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THE EFFECTS OF OLIGOMYCIN ON ENERGY METABOLISM AND CATION TRANSPORT IN SLICES OF RAT LIVER

INHIBITION OF OXIDATIVE PHOSPHORYLATION AS THE PRIMARY ACTION

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SUMMARY

1. In slices of rat liver, oligomycin inhibited the net transport of Na^+ and K^+ by a maximum of 30 % and endogenous respiration by 25 %. These effects were not increased by a number of modifications in the incubation conditions.

2. Mitochondria isolated from the slices after incubation showed respiratory control ratios that were somewhat less than in mitochondria from fresh liver, but state 3 respiration retained normal sensitivity to oligomycin.

3. Low concentrations of oligomycin or cyanide reduced respiration and ATP levels of the slices but did not affect ion transport unless these levels fell below a definite critical value. In contrast, ouabain and atractyloside each caused substantial degrees of transport inhibition at ATP levels which were in excess of the critical value.

4. High concentrations of cyanide and oligomycin reduced ATP contents maximally by 90 % and 65 %, respectively. Studies of lactate production, and of the effects of arsenite on respiration and ATP levels, suggested that substrate-level phosphorylation in the citric-acid cycle was the major source of the oligomycin-resistant ATP synthesis.

5. The results suggest that oligomycin acts in the liver slices primarily as an inhibitor of oxidative phosphorylation, and that this is the cause of the partial inhibition of ion transport. The oligomycin-resistant ion-transporting activity is consistent with the persisting level of ATP synthesis.

INTRODUCTION

Oligomycin causes a partial inhibition of respiration and of the energy-dependent transport of Na^+ and K^+ in slices of rat liver, and it was suggested previously that the oligomycin-resistant transport may be supported by high-energy intermediates of oxidative phosphorylation [1, 2]. However, oligomycin is known to inhibit two distinct enzyme systems, namely, mitochondrial oxidative phosphorylation

[3] and, at higher concentrations, the Na^+ and K^+-stimulated adenosine triphosphatase of the plasma membrane [4, 5]; each of these two actions has been invoked to account for the effects observed in whole cells [1, 6–8].

We have now attempted to determine which inhibition is the major one in liver slices, by comparing the effects of oligomycin and other inhibitors of the two enzyme systems on the levels of adenine nucleotides and the transport activity. The results indicate that the inhibition of oxidative phosphorylation can account for the effects of oligomycin seen in this preparation, and that the oligomycin-resistant transport of ions is supported by a small amount of continuing ATP synthesis from mitochondrial sources. Preliminary accounts of some of this work have been published [9, 10].

METHODS

Work with liver slices. The incubation procedures and analytical methods used in the experiments with slices of rat liver have been described previously [11, 12]. Male, albino rats weighing 200–300 g were used.

Work with isolated mitochondria. Mitochondria were prepared both from fresh tissue and from incubated liver slices taken from the same animals. In the latter case, the contents of the incubation flasks were poured onto a coarse filter paper in a Büchner funnel under suction, thus separating the slices from the medium. The slices on the filter were rapidly rinsed with mitochondrial-isolation medium and then transferred to a tared beaker containing the medium. Mitochondria were then isolated by the method of Johnson and Lardy [13], modified by the use of isolation medium containing, 250 mM mannitol, 75 mM sucrose, 2.5 mM Tris (pH 7.4) and 0.1 mM EDTA. The "fluffy layer" obtained by two washings of the surface of the mitochondrial pellet was collected as a loose pellet by centrifugation at 17 300 $\times g$ for 15 min. Mitochondrial respiration was determined with a Clarke-type oxygen electrode, using as a suspension medium the isolation medium to which had been added 5 mM Mg^{2+} and 5 mM phosphate (pH 7.4). In each run, approximately 10 mg protein were added to 2.1 ml medium. Substrates and ADP were added as indicated. Sensitivity to oligomycin was tested by comparing the effect of ADP on the rate of respiration before and after addition of the inhibitor, 5 min being allowed for the inhibition to develop fully. Oligomycin titration curves were constructed from the results of multiple runs with each mitochondrial preparation. In most cases, each preparation was tested at 5–7 different oligomycin concentrations, with duplicate runs at each concentration.

RESULTS

Influence of incubation conditions on effects of oligomycin.

(*a*) *Slices.* In view of the much smaller maximal inhibition by oligomycin of the respiration of liver slices than of isolated liver-mitochondria [1, 3], the influence of incubation conditions on the sensitivity of the slices was first studied. The basic experimental procedure consisted of a pre-incubation in phosphate-buffered Ringer solution for 90 min at 1 °C (to deplete the cells of K^+ and load them with Na^+) followed by incubation at 38 °C in oxygenated medium (when the ion changes were

TABLE I

EFFECTS OF DELAYED ADDITION OF OLIGOMYCIN TO LIVER SLICES

The standard incubation procedure consisted of pre-incubation for 90 min at 1 °C followed by incubation for 60 min at 38 °C in a Warburg manometric apparatus gassed with O_2. The phosphate-buffered Ringer solution contained: Na^+, 161 mM; K^+, 5 mM; Ca^{2+}, 1.2 mM; Mg^{2+}, 1 mM; Cl^-, 153.4 mM; SO_4^{2-}, 1 mM; phosphate, 10 mM (pH 7.4). For the Tris-buffered Ringer solution, 10 mM Tris · HCl (pH 7.4) replaced the 10 mM sodium phosphate. Oligomycin (10 μg/ml) addition was either made after 30 min at 1 °C, or was delayed until after 5 min at 38 °C when it was added from the side-arm of the Warburg vessel. All flasks contained 0.3 % ethanol (the solvent for oligomycin). Values are Mean±SEM.

Incubation	Respiration (mmol/kg protein/h)		Tissue K^+ (mmol/kg protein)	
	Phosphate Ringer	Tris Ringer	Phosphate Ringer	Tris Ringer
90 min at 1 °C	–	–	134± 8	149±13
Then 60 min at 38 °C:				
Without oligomycin	372±9	342±8	419±18	381±12
Oligomycin added at 1 °C	326±4	290±16	351±17	322±23
Oligomycin addition delayed	351±7	312±12	371±17	343±25
(*n*)	(12)	(8)	(11)	(8)

reversed by active transport). Restriction of the pre-incubation to a 3-min period (the minimum practical) in a medium at 20 °C gassed with O_2 did not significantly increase the sensitivity of respiration to a high concentration (10 μg/ml) of oligomycin. Further, in contrast to the results of Currie and Gregg [14] with various isolated cells, neither respiration nor K^+ accumulation of liver slices showed any greater sensitivity to oligomycin when the addition of inhibitor was delayed until incubation at 38 °C had proceeded for 5 min (Table I). Use of Tris · HCl as the sole buffer in the Ringer, or of dimethyl sulphoxide (0.033 % v/v) instead of the usual ethanol (0.3 % v/v) as solvent for the oligomycin, did not alter any of these results.

(*b*) *Mitochondria isolated from slices.* In order to see if the incubation procedures in vitro had affected the oligomycin sensitivity of the mitochondria, these organelles were isolated from fresh liver and from liver slices that had been incubated for 90 min at 1 °C and for a further 30 min at 38 °C in oxygenated, phosphate-buffered Ringer solution. The rates of respiration in states 1, 2 and 4 (as defined in ref. 15) with glutamate (8 mM) plus L-malate (0.8 mM) as substrate were not affected by the pre-treatments of the tissue; and while the rate in state 3 appeared to be reduced about 20 % after incubation at 38 °C, the effect was not statistically significant. However, the respiratory-control ratios were successively reduced, from 5.5±0.1 in mitochondria from fresh liver to 4.7±0.1 and 3.5±0.2 after incubation of slices at 1 °C and 38 °C, respectively. Nevertheless, titration with oligomycin totally abolished the respiratory control response to ADP in each preparation, and the concentrations needed for half-maximal effects were very similar in each, namely: 0.15, 0.13 and 0.33 μg oligomycin/mg mitochondrial protein, respectively. The recovery of protein in the mitochondrial pellet fell substantially after incubation of the slices while that

of the "fluffy layer" fraction increased correspondingly; the properties of the latter were therefore also studied. The respiratory-control ratios were 3.3 ± 0.2 and 2.6 ± 0.1 for the "fluffy layer" from slices incubated at 1 °C and then at 38 °C, respectively, the reduction from the values found with the mitochondrial pellets being entirely due to a fall of the rates of respiration in state 3. These ratios were also reduced to unity by oligomycin, with concentrations for half-maximal effects being 0.08 and 0.07 μg oligomycin/mg fraction protein, respectively. In summary, the mitochondria of the slices underwent no change of properties which could account for the unexpectedly small effect of oligomycin on the respiration of the whole slices.

Effects of inhibitors on adenine nucleotides in slices

The effects of oligomycin and other inhibitors on oxidative phosphorylation in the intact slices was estimated by determining the adenine nucleotide content existing at the end of a standard 60-min period of incubation at 38 °C. Previous work has shown that the nucleotides remain in a steady state from the 10th to the 70th minute at 38 °C, while the ions attain a steady state after 45 min [12]. Fig. 1 shows

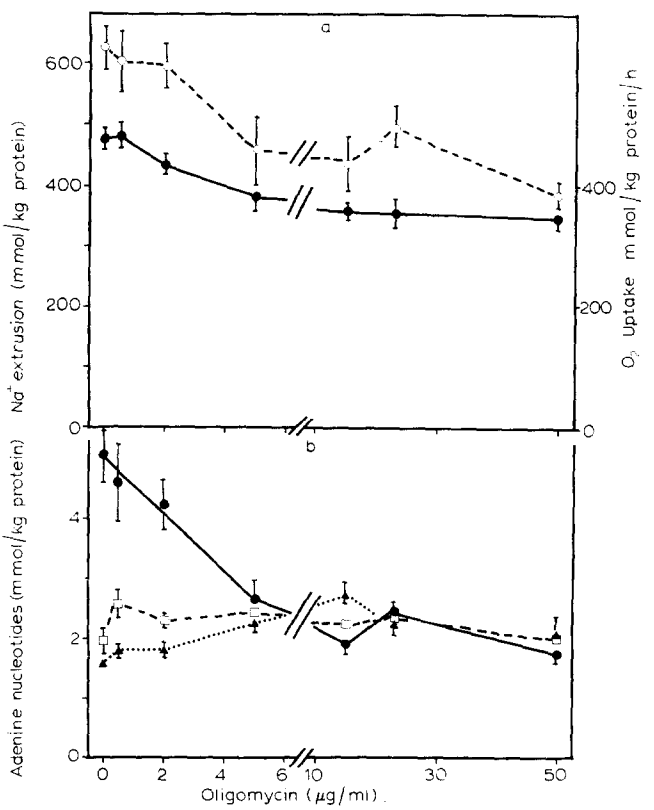

Fig. 1. Effects of oligomycin on liver-slice activities; 0.3 % ethanol was present in all incubation media. General details as for Fig. 2. (a) Rate of respiration (●) and the net extrusion of Na^+ (○) during 60 min at 38 °C; $n = 14$. (b) Adenine nucleotide contents of the slices after 60 min at 38 °C. ●, ATP; □, ADP; ▲, AMP; $n = 14$.

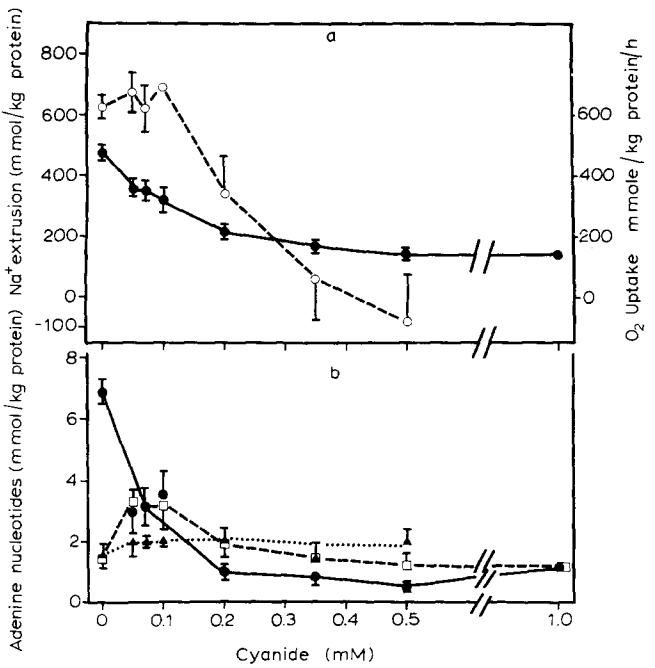

Fig. 2. Effects of cyanide on liver-slice activities 0.3 % ethanol was present in all incubation media. (a) Rate of respiration (●) and net extrusion of Na^+ (○) during incubation at 38 °C for 60 min; $n = 8$. General experimental details as described in Table I. The centre wells of the Warburg manometric flasks contained mixtures of KCN and KOH appropriate to maintain the concentration of cyanide in the incubation medium [11]. (b) Adenine nucleotide contents of the slices after 60 min at 38 °C. ●, ATP □, ADP; ▲, AMP; $n = 8$. The points and bars represent mean ± standard error of the mean.

that the lowest concentration of oligomycin used (0.5 μg/ml) caused a 10 % fall of ATP and a small increase of ADP. The maximal reduction of ATP (65 %) occurred at 5–10 μg oligomycin/ml, when respiration was inhibited by only 25 %. There was also a small increase of AMP, but most of the loss of ATP was accounted for by a decrease of total adenine nucleotides [12]. In the presence of the concentration of ethanol used as solvent for oligomycin, cyanide caused up to 90 % reduction of ATP content and some increase of ADP and AMP (Fig. 2). About 30 % of the respiration was resistant to cyanide, but this probably represents the activity of microsomal oxidases [16] in which case the residual 10 % of ATP was presumably derived from glycolysis. Plots of the relation of "energy charge" [17] versus rate of respiration obtained with these two inhibitors (not illustrated) followed a closely parallel course, suggesting that both agents acted at closely related sites to inhibit oxidative phosphorylation.

Both ouabain and atractyloside caused a rather similar fall of respiration to that seen with oligomycin (Fig. 3), but whereas ouabain reduced neither the ATP content nor the "energy charge", atractyloside reduced them both slightly (but not to the same extent as oligomycin did). The lowest concentrations of atractyloside

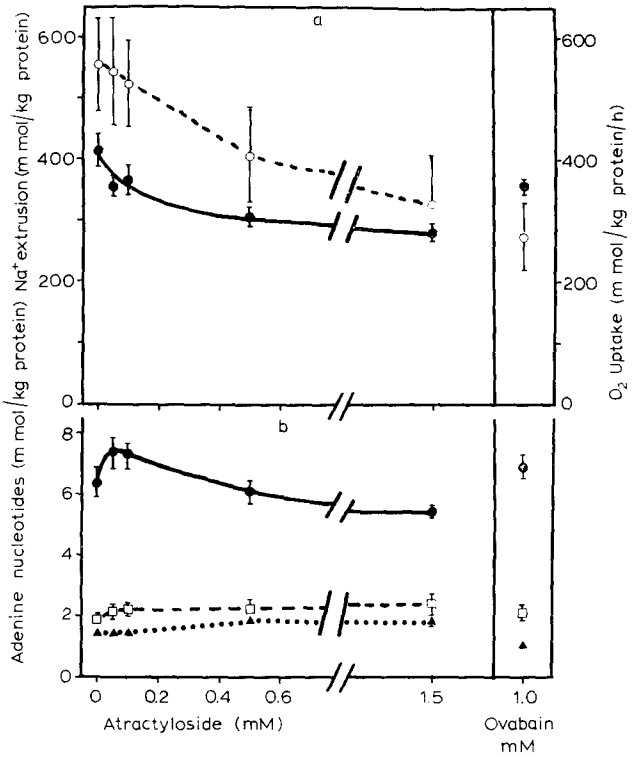

Fig. 3. Effects of atractyloside and ouabain on liver-slice activities. General details as in Fig. 2. The effects of ouabain at a single concentration (1 mM) are shown by the individual points in the right-hand portion of the figure. (a) Rate of respiration (●) and the net extrusion of Na^+ (○) during 60 min at 38 °C; $n = 12$. (b) Adenine nucleotide contents of the slices after 60 min at 38 °C. ●, ATP; □, ADP; ▲, AMP; $n = 12$. In the case of AMP, the standard errors of the mean were smaller than the size of the symbols.

showed some tendency to increase the ATP content of the slices, although the effect was not statistically significant.

Effects of inhibitors on ion transport

Figs 1–3 include measurements of the net transport of Na^+ from the same experiments. The results may be summarised as follows: (i) Cyanide totally inhibited Na^+ extrusion at ATP levels that were 10 % of control. (ii) On the other hand, ouabain caused a large (60 %) inhibition of Na^+ extrusion (Fig. 3) and total inhibition of K^+ accumulation [18] despite the persistence of high levels of ATP. The results with cyanide and ouabain are clearly consistent with their known primary sites of inhibitory action. (iii) The 65 % reduction of ATP content caused by oligomycin was associated with only a 30 % fall of Na^+ extrusion (Fig. 1) and K^+ reaccumulation (Table I). (iv) Atractyloside inhibited Na^+ transport (by 40 %; see Fig. 3) and K^+ transport (by 75 %; not illustrated) even more than oligomycin did.

Further information on the mechanism of action of the inhibitors was obtained by comparing plots of the transport activity and ATP content of individual samples

against the differing rates of respiration induced by increasing concentrations of the agent studied. Thus, Fig. 4, shows that in the presence of ethanol increasing concentrations of cyanide did not reduce the net transport of Na^+ until the respiration had fallen below 350 mmol O_2/kg protein/h, at which point the slices had a mean ATP content of 3.4 mmol/kg protein and an "energy charge" of 0.59 [cf. ref. 12]. Similarly, Fig. 5 indicates that the minimal ATP content consistent with full transport activity in the presence of oligomycin was 2.9 mmol/kg, the corresponding "energy charge" being 0.57. These correspond closely to the values seen in the titration with cyanide, and it may be concluded that the reduction of energy levels caused by oligomycin is alone sufficient to account for the onset of transport inhibition.

The question then arises as to whether the level of ATP that is maintained in the presence of the higher concentrations of oligomycin is sufficient to support the large, oligomycin-resistant portion of the Na^+ transport. From Fig. 5, this fraction was about 70 % of the control extrusion (i.e. 470 mmol Na^+ lost per kg protein, from a control loss of 620 mmol/kg) and was associated with a minimal ATP level of 1.9 mmol/kg protein and "energy charge" of 0.43. From Fig. 4 it can be seen that the same level of ATP, in the presence of cyanide as inhibitor, was associated with a net extrusion of 500 mmol Na^+/kg protein, compared to a control extrusion of some 680 mmol/kg, i.e. approximately 70 % again. Thus, both the onset of transport inhibition at low oligomycin concentrations, and the degree of resistance of transport to the inhibition, are closely related to the level of ATP in the tissue. This is in contrast to

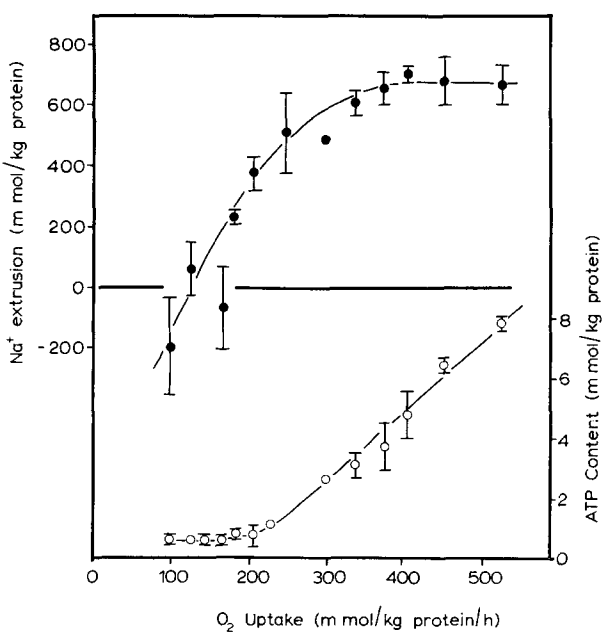

Fig. 4. Relation of ATP content and net extrusion of Na^+ to the rate of respiration of liver slices incubated in the presence of different concentrations of cyanide. Results from Fig. 1 have been grouped according to the varying rates of respiration that were induced by the different concentrations of cyanide. ●, net extrusion of Na^+; ○, final ATP content.

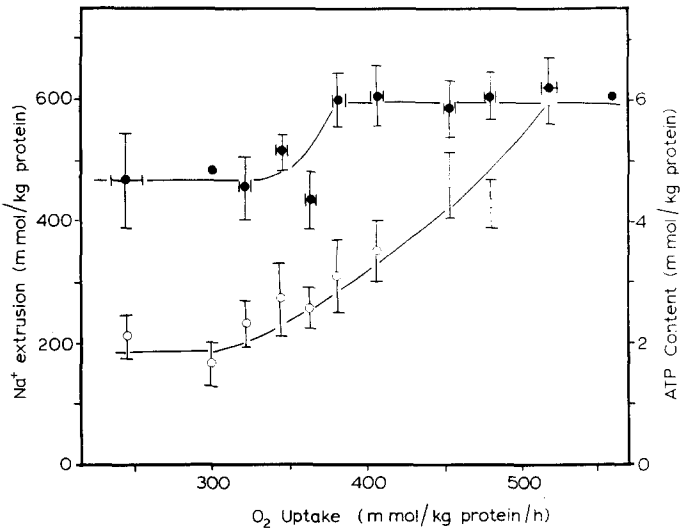

Fig. 5. Relation of ATP content and the net extrusion of Na^+ to the rate of respiration of liver slices incubated in the presence of different concentrations of oligomycin. Results from Fig. 2 have been grouped according to the rates of respiration of the individual samples of slices. ●, net extrusion of Na^+; ○, final ATP content.

the effect of ouabain, where the larger inhibition of transport was accompanied by no such fall in energy levels.

Source of ATP in the presence of oligomycin

Since oligomycin appeared effectively to inhibit oxidative phosphorylation in the slices, it follows that the higher ATP content maintained in the presence of oligomycin than of cyanide must have been derived from the substrate-level phosphorylation associated with either glycolysis or with α-oxoglutarate oxidation in the citric-acid cycle. The former only appears able to contribute a small portion of the extra ATP, since oligomycin increased lactate production by only 30 % more than

TABLE II

EFFECTS OF OLIGOMYCIN AND CYANIDE ON THE RESPIRATION AND LACTATE PRODUCTION OF LIVER SLICES

All flasks contained 0.33 % ethanol but no other added substrates. The rats were fed ad libitum. The lactate production has been corrected for the small amount present after the pre-incubation period at 1 °C.

Inhibitor	O_2 Uptake (mmol/kg protein/h)	Lactate production (mmol/kg protein/h)
None	396±24 (7)	78.8±10.4 (9)
Oligomycin (10 μg/ml)	335±24 (8)	139.7± 7.8 (9)
Cyanide (1 mM)	137±17 (6)	101.9± 9.3 (9)
Oligomycin plus cyanide	139±39 (6)	129.9±19.0 (9)

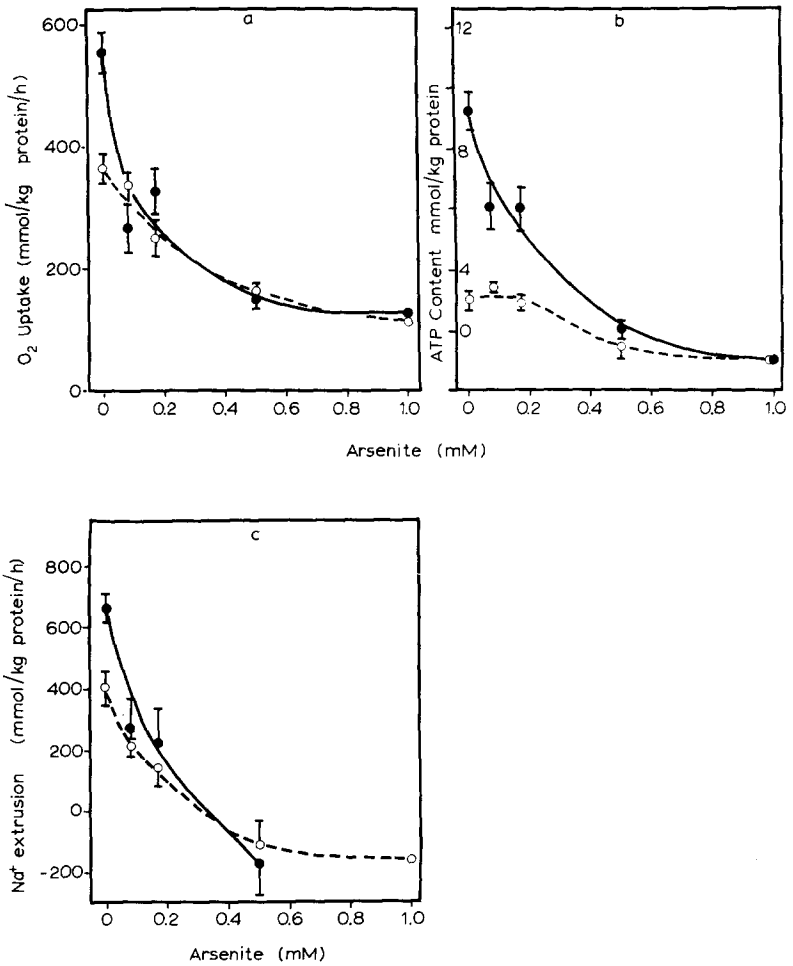

Fig. 6. Effects of arsenite on the activities of liver slices incubated with or without oligomycin; all flasks contained 0.3 % ethanol. General experimental details as for Fig. 1. ●, without oligomycin; ○, with oligomycin (20 μg/ml). (a) Rate of respiration; (b) ATP content after 60 min at 38 °C; (c) net extrusion of Na^+ during 60 min at 38 °C. $n = 6$.

cyanide did (Table II) although maintaining an ATP content three times higher (compare Figs 1 and 2). Other experiments (not illustrated) showed that lactate production was linear over the 60 min incubation period with either inhibitor.

The oligomycin-resistant respiration was found to be sensitive to cyanide (Table II), and was thus of mitochondrial origin. Further, Fig. 6 shows that the slice functions of oxidative phosphorylation and Na^+ transport were very sensitive to arsenite. Although the concentration of this inhibitor required for half-maximal inhibition was somewhat greater in the presence of oligomycin than in controls, the results suggest the great importance of the citric-acid cycle and, in particular, α-oxoglutarate dehydrogenase, as a source of energy for ion transport. On the other hand, malonate (20 mM, a maximally inhibiting concentration [19, 20]) reduced the

respiration of control liver slices incubated with 0.5 % ethanol by 25 %, but did not affect the oligomycin-resistant respiration. Malonate also failed to reduce the ATP content and Na^+ extrusion of either control or oligomycin-treated slices.

DISCUSSION

Although oligomycin is known to be an inhibitor of the Na^+ and K^+-stimulated adenosine triphosphatase and to inhibit ion transport in mammalian erythrocytes by virtue of this action [6], it is a more powerful inhibitor of mitochondrial oxidative phosphorylation [5]. In the present work, oligomycin clearly affected energy metabolism in a different manner from ouabain in that it reduced ATP levels and the "energy charge" and stimulated glycolysis (whereas ouabain reduces lactate production [21, 22]). These effects were more closely similar to those of cyanide and are clearly consistent with an action of oligomycin as an inhibitor of oxidative phosphorylation. Further, comparison of the minimal ATP levels critical for the maintenance of full Na^+ transport activity in the presence of cyanide and oligomycin shows that it is the limitation of the energy supply, rather than an inhibition of ATP utilisation, which is the cause of the inhibition of transport by oligomycin. The effect of atractyloside in causing considerable inhibition of transport in the presence of high levels of ATP is of interest. This agent does not inhibit the Na^+ and K^+-stimulated adenosine triphosphatase [23] and it is therefore suggested that the inhibition of ATP efflux from the mitochondria by atractyloside [24] prevents the cytosolic concentration of ATP from attaining the critical level required for maintenance of maximal Na^+ transport at the plasma membrane.

The persistence of a substantial transport of Na^+ and K^+ in liver slices, from adult rats, treated with oligomycin was previously suggested to be due to direct utilisation of high-energy intermediates of oxidative phosphorylation [1], but comparison of the transport activity at varying ATP contents seen in Fig. 4 and 5 indicates that the level of oligomycin-resistant synthesis of ATP is sufficient to support the observed transport activity. The major metabolic source for the oligomycin-resistant synthesis of ATP does not appear to be glycolysis (Table II). Rather, positive evidence for the importance of intra-mitochondrial respiration as the source of this ATP is provided by the inhibitory effects of cyanide and arsenite in the presence of oligomycin. The inhibition of ion transport by atractyloside also indicates the importance of an intra-mitochondrial source of ATP for the support of transport. Since state 3 respiration in mitochondria isolated from the slices was completely sensitive to oligomycin, the most likely source of the oligomycin-resistant ATP formation is the substrate-level phosphorylation associated with succinyl-CoA synthetase (EC 6.2.1.4). The sensitivity of the slice functions to arsenite provides support for this conclusion. At first sight, the inability of malonate to inhibit the oligomycin-resistant functions is not in accord with this suggestion. However, the control endogenous respiration (although not that of added succinate) is also rather insensitive to malonate [20] and, furthermore, succinate only undergoes the one-step oxidation to malate in this preparation [25]. Thus, it appears that the activity of the citric-acid cycle beyond the fumarase step is slow in liver slices, most of the respiration being due to oxidation of the endogenous substrates through the span from citrate to malate. The insensitivity to malonate thus does not necessarily invalidate the suggested importance of the

substrate-level phosphorylation in the cycle as a source of energy for the oligomycin-resistant transport of ions.

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