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THE ROLE OF ION TRANSPORT IN THE REGULATION OF RESPIRATION IN THE EHRLICH MOUSE ASCITES-TUMOR CELL

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SUMMARY

1. When suspensions of Ehrlich mouse ascites-tumor cells are chilled to 2° in media free of K^+, the cells gain large amounts of Na^+ and lose K^+.

2. When re-warmed to 23° the net active extrusion of Na^+ may be totally uncoupled from the net active accumulation of K^+.

3. Cell suspensions transitioned from low temperature to 23° in K^+-free medium respire at rates only one-sixth the rate of cells normally maintained at 23° with 6 mM K^+. Addition of K^+ to the external medium stimulates respiration.

4. The relation between respiration rate and external K^+ follows Michaelis–Menten kinetics. The K_m was 0.43 mM and v_{max} was 1.0 μmole $O_2/10^7$ cells·h.

5. Ouabain inhibited K^+-stimulated respiration at a concentration effective against the active transport of Na^+ and K^+.

6. External K^+ and 2,4-dinitrophenol affect a common source of respiratory intermediate, but act at different sites.

7. The hypothesis is proposed that components of oxidative phosphorylation participate in the transport of Na^+ and K^+ and that intermediates may communicate between cell membrane and mitochondria.

INTRODUCTION

Many cells maintain low intracellular Na^+ and high intracellular K^+ concentrations by systems of active transport which depend on an adequate supply of metabolic energy. The mammalian erythrocyte derives energy for this process exclusively from glycolysis[1,2], while tissues such as adult kidney cortex[3,4], adult liver slices [5],toad bladder[6], rabbit ileum[7], squid axon[8], and gastric mucosa[9], depend to a great extent upon respiration. Other cells like Ehrlich mouse ascites-tumor cells[10], leucocytes[11,12], duck erythrocytes[13], seminal vesicle mucosa[14], fetal liver slices[5], fetal kidney cortex slices[15], frog skin[16], frog skeletal muscle[17], and turtle bladder[18] ,derive energy from both pathways.

Considerable evidence indicates that the linkage between cell metabolism and active transport is mediated in part through ATP and that the active transport mechanism acts as an ATPase[19]. It has been hypothesized also that metabolic energy in the form of an intermediate of oxidative phosphorylation can be utilized directly for active cation transport in liver cells[5] and ascites tumor cells[20].

Since ion transport and respiratory metabolism are coupled, ion transport may be expected to influence respiratory activity. LUNDEGÄRDH[21] had observed this phenomenon when plants actively accumulated anions and coined the term "anion respiration". ZERAHN reported that the extent of Na^+ transport in the frog skin determined the respiration of the tissue (see ref. 22). CHANCE[23] intimated that any cellular process, such as active transport, acting as an ATPase was a potential source of metabolic regulation, basing his conjecture on observations that ADP could regulate respiratory activity[24]. In a series of papers, WHITTAM and his associates have provided considerable evidence that the active transport of Na^+ and K^+ regulate glycolytic activity in human erythrocytes and respiratory activity in slices of rabbit kidney cortex and guinea-pig brain cortex[25].

Recent work of WEINSTEIN AND HEMPLING[26] and HEMPLING[20] has emphasized the importance of ATP for the transport of K^+ and Na^+ in the Ehrlich mouse ascites-tumor cell. This cell lends itself readily to the activation of net fluxes of these ions against electrical and chemical gradients as well as the release of considerable unidirectional flux activity by transition experiments from 2° to 25° (refs. 27, 28). Preincubation at 2° for as short a time as 2 h in media free of K^+ is sufficient to convert cells with high K^+ and low Na^+ to cells with high Na^+ and low K^+. Reincubation at 25° is all that is needed to activate the energy-requiring extrusion of Na^+ and reaccumulation of K^+, drawing on endogenous respiration, provided that external K^+ is made available[10]. In this system then we have investigated the extent to which external K^+ may regulate respiration in the ascites-tumor cell, recognizing that external K^+ is serving as the means to initiate ion transport.

METHODS

Preparation of cell suspensions

Experiments were performed with Ehrlich mouse ascites-tumor cells that were maintained in Swiss white mice by weekly transplantation. Tumor-bearing animals with growths between 9 and 14 days were used. Cell suspensions were removed from the peritoneal cavity by aspiration and transferred to a large volume of ice-cold Tris-buffered saline (9 g NaCl, 1.21 g Tris, 0.7 ml conc. HCl, to 1 l with glass-distilled water), which contained 305 milli-osmoles/l at pH 7.3. Since the cell suspensions were usually bloody, red cells and leucocytes were removed by low-speed centrifugation and resuspension in fresh ice-cold Tris–NaCl. Two to four washes were sufficient to remove blood elements. A sample of the concentrated cell suspension was counted in a Neubauer-Levy hemocytometer. The cell suspension containing $2–4 \cdot 10^7$ cells/ml was stoppered and placed in an ice-bath to permit the loss of K^+ and the gain of Na^+ (refs. 10, 27). In some experiments a portion of the aspirated cell suspension was washed and incubated at room temperature in K^+–Na^+ Ringer's solution[27] in order to maintain normal internal K^+ and Na^+ content.

Measurement of oxygen consumption

Oxygen consumption was measured polarigraphically with a Clark oxygen electrode (Yellow Springs Instrument Co., Yellow Springs, Ohio). The output was fed into a Grass Model 5 polygraph (Grass Instruments, Quincy, Mass.) and measurements recorded continuously. The electrode was calibrated with 10 ml of Tris–NaCl equilibrated with 10% oxygen–90% nitrogen gas (v/v) and 10 ml equilibrated with room air. 1 ml of cell suspension ($2–4 \cdot 10^7$ cells) was washed in cold Tris–NaCl immediately before the measurement of oxygen consumption, and added to 9.0 ml of Tris–NaCl. Oxygen consumption expressed as μmoles/10^7 cells·h, was measured under oxygen tensions between room air and 10% oxygen. A magnetic stirrer kept the suspension well mixed. Temperatures ranged from 20° to 23°. It is important to wash the cells to reduce the external K^+ to a minimum. Further, the concentration of cells must be kept low since the exposure of cells to K^+-free medium results in a rapid loss of cell K^+ which when present in the medium can stimulate respiration. For example, in one experiment $2.2 \cdot 10^7$ cells were added to 10 ml of Tris–NaCl buffer in the respiration chamber. Initially the external K^+ was 0.035 mM but rose to 0.22 mM in 30 min. Respiration increased 4-fold over the 30-min interval. This same stimulation of respiration would also occur if the K^+ were added initially to a final concentration of 0.22 mM. This observation demonstrated that cellular K^+ upon reaching the external medium was capable of stimulating respiration.

Additions of K^+, 2,4-dinitrophenol and oligomycin

A number of studies required the measurement of oxygen consumption continuously while increments of K^+ or metabolic inhibitors were added to the cell suspension in the respiration chamber. The usual technique was to measure oxygen consumption for 5 min. While continuing to record, aliquots of 0.01–0.05 ml of test solution were injected into 10 ml of cell suspension within the chamber. Mixing and electrode equilibration were complete in 15 sec or less. The response of the cells was followed for an additional 5 min or until a constant rate of oxygen consumption was attained. This procedure was repeated with each increment added. Cell suspensions were so dilute that oxygen consumption could be followed continuously for 1 h without reducing the tension within the suspension below 10%. Oligomycin was obtained from the Wisconsin Alumni Research Institute and 2,4-dinitrophenol from Eastman Kodak (Rochester, N.Y.).

Electrolyte measurements concomitant with oxygen consumption

It was often desirable to follow changes in electrolyte content of the cells as increments of K^+ were added to the respiration chamber. To do so, a comparable system on a larger scale was employed. Thus 10 ml of cell suspension, washed in cold Tris–NaCl, were added to 90 ml of Tris–NaCl in a 500-ml flask at room temperature. Since the cell suspension was dilute and the large flask provided a thin layer of suspension with a large surface area, adequate oxygenation with room air was assured.

During the measurement of respiration when no extra K^+ was added, two aliquots of 10 ml each were removed. Subsequently, whenever an aliquot of KCl was injected into the respiration chamber, a comparable aliquot was added to the erlenmeyer flask. Care was taken to assure that the final concentration of external K^+ in each container was identical. Immediately upon the removal of the two

samples, the aliquots were centrifuged at maximal speed in the International centrifuge for 30 sec. The supernatants were quickly decanted and saved for analysis. One sample was washed two more times with ice-cold isosmotic choline chloride and saved for K^+ and Na^+ analysis of cells. To the second sample was added 0.5 ml of 6% (v/v) perchloric acid. Electrolyte analysis was done by flame photometry.

Analysis of ATP was carried out enzymatically with glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.12) and 3-phosphoglyceric acid kinase (EC 2.7.2.3) (ref. 26) on the perchloric acid extract.

RESULTS

Effect of external K^+ on cellular respiration

To determine how the respiration of the ascites-tumor cells depended on the

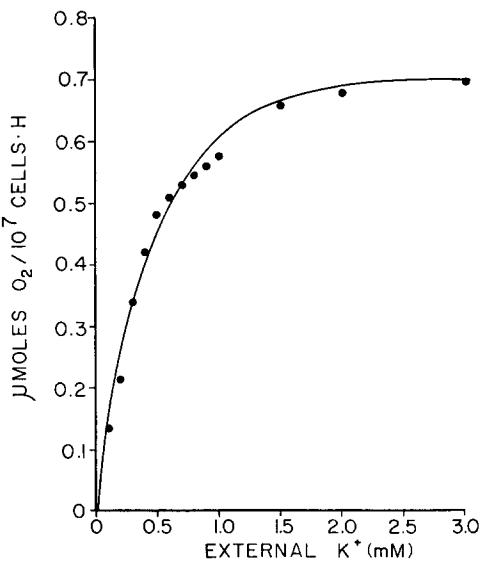

Fig. 1. The effect of increasing external K^+ concentration on respiration. Cells were incubated at 2° in Tris–NaCl for 90 min. After washing, the cells were warmed to 23° and the rate of oxygen consumption was measured as increments of K^+ were added.

external K^+ concentration, the cells were followed continuously as successive increments of K^+ were added. The variation of the rate of respiration with external K^+ is shown in Fig. 1. Between 0.10 mM and 2.0 mM K^+ stimulated respiration with a half-maximum at 0.40 mM. The K_m was evaluated from a plot of $[K^+_{ext.}]$/respiratory rate *versus* $[K^+_{ext.}]$ (ref. 29) and in five separate experiments was equal to 0.43 ± 0.098 mM (S.E.).

Oxygen consumption and Na^+ and K^+ transport

The effect of external K^+ on Na^+ and K^+ transport and on oxygen consumption is illustrated in Fig. 2. When the cells were transferred to 23°, at time zero, Na^+ was extruded from the cell almost immediately, at an external K^+ concentration between 0.075 mM and 0.322 mM. In contrast, at these low external K^+ concentrations, K^+

was lost from the cell. At this same time densimeter measurements[30] indicated a maximal decrease in cell water of 25%. Taking this into account, the Na^+ concentration was initially 85–87 mequiv per kg cell water and continued to fall while the K^+ concentration was 70–73 mequiv per kg cell water. Since external Na^+ was 155 mequiv per l water and $[Cl^-_i]/[Cl^-_0]$ was less than 1, indicative of a potential difference with inside negative to outside, the movement of Na^+ was against an electrochemical gradient throughout the entire course of the experiment. On the other hand, K^+

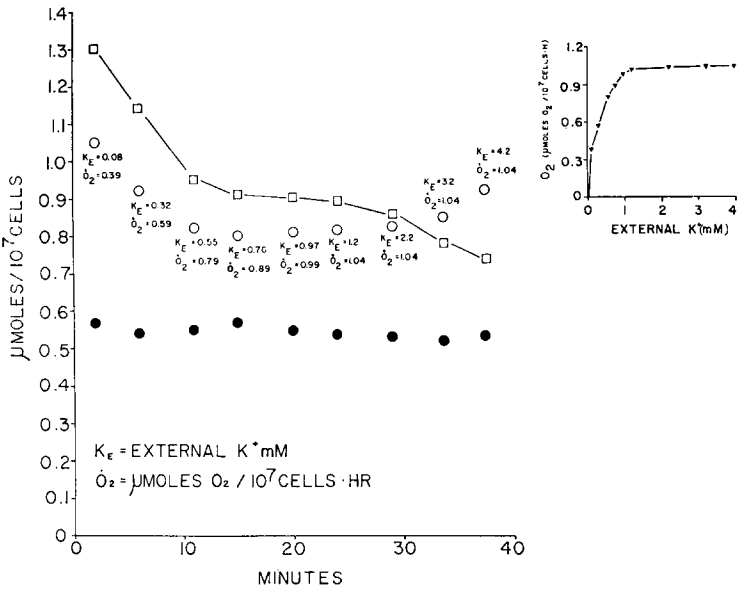

Fig. 2. The effect of increasing external K^+ concentration on respiration (insert at upper right); net Na^+ flux (□—□); net K^+ flux (○—○), and ATP content × 10 (●—●). Respiration, Na^+, K^+ and ATP content were simultaneously measured on the same population of cells. (See text for details.)

left the cell down a concentration gradient during the early periods of the experiment. Note that net Na^+ extrusion was not balanced by net K^+ accumulation. Rather, at external K^+ concentrations between 0.075 mM and 0.55 mM both Na^+ and K^+ were leaving the cell with a concomitant loss of water. However, when the external K^+ concentration was increased to 1.2 mM, K^+ changed direction and entered the cell against an electrochemical gradient. Na^+ continued to move against an electrochemical gradient, although not coupled to K^+ in a 1:1 ratio. Thus over the range of external K^+ concentrations tested, the net outward movement of Na^+ and the net inward movement of K^+ could vary from complete uncoupling to ratios as high as 2 Na^+ to 1 K^+.

Net flux of Na^+ was highest at low external K^+ and fell progressively with time as the cell approached the steady state. The addition of increments of K^+ did not produce any transient changes in the net flux of Na^+. Nevertheless, respiration increased with an increase in external K^+, with the same pattern as Fig. 1. Therefore no positive correlation between net active extrusion of Na^+ at any particular time

and the stimulation of respiration at that same time was demonstrable. Similarly, even though K^+ left the cell, respiration was stimulated. Further, no extra stimulation occurred when a net loss of K^+ was converted into a net gain, at 1.2 mM external K^+.

It is important to emphasize, however, that a relation between the rate of respiration and the unidirectional active efflux of Na^+ or unidirectional active influx of K^+ is not ruled out by the results summarized in Fig. 2. This relationship can only be tested with simultaneous measurements of isotopic fluxes and respiration, and the selection of some operational definition to fragment the unidirectional fluxes into active and passive components. It must be further emphasized that the absence of coupling between the net movements of Na^+ and K^+ does not rule out the possibility of coupling between the unidirectional fluxes of Na^+ and K^+, again after defining the fluxes as active and exchange or leak components by some operational definition.

What is significant, however, is that if Na^+ and K^+ can be uncoupled in their net movements and a net loss of Na^+ occurs against an electrochemical gradient, then one must look for some other cation in the medium for exchange or hypothesize an electrogenic phenomenon, at least on a gross level.

The ATP content of the cells was measured to determine whether a fall in ATP preceded the stimulation of respiration by external K^+. A linear relationship between ADP concentration and the rate of oxygen consumption had been described by CHANCE AND WILLIAMS[24] in mitochondria and more recently by BLOND AND WHITTAM[31] in kidney cortex homogenates. As evidenced in Fig. 2, the ATP content of the cells did not change during the course of the experiment. However, we cannot rule out a stimulation of respiration mediated by ADP because the levels of of ADP necessary to stimulate oxygen consumption might have been too low to detect as a decrement in cell ATP content. For example, the maximal rate of oxygen consumption in response to added K^+ was 1.04 μmoles/10^7 cells·h or 5.7 μl per mg dry wt. per h (based upon 4 mg dry wt. per 10^7 cells[27]). Using the data presented by BLOND AND WHITTAM, 0.06 mM ADP would be required to produce this rate of respiration. Since 0.06 mM ADP corresponds to $0.8 \cdot 10^{-3}$ μmole ADP per 10^7 cells and the mean ATP content was $56 \cdot 10^{-3}$ μmoles per 10^7 cells, less than 2% of the cellular ATP would have been needed to provide the ADP to stimulate respiration. This decrement in ATP content falls within the experimental error of the enzymatic method used.

Effect of ouabain on K^+-stimulated respiration

Since it was possible that external K^+ could stimulate respiration because the ion was involved directly or indirectly in transport processes, the effect of ouabain on K^+-stimulated respiration was investigated. The cardiac glycosides have been considered by many workers to interfere with the mediated transport of a number of substances[32]. Its action on the tumor cell was reported by MAIZELS, REMINGTON AND TRUSCOE[10] and by BITTNER AND HEINZ[33].

Fig. 3 summarizes the effect of external K^+ on the respiration of cells, previously kept at 2° in Tris–NaCl for 2 h and then transferred to a Tris–NaCl solution at 23° containing ouabain at $4.5 \cdot 10^{-4}$ M (Sigma Chemical Co., St. Louis, Mo.). Control cells were in Tris–NaCl. All cells were washed quickly in ice-cold Tris–NaCl

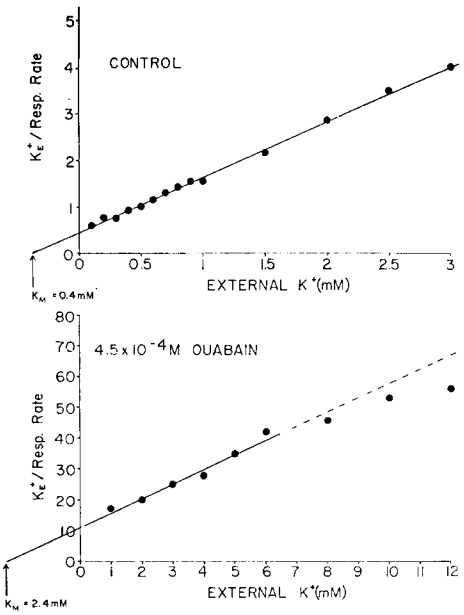

Fig. 3. The evaluation of the K_m for K^+-stimulated respiration in the presence and absence of ouabain. Fig. 3a (upper) represents the effect of external K^+ on the respiratory rate under control conditions (see Fig. 1). Fig. 3b (lower) is the effect of external K^+ on the respiratory rate in the presence of $4.5 \cdot 10^{-4}$ M ouabain. The same population of cells was used for both experiments.

before the transfer to the respiration flask to reduce external K^+ to a minimum. External K^+ concentrations were then increased in a step-wise fashion. Although under control conditions (Fig. 3a) a plot of $[K^+_{ext.}]$/respiratory rate *versus* $[K^+_{ext.}]$ produced a straight line compatible with Michaelis–Menten kinetics, the response to ouabain was complex (Fig. 3b). With external K^+ between zero and 5 mM, comparable to controls, ouabain increased the apparent K_m from 0.50 mM to 2.4 mM. Likewise in the same range, v_{max} was lowered from 1.06 μmoles $O_2/10^7$ cells·h to 0.20 μmole $O_2/10^7$ cells·h. Note also that the control v_{max} compared well with that respiratory rate observed when the cells were uncoupled with 2,4-dinitrophenol. In the presence of ouabain, respiratory rates were not zero but rather were comparable to values observed at very low external K^+ concentrations.

Fig. 4 indicates that under comparable experimental conditions, ouabain at $4.5 \cdot 10^{-4}$ M inhibited the net extrusion of Na^+ and accumulation of K^+ at an external K^+ concentration of 3 mM. The evidence would indicate, therefore, that the effect of external K^+ to stimulate respiration may involve its participation in some process sensitive to ouabain.

The effect of 2,4-dinitrophenol and oligomycin on K^+-stimulated respiration

Since it was possible that external K^+ was acting either indirectly as an uncoupler of oxidative phosphorylation or directly as an ATPase, its effect on respiration was compared to that of 2,4–dinitrophenol. The question might be asked: "Could 2,4-dinitrophenol stimulate respiration further after it was already stimulated by increments of external K^+"? In these experiments, 1 ml of cell suspension,

equilibrated at zero to 2° for about an hour in Tris–NaCl solution and after a preliminary wash, was transferred to the respiration chamber containing 9.0 ml of the same solution, at 20–23°. After approx. 3 min to establish a control rate of respiration, K^+ was added to produce the lowest external concentration reported in Table I (0.1 mM). After recording the stimulation which occurred, 0.05 ml of 2,4-dinitrophenol, sufficient to produce a final concentration of $5 \cdot 10^{-5}$ M, was added to assess whether further respiratory activity could be obtained. After recording for sufficient time to establish any change in respiratory rate, the run was terminated. A new run was then begun in which the final concentration of external K^+ was raised prior to the addition of the same dose ($5 \cdot 10^{-5}$ M) of 2,4-dinitrophenol. The results of five successive runs on the same population of cells is summarized in Table I. Included for comparison are the respiratory rates of cell suspensions from a separate, representative experiment on a different population of cells in K^+–Na^+ Ringer's solution without any prior history of cooling or low K^+.

TABLE I

THE EFFECT OF 2,4-DINITROPHENOL ON K^+-STIMULATED RESPIRATION

[$K^+_{ext.}$] (mM)	*Respiration rate (μmoles/10^7 cells · h)*	*2,4-Dinitrophenol (M)*	*Respiration rate (μmoles/10^7 cells · h)*	*2,4-Dinitrophenol/ [$K^+_{ext.}$]*
0.1	0.187	$5 \cdot 10^{-5}$	1.00	5.35
0.5	0.374	$5 \cdot 10^{-5}$	1.06	2.84
1.0	0.500	$5 \cdot 10^{-5}$	1.06	2.12
2.0	1.12	$5 \cdot 10^{-5}$	1.18	1.05
4.0	1.18	$5 \cdot 10^{-5}$	1.20	1.02
Control	0.333	$+5 \cdot 10^{-5}$ M 2,4-dinitrophenol	0.525	1.59
Control	0.318	+4 mM KCl	0.337	1.06

The results in Table I indicate that respiratory activity may be stimulated to a constant level either by successive additions of external K^+ or a single dose of 2,4-dinitrophenol. Thus, for example, at 0.1 mM, external K^+ concentration was not sufficient to elicit maximal respiratory activity so that the addition of 2,4-dinitrophenol increased oxygen consumption 5-fold. On the other hand, at 4.0 mM external K^+ almost all of the respiratory activity was released and little more remained to be elicited by the addition of 2,4-dinitrophenol. Therefore, the ratio 2,4-dinitrophenol rate/external K^+ rate was only 1.02.

To determine whether oligomycin would serve to differentiate the stimulation produced by 2,4-dinitrophenol from that by external K^+, a population of cells was kept at 0–2° in Tris–NaCl solution and then after brief washing, was transferred to the same solution at 23° for measurements of respiration. Small amounts of external K^+ (0.09–0.25 mM) were present at the start of the measurement. In Table II (a) the characteristic stimulation of respiration by external K^+ is recorded. In Table II (b), stimulation of respiration by K^+ was observed. Then when oligomycin was added, inhibition occurred. If 2,4-dinitrophenol was added on top of oligomycin,

this inhibition was reversed and the resulting respiration rate was higher than that recorded prior to the addition of any K^+. If a very low dose of oligomycin was added before K^+ (Table II (c)), then after a short period of stimulation, inhibition set in and continued after the addition of K^+. Nevertheless, 2,4-dinitrophenol could reverse the inhibition partially. Finally, in Table II (d) evidence is presented to show that 2,4-dinitrophenol could relieve the inhibition produced by oligomycin without prior addition of 4.0 mM K^+. We may conclude therefore, that 2,4-dinitrophenol and external K^+, although equally effective in stimulating respiration (Table I), behave differently toward an oligomycin-inhibited system.

TABLE II

THE EFFECT OF K^+, OLIGOMYCIN AND 2,4-DINITROPHENOL ON RESPIRATION

	[$K^+_{ext.}$] (mM)	*Respiration rate (μmole/10^7 cells · h)*	*Addition: total amount or concentration in respiratory chamber*	*Respiration rate (μmole/10^7 cells · h)*
(a)	0.09	0.162	4 mM KCl	0.486
(b)	0.25	0.262	4 mM KCl	0.525
		then	2.5 μg oligomycin	0.061
		then	$5 \cdot 10^{-5}$ M 2,4-dinitrophenol	0.324
(c)	0.25	0.242	0.5 μg oligomycin	0.324 → 0.14
		then	4 mM KCl	0.101
		then	$5 \cdot 10^{-5}$ M 2,4-dinitrophenol	0.252
(d)	0.25	0.229	2.5 μg oligomycin	0
		then	$5 \cdot 10^{-5}$ M 2,4-dinitrophenol	0.250

DISCUSSION

When mouse ascites-tumor cells were cooled to 2° in normal K^+–Na^+ Ringer's solution, they lost K^+ and gained Na^+. Significant losses of K^+ may occur within 30 min and more than half of the cell K^+ may exchange with Na^+ in 2 h. These times are short when compared to the overnight low-temperature incubation required for muscle[17] or the days to weeks required for human erythrocytes[1] to achieve the same changes.

When the cells were rewarmed to 23°, Na^+ was pumped out and K^+ pumped in, against electrochemical gradients[10,27]. The use of K^+-free solutions for preincubation at 2° and later for the first phase of temperature transition to 23° enabled us to study the relationship between net Na^+ fluxes and net K^+ fluxes, and the relation of the net fluxes to the extracellular K^+ concentration. Fig. 2 shows that at low extracellular K^+ concentrations, below 1 mM, there was a net efflux of Na^+ against an electrochemical gradient in the face of a passive loss of K^+. However, when the external K^+ concentration was increased to 1.2 mM the direction of net K^+ movement was reversed and the cells accumulated K^+. Na^+ extrusion continued although it was not tightly linked to K^+ accumulation. We may conclude from these results that the net active components of Na^+ and K^+ transport are not coupled obligatorily and, indeed, are uncoupled completely at low external K^+ concentrations.

Warming the cells from 2° to 23°, in addition to promoting the characteristic

asymmetrical distribution of Na^+ and K^+, results in a large stimulation of the unidirectional fluxes of these ions[27,28]. The unidirectional fluxes may exceed net fluxes by 5-fold or more and are not associated with detectable changes in Na^+ and K^+ content.

The hypothesis has been proposed[28] that at low temperatures the transport system is converted from a form capable of net K^+ gain to one lacking this asymmetric specificity and capable only of exchange. The existence of the K^+ exchange system is revealed upon rewarming as an accelerated exchange flux which far exceeds concomitant net flux. With the passage of time, however, the exchange system reverts almost entirely to the pump system and a balance is reached between unidirectional influx and efflux which is characteristic of steady-state conditions at room temperature.

It was suspected that changes which occurred in the transport system as a consequence of low to room temperature transition might be correlated with changes in energy metabolism. The ATP content of the cells did not change after the transition from 2° to 23° (Fig. 2), which suggested that an adequate supply of energy was derived from respiration. Since we had observed that the net fluxes of Na^+ and K^+ were sensitive to the external K^+ concentration, the effect of this ion on respiration was examined.

When the external K^+ concentration was kept below 0.1 mM, oxygen consumption was far less than that of cells in the steady state with the normal concentration of K^+ (4 mM) in the medium. However, external K^+ concentrations as low as 0.1 mM or greater were stimulatory and the respiratory response to increasing concentrations of external K^+ followed the kinetics of a saturable system with a K_m of 0.43 mM and a $v_{max} = 1.0$ μmole $O_2/10^7$ cells·h (Fig. 3a).

The observation that the cardiac glycoside, ouabain, effectively inhibited K^+-stimulated respiration (Fig. 3b) and at the same time inhibited K^+ accumulation

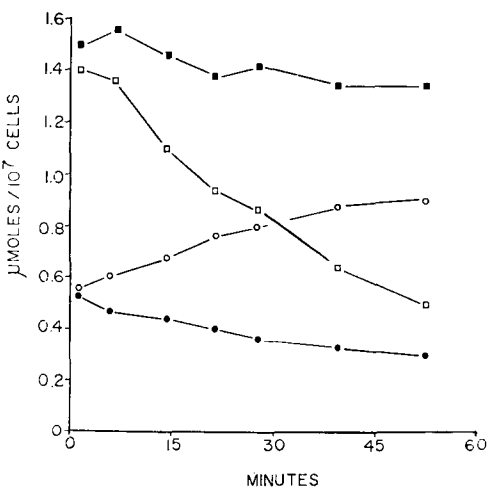

Fig. 4. The effect of $4.5 \cdot 10^{-4}$ M ouabain on net Na^+ and net K^+ flux. Cells were incubated for 90 min at 2° in Tris–NaCl. At zero time the cell suspension was warmed to 23° in the presence (dark figures) or absence (open figures) of ouabain. Na^+ content is represented by squares, K^+ content by circles.

(Fig. 4) favors the view that a linkage exists between K^+-stimulated respiration and transport. Although relatively high concentrations of ouabain ($4.5 \cdot 10^{-4}$ M) were required, it is well known that rodent cells are highly resistant to the drug and this level must be used to demonstrate inhibition of transport processes at 23° (refs. 32, 33).

Granting that K^+-stimulated respiration and transport are intimately associated, the question that must be answered is: "By what mechanism does external K^+ stimulate tumor cell respiration, and in so doing make energy available to the transport system"? The answer to this question may be found in the interrelationship between the enhanced respiratory effects of 2,4-dinitrophenol and external K^+ and the inhibitory action of oligomycin.

It is generally held that 2,4-dinitrophenol leads to loss of respiratory control because it dissociates a high-energy compound formed either by the reaction of substrate with intermediate ($C \sim I$) or between two unidentified intermediates ($I \sim X$), releasing the free energy as heat and allowing the intermediates to participate once again in the transfer of electrons from substrate to oxygen[34]. Oligomycin, on the other hand, inhibits oxygen consumption by blocking the interaction of $I \sim X$ with P_i to form $X \sim P$.

The data in Table I provided evidence that external potassium and 2,4-dinitrophenol affected a common source of respiratory intermediate so that every increment of external K^+ produced an increment in respiration and 2,4-dinitrophenol contributed less to the total increase in respiration. However, with additions of external K^+, the cellular ATP content remained normal (Fig. 2), while we know that 2,4-dinitrophenol depresses the ATP content of the tumor cell[20]. It required oligomycin (Table II), however, to demonstrate unequivocally that external K^+ and 2,4-dinitrophenol do not share the same site of action. Since 2,4-dinitrophenol relieved the inhibition of respiration produced by oligomycin, but external K^+ did not, then external K^+ and 2,4-dinitrophenol have to be acting on two different forms of respiratory intermediate. Thus, 2,4-dinitrophenol acts above the oligomycin-sensitive site and external K^+ below it.

POST, SEN AND ROSENTHAL[35], SKOU[36], and WHITTAM[37], have evidence that external K^+ promotes the breakdown of a phosphorylated intermediate formed from ATP and membrane fragments. From such evidence we might speculate that the intermediate upon which K^+ acts in the tumor cell membrane is some such constituent. Since many investigators[38] feel that oligomycin blocks between $I \sim X$ and $X \sim P$, this choice of intermediate is consistent with the scheme that the intermediate be below oligomycin in the chain of oxidative phosphorylation.

The proposed scheme based upon mitochondrial function depends upon our willingness to accept the possibility that components of the oxidative phosphorylation system are part of the membrane of the tumor cell and that intermediates may communicate between cell membrane and mitochondria. Several lines of evidence suggest that such a hypothesis might be reasonable. First, stimulation of respiration was produced by an external ion and the stimulation was not secondary to changes produced in the internal environment. We have not been able to correlate K^+-stimulated respiration with any particular level of intracellular Na^+ or K^+. In fact, we have observed the phenomenon when cells were low or high in K^+ subsequent to transition. Second, the effect of 2,4-dinitrophenol and various levels of external K^+

on cell respiration suggests that a common pool of intermediate is involved, accessible both to external K^+ and 2,4-dinitrophenol.

Third, ouabain was effective in depressing K^+-stimulated respiration at a concentration which effectively blocked net transport of Na^+ and K^+. For several other systems, the site of action of the cardiac glycoside has been localized at the external surface of the cell where it competes with K^+ for carrier sites[32]. Taken together, the evidence suggests a close relation between classical mitochondrial function and external transport sites .

Perhaps it appears puzzling to draw on a scheme for mitochondrial membrane function to explain a phenomenon elicited by an ion external to the cell. Whether we choose to think of intermediates shuttling between mitochondrion and membrane transport sites or prefer a patch in the plasma membrane with mitochondrial properties, we are faced with the fact that external K^+ is acting upon a pool of high-energy intermediates accessible to 2,4-dinitrophenol, but separated from it through an oligomycin-sensitive site.

Finally, now that we have evidence that considerable energy turnover occurs when we release extensive unidirectional flux activity in a transition experiment, the next step will be to examine the potential coupling between these two processes.

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