Biochimica et Biophysica Acta, 600 (1980) 432—447

BBA 78808

ELECTRICALLY SILENT COTRANSPORT OF Na^+, K^+ and Cl^- IN EHRLICH CELLS

P. GECK, C. PIETRZYK *, B.-C. BURCKHARDT **, B. PFEIFFER and E. HEINZ ***

Gustav-Embden Zentrum der Biologischen Chemie, Theodor-Stern-Kai 7, J.W. Goethe-Universität, D-6000 Frankfurt am Main 70 (F.R.G.)

(Received November 13th, 1979)

Key words: Na^+; K^+; Cl^-; Cotransport; Diuretic; (Ehrlich cell)

Summary

A cotransport system for Na^+, K^+ and Cl^- in Ehrlich cells is described. It is insensitive towards ouabain but specifically inhibited by furosemide and other 'high ceiling' diuretics at concentrations which do not affect other pathways of the ions concerned. As the furosemide-sensitive fluxes of these ions are not affected by changes in membrane potential, and as their complete inhibition by furosemide does not appreciably alter the membrane potential, they appear to be electrically silent. Application of the pulse-response methods in terms of irreversible thermodynamics reveals tight coupling between the furosemide-sensitive flows of Na^+, K^+ and Cl^- (q close to unity for all three combinations) at a stoichiometry of 1 : 1 : 2. The site for each of the ions appears to be rather specific: K^+ can be replaced by Rb^+ but not by other cations tested whereas Cl^- can be poorly replaced by Br^- but not by NO_3^-, in contradistinction to the Cl^--OH^- exchange system. The cotransport system appears to function in cell volume regulation as it tends to make the cell swell, thus counteracting the shrinking effect of the ouabain-sensitive (Na^+, K^+) pump.

The experiments presented could not clarify whether the cotransport process is a primary or secondary active one; while incongruence between transport and conjugated driving force seems to indicate primary active transport, it is very unlikely that hydrolysis of ATP supplies energy for the transport process, since there is no stimulation of ATP turnover observable under operation of the cotransport system.

* Present address: Institut für Klinische Chemie, D-8000 Muenchen-Grosshadern, F.R.G.
** Present address: Max Planck-Institut für Biophysik, D-6000 Frankfurt 70, F.R.G.
*** Present address: Department of Physiology, Cornell University Medical College, New York, NY, U.S.A.

Introduction

There is evidence of an electrogenic Na^+ pump in Ehrlich cells which is specificially activated by extracellular K^+ and intracellular Na^+, and inhibitable by ouabain [1]. This pump may, under suitable conditions, strongly raise the membrane potential (hyperpolarization) as can be shown by various methods, such as the distribution of the lipid-soluble cation, tetraphenylphosphonium, or the change in fluorescence of carbocyanine dyes, and by the enhancement of Na^+-linked amino acid transport. The distribution of Cl^-, however, formerly also used as a potential monitor did not respond to changes in membrane potential, such as induced by the activation of the pump. On the contrary, after activation of the pump the Cl^- distribution often changes in the direction opposite to the expected one. This paradoxical behavior was initially attributed to the sluggishness of the Cl^- net flow not allowing equilibration within the time of the experiment [2], until evidence was found of a presumably electrically silent pathway, which is inhibitable by furosemide and activated by extracellular K^+, by which Cl^- may be moved into the cell against a membrane potential [3—5]. At first it was assumed that this 'paradoxical' Cl^- movement was caused by exchange with OH^-, driven by a K^+-activated proton pump. Even though evidence of active proton extrusion in Ehrlich cell could be produced, it was not activated by K^+ and appeared to be too slow to account for the observed Cl^- fluxes [6]. Since, on the other hand, this paradoxical Cl^- influx was always accompanied by an ouabain-insensitive and almost equivalent K^+ influx, and since both these fluxes were sensitive to furosemide and unaffected by changes in electrical potential difference, a kind of KCl cotransport appeared to be the most appropriate interpretation. Meanwhile, this hypothesis has been supported by more evidence, especially of a tight and ion-specific coupling between fluxes of K^+ and Cl^-, respectively, and of its likely function in the volume regulation of the cell. Contrary to earlier expectations, this cotransport appears to involve also Na^+, as will be shown below.

Materials and Methods

The media employed throughout were Krebs-Ringer phosphate buffers of pH 7.4 with 1% albumin. Variations of Na^+, K^+ or Cl^- concentrations are indicated in the individual experiments, respectively. The chemicals used were analytical grade and were obtained from E. Merck, Darmstadt. Furosemide was a generous gift of the HOECHST AG Frankfurt/Main. [^{3}H-]Tetraphenylphosphonium (0.16 TBq/mol) was labelled by the HOECHST AG.

Ehrlich ascites tumor cells were propagated and collected as described previously [1,7]. K^+-depleted cells were obtained by preincubating the cells in K^+-free Krebs-Ringer phosphate buffer for 20 min at 0° C and again for 20 min at 37° C. After this procedure, the cells contained 10—20 mM K^+ and about 200 mM Na^+, as compared to 180 mM K^+ and 30 mM Na^+ after the normal preincubation in K^+-containing media for 30 min at 37° C.

These K^+-depleted cells were resuspended in a K^+-free buffer and preincubated for 5—10 min. The main incubation was started by addition of K^+. If the Na^+ or Cl^- concentration in the incubation medium was varied, Na^+ was

replaced by tetraethylammonium and Cl^- by gluconate. The transport processes were stopped by cooling the samples in an ice bath (−4° C). The analytical procedures were as described previously [1].

Alterations in membrane potential were determined using the distribution ratio of the lipid-soluble cation tetraphenylphosphonium, which distributes passively, showing proportionally between the distribution ratio (R_{TPP}) and the electrochemical activity coefficient ($\xi = \exp(-F\psi/RT)$). The validity of this procedure to indicate alterations in membrane potential of Ehrlich cells was shown previously [2,8].

Results

Furosemide-sensitive ion flows

The furosemide-sensitive flow of a given ion has been defined as the difference between this flow in the absence of furosemide and that in the presence of furosemide. To separate this flow from the (Na^+, K^+) pump, it is usually studied in the presence of ouabain. Fig. 1a shows that upon the addition of K^+, furosemide-inhibitable fluxes of all three ions, K^+, Na^+, and Cl^-, are induced with an apparent stoichiometry of 1 : 1 : 2. If, for instance, the furosemide-sensitive movements of Cl^- and Na^+, respectively, are plotted against the concomitant movements of K^+, one obtains straight lines the slopes of which confirm the stoichiometry suggested above (Fig. 1b).

The question arises as to whether this apparent association between the three fluxes, especially that between the cation fluxes and the concomitant Cl^- flux, is merely caused by electrostatic interaction, i.e., in order to maintain electroneutrality, or whether it is based on true (chemical) coupling. To answer this question the interdependence between these flows and the membrae potential has been studied.

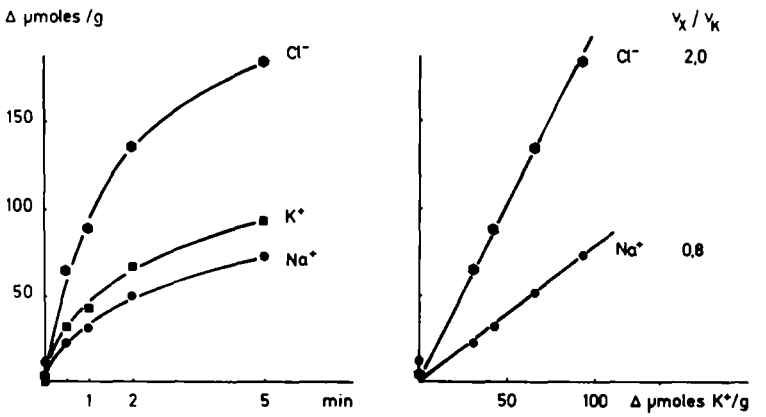

Fig. 1. Furosemide-sensitive ion movements. K^+-depleted, Na^+-rich cells after preincubation for 5 min at 37° C in K^+-free medium were incubated for up to 5 min in Krebs-Ringer phosphate buffer with 38 mM K^+ and 98 mM Na^+. The furosemide-inhibitable ion movement, defined as the difference in intracellular ion content (per g dry wt.) between cells incubated in the presence of 1 mM ouabain and those incubated with 1 mM ouabain and 2 mM furosemide are plotted versus incubation time (left side). The movements of Na^+ and Cl^- are plotted versus that of K^+ (right side).

Membrane potential and furosemide-sensitive ion flows

Whether or not Cl^- is transported actively, the electrical potential difference should gradually decrease with increasing K^+ influx to the extent that the latter is rheogenic.

To test this, the furosemide-sensitive K^+ influx was gradually enhanced by increasing the extracellular K^+ concentration in the presence and absence of ouabain, and the membrane potential was monitored by tetraphenylphosphonium ion (Fig. 2). Whereas in the control, i.e., in the absence of ouabain, the addition of K^+ hyperpolarized the cell, which is attributed to the activation of the elctrogenic (Na^+, K^+) pump (upper curve), K^+ fluxes of the same magnitude failed to do so in the presence of ouabain; the tetraphenylphosphonium ion distribution remained almost unchanged over the whole range of the induced K^+ influxes. This indicated that the magnitude of the ouabain-insensitive and furosemide-sensitive K^+ movements has no effect on the membrane potential. Likewise, the variation of the furosemide-sensitive flow of K^+, Na^+ or Cl^- in wide ranges, each induced by increasing its concentration gradient, had not appreciable effect on the distribution of tetraphenylphosphonium ion (Table I). Hence, the furosemide-inhibitable and ouabain-insensitive flows of none of the three ions appears to have a direct effect on the membrane potential. In other experiments it was investigated whether the converse is also true, namely whether arbitrary changes in the electrical potential do not affect the furosemide-sensitive flow of K^+, Na^+, and Cl^-. The change in electrical potential

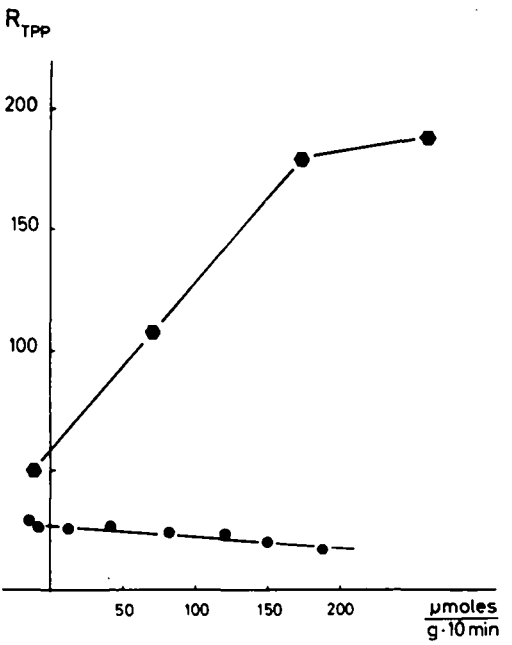

Fig. 2. Lack of influence of the ouabain-resistant K^+ flux on the membrane potential. K^+-depleted cells were incubated for 10 min at 37°C in buffers of different K^+ concentration containing 10 μM [^{3}H] tetraphenylphosphonium. The distribution ratio (R_{Tpp}) of [^{3}H]tetraphenylphosphonium was plotted versus the associated K^+ movements in the absence (•) and presence of 1.25 mM ouabain (•).

TABLE I

INFLUENCE OF FUROSEMIDE-SENSITIVE TRANSPORT ON MEMBRANE POTENTIAL

Na^+-rich, K^+-depleted cells were incubated for 5 min at 37°C in media of different ion composition in the presence of 1 mM ouabain with and without 2 mM furosemide. The furosemide-sensitive fluxes are represented by its associated water movements (ml H_2O/dry wt., 5 min). Q_{TPP} gives the ratio of the tetraphenylphosphonium ion distributions in the absence and presence of 2 mM furosemide.

Extracellular ion concentration (mM)			Furosemide-sensitive water movements (ml/g dry wt., 5 min)	Q_{TPP}
Na_f^+	K_f^+	Cl_f^-		
126	27	108	0.43 ± 0.02	0.90 ± 0.07
119	0.2	109	0.02 ± 0.13	0.89 ± 0.05
5	26	106	0.05 ± 0.02	0.89 ± 0.01
119	27	5	0.03 ± 0.06	0.96 ± 0.07

was produced in two different ways: (1) by partial inhibition of the electrogenic Na^+ pump using varying concentrations of ouabain and (2) by depolarization of the potential through graduated addition of the amino acid glycine, which via the amino acid-Na^+ cotransport acts like an ionophore for Na^+ (Table II). From Table II it follows that there is no membrane potential dependence of the fluxes. The fluxes of each of these ions remain rather constant if the membrane potential is varied within a wide range. These and the preceding observations appear to indicate that there is no mutual influence between the electrical potential and the flow of any of these ions, i.e., that the furosemide-sensitive fluxes of all three ions are electrically silent.

Stoichiometry and degree of coupling between the three ions

Since the previous observations of the lack interdependence between potential changes and changes in flux rate of these three ions appear to exclude that any linkage between these ion fluxes to the extent that they are furosemide-sensitive is merely due to electrostatic interaction, i.e., to maintain electro-

TABLE II

INFLUENCE OF CHANGES IN MEMBRANE POTENTIAL ON FUROSEMIDE-SENSITIVE TRANSPORT

Na^+-rich, K^+-depleted cells were incubated for 5 min at 37°C in Krebs-Ringer phosphate buffer with 50 mM K^+ in the presence or absence of 2 mM furosemide. The furosemide-sensitive ion transport is defined as the difference in ion content of cells incubated for 5 min in the absence and presence of furosemide. The membrane potential was varied in two different ways: (a) by varying the extracellular glycine concentration to shunt gradually the pump potential via the rheogenic Na^+-glycine cotransport and (b) by gradual inhibition of the (Na^+, K^+) pump by ouabain. The slopes of the regression lines are not significantly different from zero: (a symmetrical Eyring's mechanism for transport of monovalent ions would lead to a slope of 0.5).

	a	b
$(\partial \ln J_{Na}/\partial \ln R_{TPP})_{[Na^+]}$	−0.03 ± 0.32	0.10 ± 0.13
$(\partial \ln J_K/\partial \ln R_{TPP})_{[K^+]}$	−0.03 ± 0.02	0.02 ± 0.01
$(\partial \ln J_{Cl}/\partial \ln R_{TPP})_{[Cl^-]}$	−0.09 ± 0.01	0.12 ± 0.05
$(\partial \ln J_{H_2O}/\partial \ln R_{TPP})$	−0.11 ± 0.06	0.12 ± 0.21

neutrality, it may be followed that they are coupled with each other in a more direct way, possibly by carrier-mediated cotransport. As used for irreversible thermodynamics, the degree of such coupling [9], the parameter q_{ij}, has been introduced which is defined in terms of phenomenological coefficients by the following equation:

$$q_{ij} = \frac{L_{ij}}{\sqrt{L_{ii} \cdot L_{jj}}}$$

For each pair of coupled flows, q_{ij} can be determined by the dependence of each flux on the arbitrarily induced flux of the other.

$$q_{ij}^2 = \left(\frac{\partial J_i}{\partial J_j}\right)_{X_m} \cdot \left(\frac{\partial J_j}{\partial J_i}\right)_{X_n} \qquad n \neq i,\ m \neq j$$

These equations are only valid, i.e., giving the true degree of coupling, if the electrochemical potential difference (X_i) of the dependent ion remains constant while the flux of the independent ions species is induced by variation of the driving force of the ion.

This condition can hardly be maintained with ions because the change in concentration difference of any species between both intra- and extracellular space is likely to entail a change in electrical potential, which would then alter the electrochemical potential differences of all ions present, and hence that of the independent ion species. The determination of q would then be incorrect. The derivation of the above equation, however, may be modified such as to provide conditions under which the electrical potential difference of the dependent ion species can be neglected [10].

It is seen from these equations that under the condition that the furosemide-sensitive fluxes of each ion species are non-rheogenic, the electrical potential difference is of no avail, i.e., provided that the differentials of the potential vs. the imposed ion fluxes are zero or negligible, it suffices that merely the chemical potential difference (or simply the concentration of the dependent species) has to remain constant during the pulse to obtain valid q values which give the degree of true coupling. To get a complete picture, the coupling between any two for the furosemide-sensitive flows of any pair of the three ions concerned had to be determined by this method, and in the case of true coupling, q values close to unity should be expected with each of the three combinations. The experiments were carried out as follows: the extracellular concentrations of two of the three ion species were kept constant at a time while the flow of the third ion species was pulsed by varying its concentration in the medium. The resulting net flows of each of the two constant ion species were then plotted against the imposed flow of the varied ion species. The results are shown in Fig. 3A—C. It is seen that in all cases straight lines are obtained, indicating the existence of true coupling with a fixed stoichiometry which can be derived from the slope of this graph. The results of these experiments are summarized in Table III. It is seen that for all three possible combinations of furosemide-sensitive flows, the degree of coupling, q, it close to unity. The stoichiometry derived from the slopes is also close to what was expected from previous observations, namely that within the cotransport the

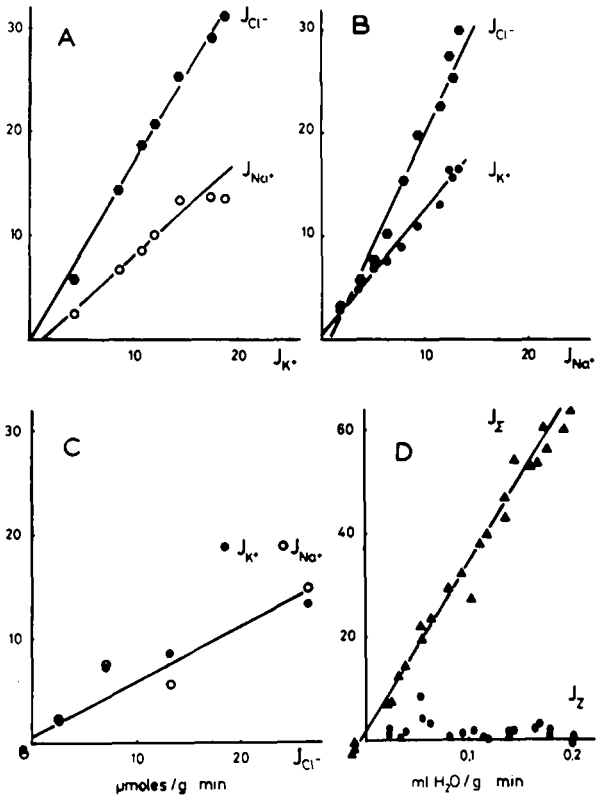

Fig. 3. Pulse-response experiments with Na^+, K^+ and Cl^-. Na^+-rich, K^+-depleted cells were incubated for 5 min at 37° C in Krebs-Ringer phosphate buffers with varied Na^+ and Cl^- content, which were substituted by tetraethylammonium or gluconate, respectively. Furosemide-sensitive ion fluxes were initiated by addition of K^+. The furosemide-sensitive transport (μmol/g per min) of the responding (constant) ion is plotted versus that of the pulsed one. The furosemide-sensitive ion movements are defined as the uptake with 1 mM ouabain minus that with 1 mM ouabain and 2 mM furosemide. For the different types of experiment the following ion concentrations were kept constant: (A) 84 mM Na^+, 135 mM Cl^-; (B) 28 mM K^+, 135 mM Cl^-; (C) 116 mM Na^+, 29 mM K^-. The slopes of the regression lines are given in Table III. (D) The pooled results of all three types of experiment. The triangles show the sum of the three furosemide-sensitive ion fluxes ($J_{Na} + J_K + J_{Cl}$) (μmol/g per min) plotted vs. the associated water movements (ml/g per min). The slope of the line indicating the osmolarity of the transported mixture in these three ion species is 321 ± 8 mM. The points give the flux of the electrical charge ($J_{Na} + J_K - J_{Cl}$) (μmol/g per min). The slope of the regression line is not significantly different from zero (1 ± 5 mM).

ration of Na^+ to K^+ to Cl^- is equal to 1 : 1 : 2. Hence, it appears that there is a cotransport in which the three ion species are carried simultaneously. If the sum of the three furosemide-sensitive ion flows is plotted against the concomitant volume flow (Fig. 3D), the slope of the line indicates that the transported mixture has a tonicity of 330 mosmol/l. Since this is isotonic with the medium, it seems to exclude that in this cotransport the flow of a species other than the three ion species is appreciably involved. The total net charge transferred by the transported species, plotted against the ion flow, is zero throughout. This indicates that no other ion species appreciably accompanies this transport.

TABLE III

PULSE-RESPONSE EXPERIMENTS WITH (Na^+, K^+, Cl^-) COTRANSPORT

Experimental conditions are given in the legend to Fig. 3. The subscripts 1, 2 and 3 refer to the furosemide-sensitive net flows at Na^+, K^+ and Cl^-, respectively. The subscripts, X_i^c, refer to the chemical potential differences of Na^+, K^+ or Cl^-, respectively, kept constant during the pulse response measurements. (a) The slopes of the pulse-response curves between Na^+ (1), K^+ (2) and Cl^- (3) transport. (b) Degree of coupling in the (Na^+, K^+, Cl^-) cotransport. (c) Stoichiometry of the (Na^+, K^+, Cl^-) cotransport.

(a) $(\partial J_1/\partial J_2)X_1^cX_3^c = \nu_1/\nu_2 = 0.80 \pm 0.09$
$(\partial J_2/\partial J_1)X_2^cX_3^c = \nu_2/\nu_1 = 1.20 \pm 0.07$
$(\partial J_1/\partial J_3)X_1^cX_2^c = \nu_1/\nu_3 = 0.51 \pm 0.13$
$(\partial J_3/\partial J_1)X_2^cX_3^c = \nu_3/\nu_1 = 2.36 \pm 0.14$
$(\partial J_2/\partial J_3)X_1^cX_2^c = \nu_2/\nu_3 = 0.49 \pm 0.05$
$(\partial J_3/\partial J_2)X_1^cX_3^c = \nu_3/\nu_2 = 1.75 \pm 0.05$

(b) $q_{1,2} = 0.98 \pm 0.06$
$q_{1,3} = 1.10 \pm 0.16$
$q_{2,3} = 0.93 \pm 0.05$

(c) $\nu_2/\nu_1 = 1.22 \pm 0.08$
$\nu_3/\nu_1 = 2.15 \pm 0.28$
$\nu_3/\nu_2 = 1.89 \pm 0.10$

Specificity of cotransport

If there is a firm coupling between the three ion species concerned, we would also expect some specificity of the process. Table IV shows that this is indeed the case. As for the anions, it seems that besides Cl^- only Br^- appears to have some affinity to the transport systsem. If Cl^- is replaced by nitrate or methylsulfate there is no transport, presumably because these ion species have no affinity for the cotransport system or, whenever they bind, the resulting complex is no longer transportable. The failure of NO_3^- to replace Cl^- (Fig. 4) is of special significance, since it shows that the system under consideration is distinctly different from the well known anion exchange mechanism which does not discriminate between Cl^- and NO_3^- [11,12]. As presented in Table V, also the K^+ site shows some specificity, though it does not appear to distinguish between K^+ and Rb^+. If K^+ is replaced by Cs^+, the transport is reduced and, if

TABLE IV

ANION SPECIFICITY OF THE FUROSEMIDE-SENSITIVE COTRANSPORT

Na^+-rich, K^+-depleted cells were incubated for 10 min at 37°C in media of different anions replacing Cl^- (112 mM). After elevating the K^+ concentration to 28 mM the cells were incubated for 5 min in the presence of 1 mM ouabain with or without 2 mM furosemide. Furosemide-sensitive ion and water movements are determined as the difference of the ion and water content in cells with and without furosemide.

Cation	Furosemide-sensitive movement		
	Na^+	Cl^-	H_2O
	(μmol/g dry wt., 5 min)		(ml/g dry wt., 5 min)
Cl^-	80	80	0.94
Br^-	45	59	0.62
Nitrate	16	14	0.16
Methylsulfate	1	1	—0.01

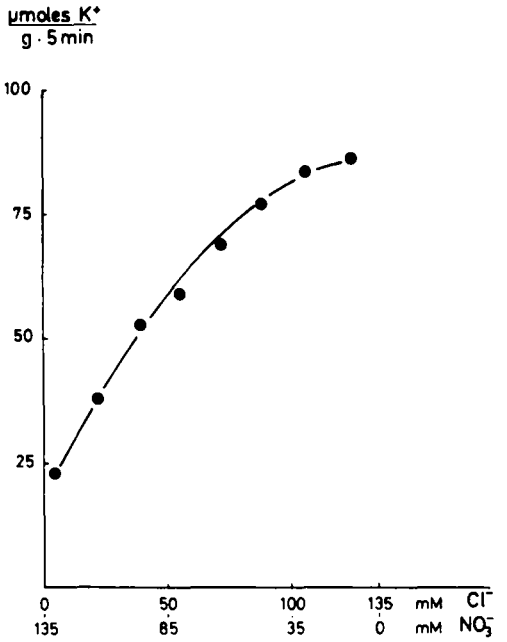

Fig. 4. Anion specificity of the furosemide-sensitive ion transport. K^+-depleted cells were preincubated for 10 min at 37°C in K^+-free buffers of different Cl^- concentration, which was substituted partially by nitrate. The furosemide-sensitive uptake for 5 min initiated by addition of 54 mM K^+ is plotted vs. the Cl^- concentration in the incubation medium.

K^+ is replaced by Li^+, transport stops altogether. Apparently Li^+ is not accepted by the transport mechanism as a substitute for K^+. Table VI shows that Li^+ may serve as a substitute for Na^+.

Energetics of cotransport

In order to find out whether the cotransport is secondary active, i.e., whether the combined electrochemical potential differences of all the ions

TABLE V

SPECIFICITY OF THE FUROSEMIDE-SENSITIVE COTRANSPORT WITH RESPECT TO K^+

Na^+-rich, K^+-depleted cells were incubated for 5 min at 37°C in K^+-free Krebs-Ringer phosphate buffer. Transport was initiated by adding different ions (final concentration 15 mM). The difference in ion and water content after a 5 min incubation in cells without and with 2 mM furosemide in the presence of 1 mM ouabain is taken as the furosemide-sensitive transport.

Anion	Furosemide-sensitive movement		
	Na^+	K^+	H_2O (ml/g dry wt., 5 min)
	(µmol/g dry wt., 5 min)		
K^+	52	134	0.80
Rb^+	50	119	0.67
Na^+	−4	1	−0.01
Li^+	−4	−11	0.00
Cs^+	40	89	0.56

TABLE VI

SPECIFICITY OF THE FUROSEMIDE-SENSITIVE COTRANSPORT WITH RESPECT TO Na^+

Na^+-rich, K^+-depleted cells were incubated for 10 min at 37°C in K^+-free media with different cations replacing Na^+ (10 mM). After elevating the K^+ concentration to 29 mM the cells were incubated for 5 min in the presence of 1 mM ouabain without and with 2 mM furosemide. The difference in ion and water content in cells incubated without and with 2 mM furosemide gives the furosemide-sensitive transport.

Cation	Furosemide-sensitive movement		
	K^+	Cl^-	H_2O (ml/g dry wt., 5 min)
	(μmol/g dry wt., 5 min)		
Na^+	84	163	1.00
Li^+	78	147	0.92
Cs^+	7	−8	0.06
Tetramethylammonium	20	23	0.13

concerned is the only driving force involved, the overall flows has been plotted against the overall driving force of the cotransport [7], i.e., the sum of the individual electrochemical potential differences of the three ion species, each multiplied by its stoichiometric coefficient ($X_{Na} + X_K + 2 \cdot X_{Cl}$). In Fig. 5 it is seen that the resulting line does not pass through the origin, indicating that even in the absence of the conjugated driving force, substantial inward movement of the complex takes place, and that to stop this movement an opposing electrochemical potential is necessary of a magnitude of at least 4—5 kJ/mol as can be estimated from the intercept of the curve on the abscissa. This apparent lack of congruence between flow and force suggests primary active transport, i.e., direct coupling of the cotransport to a metabolic reaction, as

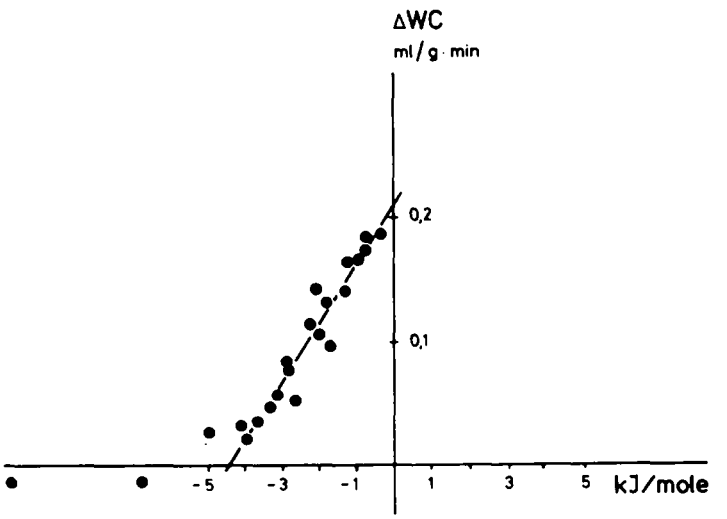

Fig. 5. Driving force for the furosemide-sensitive transport. The furosemide-sensitive water flow (Δ *WC*) as a measure of the furosemide-sensitive solute transport obtained from Fig. 3 is plotted vs. its conjugated driving force resulting from the degree ion gradients ($X = X_K + X_{Na} + 2 \cdot X_{Cl}$).

would agree with the previously reported observation [5] that the cotransport disappears during metabolic inhibition.

This deduction, however, is not absolutely conclusive, since the calculations of the driving force rest on two assumptions which are difficult to verify: that the activity coefficients for the three ion species in cytoplasm and incubation medium do not differ, and that the ions are not compartmentalized inside the cell thus leading to an overestimation of the cytoplasmic ion concentrations. If there are pronounced deviations from these assumptions, it might be possible that the driving force is underestimated to such an extent that there is no incongruent transport observable and that the transport process is a secondary active one. In Fig. 3D, however, no indication is given for activity coefficients of the ions to be lower inside the cell than in the incubation medium, since a tonicity of the transported mixture of 321 ± 8 mM implies that between incubation medium and cytoplasm the mean activity coefficients for these ions only seem to differ by less than 10%. Whether compartmentalization of the three ions, e.g., in the nucleus, would play a significant role is controversial; while fractionation of Ehrlich cells into subcellular components in non-aqueous media seemed to indicate an enrichment of Na^+ and Cl^- in the nucleus [13], investigations using the electron microprobe analysis for other cell types showed no such compartmentalization (e.g., Ref. 14). Hence, the experiment cannot help us to decide whether the transport process under investigation is a primary or a secondary active one.

The following experiments were carried out to test whether the hydrolysis of ATP might supply energy for the cotransport process. If this is the case, operation of the cotransport system should stimulate the hydrolysis of ATP. This was investigated in two different ways. Firstly: the respiratory synthesis of ATP in the cell was blocked by antimycin A, and the rate of ATP disappearance was related to the cotransport rate. In Fig. 6 it is seen that in the ouabain-free control, viz. under an operating (Na^+, K^+)pump, the ATP hydrolysis is greatly accelerated as compared to cells with an ouabain-blocked pump. No such deceleration of ATP consumption can be observed after blocking the cotransport system with furosemide in the presence or absence of ouabain; the curves with or without furosemide are almost the same, thus indicating that the furosemide-sensitive cotransport does not appear to consume any ATP. Secondly, this observation has been confirmed by showing that the rate of glycolysis as a measure of the glycolytic ATP production, which is strongly stimulated by operation of the (Na^+, K^+)pump, is entirely unaffected by furosemide (Fig. 7). It may be concluded that no ATP energy appears to be consumed by the furosemide-sensitive cotransport, whether the ATP is derived from respiration or from glycolysis, unless the ratio of transport rate to ATP splitting is unreasonably high. Comparing the results shown in Figs. 3 and 7, one obtains a ratio for the furosemide-sensitive lactate production to the furosemide-sensitive K^+-transport of 0.04 ± 0.10. Hence, from the presented experiments one cannot decide whether the transport process under investigation is a secondary or a primary one.

Cotransport in volume regulation

Whenever K^+ is added to the K^+-free medium of cells previously depleted of

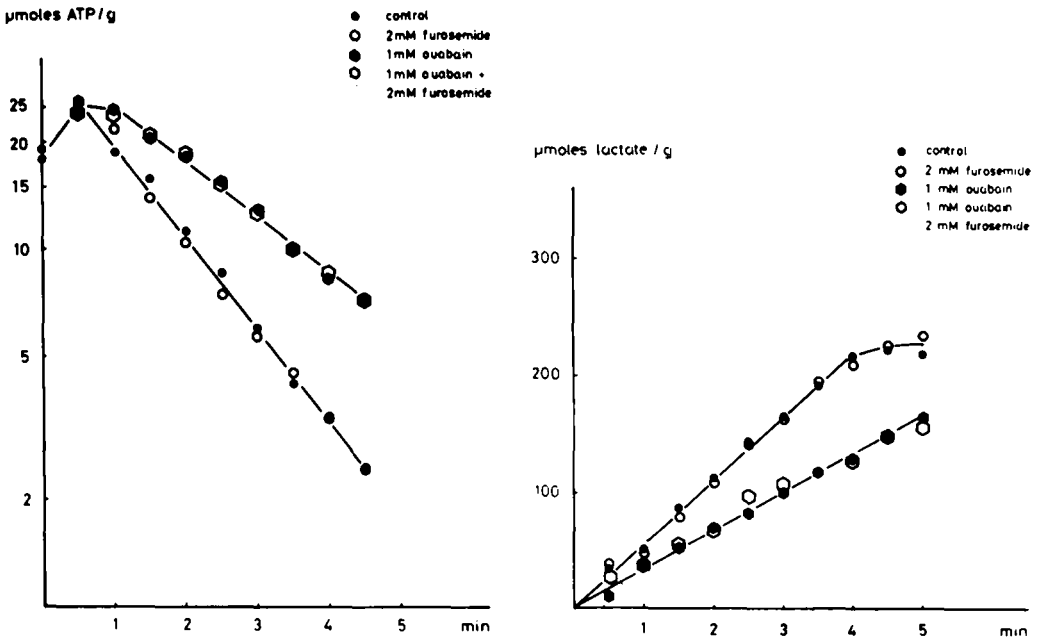

Fig. 6. Influence of ouabain and furosemide on the ATP breakdown. The ATP content of Na^+-rich, K^+-depleted cells at 37°C was determined at different times after addition of 15 mM K^+ and 0.6 µM antimycin A. 1 mM ouabain and 2 mM furosemide were added as indicated.

Fig. 7. Influence of ouabain and furosemide on the glycolytic rate. Lactate production at 37°C of Na^+-rich, K^+-depleted cells after addition of 1.25 µM oligomycin, 1 mM glucose, 15 mM K^+ and 1 mM ouabain and/or 2 mM furosemide as indicated was measured for 5 min.

K^+ and enriched with Na^+, the cell takes up K^+ and loses Na^+, but the K^+ uptake exceeds the Na^+ loss while electroneutrality is maintained by the uptake of Cl^- (Fig. 8). Due to this extra uptake of osmotically active solutes the cell swells.

In the presence of ouabain the same cells stop extruding Na^+, but still take up Cl^- together with an approximately equivalent amount of K^+. Now the increase in osmolarity is still greater than before, and the cells swell more strongly than in the control.

If instead of ouabain, furosemide is added under otherwise similar conditions, the K^+ uptake is strongly reduced while the Na^+ exit is slightly increased. Now the Na^+ loss exceeds the K^+ uptake and electroneutrality now being maintained by an exit of Cl^-. As can be expected the cell shrinks.

If ouabain and furosemide are added at the same time, the flows of all the mentioned ions are strongly reduced.

The observations with these two inhibitors are best interpreted by the assumption of two pathways for the entry of K^+, the one by the (Na^+, K^+)pump which is inhibitable by ouabain and the other one by the cotransport which is inhibitable by furosemide. These two processes are antagonistic with respect to volume regulation, the first acting against cell swelling, and the second against cell shrinking.

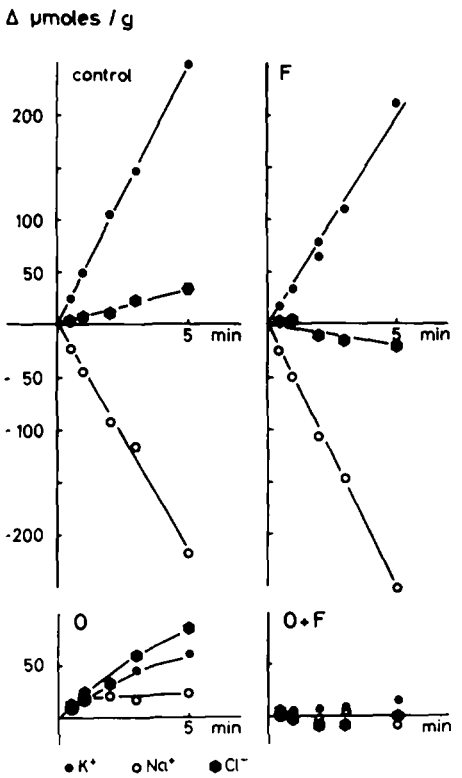

Fig. 8. Influence of ouabain and furosemide on ion movements. The movements of K^+, Na^+ and Cl^- into (positive ordinate) and out of Na^+-rich K^+-depleted cells (in $\Delta\mu$mol/g dry wt.) are plotted vs. incubation time (in Krebs-Ringer phosphate buffer at 37°C) in the presence or absence of the two inhibitors. Upper left: without inhibitor; upper right: with 2 mM furosemide (F); lower left: with 1 mM ouabain (O); right: with both inhibitors (O + F). The associated water movements per g dry wt. after 5 min were: control, +0.17 ml; with furosemide, +0.35 ml; with ouabain, —0.23 ml; with furosemide + ouabain, —0.07 ml.

Discussion

The distribution of Cl^-, previously used as a monitor of the membrane potential based on the assumption that it freely and rapidly reaches equilibrium, has been found not to follow the Nernst equation. If K^+ is added to the medium of K^+-depleted Ehrlich cells Cl^- moves into the cells against its electrochemical potential gradient [3] as if the Cl^- transport into the cell were either primary active or coupled to the flow to other ions. This 'paradoxical' Cl^- flow is always accompanied by a parallel flow of K^+ and both are insensitive towards ouabain but specifically inhibited by furosemide [4,5]. In the present paper evidence has been presented that the furosemide-sensitive flows of Cl^- and K^+ are part of an electrically silent cotransport of four ions: Na^+, K^+ and two Cl^-. This evidence is based on the following observations:

The furosemide-sensitive net fluxes of each Na^+, K^+ and Cl^- are not affected by a change in membrane potential, whether such a change is induced by modulating the Na^+ pump or by depolarizing the membrane of diffusion

potential (Table II). On the other hand, arbitrary variation of any of these fluxes does not appreciably change the potential as indicated by an uninfluenced tetraphenylphosphonium ion distribution (Fig. 2). Hence it appears that each of these ion species passes the barrier by a coupled co- or counter transport, which is either electrically silent or electrically matched by an equivalent leakage in the opposite direction. Such precise matching for each of these flows, however, appears to be highly unlikely. To show that all these flows are truly coupled to each other, the 'degree of coupling', q, based on the principles of irreversible thermodynamics, has been determined for all three individual ion flows involved. By using the difference between the flows in the presence and absence of furosemide, the furosemide-insensitive fractions of the flows which are not affected by alterations in membrane potential are 'filtered out'. Therefore, the resulting furosemide-sensitive flows of Na^+, K^+ and Cl^- can be treated like those of neutral solutes, for which proper q values can be obtained if only the chemical potential differences of the dependent ion species are kept constant. All three q values obtained this way are very close to unity, which appears to indicate that the furosemide-sensitive leakages are very small. Hence, also the stoichiometry derivable from the coupling equations cannot be appreciably distorted by leakages. It follows that all three ion species move simultaneously as a neutral complex with the stoichiometry of 1 Na^+, 1 K^+ and 2 Cl^- (Table III).

Further support to the view of coupling is given by the specificity of cotransport with respect to Na^+, K^+ and Cl^- which can be replaced by Li^+, Rb^+ and Br^-, respectively (Tables IV—VI), but not at all or only poorly so by several other related ions tested. The fact that NO_3^- cannot replace Cl^- strongly argues against the anion exchange system to be involved, since this cannot distinguish between Cl^- and NO_3^- [11,12].

The view of the quarternary cotransport seems to disagree with previous observations of an approximate equivalent between furosemide-sensitive K^+ influx and Cl^- influx [3], whereas on the basis of our present view, a clear excess of Cl^- over the K^+ movement should have to be expected. Since, however, in the previous experiments the ion movements were determined only after 10 min following the stimulation of the cotransport, some of the originally transported Na^+ and Cl^- may already have left the cell via some other route, especially since these cells were highly preloaded with Na^+ salts.

The energetics of the cotransport system are not clear up to now. On the one hand, incongruence between furosemide-sensitive cotransport and conjugated driving force resulting from the electrochemical potential differences of the three ion species concerned seems to indicate primary active transport. This incongruence, however, might be an artifact resulting from an underestimation of the driving force as the consequence of an overestimation of the cytoplasmic activity of the ions, due to compartmentalization of the ions inside the cell, or due to low cytoplasmic activity coefficients for the cotransported ion species. On the other hand, no enhancement of ATP hydrolysis by operation of the cotransport system could be shown, i.e., this reaction does not supply energy for the cotransport process, indicating that the cotransport process might be a secondary active one or, if primary active, energized by a chemical reaction other than the hydrolysis of ATP.

Several other cotransport systems with similar functions have been described or postulated for these and other cells: in Ehrlich cells it was postulated that furosemide inhibits the self-exchange of Na^+ and K^+ [15,16] and of Cl^- [17, 18] (in our view these observations may represent the behavior of the cotransport system under steady-state conditions). For the cornea it has been postulated that furosemide inhibits an active Cl^- pump [19,20]. In red blood cells an (Na^+, K^+)cotransport as target for the furosemide action was shown (e.g., Refs. 21, 22). For several epithelia, e.g., thick ascending limb of the loop of Henle [23], fish intestine, rectal gland of the shark and gallbladder, an Na^+Cl^- transport sensitive to high ceiling diuretics was shown (for review see Ref. 24). It might well be possible that all these cotransport systems are referring to a system similar to that described in this paper, i.e., that all these various systems of cotransport may represent an (Na^+, K^+, Cl^-)cotransport system, looked at from different sides. Though this view has not been proved, we are not aware of anything which clearly contradicts it.

Like other cotransport systems [25,26], also the present one can be related to cellular volume regulation. It has been shown capable of effectively increasing the amount of intracellular osmolar units and of making the cell swell. This cotransport can therefore function as an antagonist of the (Na^+, K^+)pump, which for obvious reasons tends to reduce the amount of osmotically active particles inside the cell and make it shrink. The maintenance of normal cellular volume thus appears to depend on a regulated balance between these two opposing effects. By its stoichiometry the present cotransport seems to serve as an effective osmolar regulator as four ions enter simultaneously by a single step. So the movement of 1 mol of Na^+ across the membrane in the end amounts to transferring 4 osmol from the extracellular to the intracellular medium. medium.

Acknowledgements

The work has been supported by Grants He 102/15 and Ge 424/1 from Deutsche Forschungsgemeinschaft and by USPHS, NIG-Grant ROI GM 26554-01.

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