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THE EFFECTS OF PHOSPHATE AND ELECTRON TRANSPORT ON THE CARBONYL CYANIDE *m*-CHLOROPHENYLHYDRAZONE-INDUCED ATPase OF RAT-LIVER MITOCHONDRIA

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SUMMARY

1. The effects of phosphate and electron transport on the ATPase induced in rat-liver mitochondria by the uncoupler carbonyl cyanide *m*-chlorophenylhydrazone have been measured at different uncoupler concentrations and compared with those of ATP, oligomycin and aurovertin.

2. The inhibitory action of respiratory-chain inhibitors on the ATPase activity, which is independent of the actual inhibitor used, is greatly delayed or prevented by the presence of uncoupler, and, in the case of rotenone, can be reversed completely by the subsequent addition of succinate (in the absence of uncoupler). These results can be explained on the basis of the proposal previously made by others that coupled electron transfer causes a structural change in the ATPase complex that results in a decreased affinity of the ATPase inhibitor for the mitochondrial ATPase.

3. Inorganic phosphate specifically stimulates the ATPase activity at high uncoupler concentrations ($> 0.2\ \mu M$), but has no effect at low concentrations. The stimulation is prevented or abolished by sufficiently high concentrations of aurovertin.

4. Aurovertin prevents the inhibition of the uncoupler-induced ATPase by high uncoupler concentrations.

5. It is proposed that the steady-state concentration of endogenous P_i may be an important regulator of the turnover of the ATPase in intact mitochondria and that the inhibition of ATPase activity by high concentrations of uncoupler is at least partially mediated via changes in the concentration of endogenous P_i.

INTRODUCTION

Intact mitochondria catalyse an uncoupler-induced ATPase [1]. The characteristics of this reaction have been thoroughly studied, in particular the possible involvement of respiratory-chain components [2–7], the inhibition by specific inhibitors (oligomycin [8–11], aurovertin [9, 11] and atractyloside [10]) and its dependence on

Abbreviation: CCCP, carbonyl cyanide *m*-chlorophenylhydrazone.

the presence of cations [12–15]. At least two enzymes are involved in the catalysis of this reaction: (i) the adenine nucleotide translocator, exchanging exogenous ATP for endogenous ADP and (ii) the mitochondrial ATPase, catalysing the hydrolysis of endogenous ATP to endogenous ADP and P_i. It has been shown that the translocator governs the affinity of ATP for the overall reaction [16]. The actual rate of product formation, however, is dependent on the kinetic parameters of both enzymes and on the uncoupling capacity of the ATPase-inducing agent.

Two interesting properties of the mitochondrial ATPase have been recently reported, viz. (i) coupled electron transport regulates the maximal turnover of the mitochondrial ATPase by decreasing its affinity for the endogenous polypeptide inhibitor [17, 18], and (ii) Mitchell and Moyle demonstrated that the ATPase activity of submitochondrial particles can be greatly enhanced by P_i and that this P_i activation is sensitive to aurovertin [19,20]. Since intact mitochondria contain both endogenous substrates and endogenous P_i, it is of interest to consider their possible contribution to the regulation of the uncoupler-induced ATPase activity. In this paper the effects of P_i and electron transport on the uncoupler-induced ATPase activity will be compared with those of known effectors of the adenine nucleotide translocator (effective ATP concentration) and mitochondrial ATPase (oligomycin, aurovertin). To exclude the possibility of a rate limitation of the ATPase activity by the relative inefficiency of the uncoupling agent, CCCP was used instead of the classical 2,4-dinitrophenol. A complicating factor in the use of the more efficient uncouplers is that they inhibit ATPase activity at concentrations slightly higher than necessary for inducing ATPase activity. Because no satisfactory explanation of this phenomenon is available, the effects of P_i and electron transport on the uncoupler-induced ATPase were studied as a function of the uncoupler concentration.

RESULTS

Effective ATP concentration

Fig. 1 shows the uncoupler-effect curves for the CCCP-induced ATPase at different levels of free ATP. At a saturating free ATP concentration (3.00 mM ATP, 1.8 mM $MgCl_2$) the curve is characterized by an optimum ATPase activity at 0.20–0.25 μM CCCP. When the free ATP concentration is about equal to the K_m of ATP for ATP transport and uncoupler-induced ATPase (obtained with 1.00 mM ATP and 1.8 mM $MgCl_2$ [16]) optimum ATPase activity is obtained at significantly lower CCCP concentrations (0.15 μM). In fact it appears that a decrease in the effective exogenous ATP concentration (i.e. an increase in the K_m (ATP)/[ATP] ratio) results in the ATPase activity becoming more sensitive to inhibition by high uncoupler concentrations (see also Kraayenhof and Van Dam [21]). It should be recalled that free Mg^{2+} itself has no effect on the uncoupler-induced ATPase activity and that Mg^{2+}- or K^+-complexed ATP cannot act as a substrate for the ATP-transport system [16].

A different method of decreasing the effective outside ATP concentration is to bring about an increase in the K_m for ATP at constant free ATP concentration, for example by the addition of a competitive inhibitor of ATP transport (ADP or atractyloside [10]) or by decreasing the KCl concentration [16]. Fig. 2 shows the uncoupler-effect curves for the CCCP-induced ATPase at three different KCl concentrations (ranging from close to zero to 50 mM). With decreasing KCl, when the K_m for ATP

increases from 7 μM to 1.0 mM without affecting the maximal turnover of the system [16], the optimum ATPase activity is reached at lower CCCP concentrations. Essentially the same results can be obtained by introducing increasing concentrations of atractyloside or ADP (not shown).

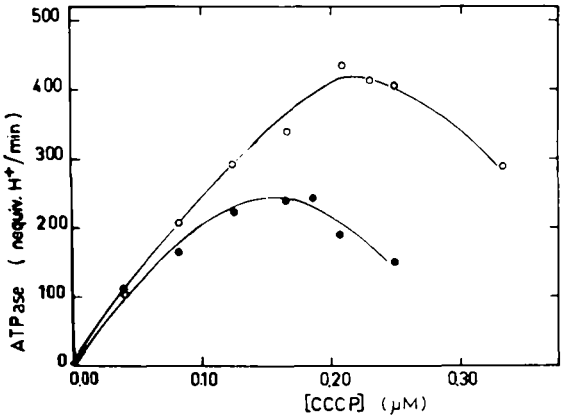


Fig. 1. Effect of the ATP concentration on the CCCP-effect curve for the CCCP-induced ATPase. Final volume, 6.16 ml; pH, 7.30; protein 0.74 mg; 25 °C. ○—○, 3.00 mM ATP, 1.8 mM $MgCl_2$; ●—●, 1.00 mM ATP, 1.8 mM $MgCl_2$.

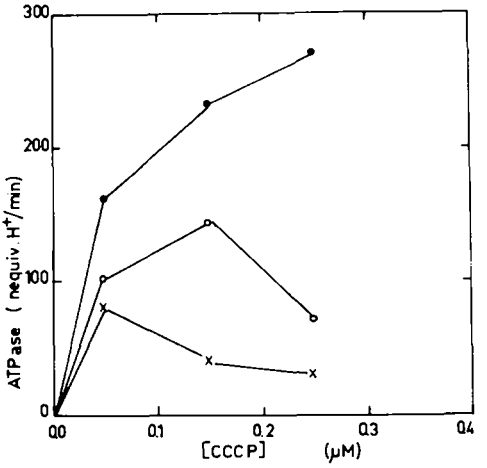


Fig. 2. Effect of the KCl concentration on the CCCP-effect curve for the CCCP-induced ATPase. Medium: 3.3 mM glycylglycine buffer (pH 7.10), 0.5 mM EDTA and 2.5 mM ATP (neutralized with Tris). Final volume, 1.88 ml; protein, 0.70 mg; 25 °C. ×—×, 0 mM KCl; ○—○, 10 mM KCl; ●—●, 50 mM KCl.

Oligomycin and aurovertin

It has been shown by Hammes and Hilborn [22] that oligomycin behaves as a non-competitive inhibitor of the ATPase activity of inner-membrane-bound mitochondrial ATPase. This makes oligomycin a suitable tool to vary the maximal turnover of

the mitochondrial ATPase in intact mitochondria. Conflicting reports have appeared concerning the nature of inhibition by aurovertin. Chang and Penefsky [23] showed

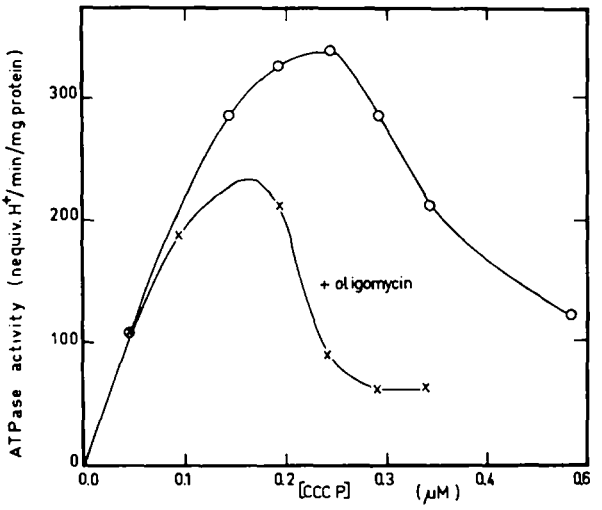


Fig. 3. Effect of a partially inhibitory concentration of oligomycin on the CCCP-effect curve for the CCCP-induced ATPase. Mitochondria were pre-incubated for 2 min in the presence (× — ×) or absence (○ — ○) of 0.1 μg oligomycin/mg protein. 3 mM ATP; final volume, 7.10 ml; pH, 7.40; 2.00 mg protein; 25 °C.

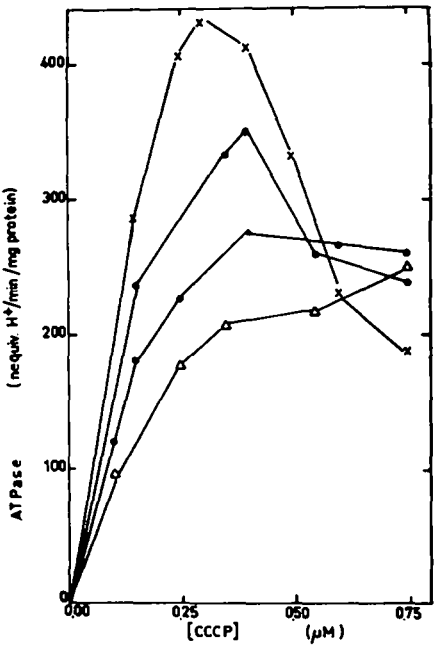


Fig. 4. Effect of aurovertin on the CCCP-effect curve for the CCCP-induced ATPase. Mitochondria were pre-incubated 2 min in the presence of aurovertin and 2.5 mM ATP. Final volume, 2.00 ml; pH, 7.19; protein, 1.0 mg; 25 °C. × — ×, without additions; ● — ●, 0.72 μg aurovertin/mg; ○ — ○, 1.39 μg aurovertin/mg; △ - △, 2.65 μg aurovertin/mg.

that inhibition of the isolated mitochondrial ATPase is non-competitive, whereas Mitchell and Moyle, using submitochondrial particles, clearly demonstrated that at pH 7.0 aurovertin decreases the K_m of ATP and increases the K_i of ADP without any effect on the rate at infinite ATP concentrations [24, 25].

Figs 3 and 4 show that in intact rat-liver mitochondria oligomycin and aurovertin have completely different effects on the CCCP-induced ATPase. Oligomycin lowers the optimum ATPase activity and the optimum CCCP concentration (cf. the effect of decreasing the effective ATP concentration in Figs 1 and 2). Increasing aurovertin concentrations, however, gradually remove the optimum from the CCCP-effect curve. At high uncoupler concentrations aurovertin even slightly stimulates the ATPase activity (see also ref. 9). A stimulation of the ATPase activity by aurovertin has also been observed for the atractyloside-insensitive Mg^{2+}-induced ATPase of aged rat-liver and house-fly mitochondria (Verdouw, H. and Bertina, R. M., unpublished observations).

Phosphate

It has been reported by Mitchell and Moyle [19, 20] that the addition of relatively large concentrations of P_i stimulates the rate of ATP hydrolysis in submitochondrial particles: the turnover of the ATPase is increased 7-fold in the presence of 20 mM P_i at pH 7.0. Similar results were obtained with Mg-ATP particles [18], whereas no stimulatory effect of P_i can be detected in ammonia-Sephadex particles at pH 8.0

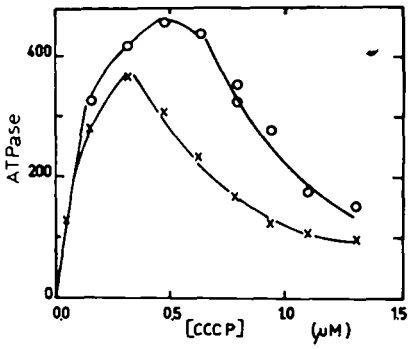


Fig. 5. Effect of P_i on the CCCP-effect curve for the CCCP-induced ATPase. P_i was added before the mitochondria. 2.5 mM ATP. Final volume, 1.88 ml; pH, 7.20; protein, 0.75 mg; 25 °C. ○—○, no P_i; ×- - -×, 1 mM potassium phosphate added.

(Verdouw, H. and Bertina, R.M., unpublished observations).

In intact mitochondria the steady-state concentration of endogenous phosphate during the uncoupler-induced ATPase is a function of the final rate of ATP hydrolysis. To study the possible involvement of endogenous P_i in the regulation of ATP hydrolysis the effect of extramitochondrial P_i on the uncoupler-induced ATPase was measured. Fig. 5 shows that the addition of 1 mM potassium phosphate stimulates ATP hydrolysis appreciably only at the postoptimal CCCP concentrations. In fact the effect of P_i can be described as a displacement of the optimum in the CCCP-effect curve both to a higher rate of ATP hydrolysis and to a higher CCCP concentration. The observation that the stimulatory action of P_i is only observed at CCCP concentrations

that inhibit ATPase activity in the absence of added P_i may explain why P_i has no effect on the 2,4-dinitrophenol-induced ATPase (not shown here), since in this case the uncoupler-effect curve is completely hyperbolic [16].

Fig. 6 shows that at 1.0 μM CCCP ATPase activity is stimulated about 4-fold by

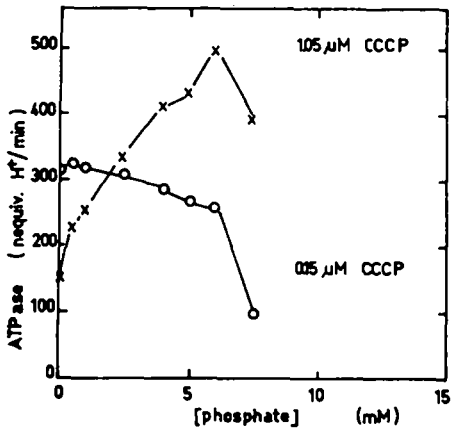


Fig. 6. Effect of the phosphate concentration on the CCCP-induced ATPase at constant CCCP concentration. Conditions as described in Fig. 5 except that the P_i concentration was varied. Protein, 1.50 mg; 25 °C. × – ×, 1.05 μM CCCP; ○ – ○, 0.15 μM CCCP.

TABLE I

EFFECT OF ROTENONE, KCN AND AUROVERTIN ON THE STIMULATION OF THE CCCP-INDUCED ATPase BY P_i

Conditions as described in Fig. 5, except that 1 μM CCCP was used as uncoupler. Rotenone (3 μg), KCN (1.5 mM) and aurovertin (2.4 μg) were pre-incubated with the mitochondria in the presence of P_i and 2.5 mM ATP. Protein: Expt 1, 0.81 mg; Expt 2, 0.81 mg; Expt 3, 1.11 mg. pH, 7.20.

Expt	Additions	ATPase activity (nequiv. H^+/min)					
		P_i	0 mM	1 mM	2 mM	5 mM	7.5 mM
1	–		136	88	170	245	246
	Rotenone		81	68	81	152	145
2	–		122	165	204	290	323
	Cyanide		84	92	105	145	132
3	–		126	132	213	288	308
	Aurovertin		140	125	130	148	145

6 mM P_i, while a further increase in P_i concentration tends to inhibit it. Since, however, at these concentrations P_i also inhibits ATPase activity at low CCCP concentration (see Fig. 6), this might be a non-specific anionic effect cf. ref. (26). Experiments similar to those described in Fig. 6, using citrate, malonate, acetate, arsenate orKC 1 instead of P_i, did not reveal any stimulatory action of these compounds on the ATPase activity, which makes it unlikely that the effect of P_i is related to anion transport in general.

Table I summarizes the effect of respiratory-chain inhibitors and aurovertin on the stimulatory action of P_i on the CCCP-induced ATPase. Even in the presence of rotenone or cyanide ATP hydrolysis can be stimulated 2-fold by 5–7 mM P_i. In the presence of aurovertin, however, P_i is completely without effect, which is in agreement with the experiments of Mitchell and Moyle in submitochondrial particles [20]. When compared with the control experiment aurovertin either slightly stimulates the ATPase activity in the absence of added P_i or inhibits strongly at 7.5 mM P_i. This suggests that the inhibitory action of aurovertin might be dependent on the endogenous P_i concentration.

Electron transport

Owing to the presence of endogenous substrates in intact rat-liver mitochondria, coupled electron transport takes place during the incubation of mitochondria with ATP. According to the findings of Ernster [17] and Van de Stadt et al. [18] in submitochondrial particles, coupled electron transport decreases the affinity of the polypeptide inhibitor [27] for the mitochondrial ATPase, which results in a largely enhanced ATPase activity after the addition of uncoupler. The existence of such a regulatory mechanism in intact mitochondria can be tested by studying the effect of respiratory-chain inhibitors and of enhanced electron transport on the uncoupler-induced ATPase.

Veldsema-Currie and Slater [28] reported that the inhibitory action of KCN on the dinitrophenol-induced ATPase depends completely on the experimental assay system: inhibition of ATPase activity is favoured by long reaction times (cf. Chefurka [5]) and dilute proteins solutions (0.3 mg/ml), while no inhibitory action was observed

TABLE II

EFFECT OF TIME OF PRE-INCUBATION WITH ROTENONE, IN PRESENCE OR ABSENCE OF UNCOUPLER, ON THE CCCP-INDUCED ATPase

In the control, mitochondria were pre-incubated 4 min with 3.5 mM ATP before ATPase activity was started with 0.3 μM CCCP. Final volume, 1.70 ml; pH, 7.30; protein, 0.92 mg; 4 μg rotenone; 25 °C.

Pre-incubation time (min) with		ATPase activity (nequiv. H^+/min)	%
ATP	Rotenone		
4	0	405	100
4	0.1	234	63.5
4	0.25	181	49
4	2	165	45
4	4	176	48
Incubated with rotenone (in the presence of uncoupler)			
0 min		405	100
1 min		320	86
2 min		320	86

with short reaction times (5 s preincubation, 1 min reaction time) and high protein concentration (3 mg/ml). Although under both sets of conditions the inhibition of electron transport is complete, there appears to be a marked difference in the uncoupler-induced ATPase activity.

Table II shows the effects of time of preincubation with rotenone, in the presence or absence of uncoupler, on the CCCP-induced ATPase. Preincubation for 15 s of

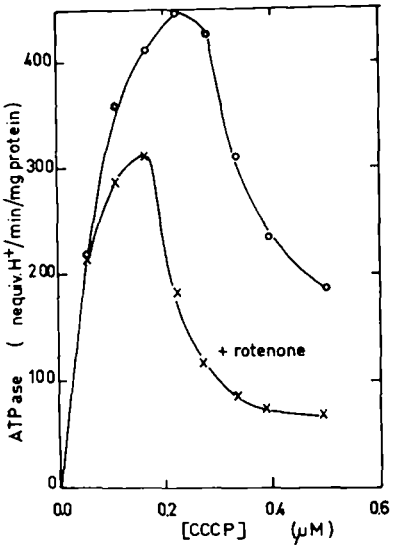


Fig. 7. Effect of rotenone on the CCCP-induced ATPase. Mitochondria were pre-incubated 2 min in the presence (× ×) or absence (○ ○) of 0.75 μg rotenone/mg protein and 2.8 mM ATP. Final volume, 1.78 ml; pH, 7.49; protein, 0.67 mg; 25 °C.

the mitochondria in the presence of ATP and rotenone causes a decrease in ATPase activity of 50 %, whereas only 15 % inhibition is obtained 2 min after addition of rotenone during the CCCP-induced ATPase. Thus it appears that the presence of uncoupler strongly delays or prevents the inhibitory action of rotenone.

Similar observations were made for antimycin and KCN, although a longer preincubation time with these inhibitors is necessary to obtain maximal inhibition of ATPase activity, which may explain the results earlier reported by Veldsema-Currie and Slater [28]. The degree of inhibition was found to be independent of the particular inhibitor used: 71 % for antimycin, 68 % for rotenone and 72 % for KCN (at 0.25 μM CCCP). This shows that the redox state of the respiratory-chain carriers, although possibly of importance for the kinetics of inhibition, has no effect on the final inhibition level. This suggests that it is the complete absence of electron transport that causes the inhibition of ATPase activity. Similar results were obtained for the dinitrophenol-induced ATPase.

It should be noted that the degree of inhibition of uncoupler-induced ATPase by a respiratory-chain inhibitor is dependent on the uncoupler concentration. Fig. 7 shows the effect of rotenone on the CCCP-induced ATPase at different uncoupler concentrations. Again (cf. Figs 1 and 3) the net effect appears to be a shift in the optimum of the CCCP-effect curve to lower ATPase activity and uncoupler concentration.

That the inhibition of ATPase activity by respiratory-chain inhibitors (i.e. by the complete absence of electron transport) is a reversible process, has been demonstrated already by Chefurka [5], who showed that the inhibition of the dinitrophenol-induced ATPase by anaerobiosis is completely reversible on aerobiosis. Table III fur-

ther demonstrates this reversibility: addition of 0.6 mM succinate during the preincubation, 2 min after rotenone (Line 3), completely restores ATPase activity when compared with the control (Line 4). The substitution of succinate by malonate (Line 5) has no effect on the rotenone-inhibited activity, showing that the effect of succinate is due to its ability to restore electron transport. The experiment in Line 7 shows that the presence of uncoupler completely prevents the reactivation of the rotenone-inhibited ATPase by succinate. ATPase activity is even further inhibited under these conditions, due to

TABLE III

REACTIVATION OF ROTENONE-INHIBITED ATPase BY SUCCINATE

Conditions as described in Table II. Succinate, 0.6 mM; malonate, 1 mM. R = rotenone, A = ATP, C = CCCP, S = succinate and M = malonate.

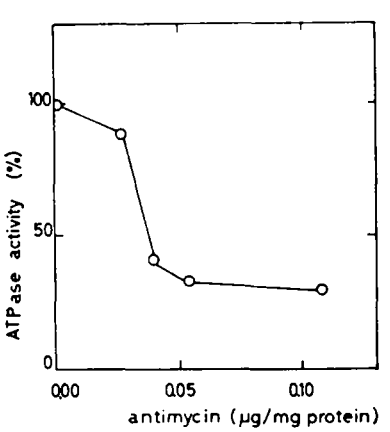
Conditions	ATPase activity (nequiv. H^+/min)
1. A —4 min— C	405
2. AR —4 min— C	176
3. AR —2 min— S —2 min— C	328
4. A —2 min— S —2 min— C	334
5. AR —2 min— M —2 min— C	176
6. AR —4 min— C	176
7. AR —4 min— C —30 s— S	145

the introduction of a concurrent energy-generating system (cf. Lines 1 and 4 with Lines 6 and 7). Thus, both the inhibition of ATPase activity by rotenone and its release by the addition of succinate are completely dependent on the presence of coupled conditions.

For a further quantitation of the relationship between electron transfer and ATPase activity, electron flow can be modified (i) by addition of different amounts of specific inhibitors and (ii) by the addition of suitable substrates. In the experiment of Fig. 8 the effect of varying concentrations of antimycin on the ATPase activity was studied. Although the resulting sigmoidal curve closely correlates with the antimycin-effect curve on endogenous respiration it cannot be excluded that the relation between electron transport and ATPase activity shows an 'all or nothing' transition.

In the study of enhanced electron transport (via the addition of substrates) a number of difficulties will be met: (a) At very low uncoupler concentrations ATPase activity is slightly underestimated by concomitant ATP synthesis. (b) At sub-optimal uncoupler concentrations the ATPase activity is limited by the energy-dissipating capacity of the uncoupler. Addition of even low substrate concentrations provides an alternative energy-generating pathway, thus resulting in a decrease in the rate of ATP splitting (see Fig. 9). (c) High concentrations of anionic substrates may result in a decrease of the steady-state endogenous P_i concentration, thus effecting a decrease in the ATPase activity. This effect will be more pronounced at low uncoupler concentrations (low rate of P_i production) than at high uncoupler concentrations, the net result of which will be an inhibition of the mixed competitive type (see also refs 28,29). (d) The ATPase activity of ATPase bound to the inner membrane may be masked by the limit-

ing capacity of the adenine nucleotide translocator. These complications appear to make it less appropriate to study the effect of enhanced electron transport on the uncoupler-induced ATPase in intact mitochondria.



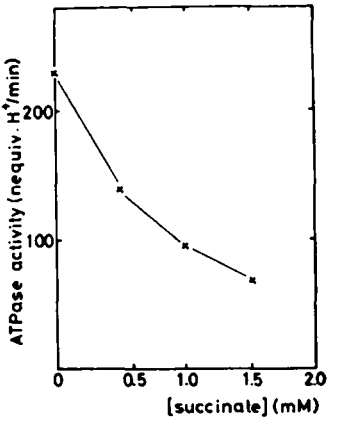


Fig. 8. Inhibition of the CCCP-induced ATPase by antimycin. Mitochondria were pre-incubated 2 min with the indicated amounts of antimycin in the presence of 3 mM ATP and 1.8 mM $MgCl_2$. 0.26 μM CCCP. ATPase activity is plotted as the percentage of that in the control, where only ethanol was added (which causes up to 15 % inhibition). Final volume, 6.70 ml; pH, 7.50; protein, 2.63 mg; 25 °C.

Fig. 9. Effect of succinate concentration on the CCCP-induced ATPase at suboptimal CCCP concentration. Succinate was added 30 s after the addition of 0.01 μM CCCP. 3.5 mM ATP. Final volume, 1.70 ml; pH, 7.30; protein, 0.92 mg; 25 °C.

DISCUSSION

Comparison of the experiments from Figs 1 and 3 shows that the uncoupler-effect curve for the CCCP-induced ATPase is equally affected by changes introduced in the turnover of the adenine nucleotide translocator (Fig. 1) and the mitochondrial ATPase (Fig. 3). At low uncoupler concentrations the initial slope of the uncoupler-effect curve (nequiv. $H^+ \cdot min^{-1} \cdot mg^{-1} \cdot \mu M\ CCCP^{-1}$) is independent of changes brought about in the maximal turnover of the system (at constant volume and protein concentration). This can be explained by the observation that the binding of CCCP to mitochondria is linearly related to the added CCCP concentration and is governed only by the partition coefficient for distribution of the uncoupler over mitochondrial and aqueous phases [30]. Increasing uncoupler concentrations then simply titrate the energy-generating capacity of the mitochondria, which is mainly determined by the turnover of the ATP-hydrolysing system (in the absence of added substrates). In the case of the uncoupler-induced ATPase the endpoint of this titration is masked by the inhibitory action of higher uncoupler concentrations. It may be concluded, however, from Figs 1 and 3 that the onset of inhibition by CCCP occurs at lower uncoupler concentrations when the ATP-hydrolysing capacity of the mitochondria decreases, and vice versa.

The effects of P_i and electron-transport inhibitors on the CCCP-induced ATPase, as described in Figs 5 and 7, then suggest that P_i enhances the maximal turnover of the ATPase system while electron-transport inhibitors decrease it.

The inhibitory action of electron-transport inhibitors on the CCCP-induced ATPase shows the following characteristics: (i) the maximal expression of the inhibitory effect requires a longer time than inhibition itself; (ii) the titre of electron-transport inhibitors for maximal inhibition of substrate oxidation and ATPase activity is the same; (iii) at constant uncoupler concentration the degree of inhibition of the ATPase activity is independent of the inhibitor actually used (rotenone, antimycin, cyanide); (iv) the inhibitory action of rotenone can be completely reversed by low concentrations of succinate; and (v) both the inhibition of ATPase activity by respiratory-chain inhibitors and its release by succinate (in the case of rotenone) require coupled mitochondria (or a high endogenous ATP/ADP ratio).

These characteristics (in particular, Points i–iii and v) of the inhibitory action of electron-transfer inhibitors are those to be expected if the rat-liver ATPase, like the ATPases of other energy-transducing membranes, contains an inhibitory polypeptide with the properties described by van de Stadt et al. [18] for the heart enzyme. These authors have shown that coupled electron transfer induces a conformational change in the ATPase complex that facilitates the dissociation of the inhibitor. The inhibitory polypeptide has been isolated from beef heart [27, 31] and yeast mitochondria [32, 33]. Pedersen et al.[34] have recently reported that fractionation of rat-liver mitochondria by the same procedure as used for beef-heart mitochondria by Horstman and Racker [31] yields a protein soluble in trichloroacetic acid that inhibits membrane-bound ATPase. Although a further characterization of this protein is necessary, it seems likely that this protein is the homologue for rat liver of the inhibitory polypeptide found in other energy-transducing membranes.

It is to be expected, on the basis of the model of van de Stadt et al. [18], that it would be easier to reverse the inhibition in intact mitochondria than in suspensions of submitochondrial particles. Since the ATPase is on the outside of these particles, the polypeptide, after dissociation from the ATPase, will appear in the suspension medium where its concentration will be so low as to make a reassociation more difficult. In intact mitochondria, where the ATPase complex is not exposed to the outer phase, it is likely that the inhibitor will either pass into the matrix or remain loosely bound to the ATPase without any inhibitory action on the ATPase activity (cf. Intermediate $F_1 \cdot I$ in the scheme of Van de Stadt et al.[18]). Addition of an electron-transport inhibitor to the mitochondria, then, would be expected to bring about an association of the polypeptide with the ATPase complex in such a way as to yield an inhibited complex.

The experimental data on the stimulatory action of inorganic phosphate on the CCCP-induced ATPase confirm the earlier observations of Mitchell and Moyle that phosphate can be considered as an important activator of the mitochondrial ATPase [19, 20]. The relatively high concentrations of endogenous P_i that apparently are necessary to induce the stimulatory effect in mitochondria correspond fairly well with the concentration range effective in submitochondrial particles. The observation that aurovertin specifically prevents the P_i effect suggests that P_i interacts directly with the mitochondrial ATPase. The involvement of endogenous P_i in the regulation of ATPase activity has as a consequence that the uncoupler-induced ATPase will be sensitive to those external conditions that influence P_i accumulation (for instance the presence of high concentrations of permeable anions and the tonicity of the external medium). This might provide an explanation for the inhibitory action of high CCCP concentrations on the ATPase activity.

It has been reported by Tsou [35] that relatively high uncoupler concentrations can decrease the accumulation of anionic substrates, while the mitochondrial K^+ content remains constant. The explanation given for this observation is that in de-energized mitochondria the rate of anion outflow is greater than in energized mitochondria. If this is so, increasing uncoupler concentrations will first saturate the energy-generating capacity of the mitochondria, after which they will tend to decrease the endogenous P_i concentration and thus the ATPase activity. This would explain why the presence of sufficiently high aurovertin concentrations removes the optimum from the CCCP-effect curve (see Fig. 4), since aurovertin prevents the activation of the ATPase by P_i. The observation that the uncoupler-effect curve for the dinitrophenol-induced ATPase lacks an optimum [16] can then be explained on basis of the relatively poor uncoupling capacity of dinitrophenol: even at saturating uncoupler concentrations the mitochondria are not completely uncoupled.

The explanation now proposed for the inhibitory action of high uncoupler concentrations on the ATPase activity is quite different from that proposed by Kraayenhof and Van Dam [21], namely the competition between anionic uncoupler and ATP for ATP entry. The latter explanation has been made unlikely by the observation (unpublished observations) that the inhibitory action can still be observed at 0 °C, since Pfaff and Klingenberg [36] find no inhibition of CCCP on ATP transport at this temperature.

METHODS AND MATERIALS

Rat-liver mitochondria were isolated according to the method of Hogeboom [37] as described by Myers and Slater [38].

Protein was determined by the biuret method as described by Cleland and Slater [39].

ATPase activity at 25 °C was measured by measuring pH changes in the medium with sensitive pH recording (Electrofact combined electrode connected with a E.I.L. Vibron Electrometer). Unless indicated otherwise the medium contained 100mM sucrose, 0.5 mM EDTA, 50 mM KCl and 10 mM Tris · HCl buffer. Mitochondria were preincubated for 2 min with ATP, before the ATPase reaction was started with uncoupler. Initial rates of H^+ formation were calculated after determination of the buffer capacity with standardized oxalic acid. Extra additions to the ATPase system were made before addition of the mitochondria, so that adjustment of the pH to its original value was possible. Experimentally an H^+/P_i ratio of 0.61 was obtained (insensitive to uncoupler, cation, ATP and protein concentration). ATPase activities are reported in nequiv. H^+/min per mg protein. The advantage of this method is the allowance of accurate measurements of initial rates also in the presence of P_i or in the absence of Mg^{2+} or K^+.

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