The Relation of Sodium and Potassium Ion Transport to the Respiration and Adenine Nucleotide Content of Liver Slices Treated with Inhibitors of Respiration

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1. The dependence of the net transport of Na^+ and K^+ by rat liver on the respiration has been determined by incubating slices in the presence of varying concentrations of respiratory inhibitors. 2. Neither the rate of net transport nor the total amount of each ion transported was inhibited unless the rate of endogenous respiration was decreased below a critical value of about 330 mmol of O_2/h per kg of protein (i.e. 50% of the total endogenous respiration). 3. The uninhibited rate of respiration could be varied over a twofold range (380–770 mmol of O_2/h per kg of protein) by the use of different substrates, but the critical value for the onset of transport inhibition was quite constant (290–360 mmol/h per kg of protein) under these different conditions. 4. Slices incubated at 38°C without inhibitors showed an increase of their ATP content and the concentration ratio ATP/ADP. The final ATP content and concentration ratio, ATP/ADP, of slices treated with different concentrations of inhibitors were closely related to the rate of respiration. 5. The increased ATP content of the control slices during incubation was equal to the increase of total adenine nucleotides. At increasing degrees of respiratory inhibition the relative contributions of ADP and AMP to the total adenine nucleotide content increased. 6. The critical rate of respiration for the onset of inhibition of ion transport and the corresponding contents of adenine nucleotides provide estimates of the maximal values of certain parameters of energy metabolism required for the support of alkali-cation transport in the liver slices.

The net transport of Na^+ and K^+ ions by liver slices from adult rats is almost entirely dependent on the energy made available by oxidative phosphorylation (Flink *et al.*, 1950; van Rossum, 1963; Elshove & van Rossum, 1963). A quantitative estimate of the energy required to support the transport mechanism itself may be obtained from thermodynamic considerations, by calculating the rate of energy production required to support the observed rate of transport (Ussing, 1960). Such an estimate has been made for Na^+ transport in liver slices (van Rossum, 1970a, 1971a). However, in the living cell one must also take into account the possibility that other ATP-utilizing processes compete for the available ATP. The minimal rate of respiration and ATP production actually required to maintain the observed transport activity in the cell is thus likely to be greater than that calculated as above. The experiments here described represent an attempt to approach this latter aspect.

The activity of an energy-requiring process in the presence of others competing for available ATP will be partly a reflection of the apparent K_m values for ATP. Lowering the production of ATP by degrees (e.g. by titration with respiratory inhibitors) may therefore cause inhibition of a particular energy-

dependent activity only when the intracellular ATP concentration has fallen below a value determined by the apparent K_m , and other activities may still be able to continue at, or near, their maximal rates. The present work shows that titration of liver slices with inhibitors of respiration had no effect on the net transport of Na^+ and K^+ until the activity of the energy metabolism, as indicated by the rate of respiration and the contents of adenine nucleotides, was decreased below a critical point. The values of the parameters of energy metabolism pertaining at the critical point represent estimates of the values that are required to maintain ion transport in the whole liver cell in the presence of other activities, such as the synthesis of urea (Hems *et al.*, 1966), protein (Peters & Anfinsen, 1950; Bhargava & Bhargava, 1962) and RNA (Shanmugam & Bhargava, 1968; Jackson & Sells, 1967), which are known to proceed under conditions similar to those used here.

Preliminary reports of some of this work have been published (van Rossum, 1971a,b).

Methods

Experimental procedure

Liver slices from male albino rats were preincubated at 1°C for 90 min, followed by incubation at 38°C

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for the times indicated, in a phosphate-buffered saline medium contained in Warburg manometric apparatus gassed with O_2 . Details of the incubation procedures were given by Elshove & van Rossum (1963) and van Rossum (1969). In some experiments the volume of distribution of inulin was measured as described by Parsons & van Rossum (1962a); the preincubation was then continued for 120 min to ensure complete equilibration of the marker with the slice water. In all experiments the slices were collected by filtration on hardened paper (Whatman no. 54), as described by Heckmann & Parsons (1959), and subsequently treated in one of three ways.

(i) Slices were transferred to tared, hard-glass weighing bottles and analysed for their water, fat-free solids and ionic contents (Elshove & van Rossum, 1963).

(ii) When the inulin distribution was measured, the slices from each incubation vessel were divided into two groups. One group was treated by method (i). The other was weighed on a torsion balance, dropped into perchloric acid (8%), homogenized and centrifuged. The acid supernatant was assayed for inulin. A sample of the medium from each flask was also deproteinized and assayed. In each experiment, appropriate blank analyses were performed on slices incubated in inulin-free medium.

(iii) When adenine nucleotides were analysed, the slices were dropped into 8% perchloric acid in 40% ethanol at -20°C and were briefly agitated on a mechanical mixer. The time elapsing between removal of the slices from the incubation flasks and completion of the agitation was 10–15 s. The slices were then homogenized and centrifuged at 0°C . A sample of the supernatant was taken for Na^+ analysis (analysis of tissue K^+ was impossible in these experiments because of the insolubility of $KClO_4$); the remainder was neutralized with a mixture of K_2CO_3 and triethanolamine (Williamson & Corkey, 1969) and analysed for adenine nucleotides. The perchloric acid-insoluble material was analysed for protein.

Analytical procedures

Na^+ and K^+ were analysed by flame photometry on the Beckman DU spectrophotometer with flame attachment (Beckman Instruments Inc., Fullerton, Calif., U.S.A.), appropriate amounts of perchloric acid and ethanol being added to the standards for slices extracted by method (iii). Cl^- was estimated by potentiometric titration with Ag^+ on the Aminco Chloride Titrator (Aminco Instrument Co., Silver Spring, Md., U.S.A.) and inulin was analysed by the method of Kulka (1956). Adenine nucleotides were determined by enzymic methods (Williamson & Corkey, 1969) in a metabolite fluorimeter (Mayer *et al.*, 1969). AMP estimates were corrected for the small amounts of this substance present in some

solutions of NADH by running reagent blank determinations. Since the pyruvate kinase (EC 2.7.1.40) required for analysis of ADP is not specific for the adenine nucleotide, the total amount of other nucleotide diphosphates present in the tissue extracts was estimated after removal of ADP by creatine phosphokinase (EC 2.7.3.2) and excess of creatine phosphate (Yushok, 1971). In samples taken at random from several experiments, the reaction obtained with pyruvate kinase after removal of ADP was $5.4 \pm 1.4\%$ (16) of that seen in its presence. The results in the tables are uncorrected for this factor. The protein precipitated in procedure (iii) was washed with ethanol (96%) and was determined by the biuret reaction (Gornall *et al.*, 1949) as modified for turbid solutions (Szarkowska & Klingenberg, 1963). Bovine serum albumin (Cohn fraction V; Sigma Chemical Co., St. Louis, Mo., U.S.A.) was used as protein standard.

Expression of results

The net transport of Na^+ or K^+ was determined as the difference between the ionic content of slices analysed after the preincubation at 1°C and of slices analysed after further incubation at 38°C . All values given are means $\pm$ S.E.M. with the number of observations in parentheses. For the experiments in which slices were treated by procedure (iii) the measured protein content has been used as the reference basis. For slices treated according to procedures (i) and (ii) the measured weight of fat-free solids has been converted into the weight of protein by using a mean value for the ratio protein/fat-free dry weight. This ratio was determined in preliminary experiments on groups of slices that had been incubated under four conditions: for 90 min at 1°C in the presence or absence of a high concentration of Amytal (4 mM), or at 1°C followed by 70 min at 38°C in the presence or absence of 4 mM-Amytal. No significant difference was found between the groups, and the mean value for the pooled estimates of the ratio was $604 \pm 17\text{g}$ of protein/kg fat-free dry wt. (27 observations).

Results

Effects of respiratory inhibitors on net movements of Na^+ and K^+

During preincubation at 1°C the liver slices lost K^+ and gained Na^+ , Cl^- and water to an extent that was not influenced by any of the inhibitors or substrates used in this work. The changes were largely reversed during subsequent incubation at 38°C without inhibitors, and the ionic composition of the intracellular compartment (as determined from inulin-space measurements) attained a new steady state after 50–60 min (Fig. 1). In most subsequent experiments a

Fig. 1. Effect of 2.0 mM-Amytal on the time-course of ion transport at 38°C

Liver slices were preincubated for 90 min at 1°C before incubation at 38°C for the times indicated. No substrates were added. Incubation conditions are further described in the Methods section. The net transport of ions represents the change in tissue composition from that of slices incubated for 90 min at 1°C. ●, ○, K⁺; ▲, △, Cl⁻; ■, □, Na⁺; filled-in symbols represent slices incubated without Amytal, and open symbols slices incubated with Amytal. Vertical lines represent $\pm$ S.E.M., where these exceed the size of the symbols. Each point is the mean of six observations.

standard incubation time of 70 min was used at 38°C, and measurements of inulin space were omitted. Fig. 2 shows that 5 mM-Amytal caused maximal inhibition of respiration and almost completely prevented the recovery of the ionic composition of slices utilizing only endogenous substrate. Low concentrations of this inhibitor (1 mM or less) gave a relatively smaller inhibition of transport than of respiration, an effect which is most clearly seen when the individual observations of the net movement of cations are grouped according to the rate of respiration of the same samples of slices, as in Fig. 3. Results of experiments with cyanide and arsenite are also illustrated in Fig. 3, and numerical values relevant to the following discussion are summarized in Table 1. With each inhibitor, the rate of respiration had to be decreased below about 350 mmol of O₂/h per kg of protein (Table 1, column 7) before any consistent inhibition

Fig. 2. Effect of different concentrations of Amytal on the rate of oxygen consumption and on the net transport of ions by liver slices utilizing only endogenous substrate

The slices were preincubated for 90 min at 1°C and then incubated for 70 min at 38°C, as described in the Methods section, in the presence of the indicated concentrations of Amytal. Respiration was measured from 10 to 60 min of incubation at 38°C and was constant during this time; net transport of ions represents the change of tissue composition occurring during the 70 min at 38°C. ○, Oxygen consumption; ●, K⁺ transport; □, Na⁺ transport. Vertical lines represent $\pm$ S.E.M.; each point is the mean of six to eight observations.

of cation movement occurred. This was true for both Na⁺ and K⁺ despite the greater net transport of the former (Table 1, columns 3 and 4), an observation consistent with other experiments indicating a coupling of the transport of these ions in the liver (Elshove & van Rossum, 1963; van Rossum, 1966b). In all of these experiments there was a residual, inhibitor-insensitive O₂ uptake. This inhibitor-resistant respiration varied little (150–180 mmol/h per kg of protein) with the inhibitor used and was also little affected by either added substrates (Table 1) or oligomycin (van Rossum, 1964). These findings, together

Fig. 3. Relation of the net transport of ions to the different rates of respiration caused by various concentrations of inhibitors

Liver slices utilized only endogenous substrates. The results are taken from experiments of the type illustrated in Fig. 2, individual values of the net transport being grouped according to the rate of respiration of the same sample slices (at intervals of 32.5 or 65.0 mmol of O_2 /h per kg of protein), irrespective of the inhibitor concentration. (a) K^+ accumulation in the presence of Amytal (0.2–5.0 mM); (b) K^+ accumulation in the presence of arsenite (0.1–5.0 mM); (c) Na^+ extrusion in the presence of cyanide (0.01–10.0 mM); (d) K^+ accumulation in the presence of cyanide (0.01–10.0 mM). Vertical lines represent $\pm$ S.E.M.; numbers of observations are indicated on the bars.

Table 1. Requirement of respiration for the support of ion transport in liver slices incubated with various substrates

The results are derived from titration experiments of the type shown in Fig. 2. The medium contained varying concentrations of the inhibitors noted in column (1), and the substrates mentioned in column (2). Columns (3) and (4) show the net accumulation of K^+ and extrusion of Na^+ occurring during incubation of the slices for 70 min at $38^\circ C$ in the absence of inhibitor. The rates of respiration shown in columns (5) and (6) were obtained in each experiment from slices incubated with and without the exogenous substrates, in the absence of inhibitor; where the rate tended to fall during incubation the final rate is shown, as discussed in the text. Column (8) shows the respiration observed with each substrate in the presence of maximal concentration of inhibitor. Column (7) shows the approximate inflexion points of plots relating net ion transport to rate of respiration, as illustrated by Fig. 3; the inflexion points for Na^+ and K^+ were indistinguishable. Column (9) represents (column 6 minus column 8); column (10) represents (column 7 minus column 8). Incubation conditions were as described in the text, except that the medium contained $2.5 \text{ mM-}Ca^{++}$ in the experiments with citrate. Values shown are the means for four to eight experiments.

Inhibitor	Substrate or malonate	Net cation movement without inhibitor (mmol/kg of protein)		Uninhibited, endogenous substrate	Uninhibited, with exogenous substrate	Rate above which transport is not inhibited	Inhibitor insensitive	'Mitochondrial' respiration [(6)-(8)]	'Mitochondrial' respiration above which transport is not inhibited [(7)-(8)]
		K^+	Na^+						
(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)
Cyanide	Endogenous	303 ± 20	600 ± 31	735 ± 40	—	358	182 ± 7	554	176
Arsenite	Endogenous	440 ± 13	695 ± 36	571 ± 30	—	324	147 ± 8	424	177
Amytal	Endogenous	350 ± 23	531 ± 53	514 ± 33	—	292	177 ± 28	338	115
Amytal	Pyruvate (20 mM)	401 ± 17	598 ± 46	530 ± 18	700 ± 28	389	236 ± 10	461	153
Amytal	L-Malate (20 mM)	350 ± 30	475 ± 74	574 ± 26	699 ± 43	324	228 ± 17	469	96
Amytal	Citrate (3 mM)	389 ± 8	781 ± 22	499 ± 13	610 ± 20	358	167 ± 12	441	191
Amytal	Octanoate (2 mM)	298 ± 23	696 ± 45	544 ± 22	770 ± 41	358	182 ± 30	588	176
Amytal	Malonate (20 mM)	330 ± 31	408 ± 89	600 ± 17	384 ± 22	324	137 ± 26	246	187

with the work of Thurman & Scholz (1969), suggest that the resistant respiration was probably due to the microsomal, mixed-function oxidases and may thus be subtracted from the total O_2 utilization to obtain an estimate of the mitochondrial respiration, which alone is coupled to energy-conserving reactions. In the experiments with endogenous substrates the total 'mitochondrial' O_2 uptake in the absence of inhibitors varied from 338 to 554 mmol/h per kg of protein whereas the minimal rate required to maintain the full net transport of the ions was 115–177 mmol of O_2 /h per kg (Table 1, columns 9 and 10).

To see whether the minimal 'mitochondrial' O_2 uptake required to maintain transport could be varied, titrations with Amytal were done in the presence of exogenous substrates that stimulate respiration without affecting the net transport, and in the presence of malonate, which partially inhibits respiration without affecting transport (van Rossum, 1970a). Octanoate, citrate and, unexpectedly, malonate [probably as a result of its activation to malonyl-CoA; Nakada *et al.* (1957); Spencer & Lowenstein (1962)], offered some protection of the respiration against the effects of 1–5 mM-Amytal, since the initial rate of respiration was higher than that obtained with endogenous substrate at the same Amytal concentration. However, the rate quickly declined and reached a constant low value within 30 min. A study of the early time-course of ion transport in the presence of 2 mM-Amytal showed that the initial 'protection' of respiration by 2 mM-octanoate was not accompanied by a corresponding increase in the initial rate of net transport. The later, constant rate of respiration thus gives an adequate indication of the O_2 consumption supporting ion transport; it has been assumed that this is also true of the effects of citrate and malonate. The minimal rates of O_2 consumption

required to support full ion-transport activity in the presence of the various substrates and malonate were determined from plots of the type shown in Fig. 3, and a summary of the results is included in Table 1. The minimal 'mitochondrial' O_2 uptake required for the maintenance of net transport was found to be quite constant, lying between 153 and 191 mmol/h per kg in six out of eight series of experiments (Table 1, column 10). It showed no correlation with the much greater variation in the uninhibited 'mitochondrial' respiration, which ranged from 246 to 588 mmol of O_2 /h per kg (Table 1, column 9).

In the experiments summarized in Table 1, the total net movement of Na^+ and K^+ during 70 min at 38°C was taken as the index of transport activity. It was important to see whether the rate of the net transport showed a similar relation to the tissue respiration. The time-course of the ion movements was studied in the presence of four concentrations of Amytal, two of which were expected to give less than, and two more than, the degree of respiratory inhibition found to be critical for the maintenance of the maximal total ion movement in 70 min. No substrates were added in these experiments. An example of the results is illustrated in Fig. 1; the maximal rate of Na^+ extrusion always occurred in the first 10 min at 38°C, but the maximal rate of K^+ accumulation occurred later, after a lag period of 10–15 min (cf. Judah & McLean, 1962; Elshove & van Rossum, 1963; Kalant & Hickie, 1968; van Rossum, 1970b). Table 2 contains a summary of the results with all four Amytal concentrations. In no case was the maximal rate of net transport more sensitive than the total movement of ions to the respiratory inhibitor. Thus the two lowest concentrations of Amytal had no significant effect on the ion movements despite inhibiting respiration by 20 and 36% respectively. At

Table 2. *Effect of Amytal on the maximal rates of net Na^+ extrusion and K^+ accumulation by liver slices*

The results are taken from experiments of the type illustrated in Fig. 1. The rates of transport are calculated for the 10 min interval during which maximal transport was observed; for Na^+ this was always the first 10 min at 38°C, and for K^+ was at 10–20 or 20–30 min. Rates of respiration are the average rates over 50 min.

Amytal (mM)	Uptake of O_2		Maximal rate of net transport (mmol/h per kg of protein)		
	Rate (mmol/h per kg of protein)	Inhibition by Amytal (%)	Na^+	K^+	(n)
0	536 ± 22	—	1660 ± 149	427 ± 70	(6)
0.75	427 ± 22	20.4	1510 ± 208	457 ± 30	(6)
0	504 ± 12	—	1420 ± 516	526 ± 89	(6)
1.25	324 ± 20	35.5	1302 ± 109	457 ± 99	(6)
0	506 ± 20	—	1252 ± 397	437 ± 103	(6)
2.0	202 ± 13	60.1	1263 ± 189	294 ± 124	(6)
0	471 ± 36	—	1620 ± 466	621 ± 104	(3)
5.0	182 ± 7	61.4	783 ± 50	268 ± 99	(3)

2mM, Amytal caused a small (30%) reduction in the amount and rate of K^+ accumulation, but the effect on the rate was not statistically significant. It had no effect on the maximal rate of Na^+ transport, but caused the net extrusion to cease sooner than in the controls so that a higher, constant Na^+ concentration was established. Only at the highest concentration used (5mM) did Amytal cause a significant inhibition of the rate of transport. Net movements of Cl^- and water showed a similar behaviour to the Na^+ movement in response to inhibition of respiration, in accord with earlier results on the effects of altered metabolic activity in liver slices (e.g. Leaf, 1956; Heckmann & Parsons, 1959; Parsons & van Rossum, 1962b). These results thus confirm the conclusion that net transport activity was not affected unless the rate of respiration was inhibited by more than 50%.

Effect of respiratory inhibition on adenine nucleotides

The effects of inhibitors of respiration on the adenine nucleotides of the liver slices were studied with the following points in mind: (i) since a considerable proportion of the respiration could be inhibited without ion transport being affected, it was important to know whether this fraction of the O_2 uptake was concerned in the maintenance of concentrations of cellular ATP; (ii) the results would give an indication of the ATP concentrations and values of the concentration ratio, ATP/ADP, associated with various levels of transport activity.

The adenine nucleotide content of liver slices that had been incubated for 90min at $1^\circ C$ was not affected by the presence of cyanide (1mM) or Amytal (2mM), suggesting that metabolism was totally arrested at $1^\circ C$ so that ATP was neither synthesized nor utilized. In the absence of inhibitors, incubation at $38^\circ C$ for 70min led to a two- to four-fold increase of the ATP content, indicating that the rate of the restored synthesis of ATP exceeded the rate of its utilization; the increased ATP content was almost entirely accounted for by an increase of total nucleotides in the slices, there being little change in the amounts of ADP or AMP. Concentrations of cyanide up to 0.1mM caused a reduction of net ATP synthesis without decreasing total nucleotide synthesis, the difference being largely accounted for by an increase in ADP (Fig. 4). At higher cyanide concentrations there was a smaller net synthesis of total nucleotides and an increase in the proportion of AMP. Similar results were obtained in the experiments with intermediate concentrations of Amytal, the smaller ATP synthesis caused by Amytal concentrations of up to 0.8mM being reflected in increased amounts of ADP, whereas the greater decline of ATP synthesis at higher inhibitor concentrations was reflected in greater amounts of AMP and by smaller net synthesis of total nucleotides. Clearly,

Fig. 4. *Effect of cyanide on the adenine nucleotide content of liver slices incubated with endogenous substrate only*

Values represent the slice composition after 70min at $38^\circ C$; the net change of composition during the incubation may be determined by subtracting the values existing after preincubation at $1^\circ C$: ATP (●), 1.6 ± 0.3 ; ADP (○), 1.9 ± 0.4 ; AMP (▲), 1.7 ± 0.1 ; total nucleotides (□), 5.2 ± 0.5 mmol/kg of protein. Vertical lines represent $\pm$ s.e.m.; each point is the mean of four to ten observations.

the liver slices were able to synthesize adenine nucleotides from endogenous precursors (possibly by operation of a 'purine nucleotide cycle' as described by Tornheim & Lowenstein, 1972) but the degree of their phosphorylation depended on the availability of energy conserved by oxidative phosphorylation.

It has previously been shown that the increase in ATP content of liver slices during incubation at $38^\circ C$ takes place during the first 40min, after which time the new steady-state level is maintained at least up to 80min (van Rossum, 1969). Fig. 5 shows that in the presence of various concentrations of Amytal that induced a decline of the ATP content, the new low steady-state concentration was established very rapidly, little change occurring after 5min at $38^\circ C$.

Grouping the results of the experiments illustrated in Fig. 4 according to the respiratory activity of the slices shows a close relation between the ATP content and the rate of inhibitor-sensitive O_2 consumption, until the latter was reduced below about

Fig. 5. Change of ATP content during incubation of slices at 38°C with endogenous substrate and various concentrations of Amytal

The origin represents the ATP content of the slices after preincubation for 90 min at 1°C; this value was 4.54 ± 0.30 (18 observations), which, for a reason that is not understood, was rather higher than the corresponding value obtained in any other series of experiments reported in the paper. Slices were incubated with: ●, 1.25 mM-Amytal (mean respiration 216 ± 18 mmol of O_2 /h per kg of protein); ○, 2.0 mM-Amytal (respiration 188 ± 23 mmol of O_2 /h per kg of protein); ▲, 4.0 mM-Amytal (respiration 161 ± 50 mmol of O_2 /h per kg of protein). Control slices incubated without Amytal respired at a rate of 500 ± 37 mmol of O_2 /h per kg of protein. Vertical lines represent $\pm$ S.E.M., but have been omitted from the values with 2 mM-Amytal for the sake of clarity.

135 mmol of O_2 /h per kg of protein (Fig. 6). Quantitatively similar results were obtained in experiments with Amytal as inhibitor. Thus the whole of the respiration sensitive to inhibitors contributed equally to the maintenance of amounts of ATP. A significant ATP content, of about 0.7 mmol/kg of protein, was maintained in the presence of the maximal inhibition of respiration, presumably by the activity of anaerobic glycolysis. Plots of the concentration ratio, ATP/ADP, versus O_2 uptake ran closely parallel to the curves of the ATP content. The maximal values of this ratio were somewhat variable; the mean values were 2.9 ± 0.6 (7) in the experiments with cyanide shown in Fig. 6 and 1.7 ± 0.1 (6) in the experiments with Amytal. In each case the ratio fell to 0.35 at maximal inhibition of respiration.

Relation of transport activity to ATP contents

Fig. 6 also illustrates the net extrusion of Na^+ in the same experiments as a function of the varying rate

Fig. 6. Relation of net transport of Na^+ and tissue content of ATP to the different rates of respiration induced by various concentrations of cyanide

Values for the net extrusion of Na^+ during incubation for 70 min at 38°C (●), and for the final content of ATP (○), have been grouped according to the rate of respiration of the same slices, irrespective of inhibitor concentration. Horizontal lines on the ATP points represent $\pm$ S.E.M. of the rate of respiration at each point. Values are the means of three to ten observations.

of respiration induced by cyanide; almost identical results were obtained when Amytal was used as the inhibitor. Dropping perpendicular lines from the curves of Na^+ extrusion to those of the adenine nucleotide content allows one to obtain estimates of the ATP content that was associated with varying levels of transport activity. Thus in Fig. 6 the amount of Na^+ extruded in 70 min was only decreased when the ATP content of the slices was less than 1.6 mmol/kg of protein. The latter value may thus be taken as the minimal ATP content compatible with the full Na^+ extrusion in this series of experiments. The ATP content pertaining at half-maximal Na^+ extrusion may be similarly estimated. From estimates of the intracellular water content of slices showing differing rates of respiration (based on measurement of the inulin space in the experiments illustrated in Table 2) the apparent concentrations of ATP in the cell water required for maximal and half-maximal Na^+ extrusion may be calculated. These values are summarized in Table 3, together with corresponding estimates of the concentration ratio, ATP/ADP, obtained from plots of this ratio against the rate of respiration.

Discussion

The results indicate that although net transport of Na^+ and K^+ by liver slices from adult rats is almost entirely dependent on cell respiration and the main-

Table 3. *Tissue adenine nucleotide contents associated with maximal and half-maximal extrusion of Na⁺*

The values recorded for experiments with Amytal and cyanide were derived from the experiments illustrated in Fig. 6 and from similar results with Amytal, as described in the text. Other values were obtained from similar experiments in which liver slices were titrated with either cyanide (0.01–1 mM) or oligomycin (1.25 μ M–0.75 mM) in the presence of 85 mM-ethanol (van Rossum, 1971*b*). Values of intracellular concentrations were based on the inulin-free water compartment of slices having rates of respiration similar to those of the slices shown in the table; these measurements of inulin-free water were obtained from the experiments summarized in Table 2.

Na ⁺ extrusion	...	...	Content of ATP (mmol/kg of protein)		Concn. of ATP (mmol/kg of intracellular water)		Ratio ATP/ADP	
			Maximal	Half- Maximal	Maximal	Half- Maximal	Maximal	Half- Maximal
Inhibitor								
Amytal			2.85	1.20	0.85	0.32	0.66	0.40
Cyanide			1.60	0.73	0.48	0.24	0.80	0.60
Cyanide+ethanol			3.35	0.70	1.00	0.19	1.10	0.65
Oligomycin+ethanol			2.85	—	0.85	—	1.48	—

tenance of an adequate content of ATP, each of these aspects of energy metabolism must be decreased substantially before transport is affected. This is true both of the maximal rate of the net movement of Na⁺ and K⁺, and of the total amount of each ion moved in attaining the final steady-state concentrations. In what follows, the results will be used to deduce some features of the energy requirements of the mechanism for the active transport of alkali metal ions in the liver cells, and it is therefore necessary to consider briefly the relation of the measured, net ion movements to the activity of the transport mechanism.

At any time during the recovery process at 38°C the ionic content of the liver cells will depend on the relative rates of the active transport mechanism and the backward passive leak, the latter being determined by the electrochemical potential for the ion at that moment and the passive permeability properties of the membrane. If the passive permeability is little affected by varying conditions (in particular, duration of incubation and rate of respiration) then both of the measured quantities (i.e. maximal rate of ion movement and total net movement) will be closely related to the rate of the active-transport mechanism. Earlier work (van Rossum, 1966*a,b*) on the efflux of ²⁴Na from liver slices incubated under conditions in which active transport is largely inoperative (anaerobic medium or K⁺-free medium) does indeed suggest that the rate constant for passive Na⁺ efflux (which is an indication of the passive permeability of the membranes) changes only slightly from 20 to 80 min of incubation at 30°C. Also the passive rate constant was little different whether respiration was inactive (incubation in anaerobic medium) or active (incuba-

tion in K⁺-free aerobic medium). It is therefore concluded that the results on the net movements of Na⁺ and K⁺ reflect the activity of the active-transport mechanism.

Energy requirements of Na⁺ and K⁺ transport

The results allow approximate quantitative conclusions to be drawn concerning the relation of various levels of transport activity to some features of energy metabolism. First, values of various parameters of adenine nucleotide metabolism may be determined at different levels of transport activity, as shown in Table 3. Results from two other series of experiments with cyanide and oligomycin as inhibitors in the presence of ethanol (van Rossum, 1971*b*) are also included in the table, and are generally close to the values reported in detail in this paper. The values of the apparent concentration of ATP at half-maximal transport activity, 0.19–0.32 mmol/kg of intracellular water, may be compared with the ATP concentration required for half-maximal activity of a variety of preparations of Na⁺+K⁺-activated adenosine triphosphatase. The latter concentrations fall in the range 0.5–1.5 mM (Post *et al.*, 1960; Whittam & Wheeler, 1961; Skou, 1957; Dunham & Glynn, 1961; Landon & Norris, 1963). But it must be emphasized that the concentrations of ATP in the intracellular water shown in Table 3 are, at best, only an approximate guide to the concentration of ATP existing at the plasma membrane, since the calculation necessarily assumes an even distribution of ATP throughout the intracellular water.

Secondly, the results of Fig. 6 and Table 1 offer estimates of the rates of 'mitochondrial' O₂ uptake

Table 4. *Estimates of the oxygen consumption required to maintain transport of Na⁺ and K⁺ in slices of rat liver*

Method 1: values represent the 'mitochondrial' respiration just sufficient to maintain maximal transport (calculated as in column 10 of Table 1). Method 2: values represent the reduction in rate of O₂ uptake when transport is specifically inhibited by addition of ouabain or omission of K⁺, the apparently direct effects of low intracellular K⁺ concentrations on the respiration having been eliminated (van Rossum, 1970a). Method 3: calculation of the rate of energy production required to maintain the observed, maximal rate of Na⁺ extrusion (Ussing, 1960) by using values and assumptions described by van Rossum (1970a, 1971a).

	Uptake of O ₂ required for transport (mmol/h per kg of protein)
Method 1 Titration with inhibitors of respiration (results from Table 1 and Fig. 6)	88–190
Method 2 Effect of specific inhibition of transport (results recalculated from van Rossum, 1970a)	42–75
Method 3 Calculated from rate of net transport:	
(a) assuming 100% efficiency of utilization of energy of ATP for transport	60
(b) assuming 50% efficiency	120

and (by assuming a value for the ratio P/O) of the rate of ATP synthesis required to maintain the full transport activity. The necessary rates of 'mitochondrial' respiration taken from titrations with Amytal and cyanide (Fig. 6) are 128 and 88 mmol of O₂/h per kg of protein and, assuming that the P/O ratio is 2.5, the corresponding rates of ATP synthesis by oxidative phosphorylation would be 640 and 440 mmol/h per kg of protein. These values of the O₂ uptake, together with those from Table 1, are summarized in Table 4. The overall range is 88–190 mmol of O₂/h per kg of protein, but in seven of the ten series of experiments the range is 128–190 mmol/h per kg. In considering the applicability of these estimates to the liver *in vivo*, or to perfused liver, it must be borne in mind that, as recently pointed out by Woods & Krebs (1971), liver slices have a considerably lower rate of glycolysis than the perfused liver preparation. The ion transport of the latter is thus likely to be even less sensitive to respiratory inhibitors than is transport in the slices, since the greater anaerobic glycolysis will compensate more effectively for the decrease in ATP production by oxidative phosphorylation. However, the estimates of the apparent ATP concentrations required for maximal and half-maximal activity of the transport system (cf. Table 3) are likely to be similar in both types of preparation.

The rates of 'mitochondrial' respiration and oxidative phosphorylation calculated above to be just sufficient to maintain full transport activity in the slices are maximal estimates of the energy required by the transport process itself in this preparation. This is because there may be other processes with K_m values for ATP equal to, or less than, that of the

transport-enzyme system, which compete with transport for the limited supply of ATP existing when respiration has been partially inhibited (see below). Conversely, a minimal estimate of the O₂ uptake required for transport *per se* has been obtained from the reduction of respiratory activity that is seen when the transport process is specifically inhibited by, for example, ouabain (van Rossum, 1970a). The results, recalculated on a protein basis, are shown in Table 4 (method 2); they are a minimal estimate since it is possible that when transport is inhibited some of the ATP it normally utilizes is diverted to other reactions. Although the range of results obtained to date from each of these maximal and minimal estimates is rather large, they do appear to establish absolute limits to the respiratory activity that is required to support the transport of Na⁺ and K⁺ in the liver slices. Estimates of the O₂ consumption required to support the transport system *per se* have also been made by calculating the work done in maintaining the observed, maximal rate of Na⁺ transport by the liver slices (Table 4, method 3). Such estimates fall within the range of the limits set by the two methods involving direct observation of O₂ uptakes.

The question arises as to how other energy-requiring processes of the liver cell respond to a partial limitation of the ATP synthesis. Relatively little relevant information seems to be available, but results of Scholz *et al.* (1966) show that urea synthesis in perfused rat liver is not affected by Amytal until the respiration has been decreased by 40%. Thus urea synthesis shows a sensitivity similar to that of ion transport and is likely to be one example of a process that competes with transport for ATP when respiration has been decreased below 50% (see above). In

contrast, recent work suggests that the incorporation of [^{14}C]glycine into the protein of liver slices is considerably more sensitive to inhibition of respiration than ion transport is, since titration with Amytal causes it to be inhibited by 96% at a rate of respiration that is still adequate to support maximal net transport of K^+ (van Rossum, 1972). However, limitation of the availability of ATP is not necessarily the major factor governing the rate of all energy-requiring processes in the liver cell when the respiration is reduced. Thus Scholz *et al.* (1966) showed that gluconeogenesis in the perfused liver was completely inhibited when respiration was decreased by 50%, and Ballard (1971) has shown that its inhibition in livers of young rats occurs at levels of hypoxia at which the ATP content and ATP/ADP ratio are not affected. The principal controlling factor in this case appears to be the redox level of the nicotinamide-adenine dinucleotides.

Comparison with other tissues

Slices of the rapidly growing Morris hepatoma 3924A incubated in a substrate-free medium show a similar relation of Na^+ and K^+ transport to endogenous respiration as that seen in liver slices, transport being unaffected until respiration has been reduced by more than 50% (van Rossum *et al.*, 1971). In the brains of rats subjected to hypoxia, the maintenance of the normal low Na^+ content of the cells was not affected until the ATP content had fallen by more than 25% (Reulen *et al.*, 1970), although Okamoto & Quastel (1970) reported an inverse linear relation of the water content (which is inversely related to ion-transporting activity) to ATP content of slices of rat brain cortex. The rate of Na^+ transport across the frog skin is roughly linearly related to the falling ATP/ADP ratio in anaerobiosis (Kristensen & Schousboe, 1969). Results of Mudge (1951) with kidney-cortex slices incubated in media containing varying concentrations of arsenite or with varying pO_2 suggest that there was a linear relation between K^+ accumulation and O_2 uptake, although treatment with a low concentration (0.1 mM) of cyanide appeared to cause a somewhat greater inhibition of respiration than of K^+ transport. However, it seems clear that the ion-transporting mechanism of the liver and hepatoma cells, and therefore the maintenance of cellular ionic composition and volume (Leaf, 1956), shows a greater resistance to a fall in the supply of metabolic energy than does the corresponding function of any of the other tissues for which information is available. This could either be due to differences of K_m for ATP or of other kinetic properties of the transporting enzymes of liver and hepatoma, or to a greater resistance of the passive permeability properties of the cell membranes of these two cell types to a limitation of the energy supply.

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