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ON THE COUPLING OF RESPIRATION TO CATION TRANSPORT IN SLICES OF RAT LIVER

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SUMMARY

1. Rat-liver slices utilizing endogenous substrate respired at a rate of 9.2 μl O_2 per mg fat-free dry wt. per h, in a medium containing 5 mM K^+ and 161 mM Na^+. Inhibition of the active transport of Na^+ and K^+ by ouabain, reduced the rate of respiration of these slices by 2.4 μl O_2 per mg per h (26 % inhibition).

2. In a medium containing 75 mM K^+ and 91 mM Na^+, the control rate of respiration was 10.1 μl O_2 per mg fat-free dry wt. per h; ouabain lowered this by only 0.8 μl per mg per h (8 % inhibition). It is suggested that the latter portion of the total O_2 uptake is coupled to the energy requirements of ion transport ('transport-coupled respiration'). The additional inhibition caused by ouabain in the 5 mM K^+ medium appears to be due to changes in the oxidative metabolism resulting from the altered cation composition of the intracellular environment ('cation-sensitive respiration').

3. Malonate and ethanol, at concentrations which are known to inhibit citric acid cycle activity, have little effect on ion transport.

4. In the presence of ethanol or malonate, inhibition of ion transport (by ouabain, or K^+-free medium) inhibits respiration only by an amount similar to the transport-coupled respiration (0.65–1.16 μl per mg per h). The cation-sensitive portion of the respiration appears to be due to the provision of reducing equivalents by the citric acid cycle.

5. Approximate calculations are made of the amount of energy made available by the transport-coupled respiration, and this is compared to an estimate of the minimal energy requirement of the Na^+ transport.

INTRODUCTION

In liver slices[1], as in a variety of other tissue preparations[2-7,36], the rate of respiration is reduced when the active transport of Na^+ and K^+ is rendered inactive. This has been interpreted as being due to a coupling of respiration to the transport

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process, *via* the availability of phosphate acceptor, *i.e.* a transition of the mitochondria within the cells from State 3 towards State 4 (refs. 4 and 8); alterations in redox states of respiratory pigments which are consistent with this view, have been observed in tissue slices[9,10]. The difference between the rates of respiration when transport is active and inactive (the so-called supra-basal O_2 consumption) has frequently been taken, in the work quoted above, to represent the energy requirement of the transport process.

The validity of this last conclusion rests on two assumptions: (i) that the whole of the respiration required to support ion transport is specifically coupled to this process, and is not available to support other energy-requiring processes of the cell when the transport mechanism is inhibited; (ii) that the changes in the cationic composition of the cytosol, which necessarily accompany transport inhibition, do not themselves affect oxidative metabolism. At present, there appears to be no evidence bearing on the first of these assumptions. With regard to the second, however, it is known that monovalent cations can affect the oxidative metabolism of isolated mitochondria[11,12], and observations of the oxidoreduction state of nicotinamide nucleotides in liver slices have also given indications of a direct effect of K^+ on substrate-level oxidation reactions in whole cells[9]. This latter effect, unlike the changes mentioned above which can be attributed to a State 4–State 3 transition of the slice mitochondria, was independent of the cation-transport process.

The work here described, shows that a considerable part of the respiratory effect associated with changing transport activity, in liver slices, can be accounted for by alteration of the cation composition of the intracellular environment. Consequently, the amount of respiration which can be considered to be tightly coupled to the phosphate acceptor which is released by the transport mechanism, is much less than was previously supposed[1], and it becomes necessary to consider whether this fraction of the respiration can provide sufficient energy to support the transporting activity of the cells.

METHODS

Liver slices from male, albino rats (200–250 g) were cut by hand and incubated, first, for 90 min at 1° in 15 ml of phosphate-buffered medium which was free of added substrates. In most experiments, the slices were transferred once to a fresh portion of medium, after the first 20 min. In experiments with K^+-free medium, however, they were transferred to fresh medium every 15 min throughout the incubation at 1°, in order to remove as much endogenous K^+ as possible. During the last 10 min of incubation at 1°, the slices were distributed over eight to twelve Warburg manometric vessels, each containing 3 ml of medium, with appropriate test agents. The centre well contained 0.2 ml of 20% KOH. The manometers were gassed for 5 min with O_2, and were then transferred to a bath maintained at 38°. After 10 min equilibration, manometric readings of O_2 uptake were started. Incubation at 38° was usually continued for a total of 70 min. At the end of the experiment, the slices were collected, blotted, and analysed as described previously[1].

Results are expressed as mean $\pm$ S.E. (number of observations), and are related to the fat-free solids of the tissue (fat-free dry wt.). Tests for statistical significance of differences were done by Student's *t* test.

RESULTS

Effect of cations on respiration when transport is inhibited

ELSHOVE AND VAN ROSSUM[1] showed that the concentration of strophanthin K which gave maximal inhibition of K^+ accumulation and Na^+ extrusion, also reduced the rate of respiration of liver slices by about 35 %. A similar result has now been obtained with ouabain, a concentration of 0.5 mM being sufficient to give maximal inhibition of transport and respiration. A typical result is seen in Table I, where ouabain reduced the rate of respiration by a mean of 27 % in the 'basic' medium (containing 5 mM K^+). During incubation at 38° in this medium, the cation content of the control slices (without ouabain) returned towards the values found *in vivo*, but that of slices treated with ouabain remained abnormal throughout. In order to see if this abnormal cation composition could itself contribute to the lower rate of respiration, experiments were done in which the transport mechanism was inhibited by ouabain, but the ionic composition of the cells was altered by substituting K^+ for varying amounts of Na^+ in the medium. Table I shows that the medium containing 75 mM K^+ permitted the slices to retain a similar composition, throughout incubation with ouabain, to the final composition of control slices that were allowed to transport cations normally in the basic medium. At the onset of incubation at 38°, the K^+ content of the slices incubated with 75 mM K^+ *plus* ouabain was, in fact, rather higher than the final content of the controls (302 mmoles per kg fat-free dry wt., compared to 236 mmoles/kg), while the Na^+ contents were nearly equal (523 compared to 457 mmoles per kg fat-free dry wt.).

TABLE I

EFFECT OF VARYING MEDIUM Na^+ AND K^+ ON THE RESPONSE OF LIVER SLICES TO OUABAIN

The medium composition was varied by substituting increasing amounts of K^+ for Na^+ in the basic medium (see Fig. 1). The slices were incubated for 20 min at 1° in basic medium before being transferred to the appropriate substituted medium for the remainder of the cold and warm incubation periods. ΔK^+ and ΔNa^+ represent the changes in tissue composition during incubation at 38°. Numbers of observations for Q_{O_2} are the same as for the corresponding K^+ values.

Medium concn. (mM)		*Incubation at 38° (min)*				
K^+	Na^+	*0 (90 min at 1°)*	*70 (+0.75 mM ouabain)*	*70*	*70 (+0.75 mM ouabain)*	*70*
		K^+ content (mmoles/kg fat-free dry wt.)			*ΔK^+*	*ΔK^+*
5.0	161.5	82 ± 6 (7)	118 ± 9 (13)	236 ± 21 (14)	+ 42 ± 9	+170 ± 11
25.0	141.5	147 ± 12 (7)	225 ± 18 (10)	287 ± 13 (10)	+ 71 ± 17	+133 ± 18
75.0	91.5	302 ± 14 (7)	353 ± 15 (9)	398 ± 13 (11)	+ 57 ± 6	+100 ± 7
		Na^+ content (mmoles/kg fat-free dry wt.)			*ΔNa^+*	*ΔNa^+*
5.0	161.5	787 ± 51 (7)	705 ± 79 (10)	457 ± 62 (9)	− 90 ± 73	−343 ± 36
25.0	141.5	714 ± 54 (6)	569 ± 71 (8)	397 ± 89 (5)	−164 ± 49	−287 ± 44
75.0	91.5	523 ± 33 (7)	489 ± 50 (10)	240 ± 58 (8)	− 25 ± 58	−260 ± 36
		Q_{O_2} (µl/mg fat-free dry wt. per h)				
5.0	161.5	—	6.81 ± 0.42	9.23 ± 0.45	—	—
25.0	141.5	—	7.71 ± 0.56	9.07 ± 0.70	—	—
75.0	91.5	—	9.32 ± 0.45	10.09 ± 0.59	—	—

The changes in medium composition had little effect on the respiration of slices incubated without ouabain (Table I). However, the respiration of slices incubated in the presence of ouabain increased from 6.8 μl per mg fat-free dry wt. per h in medium containing 5 mM K^+, to 9.3 μl per mg per h in the medium with 75 mM K^+. While this work was in progress, RANG AND RITCHIE[37] published similar experiments on the basal O_2 uptake of non-myelinated nerve fibres. They also found a stimulation of O_2 uptake upon increasing the external K^+ concentration in the presence of ouabain, but their results differ from the present ones in that external K^+ affected the rate of respiration of the fibres even in the absence of ouabain. Two explanations may be considered for the partial release of the ouabain-induced respiratory inhibition by high K^+–low Na^+ media: (i) It has been reported that high extracellular concentrations of K^+ reverse the inhibition of ion transport by ouabain[13], and such an effect should lead to activation of the respiration coupled to the transport process. However, Table I shows that increasing medium K^+ caused no significant increase of the net movements of either Na^+ or K^+ in the liver slices incubated with ouabain. The possibility that a release of transport inhibition occurred, but was obscured by a greater passive, 'backward' leakage of the ions in the presence of ouabain cannot, however, be excluded. (ii) A high K^+ and low Na^+ concentration in the intracellular environment may itself be required for the maximal activity of certain oxidative reactions of the cells, independently of the energy requirements of ion transport. This possibility is supported by the resistance of the ouabain-insensitive respiration of slices incubated in low K^+ media to inhibition by malonate (see below).

As the respiration in the presence of ouabain was stimulated by the high K^+–low Na^+ media, so the ouabain-sensitive portion of the respiration declined, from 2.42 $\pm$ 0.38 (10) μl per mg fat-free dry wt. per h in the basic medium, to only 0.78 $\pm$ 0.47 (10) μl per mg per h in the medium containing 75 mM K^+ (Table I). If it is accepted that the high K^+ concentration did not cause a release of the inhibition of transport by ouabain, then only that component of the ouabain-sensitive respiration that persists in the high K^+ medium (*i.e.* 0.78 μl per mg per h) may be considered to be tightly coupled to the energy requirements of the transport process. Since the net extrusion of Na^+ was similar in all ouabain-free media, it is probable that the same amount of O_2 uptake was coupled to transport in the slices in basic medium. This is not entirely certain however, because, for a reason that is not understood, the net active K^+ accumulation was significantly less in the presence of the 75 mM K^+ medium than in the 5 mM K^+ medium. It is nevertheless reasonable to conclude from these experiments that the respiratory inhibition caused by ouabain has two components, one apparently tightly coupled to transport (referred to below as 'transport-coupled respiration'), and one which is sensitive to the cation composition of the cells ('cation-sensitive respiration')*.

Inhibition of metabolic pathways

Earlier work had indicated that K^+ might directly affect the availability of reducing equivalents to the respiratory chain of the liver slices[9], and it seemed likely that this was the basis for the cation-sensitive portion of the respiration. In liver slices utilizing only endogenous substrates, the main oxidative pathways supplying

* It should be noted that ouabain itself has no direct effect on respiration, as indicated by experiments with isolated mitochondria[31].

electrons to the respiratory chain are the β-oxidation of fatty acids, and the citric acid cycle. In order to see what role these two pathways might play in the cation-sensitive respiration, as well as to obtain more information on the transport-coupled respiration, the effect of transport inhibition was studied in slices treated with ethanol or malonate. Figs. 1 and 2 show, first, the effects of these two agents themselves on respiration and cation transport.

In the case of malonate (Fig. 1), the respiration was maximally inhibited by about 25 % at a concentration of 20 mM, and there was then little or no effect on net ion movements. Under these conditions the citric acid cycle appears to be maximally inhibited, so that the principle substrate for the persisting respiration is presumably endogenous fatty acid, which undergoes β-oxidation to ketone bodies[14]. As shown previously[15], higher concentrations of malonate (40 mM) cause some inhibition of cation transport. This is not due to inhibition of the citric acid cycle, since there is no further inhibition of respiration and the effect is shared by certain other carboxylate anions (some of which stimulate respiration; ref. 15). In the particular series of experiments seen in Fig. 1, only an inhibition of Na^+ transport was apparent. This effect was largely avoided in the experiments described below, by using 20 mM malonate.

In the presence of ethanol (10 mM or higher), the activity of the citric acid cycle is greatly depressed[16–18]. In addition, the β-oxidation of endogenous fatty acids is also greatly reduced[18,19], so that the oxidation of ethanol to acetate becomes the prin-

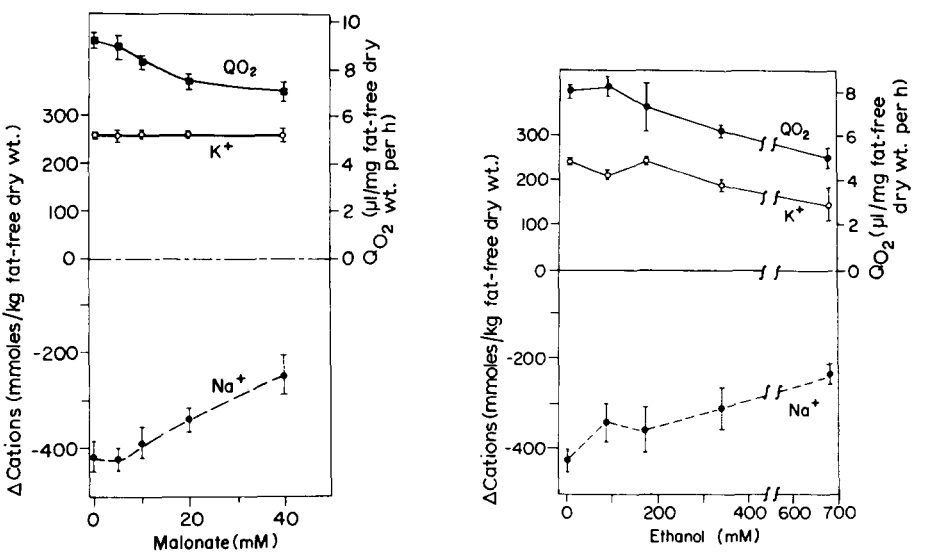

Fig. 1. Effect of malonate on respiration and net cation movements. Liver slices were incubated for 90 min at 1° and then, after gassing with O_2, for 70 min at 38°. The basic medium (without malonate) contained (mM): Na^+, 161.5; K^+, 5.0; Ca^{2+}, 1.2; Mg^{2+}, 1.0; Cl^-, 153.0; SO_4^{2-}, 1.0; phosphate (pH 7.4), 10.0; sodium malonate was added in exchange for an osmotically equivalent amount of NaCl. Each point is the mean $\pm$ S.E., of 7–10 observations. Δcations = (tissue content after incubation at 38°) – (content after incubation at 1°).

Fig. 2. Effect of ethanol on respiration and net cation movements. Details as for Fig. 1, except that ethanol was added to the basic medium without substitution for NaCl, and that each point is the mean of 3–5 observations.

ciple source of reducing equivalents for respiration in the liver slices[16,18]. Fig. 2 shows that concentrations of ethanol up to 170 mM maintained respiration and net cation transport at the same level as the endogenous substrates did alone. However, higher concentrations inhibited respiration and transport in parallel, possibly by a non-specific effect of the alcohol on cell structures.

Effects of transport inhibition in the presence of malonate or ethanol

Table II shows the effect of ouabain on the respiration of slices incubated in the presence or absence of ethanol. Although the effect on net K^+ accumulation was the same in both cases, the inhibition of respiration was significantly less ($P < 0.02$) in the presence (a fall of 0.72 ± 0.39 μl per mg fat-free dry wt. per h; Line 4 *minus* Line 5 of Table II) than in the absence of ethanol (2.17 ± 0.34 μl per mg per h; Line 2 *minus* Line 3). The former value is similar in magnitude to the transport-coupled respiration of Table I. The fact that only this portion of the respiratory inhibition caused by ouabain is seen in the presence of ethanol, suggests that the cation-sensitive portion is associated with one of the pathways of substrate-level oxidation

TABLE II

EFFECT OF OUABAIN AND ETHANOL ON RESPIRATION AND K^+ ACCUMULATION

Conditions were as for Fig. 2. Ethanol concentration was 85 mM; ouabain concentration was 0.75 mM. *n*, number of experiments.

Additions to medium	*Incubation at 38° (min)*	Q_{O_2} *(µl/mg fat-free dry wt. per h)*	K^+ *content (mmoles/kg fat-free dry wt.)*	*n*
1. Ethanol	0 (90 min at 1°)	—	81 ± 7	5
2. None	70	10.14 ± 0.42	272 ± 13	6
3. Ouabain	70	7.97 ± 0.29	106 ± 11	6
4. Ethanol	70	10.26 ± 0.48	263 ± 16	7
5. Ethanol + ouabain	70	9.35 ± 0.24	117 ± 10	7

TABLE III

EFFECTS OF OUABAIN AND MALONATE ON RESPIRATION AND K^+ ACCUMULATION

Conditions were as described for Fig. 1. *n*, number of experiments.

Additions to medium	*Incubation at 38° (min)*	Q_{O_2} *(µl/mg fat-free dry wt. per h)*	K^+ *content (mmoles/kg fat-free dry wt.)*	*n*
1. None	0 (90 min at 1°)	—	71 ± 2	5
2. None	70	7.62 ± 0.23	323 ± 6	8
3. Ouabain, 0.5 mM	70	5.81 ± 0.21	115 ± 5	10
4 Malonate, 20 mM	70	5.97 ± 0.19	282 ± 9	10
5 Ouabain + malonate	70	5.32 ± 0.13*	114 ± 6	10

* *t* test for difference (a) from malonate alone, $P = 0.02$; (b) from ouabain alone, $P > 0.05$.

TABLE IV

EFFECT OF MALONATE AND K^+ ON RESPIRATION AND ION TRANSPORT

In the K^+-free medium, Na^+ replaced the K^+ of the basic medium described in the legend to Fig. 1, the only K^+ present being derived from endogenous sources. Otherwise, conditions were as described for Fig. 1. *n*, number of experiments.

Conditions	*Incubation at 38° (min)*	Q_{O_2} *(µl/mg fat-free dry wt. per h)*	K^+ *content (mmoles/kg fat-free dry wt.)*	Na^+ *content (mmoles/kg fat-free dry wt.)*	*n*
1. K^+-free	0 (90 min at 1°)	—	48 ± 2	834 ± 18	4
2. 5 mM K^+	70	7.42 ± 0.25	272 ± 10	430 ± 34	6
3. K^+-free	70	5.08 ± 0.26	54 ± 14	711 ± 38	7
4. Malonate (20 mM) + 5 mM K^+	70	6.16 ± 0.24	263 ± 9	489 ± 45	8
5. Malonate (20 mM) + K^+-free	70	5.00 ± 0.18*	54 ± 12	775 ± 51	8

* *t* test for difference (a) from Line 4, $P < 0.01$; (b) from Line 3, $P > 0.05$.

which are inhibited by ethanol, *i.e.* β-oxidation or the citric acid cycle. Experiments with malonate allow a distinction to be made between these last possibilities.

In Table III, ouabain lowered the rate of respiration of control slices by 1.83 ± 0.18 µl O_2 per mg fat-free dry wt. per h (Line 2 *minus* Line 3). But in the presence of malonate, where it inhibited transport to the same extent as in the controls, ouabain lowered respiration by only 0.65 ± 0.21 µl per mg per h (Line 4 *minus* Line 5). Table IV shows very similar results, obtained when transport was inhibited by omission of K^+ from the medium[1]. In this case, inhibition of transport reduced O_2 uptake by 2.41 ± 0.34 µl per mg per h in the absence, and by only 1.16 ± 0.15 in the presence of malonate. In each of these tables, the respiratory effects of transport inhibition in the presence of malonate were significantly less than in its absence ($P < 0.001$). Furthermore, malonate did not cause any significant inhibition of respiration in slices whose transport was inhibited (*cf.* Line 3 with Line 5 in each of the Tables III and IV). This suggests that the citric acid cycle was already inhibited in the non-transporting slices, and points to the cycle as the site of K^+ activation (or Na^+ inhibition) of the cation-sensitive portion of the respiration.

DISCUSSION

Respiration coupled to ion transport

The results clearly indicate that the inhibition of respiration which is seen when the ion transport of liver slices in the basic medium is inactivated, cannot be entirely due to a coupling of this respiration to the transport process *via* high-energy acceptors. Thus, in each of Tables II–IV, conditions are found in which the inhibition of transport is the same as in the controls, but the accompanying inhibition of respiration is only 30–50 % of that seen in the controls. It follows that the lower values of respiratory inhibition represent the maximal portion of the respiration which can be considered to be tightly coupled to the energy requirements of cation transport. The estimates of the absolute value of this transport-coupled O_2 uptake in Tables II–IV are 0.65, 0.72 and 1.16 µl per mg fat-free dry wt. per h. The corresponding estimate from

Table I would be the value of the ouabain-sensitive respiration in the presence of 75 mM K^+, *i.e.* 0.78 μl per mg per h. However, in view of the possibility that the transporting activity (as indicated by the K^+ uptake, although not by the Na^+ extrusion) may be rather smaller in the high K^+ medium than in the basic medium, this value will not be included in the following discussion.

The question arises as to whether this amount of respiration is sufficient to provide the energy needed to support the transport mechanism. A very rough idea of this may be obtained by applying the results of ELSHOVE AND VAN ROSSUM[1] on the time-course of intracellular cation changes, to the equation for the minimum work done during ion transport[20]:

$$\text{Work done} = QRT \ln ([Na^+]_0[K^+]_i/[Na^+]_i[K^+]_0) \text{ cal per kg dry wt. per h}$$

Here, R is the gas constant, T the absolute temperature, Q is the rate of transport, which has been taken to be the initial rate of net extrusion of Na^+ during the first 10 min at 38° (1.08 moles per kg dry wt. per h; ref. 1). The values for $[Na^+]$ and $[K^+]$ have been taken as the plateau values reached after 60 min at 38°; an allowance was made for bound K^+ (see ref. 21) in the calculation of $[K^+]_i$. It was assumed that the rate of the active component of the net Na^+ movement remained constant throughout incubation at 38°. The factor most likely to limit the rate of transport is the falling intracellular Na^+ concentration. However, the cell Na^+ is calculated to be approx. 80 mmoles per kg intracellular water after 60 min at 38°, and it has been shown that the Na^+-extrusion mechanism, of frog muscle at least, is saturated at a Na^+ concentration of 30–40 mmoles per kg cell water when K^+ is present in the incubation medium (calculated from results of refs. 22 and 23). With these various assumptions, the value of the minimum rate of work done in Na^+ transport by the liver slices is calculated to be approx. 2.5 kcal per kg dry wt. per h.

The amount of energy made available by the fraction of respiration suggested above to be coupled to ion transport may be calculated if the P/O ratio, and the value for the free energy of hydrolysis ($\Delta G'$) for ATP in the cells, are known. A value of P/O = 3 may be assumed; this is probably an overestimate, particularly when only endogenous substrate is oxidised. From experiments with isolated liver mitochondria in State 4, COCKRELL *et al.*[24], and SLATER[25] have obtained values of $\Delta G' = -15$ to -16 kcal per mole ATP. However, from the analysis of liver slices incubated for 86 min at 38° (ref. 15), the assumption of an even distribution of nucleotides and phosphate throughout the intracellular water, and a value of $\Delta G'_0 = -8.7$ kcal/mole for the standard free energy of ATP hydrolysis under conditions approximating those of the cells (37°, pH 7.0, ionic strength 0.2, Mg^{2+} concn. in the range 1–10 mM; see ref. 26), an approximate estimate of $\Delta G' = -10.8$ kcal per mole ATP is obtained for the situation in the liver cells. The energy made available from the transport-coupled respiration is then calculated as follows: (i) If $\Delta G' = -16$ kcal/mole, the energy made available is between 2.8 and 5.0 kcal per kg dry wt. per h. For individual estimates of the transport-coupled respiration (from Tables II–IV), this amounts to 111, 124 and 200% of the minimal energy requirement for Na^+ transport. (ii) If $\Delta G' = -10.8$ kcal/mole, energy made available is between 1.9 and 3.4 kcal per kg per h, or 75, 83 and 135% of the minimal requirement.

Clearly, the calculated minimal energy requirement for the transport of Na^+ is in the same range as the amount of energy which appears to be made available by

the transport-coupled respiration (whichever value of $\Delta G'$ is used), so that the latter may wholly support the transport activity. On the other hand, the transport mechanism may be far from 100 % efficient in its utilization of the energy of ATP; thus it has been calculated that muscular contraction has an efficiency of 25 % in its conversion of the energy of ATP into mechanical work[27,28]. According to even the most favourable of the above estimates of energy-production (5.0 kcal per kg per h), the transport mechanism would have to be at least 50 % efficient in order to derive all its energy from the transport-coupled respiration, and the possibility is therefore raised that the Na^+ transport may depend for its energy on a greater fraction of the respiration than is actually coupled to the transporting mechanism. However, it is clear that much more precise information is required before this question can be resolved with any degree of confidence.

Other respiratory effects of transport inhibition

The results of Table I strongly suggest that much of the ouabain-induced inhibition of respiration in the basic medium, is due to a cation sensitivity of the intracellular oxidative metabolism, rather than to a direct effect on the ion-transport mechanism. The amount of O_2 uptake represented by this cation-sensitive respiration, 1.64 μl per mg fat-free dry wt. per h, is similar to the difference, (total respiratory effect of transport inhibition) *minus* (transport-coupled respiration), in Tables II–IV, the value of which varied from 1.15 to 1.45 μl per mg per h. As noted in RESULTS, the effects of malonate and ethanol indicate that this latter amount of respiration is associated with the citric acid cycle, and the failure of malonate to inhibit respiration in slices with an inactive transport mechanism, further indicates the cycle to be the site of the cation sensitivity. This is consistent with the findings of GEYER *et al.*[29] on the K^+ sensitivity of CO_2 production by liver slices, and those of VAN ROSSUM[9] on the reduction of nicotinamide nucleotides upon treatment of rat-liver slices with K^+ in the presence of ouabain. The fact that this last result was associated with raised K^+ rather than with lowered Na^+, argues that the cation sensitivity of the respiration is due to stimulation of NADH production by K^+ rather than its inhibition by high intracellular Na^+.

These results on the K^+ sensitivity of oxidation reactions in slices may be compared to observations on mitochondria isolated from rat liver. Thus, addition of K^+ to mitochondria in predominantly Na^+-containing media stimulated the oxidation of citric acid cycle intermediates, while addition of K^+, but not of Na^+, to sucrose media stimulated the oxidation of α-oxoglutarate[11]. More recently, HARRIS AND MANGER[30] failed to find a difference in the respiratory rate of liver mitochondria incubated in K^+- or Na^+-saline media; but GOMEZ–PUYOU *et al.*[12] found that rat-liver mitochondria incubated with various substrates in the presence of ADP respired more rapidly when the medium contained K^+ than when it contained Na^+. There are thus strong indications that mitochondrial oxidative reactions can be directly affected by the ionic composition of the medium in which the organelles are suspended, being favoured by the presence of K^+.

The site of the proposed cation sensitivity in the citric acid cycle remains to be ascertained, but there are indications that it is not at the level of succinate oxidation. Thus, the results with isolated mitochondria indicate succinate oxidation to be less sensitive to the absence of K^+ than is the oxidation of NAD^+-linked citric acid cycle

intermediates[11,12,31], while the oxidation of succinate by liver slices (which is largely a one-step oxidation to fumarate and malate) is also little affected when incubation is carried out in K^+-free medium containing ouabain[32].

Substrate requirement for ion transport

FIMOGNARI *et al.*[33] and COHEN *et al.*[34] suggest that some examples of Na^+ transport may derive energy from a specific section of an oxidative pathway of metabolism. However, the effects of malonate and ethanol noted here suggest that this is not the case with ion transport in rat-liver slices. Thus, inhibition of the citric acid cycle by malonate, at the succinate dehydrogenase step, had little effect on the transport; nor did ethanol-induced inhibition both of the cycle, at the level of citrate synthase (citrate–oxaloacetatelyase (CoA-acetylating), EC 4.1.3.7; ref. 18), and of β-oxidation of fatty acid affect the transport. Therefore, neither of these pathways, in its entirety, is obligatory. The oxidation of extra-mitochondrial NADH, derived from ethanol oxidation to acetate, was able fully to support the transport. The number of intramitochondrial, substrate-level oxidative reactions which could have a specific association with ion transport is thus reduced to those which are involved in the 'shuttle' mechanisms for the transport of reducing equivalents into the mitochondria, *i.e.* probably the reaction catalysed by malate dehydrogenase (L-malate:NAD oxidoreductase, EC 1.1.1.37)[35]. However, neither exogenous L-malate, nor malate produced endogenously by oxidation of succinate, have been found to have any stimulatory effect on the ion transport of liver slices[15,32]. It seems more reasonable to conclude, at present, that there is no association of a specific substrate-level reaction with cation transport in liver, and that the only requirement is a sufficient rate of respiration.

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REFERENCES

1 A. ELSHOVE AND G. D. V. VAN ROSSUM, *J. Physiol. London*, 168 (1963) 531.
2 K. ZERAHN, *Acta Physiol. Scand.*, 36 (1956) 300.
3 U. V. LASSEN AND J. H. THAYSEN, *Biochim. Biophys. Acta*, 47 (1961) 616.
4 R. WHITTAM, *Biochem. J.*, 82 (1962) 205.
5 R. WHITTAM AND J. S. WILLIS, *J. Physiol. London*, 168 (1963) 158.
6 J. S. WILLIS, *Biochim. Biophys. Acta*, 163 (1968) 506.
7 S. J. HERSEY, *Biochim. Biophys. Acta*, 183 (1969) 155.
8 R. WHITTAM AND D. M. BLOND, *Biochem. J.*, 92 (1964) 147.
9 G. D. V. VAN ROSSUM, *Biochim. Biophys. Acta*, 122 (1966) 323.
10 G. D. V. VAN ROSSUM, *Biochim. Biophys. Acta*, 153 (1968) 124.
11 B. C. PRESSMAN AND H. A. LARDY, *J. Biol. Chem.*, 197 (1952) 547.
12 A. GOMEZ-PUYOU, F. SANDOVAL, A. PEÑA, E. CHÁVEZ AND M. TUENA, *J. Biol. Chem.*, 244 (1969) 5339.
13 I. M. GLYNN, *J. Physiol. London*, 136 (1957) 148.
14 M. JOWETT AND J. H. QUASTEL, *Biochem. J.*, 29 (1936) 2181.
15 G. D. V. VAN ROSSUM, *Arch. Biochem. Biophys.*, 133 (1969) 385.
16 O. A. FORSANDER, *Biochem. J.*, 98 (1966) 244.
17 O. A. FORSANDER, *Biochem. J.*, 105 (1967) 93.
18 J. R. WILLIAMSON, M. S. OLSON, E. T. BROWNING AND R. SCHOLZ, in S. PAPA, J. M. TAGER, E. QUAGLIARIELLO AND E. C. SLATER, *The Energy Level and Metabolic Control in Mitochondria*, Adriatica Editrice, Bari, 1969, p. 207.

19 O. A. FORSANDER, N. RÄIHÄ, M. SALASPURO AND P. MÄENPÄÄ, *Biochem. J.*, 94 (1965) 259.
20 H. H. USSING, in O. EICHLER AND A. FARAH, *Handbuch der experimentellen Pharmakologie*, Vol. 13, Springer, Berlin, 1960, p. 91.
21 K. D. HECKMANN AND D. S. PARSONS, *Biochim. Biophys. Acta*, 36 (1959) 213.
22 E. J. HARRIS, *J. Physiol. London*, 177 (1965) 355.
23 M. DYDYNSKA AND E. J. HARRIS, *J. Physiol. London*, 182 (1966) 92.
24 R. S. COCKRELL, E. J. HARRIS AND B. C. PRESSMAN, *Biochemistry*, 5 (1966) 2326.
25 E. C. SLATER, in S. PAPA, J. M. TAGER, E. QUAGLIARIELLO AND E. C. SLATER, *The Energy Level and Metabolic Control in Mitochondria*, Adriatica Editrice, Bari, 1969, p. 255.
26 R. C. PHILLIPS, P. GEORGE AND R. J. RUTMAN, *J. Biol. Chem.*, 244 (1969) 3330.
27 D. R. WILKIE, *Progr. Biophys. Biophys. Chem.*, 10 (1960) 260.
28 R. J. TALLARIDA, *Thermodynamic Basis for the Interpretation of Cardiac Drug Action*, Ph.D. Thesis, Temple University, 1967.
29 R. P. GEYER, M. F. MEADOWS, L. D. MARSHALL AND M. S. GONGAWARE, *J. Biol. Chem.*, 205 (1953) 81.
30 E. J. HARRIS AND J. R. MANGER, *Biochem. J.*, 113 (1969) 617.
31 D. M. BLOND AND R. WHITTAM, *Biochem. J.*, 92 (1964) 158.
32 G. D. V. VAN ROSSUM, *Arch. Biochem. Biophys.*, 133 (1969) 373.
33 G. M. FIMOGNARI, G. A. PORTER AND I. S. EDELMAN, *Biochim. Biophys. Acta*, 135 (1967) 89.
34 J. J. COHEN, R. W. CHESNEY, P. H. BRAND, H. F. NEVILLE AND C. F. BLANCHARD, *Am. J. Physiol.*, 217 (1969) 161.
35 J. R. WILLIAMSON, R. SCHOLZ, R. G. THURMAN AND B. CHANCE, in S. PAPA, J. M. TAGER, E. QUAGLIARIELLO AND E. C. SLATER, *The Energy Level and Metabolic Control in Mitochondria*, Adriatica Editrice, Bari, 1969, p. 411.
36 P. F. BAKER AND C. M. CONNELLY, *J. Physiol. London*, 185 (1966) 270.
37 H. P. RANG AND J. M. RITCHIE, *J. Physiol. London*, 196 (1968) 163.