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THE MECHANISM OF INHIBITION BY OLIGOMYCIN OF OXIDATIVE PHOSPHORYLATION IN MITOCHONDRIA

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SUMMARY

1 The concentration of specific oligomycin-binding sites in rat-liver mitochondria is 0 12 nmole/mg protein, whereas at least 10-times more oligomycin can be bound non-specifically

2 The activity of oligomycin-inhibited processes in intact mitochondria and submitochondrial particles cannot be restored by treatment with egg lecithin or mitochondrial phospholipids

3 Analysis of the kinetics of inhibition of State-3 respiration by oligomycin reveals that (i) after a certain lag period the inhibition by oligomycin is pseudo-first order with respect to the respiratory-control ratio, defined as the ratio of the respiratory rate at time t to that of the final inhibited site, (ii) the value of the pseudo-first-order rate constant (k_0) is dependent on the oligomycin protein ratio, phospholipid protein ratio, pH and temperature, (iii) the effects of various substrates and inhibitors of electron transfer on the kinetics of oligomycin inhibition can be explained by their effects on respiratory control

4 A detailed model is proposed for the interaction of oligomycin with mitochondria It is proposed that two conformations of the oligomycin-sensitive site are present, and that oligomycin specifically binds to the conformation that is involved in the induction of respiratory control

INTRODUCTION

The antibiotic oligomycin, introduced as an inhibitor of oxidative phosphorylation by Lardy et al [1], has contributed considerably to our present views on the coupling of ATP synthesis to electron transport [2–10] It has been proposed that oligomycin inhibits the reaction of the hypothetical high-energy intermediate I~X with P_i or H_2O [6, 7, 9] or acts at the level of transduction of energy of the membrane potential to that of high-energy compounds [10] Based on experiments with submitochondrial systems Ernster has proposed that oligomycin directly titrates I~X [11]

Abbreviation CCCP, carbonyl cyanide *m*-chlorophenylhydrazone

Without specifying the nature of the energized state, we have proposed that oligomycin interacts preferentially with energized mitochondrial membranes [9,12,13] This proposal was based on the interpretation of condition-dependent changes in the shape of oligomycin-inhibition curves and the lag phase preceding oligomycin inhibition Under relatively highly energized conditions, namely those used for measurement of P_i–ATP exchange [3, 13, 14], ADP–ATP exchange [14], arsenate-induced respiration [15] or ATP-driven glutamate synthesis [8], the oligomycin-inhibition curves are completely straight, whereas sigmoidal curves are obtained for State-3 succinate oxidation [3, 8, 16] and the uncoupler-induced ATPase (at suboptimal uncoupler concentration) [8, 12] At that time we had no explanation for the linear oligomycin-inhibition curve for the uncoupler-induced ATPase at high uncoupler concentrations [15] An earlier suggestion that the sigmoidal oligomycin-inhibition curve for State-3 respiration might be a consequence of a rate-limiting step at a site other than that sensitive to oligomycin [3, 8, 16] is eliminated by the observation that, under State-3 conditions, the cytochromes, ATPase and adenine nucleotide translocator behave kinetically as a single enzyme that is rate-limiting in succinate or glutamate–malate oxidation [17] Based on the delay in the effect of oligomycin in inducing the energy-linked enhancement of the fluorescence of anilinonaphthalene sulphonate in the presence of submitochondrial particles, Ernster et al [18] have also proposed that oligomycin interacts with the 'energized state' of the mitochondrial membrane

The interesting question remains if the occurrence of sigmoidal inhibition curves can be explained on the basis of the existence of different conformational states of the oligomycin-sensitive site, as has been shown to be the case for the antimycin [19], aurovertin [20] and gummiferin [21] sensitive sites This is examined in this paper

RESULTS

Expression of the interaction of oligomycin with mitochondria

Both the kinetics of inhibition by sub-optimal concentrations of oligomycin and the final extent of inhibition are strongly dependent on the reaction studied [8, 9, 22] This is demonstrated in the experiment shown in Fig 1, in which mitochondria were pre-incubated for 2 5 min in the presence of substrate, ATP and variable amounts of oligomycin, after which either uncoupler or ADP+P_i was added, and the rate of the ATPase or the respiration, respectively, was measured as a function of time The curves in Fig 1 show that after an identical pre-incubation of the mitochondria with oligomycin (i) the degree of inhibition is dependent on the reaction measured and (ii) the inhibition of the uncoupler-induced ATPase does not change with time, whereas that of the State-3 respiration is greater after 2 min than immediately after adding the ADP and P_i The immediate and constant inhibition of the uncoupler-induced ATPase shows that the lag in reaching maximum inhibition of respiration is not due to a penetration barrier to the oligomycin, a possibility that was raised by Klingenberg [23] The finding that the expression of the interaction of oligomycin with mitochondria depends on the reaction that is used to study it suggests rather that different states of the oligomycin-sensitive site are probed in the two reactions

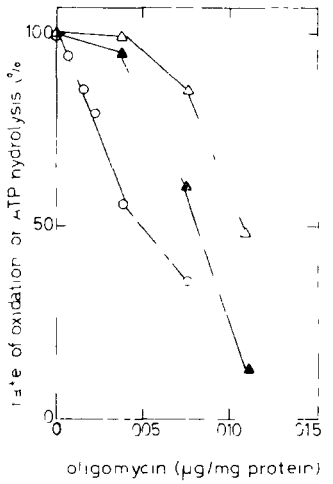

Fig 1 Comparison of the oligomycin-inhibition curves for the CCCP-induced ATPase and State-3 respiration after identical preincubation Mitochondria were incubated with oligomycin for 2 5 min in a medium containing glutamate+malate and ATP (3 mM) before State-3 respiration or ATPase activity was initiated by the addition of ADP (1 mM)+ P_i (10 mM) or CCCP (1 μM) respectively Volume, 1 55 ml (State 3) and 3 10 ml (ATPase), pH 7 3, protein, 0 9 mg/ml ○—○ ATPase activity measured initially, or 1 or 2 min after addition of uncoupler △—△ State-3 respiration measured immediately after the addition of ADP—P_i ▲—▲ State-3 respiration measured 2 min after the addition of ADP + P_i

Kinetics of inhibition of State-3 respiration by oligomycin

The kinetics of the inhibition of State-3 respiration by oligomycin were studied by measuring the decrease in $-d[O_2]/dt$ after the addition of oligomycin to State-3

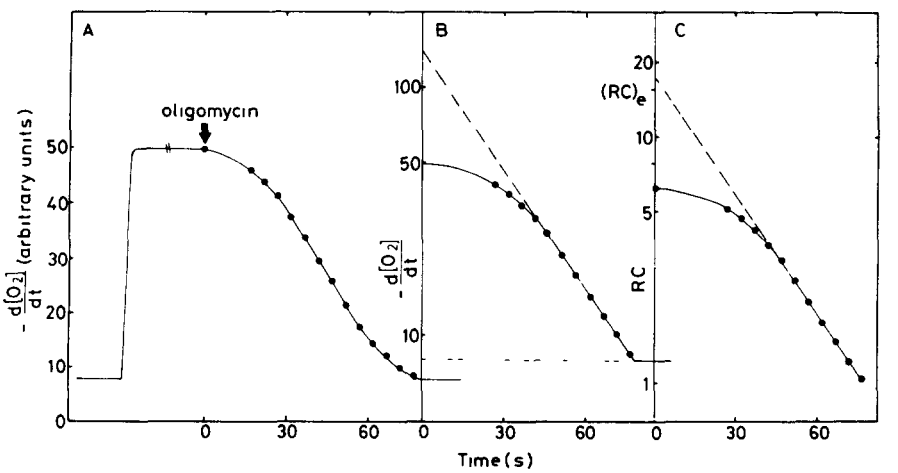

Fig 2 Analysis of the kinetics of inhibition of State-3 respiration by oligomycin At zero time 0 3 μg oligomycin/mg protein was added to mitochondria oxidizing succinate in State 3 The rate of succinate oxidation was measured continuously as $-d[O_2]/dt$ Protein 0 96 mg, volume 1 98 ml A $-d[O_2]/dt$ versus time (50 arbitrary units correspond with 280 natoms O/min), B $-d[O_2]/dt$ plotted on a logarithmic scale versus time, C, *RC* plotted on a logarithmic scale versus time, $(RC)_e$ is obtained by extrapolating the first-order phase to zero time

mitochondria Fig 2A shows such a trace at a relatively high oligomycin concentration (0 3 μg/mg protein), and Fig 2B shows the same data plotted semi-logarithmically Since no correction for the oligomycin-insensitive 'State-4' respiration was made, this curve sharply approaches a plateau when the latter rate is reached In Fig 2C, this is overcome by plotting the data semilogarithmically in terms of the respiratory-control ratio (RC), where RC = (rate at time t)/(final inhibited rate), i e

$$\log RC = \log \left[\frac{d[O_2]}{dt}\right]_{t=t} - \log \left[\frac{d[O_2]}{dt}\right]_{t=\infty}$$

It appears that, after a certain lag period, $-dRC/dt$ is linearly dependent on RC as long as $RC > 1$, or, more generally, that both $-dRC/dt$ and RC are linearly related to the same function

From the slope of the straight portion of the curve in Fig 2C a pseudo first-order rate constant can be calculated, $k_0 = 0\,693/t_{0\,5}$ Extrapolation of this straight portion to zero time (i e time of oligomycin addition) gives an intersection of the ordinate that is termed $(RC)_e$ Fig 3A shows that at high oligomycin protein ratios, when $(RC)_e$ has reached a constant value, k_0 is hyperbolically related to the oligomycin protein ratio (see Fig 3B) From an extrapolation of the straight line in Fig 3B it can be calculated that at infinite oligomycin $k_0 = 0\,2\ s^{-1}$, and k_0 is 50 % of this maximal value with 1 μg oligomycin per mg protein

The data in Table I show that when the oligomycin and protein concentrations are varied by a factor of 3, but keeping the oligomycin protein ratio constant, the value of k_0 does not change within the accuracy of the experiment Thus, at least at these oligomycin protein ratios, k_0 is strictly dependent on the oligomycin protein ratio and not on the oligomycin or protein concentration alone Essentially the same

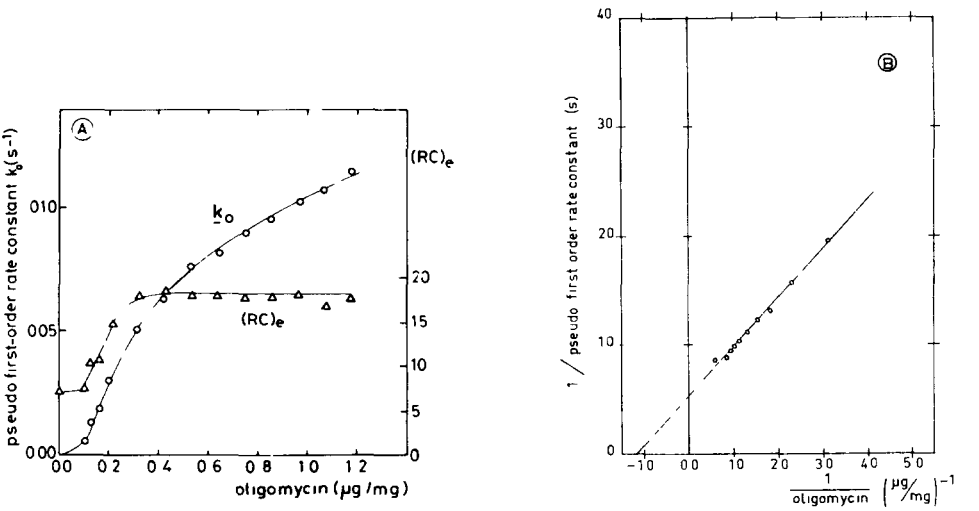

Fig 3 Effect of oligomycin protein ratio on k_0 and $(RC)_e$ Substrate, succinate, volume 1 90 ml, protein, 1 4 mg k_0 and $(RC)_e$ were calculated from plots as shown in Fig 2C, where k_0 is $0\,693/t_{0\,5}$ A, k_0 and $(RC)_e$ as function of oligomycin protein ratio, B, $1/k_0$ as a function of (oligomycin/protein)$^{-1}$ for oligomycin protein ratios higher than 0 3 μg/mg protein

TABLE I

THE EFFECT OF OLIGOMYCIN AND PROTEIN CONCENTRATION ON k_0

Conditions as described in Fig 2 Volume 1 96 ml

Oligomycin (μg)	Protein (mg)	Ratio (μg/mg)	k_0 (s^{-1})
0 066	0 48	0 14	0 0096
0 132	0 96	0 14	0 0098
0 099	0 72	0 14	0 0093
0 198	1 44	0 14	0 0098
0 099	0 48	0 20	0 027
0 148	0 72	0 20	0 028
0 198	0 96	0 20	0 027

conclusion can be drawn from experiments not shown here, using higher oligomycin protein ratios (0 25–0 8 μg oligomycin/mg protein) It may be concluded that at these oligomycin protein ratios all oligomycin added is rapidly bound to the mitochondria

The pseudo first-order character of the kinetics of oligomycin inhibition is not affected by changes in pH between 6 0 and 8 5, but k_0 increases with increasing pH, especially between 6 0 and 7 0 (Table II) This effect of pH on the kinetics of oligomycin inhibition may explain the finding (Bertina, R M , unpublished experiments) that at low pH, State-3 respiration appears to be relatively insensitive to oligomycin (when measured at the same time interval after oligomycin addition) The double-reciprocal plots in Fig 4 show that the effect of low pH on k_0 is not its maximal value but is due to an increase in the oligomycin protein ratio at which 50 % of the maximum value of k_0 is reached

The effect of temperature on the kinetics of oligomycin inhibition is shown in the Arrhenius plot of Fig 5 for different high oligomycin protein ratios ($>$ 0 3 μg/mg protein) The fact that the lines are parallel shows that increasing temperature enhances the value of k_0 at infinite oligomycin protein ratio, but has no effect on the concentration required for half-maximal k_0 From the slope of the parallel lines in Fig 5 a Q_{10} of 1 88 can be calculated for the inactivating reaction Beechey et al [6] reported a similar value (about 2) for the temperature coefficient of the inactivation by *N,N'*-dicyclohexylcarbodiimide of the ATP-dependent reduction of NAD^+ by succinate

TABLE II

THE EFFECT OF pH ON k_0 AT CONSTANT OLIGOMYCIN PROTEIN RATIO

Conditions as described in Fig 2 Oligomycin, 0 4 μg/mg protein

pH	k_0 (s^{-1})
6 10	0 026
6 50	0 036
7 00	0 046
7 30	0 049
7 85	0 052
8 30	0 046

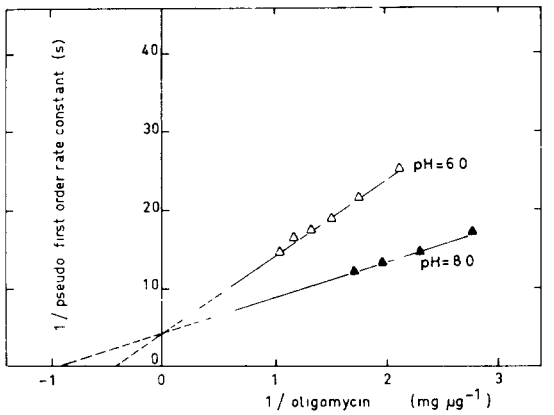

Fig 4 The dependence of k_0 on the oligomycin protein ratio at pH 6 0 and pH 8 0 Conditions as described in Fig 2 Volume 1 96 ml, protein, 1 39 mg k_0 was measured at different oligomycin protein ratios (0 35 –1 0 μg/ml protein) The results have been plotted in a double-reciprocal plot (cf Fig 3B) △ – △, pH 6 0, ▲ – ▲ pH 8 0

Effect of lecithin on the kinetics of inhibition by oligomycin of State-3 respiration

Addition of lecithin to State-3 mitochondria prior to oligomycin appreciably increases the lag phase accompanying oligomycin inhibition without affecting the final degree of inhibition An analysis of this effect reveals that the phospholipid decreases the pseudo first-order rate constant k_0 for the inactivation process (see Fig 6A) According to the extrapolation of the double-reciprocal plots in Fig 6B lecithin does not affect significantly the maximal value of k_0, but increases the oligomycin protein ratio at which k_0 equals 50 % of its maximal value In the experiment shown in Fig 7, lecithin was added at different time intervals after addition of oligomycin to State-3 mitochondria, resulting in a sharp decrease in the rate of inactivation

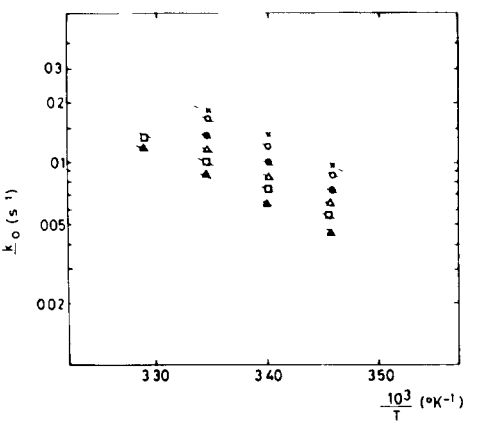

Fig 5 Effect of temperature on k_0 at different high oligomycin protein ratios Conditions as described in Fig 2 Results are plotted in an Arrhenius plot Volume 1 80, protein, 1 82 The oligomycin protein ratios (μg/mg) were, – , 1 65, ○ – ○ 1 32, ● – ●, 0 99, △ – △, 0 82, □ – □, 0 66, ▲ – ▲, 0 55

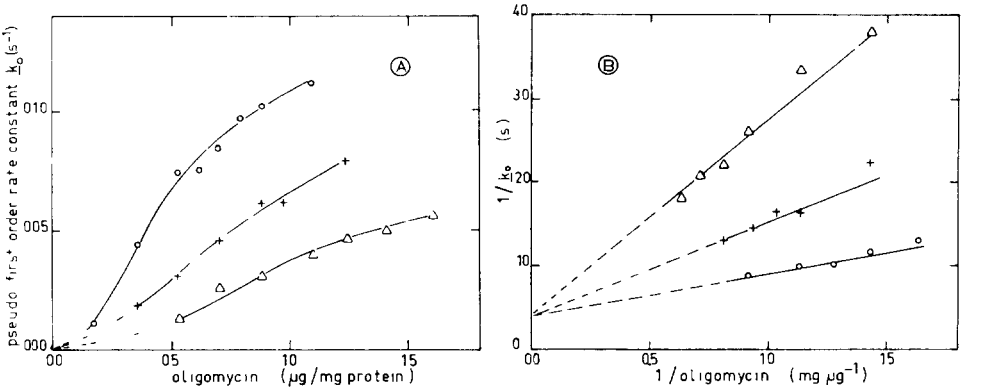

Fig 6 Effect of lecithin on the dependence of k_0 on the oligomycin protein ratio Conditions as described in Fig 2 Volume, 1 95 ml, protein, 0 78 mg Purified egg lecithin was added to State-3 mitochondria before oligomycin ○—○, in the absence of lecithin, +—+ 0 26 mg lecithin/mg protein △—△, 0 65 mg lecithin/mg protein

However, lecithin does not even partially release oligomycin inhibition under these circumstances

The observations that (i), the effect of lecithin on the kinetics of oligomycin inhibition is strictly dependent on the lecithin protein ratio (not shown here) and (ii), the pseudo first-order character of these kinetics is independent of the presence of lecithin, indicate that the effect of lecithin is not due to a change in a rate-limiting reaction (for instance the diffusion of oligomycin into the mitochondria), but is probably due to a rapid redistribution of the inhibitor between mitochondria and phospholipid without any effect on inhibitor that has already reached its inhibitory site

Similar results were obtained with phospholipids isolated from rat-liver mitochondria instead of purified egg lecithin

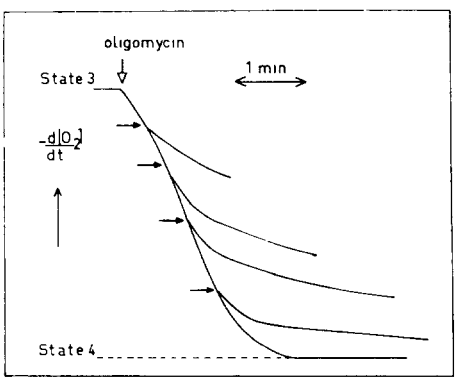

Fig 7 Effect of the addition of lecithin on the time course of oligomycin inhibition Conditions as described in Fig 2 Lecithin (1 04 mg/mg protein) was added to the mitochondria at different time intervals (given by the horizontal arrows) after the addition of 0 17 μg oligomycin/mg protein The rate of succinate oxidation was measured continuously Protein, 1 18 mg, volume, 1 88 ml

Kinetics of inhibition by oligomycin of arsenate-induced respiration

Fig 8 shows the semi-logarithmic plots of the time course of the rate of arsenate-induced respiration after the addition of different amounts of oligomycin Under these conditions a correction for the oligomycin-insensitive respiration was applied because this is probably due to irreversible damage to the mitochondria [8] It is obvious that under these circumstances the entire inhibition process can be described by a pseudo first-order process, without the 'pre-steady state' phase found with State-3 respiration (see Fig 2) It may be that this difference in 'pre-steady state' kinetics is related to the difference in the oligomycin-inhibition curve under the two conditions, viz sigmoidal for State-3 respiration and linear for arsenate-induced succinate oxidation (see Fig 12)

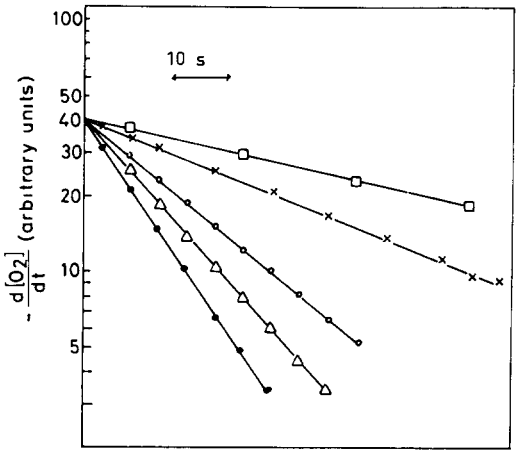

Fig 8 Kinetics of inhibition by oligomycin of the arsenate-induced succinate oxidation Volume, 2 05 ml, protein, 3 2 mg The $-d[O_2]/dt_{corr}$ was corrected for the oligomycin-insensitive respiration Oligomycin concentration (μg/mg protein) □—□ 0 094, — , 0 141, ○—○, 0 234, △—△ 0 375 and ●—● 0 47

Effects of various substrates and inhibitors on the kinetics of inhibition by oligomycin of State-3 respiration

The characteristics of inhibition by oligomycin reported above with succinate as substrate are also found with other substrates In particular the constant value of $(RC)_e$ reached at oligomycin protein ratios higher than 0 3 μg/mg protein (see Fig 3) is independent of the substrate used, although the initial *RC* values differ considerably This is demonstrated in Fig 9 The experiments shown in Figs 9 A–D and E, F respectively, represent two different mitochondrial preparations

Fig 10 shows the effect of malonate, antimycin and azide on the kinetics of inhibition In this case, $\ln -d[O_2]/dt$ is plotted on the ordinate From Fig 10A it appears that increasing malonate concentrations enhance the 'pre-steady state' phase, but have no effect on the level of the oligomycin-insensitive respiration Figs 10B and C show completely different results for the effects of antimycin and azide In these cases, the rate of decrease of the respiratory rate declines proportionally with the initial rate of State-3 respiration upon the addition of inhibitor, suggesting that anti-

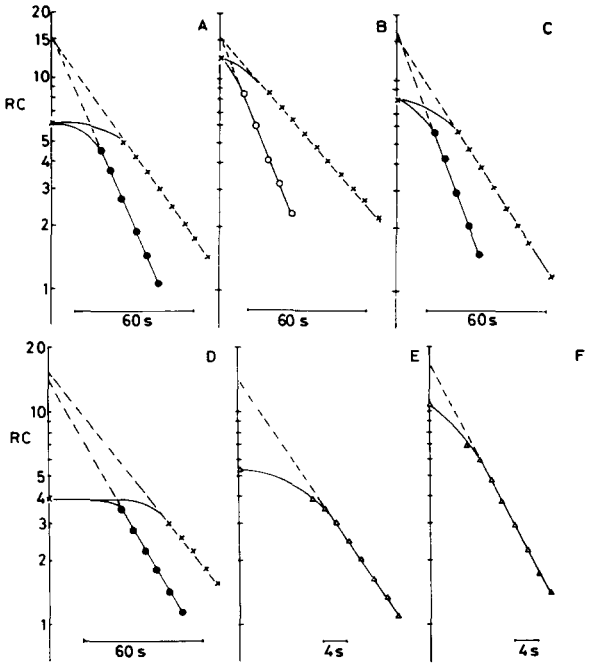

Fig 9 Effect of different substrates on $(RC)_e$ State-3 respiration was measured with different substrates Figs A–D protein 2 56 mg, volume, 2 25 ml, — , 0 24 μg oligomycin/mg protein, ● — ●, 0 36 μg oligomycin/mg protein, ○ — ○, 0 6 μg oligomycin/mg protein A β-hydroxybutyrate B, glutamate + malate, C, glutamate, D, 2-oxoglutarate + malonate Figs E and F protein, 2 2 mg, volume 2 10 ml, △ – △, 0 7 μg oligomycin/mg protein, E, glutamate + malate and F, succinate

mycin and azide inhibit the oligomycin-insensitive respiration to about the same degree as State-3 respiration, so that there is no appreciable change in respiratory control Essentially the same result has been reported by Lee et al [24] for submitochondrial EDTA particles where they found that in contrast to malonate, antimycin and azide do not affect the value of the oligomycin-induced respiratory-control ratio

These experiments clearly indicate that oligomycin can be considered as an inhibitor of respiratory control and that the extent of the 'pre-steady state' phase in the inhibition kinetics increases with decreasing respiratory control The extent of sigmoidicity of the oligomycin-inhibition curves can also be correlated with the initial respiratory-control index, sigmoidicity increases through the series glutamate + malate, succinate, glutamate, β-hydroxybutyrate and 2-oxoglutarate (Bertina, R M , unpublished experiments)

Reversibility of the inhibitory binding of oligomycin to mitochondria

Kagawa and Racker [25] demonstrated that the ATPase activity of rutamycin (a homologue of oligomycin)-inhibited submitochondrial particles is increased by subsequent washings with 0 5 % solutions of soybean phospholipids Parallel with the recovery of rutamycin-sensitive ATPase activity in the submitochondrial particles [^{3}H]rutamycin was found in the phospholipid supernatant Roberton et al [26]

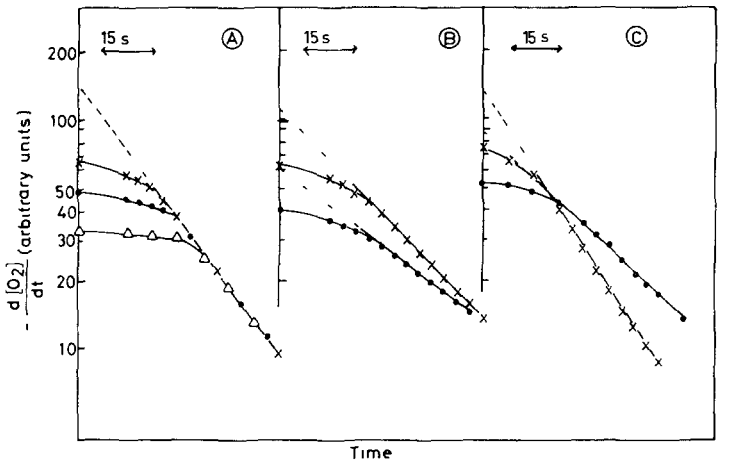

Fig 10 Effect of partially inhibitory concentrations of malonate (A) antimycin (B) and azide (C) on the oligomycin-inhibition kinetics Conditions as described in Fig 2 The time course of the oligomycin-inhibition process has been plotted as in Fig 2B Oligomycin was added at zero time A 0 30 μg oligomycin/mg protein — control, ● — ●, +0 13 mM malonate, △ - ∠ +0 33 mM malonate, B 0 30 μg oligomycin/mg protein, — , control, ● — ●, +0 07 nmole antimycin/mg protein C 0 30 μg oligomycin/mg protein, — , control, ● — ●, +0 22 mM azide Volume 1 57 ml protein, 0 44 mg

reported that, in contrast with this reversible binding of rutamycin to CF_0, *N,N'*-dicyclohexylcarbodiimide binds irreversibly More recent experiments of Palatini and Bruni [27] were interpreted in the sense that purified phospholipids can remove the effect of oligomycin on submitochondrial particles and intact mitochondria Their

TABLE III

EFFECT OF LECITHIN ON THE DINITROPHENOL-INDUCED ATPase OF OLIGOMYCIN-INHIBITED MITOCHONDRIA

Samples of 7 4 mg of mitochondria were incubated for 5 min at 25 C in 4 5 ml of a solution containing 50 mM KCl, 100 mM sucrose, 10 mM Tris-HCl buffer (pH 7 2) and 0 5 mM EDTA containing different amounts of oligomycin (see Table) The mitochondria were then removed from the medium by centrifugation, washed and resuspended in 0 25 mM sucrose The ATPase activity of these mitochondrial preparations was assayed as described under methods (at 37 °C) in the presence of 0 11 mM dinitrophenol after preincubation of the mitochondria for several minutes with the indicated amounts of lecithin

Oligomycin (μg/mg protein)	ATPase activity (nmoles ATP/min per mg protein) after preincubation with the amounts of lecithin (mg/mg protein) shown			
	0	6	11	15
0	790	—	960	—
0 35	74	—	100	—
1 5	40	86*	—	92*
3 1	26	82*	—	88*

* The addition of 1 06 μg oligomycin/mg protein after uncoupler has no effect on the rate of ATP hydrolysis

experiments suggest indeed an effect of phospholipids on the kinetics of oligomycin inhibition, but provide no evidence for a release of oligomycin inhibition by the addition of phospholipids Table III summarizes the results of an experiment to test this point The increase in ATPase activity observed in the presence of lecithin is not sensitive to oligomycin, and is thus due to a lecithin-induced oligomycin-insensitive ATPase activity and not to a release of oligomycin inhibition Titrations of the ATPase activity with oligomycin demonstrated that no significant change in the titre for oligomycin occurs in the presence of these concentrations of lecithin In agreement with the conclusion of the experiment of Table III, it was found that lecithin in concentrations up to 10 mg/mg protein cannot release the inhibition by oligomycin of State-3 respiration or dinitrophenol-induced ATPase in mitochondria (see Fig 11), or of NADH oxidation in submitochondrial 'A' particles (not shown) As with mitochondria, lecithin induces an increase in the oligomycin-insensitive ATPase activity of 'A' particles, again without significant effect on the titre of oligomycin It appears that the effect of lecithin is on the ATPase complex and not specifically on oligomycin binding (cf refs 25, 28)

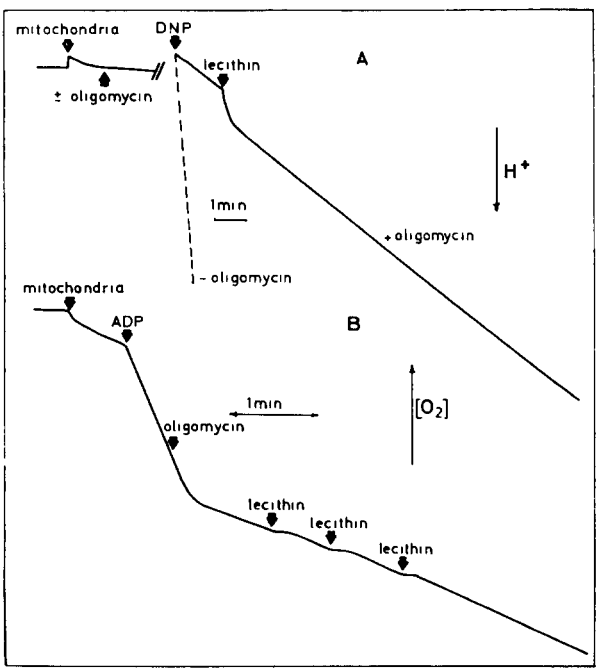

Fig 11 Effect of lecithin on oligomycin-inhibited mitochondrial processes A Dinitrophenol-induced ATPase activity ATP, 2 6 mM, protein, 0 4 mg, oligomycin, 0 75 μg/mg protein, lecithin 2 mg/mg protein, dinitrophenol (DNP), 0 08 mM, pH 7 1 B State-3 succinate oxidation ADP 1 mM, protein, 0 8 mg, oligomycin 0 4 μg/mg protein, lecithin (added at the indicated time intervals) 3 mg/mg protein

Concentration of oligomycin-binding sites in rat-liver mitochondria

The following considerations are relevant to the calculation of the concentration of mitochondrial binding sites for oligomycin from the inhibition data (1) the

degree of inhibition of mitochondrial processes by submaximal oligomycin concentrations is strictly dependent on the oligomycin protein ratio, even during the lag phase (not shown here), (ii) there is no evidence that the inactivation can be reversed at a measurable rate and (iii) from the conclusion that k_0 is related to the concentration of mitochondria-bound oligomycin, it follows that much more oligomycin can be bound than is necessary for maximal inhibition (cf Fig 3 with Fig 12) The first two points strongly suggest that low oligomycin concentrations are bound stoichiometrically to mitochondria and that their final effect on mitochondrial processes is irreversible These circumstances allow the determination of the maximal concentration of specific oligomycin-binding sites from the concentration-effect curves for different mitochondrial processes, provided that the final effect of oligomycin is measured Fig 12 shows these curves for State-3 succinate oxidation, arsenate-induced succinate oxidation, P_i–ATP exchange and dinitrophenol-induced ATPase The oligomycin titre for all four processes is 0 1 μg/mg protein, or 0 125 nmole/mg protein (using a molecular weight of oligomycin of 800 [29, 30]) Only the dinitrophenol-induced ATPase shows a 'tail' in the oligomycin-effect curve, being highly resistant to even high oligomycin concentrations (cf ref 8)

A completely independent method of estimating the concentration of specific oligomycin-binding sites is based on the finding that lecithin removes oligomycin from some mitochondrial binding sites (as shown by the decrease in k_0), but cannot remove it from the specific binding sites (as shown by its inability to release the inhibition) The concentration of specific oligomycin-binding sites can then be determined from a plot relating lecithin-bound oligomycin to added oligomycin at constant lecithin and protein concentrations Lecithin will bind oligomycin only after saturation of the specific mitochondrial oligomycin-binding sites Fig 13 shows plots from the data of the experiment shown in Fig 6 The amount of lecithin-bound oligomycin was taken to be equal to the extra amount of inhibitor that has to be added in the

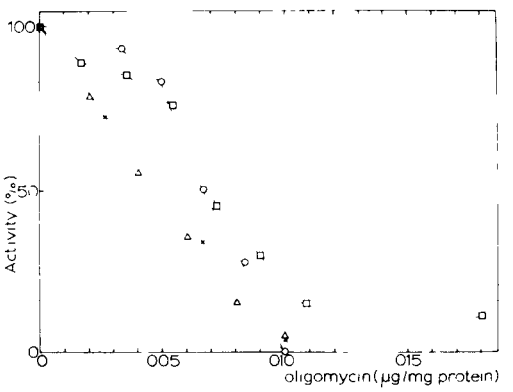

Fig 12 Oligomycin-inhibition curves for different mitochondrial processes — arsenate-induced respiration (corrected for the oligomycin-insensitive respiration) Arsenate, 40 mM Constant inhibition was reached 2 min after the addition of oligomycin ○—○, State-3 respiration (corrected for the oligomycin-insensitive respiration) with succinate Constant inhibition was reached 8 min after addition of oligomycin △—△, P_i–ATP exchange Reaction time, 4 min Mitochondria were preincubated 3 min with oligomycin □—□, dinitrophenol-induced ATPase ATP 3 mM, $MgCl_2$ 1 8 mM, pH 7 38, dinitrophenol 80 μM Mitochondria were pre-incubated 3 min with oligomycin

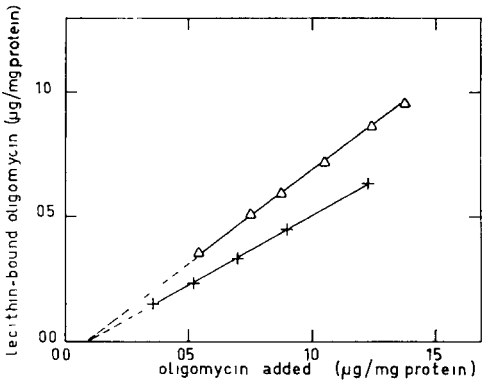

Fig 13 Lecithin-bound oligomycin as function of added oligomycin The data shown were calculated from Fig 6 The amount of lecithin-bound oligomycin was calculated as the difference in added oligomycin concentration to obtain the same value of k_0 in the presence and absence of lecithin ○ – ○, 0 26 mg lecithin/mg protein, △ – △, 0 65 mg lecithin/mg protein

presence of lecithin to obtain the same value of k_0 as in the absence of lecithin Extrapolation of the straight lines in Fig 13 results in a common intersection of the horizontal axis at about 0 1 µg oligomycin/mg protein, which is in good agreement with the result of Fig 12

DISCUSSION

Concentration of specific oligomycin-binding sites in mitochondria

From the experiments presented in the last paragraph in the Results section it may be concluded that the concentration of specific (i e inhibitory) oligomycin-binding sites in rat-liver mitochondria equals 0 1 µg/mg protein (0 125 nmole/mg protein) The possibility that at this concentration a significant amount of oligomycin is bound to non-specific oligomycin-binding sites can be excluded on basis of the observation that lecithin cannot release the inhibition of mitochondrial processes by oligomycin If the non-specifically bound oligomycin were in equilibrium with the specifically bound oligomycin, one would expect that the removal of non-specifically bound oligomycin by the addition of lecithin would cause partial release of the oligomycin inhibition The conclusion that oligomycin reaches its inhibitory site via the non-specific binding sites is based on the analysis of the kinetics of oligomycin inhibition (see below) From a comparison of Fig 3 with Fig 12 it can be estimated that at least 10 times more oligomycin can be bound to mitochondria than is necessary for maximal inhibition This conclusion has been confirmed experimentally by the observation (not shown here) that no free oligomycin can be demonstrated in a mitochondria-free supernatant (by its effect on State-3 respiration) when oligomycin concentrations up to 1 µg/mg protein were added (see also ref 31)

Kinetics of oligomycin inhibition

An analysis of the kinetics of oligomycin inhibition shows that (1) After a 'pre-steady state' phase, $-dRC/dt = k_0(RC)$, where k_0 is hyperbolically related to

the oligomycin protein ratio, i e to non-specifically bound oligomycin An implication of this observation is that oligomycin binds rapidly to the mitochondria, compared to the rate of inhibition (2) Extrapolation of the first-order phase to the time of addition of oligomycin gives a value of $(RC)_e$ that is independent of the oligomycin protein ratio (when higher than 0 3 μg/mg) and of the initial respiratory control (different substrates or presence of malonate) (3) The extent of the 'pre-steady state' phase decreases with increasing respiratory control (at constant oligomycin protein ratio), e g in the series arsenate-induced respiration ($RC = \infty$) < glutamate+malate ($RC = 12$) < succinate ($RC = 5$–8) < succinate in the presence of malonate ($RC <$ 5) (4) The extent of the sigmoidicity of the oligomycin inhibition curve decreases with increasing RC (5) The effect of the inhibitors malonate, antimycin and azide on the kinetics of oligomycin inhibition is also correlated with their effect on respiratory control (see Fig 10)

On the basis of these observations, it is proposed that oligomycin inhibition may be described by the following scheme

$$O_{free} + \text{mitochondria} \rightleftharpoons O_{ns} \tag{1}$$

$$O_{ns} + X \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} X\text{–}O \tag{2}$$

$$X\text{–}O \xrightarrow{k_2} X'\text{–}O \tag{3}$$

$$X \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} X'' \tag{4}$$

where O stands for oligomycin and O_{ns} for non-specifically bound oligomycin Eqn 1 describes the rapid binding of oligomycin to the mitochondria and Eqn 2 the also rapid equilibration of the non-specifically bound oligomycin to the oligomycin-binding site (X) involved in coupled respiration This site exists in two conformations, X and X'', in equilibrium with one another (Eqn 4), only one of which is enzymatically active in coupled respiration The equilibrium constant is dependent upon the conditions, e g type of substrate used The conversion of X'' to X (specified by the rate constant k_{-3}) is relatively slow Binding of oligomycin to X (Eqn 2) does not itself lead to inhibition, but the X–O complex is slowly and irreversibly converted to the inhibited complex, X'–O (Eqn 3) The concentration of all forms of X is given by Eqn 5

$$[X_{total}] = [X] + [X''] + [X\text{–}O] + [X'\text{–}O] \tag{5}$$

Since X and X–O are catalytically active

$$RC = \text{constant } [X + X\text{–}O] \tag{6}$$

In the absence of oligomycin, $(RC)_0$ is given by

$$RC_0 = \text{constant } (1 - \alpha)[X_{total}] \tag{7}$$

where

$$\alpha = \frac{k_3}{k_3 + k_{-3}}$$

In the presence of oligomycin the irreversible formation of X′–O is governed by Eqn 8

$$\frac{d[X'\text{–}O]}{dt} = k_2[X\text{–}O] \tag{8}$$

Since reactions 1 and 2 are postulated to be much faster than reactions 3 and 4, the micro-steady state concentration of [X–O] is given by

$$[X\text{–}O] = \frac{[X_{total}] - [X''] - [X'\text{–}O]}{1 + \frac{k_{-1}}{k_1[O_{ns}]}} \tag{9}$$

Substitution of 9 in 8 results in

$$\frac{d[X'\text{–}O]}{dt} = \frac{k_2([X_{total}] - [X''] - [X'\text{–}O])}{1 + \frac{k_{-1}}{k_1[O_{ns}]}} \tag{10}$$

where both [X″] and [X′–O] are time dependent Experimentally, oligomycin inhibition is measured by its effect on respiratory control Substitution of Eqn 5 in Eqn 7 gives

$$RC = \text{constant}\,([X_{total}] - [X''] - [X'\text{–}O]) \tag{11}$$

After the addition of oligomycin [X″] will decrease while [X′–O] will increase until [X″] becomes negligibly small when compared with $[X_{total}] - [X'\text{–}O]$ Then Eqn 10 can be approximated by

$$\frac{d[X'\text{–}O]}{dt} = \frac{k_2([X_{total}] - [X'\text{–}O])}{1 + \frac{k_{-1}}{k_1[O_{ns}]}} \tag{12}$$

and Eqn 11 by

$$RC = \text{constant}\,([X_{total}] - [X'\text{–}O]) \tag{13}$$

Integrating Eqn 12 (when $[O_{ns}] \gg [X_{total}]$), and substituting the value of [X′–O] in Eqn 13 gives

$$RC = \text{constant}\,[X_{total}]e^{-k_O t} \tag{14}$$

where

$$k_O = \frac{k_2}{1 + \frac{k_{-1}}{k_1[O_{ns}]}}$$

Then

$$\ln RC = \ln \text{constant}\,[X_{total}] - k_O t \tag{15}$$

From Eqn 15 it follows that the value of $(RC)_e$, obtained by extrapolation of the first-order phase to zero time (see Fig 2C), equals constant $[X_{total}]$ This term is independent of the $[O_{ox}]$ and of the initial *RC*, which is in agreement with the experimental observations (cf Figs 3A and 9)

The occurrence of a 'pre-steady state' phase, preceding the first-order phase, is the consequence of the simultaneous occurrence of an $X'' \rightarrow X$ and an $X\text{–}O \rightarrow X'\text{–}O$ transition (see Eqn 11) The extent of this phase will depend on the initial $[X'']/[X_{total}]$ ratio (at constant oligomycin protein ratio) and thus on the initial respiratory control

The lack of a pre-steady state phase in the kinetics of inhibition by oligomycin of arsenate-induced succinate oxidation (see Fig 8) indicates that under these conditions the ratio X/X_{total} approaches 1, which also explains the linear oligomycin-inhibition curve obtained under these conditions (see Fig 12) The explanation for the occurrence of sigmoidal oligomycin-inhibition curves for State-3 respiration is that at low oligomycin concentrations the formation of $X'\text{–}O$ is partially compensated by an $X'' \rightarrow X$ transition, so that the *RC*, defined according to Eqn 6, is hardly affected

It should be noted that the definition of *RC* according to Eqn 6 is only valid when the introduction of oligomycin does not change the rate-limiting reaction, i e when the rate limitation is localized in the complex consisting of cytochromes, mitochondrial ATPase and adenine nucleotide translocator [17], for instance during the State-3 oxidation of succinate or glutamate+malate When this is not the case, for instance in malonate-limited respiration whens succinate-dehydrogenase is rate-limiting, the kinetics of oligomycin inhibition will be masked until a change in the rate-limiting step has occurred (see Fig 10) It will be clear that this will contribute both to the extent of the 'pre-steady state' phase and to the sigmoidicity of the oligomycin-inhibition curve

Mechanism of inhibition of mitochondrial processes by oligomycin

The mechanism derived above fully explains the kinetics of the effects of oligomycin on State-3 respiration and arsenate-induced respiration The data on the inhibition of the uncoupler-induced ATPase and P_i–ATP exchange may now be fitted into the proposed model for the interaction of oligomycin with mitochondria by introducing two additional assumptions (i) ATP tends to displace the $X \rightleftharpoons X''$ equilibrium in the direction of X in an uncoupler-sensitive way, and (ii) ATPase activity is exclusively related to the concentration of X'' With these extra assumptions the model explains

(1) the observation of a linear oligomycin-inhibition curve for the P_i–ATP exchange which then is a consequence of a high X/X_{total} ratio (cf arsenate-induced respiration),

(2) the difference of oligomycin-inhibition kinetics of State-3 respiration and uncoupler-induced ATPase as a consequence of different rate-limiting reactions for the inhibitory process (see Fig 1) $X\text{–}O \rightarrow X'\text{–}O$ in the case of State-3 respiration and $X'' \rightarrow X$ in the case of uncoupler-induced ATPase,

(3) the experimental observation that the oligomycin-inhibition curve for the uncoupler-induced ATPase changes from strongly sigmoidal to completely hyperbolic at increasing uncoupler concentration [8, 15], since the addition of uncoupler

enhances the X''/X_{total} ratio In a completely uncoupled system this ratio approaches 1, so that the addition of low oligomycin concentrations immediately inhibit ATPase activity by inducing an $X'' \rightarrow X$ transition (hyperbolic inhibition curve) At suboptimal uncoupler concentration the X''/X_{total} ratio will be <1, in which case low oligomycin concentrations can bind to X without a significant effect on the X''/X_{total} ratio and thus on the ATPase activity

It is interesting now to relate the proposed X and X'' conformations of the oligomycin-sensitive site (CF_0) to the proposed schemes for energy transfer during respiratory-chain phosphorylation It is obvious that changes in the X/X_{total} ratio correspond qualitatively with changes in the presumed energy state or $I \sim X$ content However, the rate of turnover of $I \sim X$ excludes that the oligomycin-binding state (X) itself represents $I \sim X$ It is more likely that a high-energy state induces a conformational change in the CF_0 region, resulting in an $X'' \rightarrow X$ transition ATP synthesis coupled to electron transport can then only occur when the oligomycin-binding site is in the X conformation

As to the actual process that is inhibited by oligomycin, it may be of interest to consider the effects of oligomycin on the proton permeability of the mitochondrial inner membrane [32–34] The dependence of, for instance, the passive proton diffusion coefficient on the oligomycin protein ratio suggests that the decrease inproton permeability is related to the non-specifically bound oligomycin [32] However, it may be that the inhibitory action of specifically bound oligomycin is a reflection of the general properties of this antibiotic for instance the inhibition of proton translocation across some structural barrier between electron transport carriers and the mitochondrial ATPase An argument for such a hypothesis may be found in the observation of Papa et al [35] that the induction of respiratory control in submitochondrial particles parallels the inhibition of a cytochrome *b*-linked proton pump

METHODS AND MATERIALS

Rat-liver mitochondria were isolated according to the method of Hogeboom [36] as described by Myers and Slater [37]

Protein was determined by the biuret method as described by Cleland and Slater [38]

State-3 respiration was measured polarographically (using a Clark oxygen electrode) at 25 °C as has been described previously [20] Oxygen concentration and the first derivative $-d(O_2)/dt$ were recorded simultaneously The substrates used were succinate (10 mM), glutamate (10 mM), glutamate (5 mM)+malate (5 mM), 3-hydroxybutyrate (10 mM) or 2-oxoglutarate (10 mM)+malonate (5 mM) In the case of arsenate-induced respiration succinate was used as a substrate, and the ADP+P_i were replaced by 10–20 mM arsenate

Uncoupler-induced ATPase activity was measured by sensitive pH recording as described before [39]

P_i–ATP exchange activity was measured at 25 °C in a medium containing 100 mM sucrose, 25 mM Tris–HCl buffer (pH 7 4), 1 μg rotenone/mg protein, 10 mM P_i, 6 mM ATP and 2 mM EDTA The final volume was 1 0 ml After a suitable pre-incubation of the mitochondria (3 min when oligomycin was present) the

reaction was initiated by the addition of carrier-free $^{32}P_i$. The reaction was stopped by addition of 1.0 ml 10 % (w/v) trichloroacetic acid and the incorporation of ^{32}P in organic phosphate esters (ATP) present in the protein-free supernatant was measured, after extraction of the inorganic phosphate according to Nielsen and Lehninger [40], by counting a dried sample in a Nuclear Chicago gas-flow counter.

Lecithin was prepared from egg yolk as described by Pangborn [41] and suspended in ethanol or a mixture containing 0.25 mM sucrose, 1 mM EDTA and 10 mM Tris–HCl buffer (pH 7.4). Lecithin, in the concentrations used, has no significant effect on the rate of either State-3 or State-4 respiration. Mitochondrial phospholipids were prepared from rat-liver mitochondria according to a method described by Fleisher et al. [42].

Carboxyl cyanide *m*-chlorophenylhydrazone (CCCP) was received as a gift of Dr. P. G. Heytler and oligomycin was from the Upjohn Chemical Company. The sample of oligomycin used consists of 75 % of oligomycin A and about 12 % of oligomycin B and C, as determined with paper chromatography by Mr. J. Bouman.

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