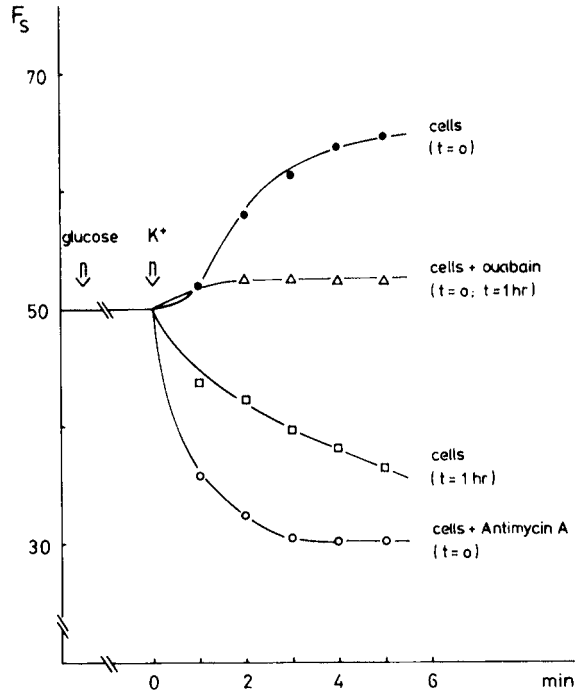


Fig. 2. Electrogenic pump potential as shown by fluorescence change. The fluorescence (F_S) of 3 μm 3, 3'-dipropylthiodicarbocyanine iodide (DiS-C_2-(5)] in a 0.3% suspension of K^+-depleted cells is plotted vs the incubation time. Cells were equilibrated with dye for 10 min. Glucose (2.5 mM), antimycin A (0.5 μM), and ouabain (1 mM) were added 5 min before the addition of K^+. The pump was stimulated by a single dose of KCl (36 mM). The immediate reaction (t = 0) is an increase in fluorescence indicating a decrease in the electrical potential (•). This p.d. decrease is presumably of mitochondrial origin and due to an increase in oxidative phosphorylation. If mitochondrial metabolism is inhibited by antimycin A the fluorescence is quenched by the addition of K^+ indicating an increase in potential. This increase in potential presumably refers to the plasma membrane potential and is due to the electrogenic pump (○). Both effects can be inhibited by the addition of ouabain (△). Also in the absence of antimycin A quenching is observed after 1 hr presumably because of partial inhibition of mitochondrial respiration due to depletion of O_2 (□).

p.d. increase attributable to the electrogenic pump p.d. This appears to be borne out by the behavior of the fluorescence with and without mitochondrial respiration: while the electrogenic pump is stimulated the mitochondrial p.d. goes down, presumably owing to the stimulation of oxidative phosphorylation. Hence our overestimation of the electrical p.d. of the plasma membrane should be smallest with maximum activity of the electrogenic pump, and therefore probably close enough to the real value to justify the assumption that the electrochemical p.d. of Na^+ is high enough to drive the active amino acid transport even during the inversion of the Na^+ and K^+ gradients.

Two questions come up in this context: Why is the electrical p.d. not shown by the Cl^- distribution, and why does Cl^- not shunt the electrogenic pump potential to a greater extent than it appears to do? To answer these questions, we have studied the behavior of Cl^-.

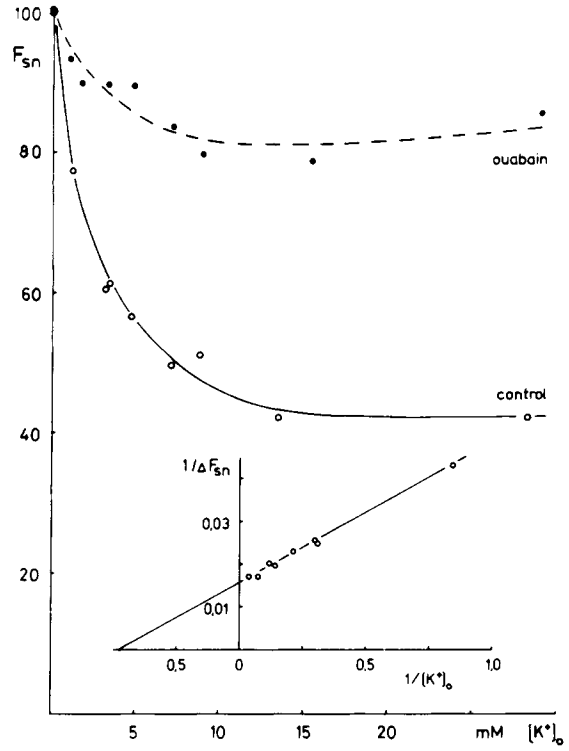


Fig. 3. Electrogenic pump potential as a function of extracellular potassium concentration, as demonstrated by the fluorescence levels of the supernatants (F_{sn}) of 0.09% cell suspensions. In K^+-depleted cells the electrogenic pump was stimulated by the addition of increasing amounts of K^+ to the extracellular medium in the presence of glucose (2.5 mM) and of antimycin A (0.5 μM) to block mitochondrial potential formation. Incubation time with K^+: 5 min. It is seen that the maximum quenching of fluorescence, indicating the maximum development of a potential, increases with extracellular K^+, reaching a maximum at about 10 mM. The shape of this curve is similar to that obtained with the distribution of TPP^+ under similar conditions. In the presence of 1 mM ouabain the potential generation is largely suppressed. In the insert the reciprocal fluorescence change ($\Delta F_{sn} = 100 - F_{sn}$) is plotted vs the reciprocal K^+ concentration suggesting a Michaelis-Menten relationship with respect to K^+.

The magnitude of the contribution of an electrogenic pump to the electrical membrane potential depends on concentration and permeabilities of the other ions, in particular of Cl^-. If these ions penetrate the cellular membrane very fast, they will shunt the pump potential and keep it to low values, as appears to be the case in some other systems. Hence it is of crucial importance in the present context to show that this mobility is indeed low enough to allow the generation of the postulated pump potential. Measurement of the actual rate of Cl^- permeability turned out to be very difficult since under different conditions the permeability coefficient for Cl^- appears to vary over wide ranges. Apparently there are several pathways of different velocities for Cl^- movement of which the more rapid ones are probably due to an electrically silent exchange between Cl^- and some base anions, most likely OH^-. In addition, the permeability coefficient of the cellular membrane for net movement of Cl^- appears to be influenced by the electrical potential of the membrane as if there were some rectification. It seems that with a normal orientation of the potential, i.e., positive outside, the movement of Cl^- is indeed very slow, but it becomes accelerated if the potential is reversed (11). Hence it seems that the electrogenic pump, by tending to raise the potential in the outward direction tends to decrease the permeability for Cl^-, thus providing the very condition necessary to avoid shunting by Cl^-. The outward net movement at least appears to be slow enough to allow the generation of a sufficiently high pump potential. For the same reason, the Cl^- distribution may in transient states depend more strongly on the pH difference than on the electrical p.d. but in the end it should nonetheless tend toward equilibrium with the electrical p.d. This, however, is not observed. On the contrary, Cl^- may under certain conditions even move into the cell steadily away from electrochemical equilibrium. (Fig. 4). This "paradoxical" Cl^- uptake is associated with an almost stoichiometric movement of K^+ as is usually observed with K^+-depleted cells if the extracellular K^+ rises above 5–10 mM (12). Since under these conditions the electrical p.d. is still strongly positive outside, the distribution of Cl^- at any time during this process should give highly erroneous p.d. estimations if the simple Nernst equation is applied. The mechanism of this paradoxical KCl uptake, which is inhibited by metabolic inhibitors but not by ouabain, is not fully understood. One explanation has been based on a hypothetical H^+-K^+ exchange pump which is stimulated by extracellular K^+ and which by raising the cellular OH^- level should favor the uptake of Cl^- via the mentioned Cl^--OH^- exchange mechanism. This pump would thus lead to a net uptake of KCl in exchange for a stoichiometric amount of water molecules. Since in the present system H^+ extrusion would be readily neutralized by OH^- via the rapid Cl^--OH^- exchange mechanism, such a proton pump cannot easily be detected. We therefore tried to catch a H^+ extrusion before neutralization using a sensitive, rapid-response pH-meter after triggering the hypothetical proton pump by a K^+ pulse. To delay neutralization via the Cl^--OH^- exchange mechanism the Cl^- in the suspending medium was replaced by gluconate. Ouabain was also added to prevent interference of the Na^+-K^+ pump with the proton pump. No evidence of a rapid H^+ extrusion, however, could be detected under these conditions. There still remains the possibility that the H^+ are not pumped directly into the medium but first into some intracellular compartment which communicates with the outside medium to let the H^+ leak out in exchange for K^+. Thus the appearance of OH^- in the cytoplasm may initially precede that of H^+ in the medium. The existence of an intracellular compartment with an acidity higher than that of the cytoplasm would be in agreement with the previously reported evidence of an intracellular inhomogeneity with respect to pH (3). It had been shown by the applica-

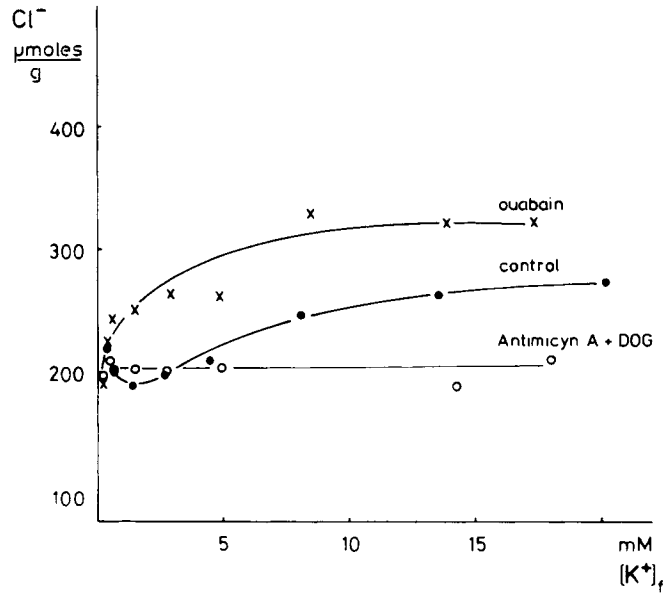


Fig. 4. Effect of extracellular K^+ on the chloride movement. The Cl^- content (μmoles/gram dry weight) of K^+-depleted Ehrlich cells incubated for 3 min in Krebs-Ringer-phosphate buffer, pH 7.4, containing different inhibitors is plotted against the extracellular K^+ concentration (mM). To K^+-depleted cells K^+-free buffers were added with and without antimycin A (0.4 μM) and 2-desoxyglucose (DOG, 5 mM) and preincubated for 5 min at 37°C before buffers with different K^+ content with and without ouabain (1.25 mM) were added for the final 3 min of incubation. In agreement with previous findings the increase of extracellular K^+ in a suspension of K^+ depleted cells causes an uptake of chloride if the extracellular K^+ exceeds about 5 mM. This uptake is strongly enhanced in the presence of ouabain. The chloride uptake is completely abolished after metabolic inhibition by antimycin A and 2-desoxyglucose.

tion of both an acidic and a basic pH probe that the distribution ratio was far from reciprocity, which should have been expected for a homogeneous compartment, whereas reciprocity is produced by metabolic inhibition (3). Further investigation is required to test this hypothesis.

A suitable tool to study this special KCl uptake may be given by the inhibitor furosemide which at 2 mM not only strongly inhibits Cl^- permeability, but also almost specifically eliminates the mentioned paradoxical Cl^- uptake and also the concomitant K^+ uptake (Fig. 5). On the other hand, furosemide does not appear to effect the ouabain-sensitive electrogenic Na^+ pump (Fig. 6).

Whatever its mechanism, the paradoxical Cl^- movement would account for inability of the Cl^- distribution to indicate the true electrical p.d. It also gives an explanation why the p.d. of the electrogenic pump is not shunted to a greater extent by electrogenic Cl^- movement.

The presented observations are all in agreement with the postulates of the gradient hypothesis, and moreover provide a rational explanation for observations seemingly arguing against this hypothesis. They are therefore regarded as a strong support of the gradient hypothesis.

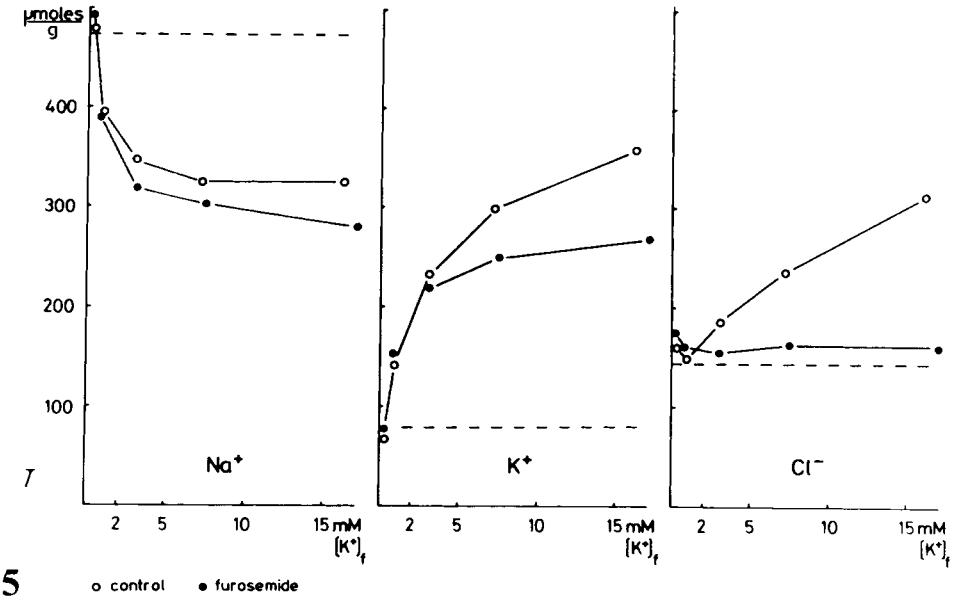
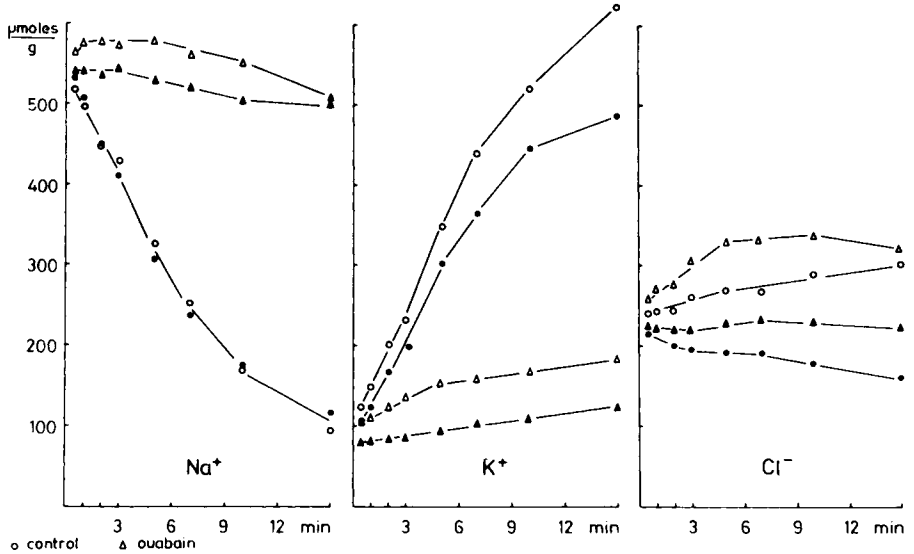


Fig. 5. The effect of 2 mM furosemide on ion transport in Ehrlich cells. The cellular content of Na^+, K^+, and Cl^- (μmoles/gram dry weight) after 5 min incubation in presence (•) and absence (○) of furosemide is plotted against the final K^+ concentration of the medium. The dotted lines give the corresponding levels at zero time. To K^+-depleted cells in K^+-free medium buffers of varied K^+ content with and without furosemide (2 mM) were added. The Na^+ extrusion, stimulated by extracellular K^+, is only slightly increased in the presence of furosemide. In agreement with previous experiments, Cl^- uptake occurs if the extracellular K^+ exceeds about 5 mM, in spite of the pump potential, which is outside positive. This "paradoxical" Cl^- uptake is almost completely inhibited by furosemide. The K^+ uptake rises with increasing extracellular K^+. As Fig. 6 shows, this K^+ uptake is composed of 2 components, one related to the Na^+-K^+ pump and an additional one which is ouabain insensitive and is about equivalent to the "paradoxical" Cl^- uptake. In the presence of furosemide this extra K^+ uptake, equivalent to the paradoxical Cl^- uptake is inhibited.

Fig. 6. Effect of ouabain and furosemide on ion transport in Ehrlich cells. The cellular content of Na^+, K^+, and Cl^- (μmoles/gram dry weight) is plotted against the incubation time. To K^+-depleted Ehrlich cells in K^+-free medium K^+-containing (15 mM) buffers with and withoug furosemide (2 mM) and ouabain (1 mM) were added. The extrusion of Na^+ is triggered and this extrusion appears not to be changed significantly by the presence of furosemide. The presence of ouabain, however, blocks this Na^+ extrusion in the presence and absence of furosemide. The addition of K^+ causes a rapid uptake of K ions, which is significantly inhibited by 2 mM furosemide. 1 mM ouabain reduces the K^+ uptake considerably without abolishing it. This ouabain insensitive residual K^+ uptake appears to be strongly inhibited by furosemide. The addition of K^+ causes first an extrusion and then at higher K^+ concentrations an uptake of Cl^-. This uptake is significantly increased in the presence of ouabain. This increase presumably presents the real, ouabain insensitive Cl^- uptake, which in the absence of ouabain is counteracted by the pump potential. In the presence of furosemide each curve appears to be displaced by about the same amount indicating that under these conditions the Cl^- movement appears to follow in the direction of the potential being outward in the absence of ouabain and remains on the same level in the presence of ouabain. ○) Control, △) ouabain, •) furosemide, ▲) ouabain + furosemide.



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