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EFFECT OF OLIGOMYCIN AND SUCCINATE ON MITOCHONDRIAL METABOLISM OF ADENINE NUCLEOTIDES

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SUMMARY

A decrease in the endogenous ATP of rat-liver mitochondria occurs in the presence of oligomycin. Such a decrease is enhanced by succinate and by arsenite, but not by α-ketoglutarate. In the presence of the latter, endogenous ATP actually increases. Exogenous ATP added to the mitochondria also undergoes a decrease upon incubation in the presence of oligomycin. Such a decrease is accompanied by a parallel increase in ADP and P_i. The ratio of P_i formed to ATP that has disappeared is significantly increased by succinate and even more by arsenite. In the presence of fluoride, PP_i accumulates. From these experiments it is concluded that the presence of oligomycin makes apparent a rapid breakdown of ATP into AMP and PP_i, which is normally masked by a concomitant synthesis due to the respiratory chain oxidative phosphorylation.

It is proposed that succinate significantly enhances the disappearance both of endogenous and added ATP in the presence of oligomycin by inhibiting ATP formation *via* substrate-level phosphorylation linked to oxidation of endogenous α-ketoglutarate.

INTRODUCTION

It has been previously reported that oligomycin, in spite of its powerful inhibitory effect on mitochondrial ATPase (refs. 1, 2), produces a rapid disappearance of intramitochondrial ATP (ref. 3).

The results reported in this communication indicate that oligomycin, by blocking the phosphorylation linked to the respiratory chain[1,2], unmasks some process that consumes ATP with the formation of AMP and PP_i, the latter being rapidly hydrolyzed into P_i. The disappearance of ATP by this route is partially counterbalanced by formation of ATP occurring during endogenous substrate-level phosphorylation.

Arsenite, and unexpectedly also succinate, were found to enhance the disappearance of ATP *via* the oligomycin-insensitive route by inhibiting the formation of ATP through the substrate-level phosphorylation.

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MATERIALS AND METHODS

Rat-liver mitochondria were isolated according to SCHNEIDER[4]. ATP was measured by determination of NADP reduction in the presence of glucose-6-phosphate dehydrogenase (EC 1.1.1.49), glucose and hexokinase (EC 2.7.1.1)[5]. ADP and AMP were measured by the Boehringer test, *i.e.* by determining the oxidation of NADH in the presence of phosphoenolpyruvate, lactate dehydrogenase (EC 1.1.1.27) and pyruvate kinase (EC 2.7.1.40), respectively, before and after the addition of adenylate kinase (EC 2.7.4.3). AMP was also determined enzymatically with AMP deaminase (EC 3.5.4.7) according to KALCKAR[6].

P_i was determined according to GOMORI[7]. PP_i was determined by measuring the P_i in two samples, one of which preincubated 15 min at 30° in the presence of yeast pyrophosphatase[8]. The difference was considered to be due to pyrophosphate. The concentration of fluoride present in some of the samples did not interfere with the enzymatic hydrolysis of PP_i. Protein was determined according to GORNALL, BARDAWILL AND DAVID[9].

$^{14}CO_2$ radioactivity was estimated in a liquid-scintillation counter (Nuclear–Chicago, Model Mark I) with a *p*-dioxane system. The scintillation fluid contained 50 g naphthalene, 5 g 2,5-diphenyloxazole, and 125 mg 1,4-bis-(5-phenyloxazolyl-2)-benzene, dissolved in 500 ml of *p*-dioxane. At the end of the incubation, the hyamine was transferred from the center wells of Warburg vessels, in counting tubes containing 18 ml of scintillation fluid, and counted at least three times for 30 sec. The method is essentially that described by BELL AND HAYES[10]. The O_2 uptake was measured manometrically by use of a conventional Warburg apparatus.

Hexokinase, glucose-6-phosphate dehydrogenase and pyrophosphatase were obtained from Sigma Chemical Co. Phosphoenolpyruvate, pyruvate kinase and adenylate kinase were from Boehringer and Soehne.

RESULTS

Disappearance of endogenous ATP

Fig. 1 summarizes the changes in the level of endogenous ATP that occur in rat-liver mitochondria incubated in the presence of oligomycin. It is seen that in the absence of any added substrate, oligomycin caused a decrease of ATP followed by a slow but consistent increase in ATP concentration. In the absence of oligomycin no substantial decrease of the endogenous ATP content was observed. It is therefore evident that the presence of oligomycin unmasks a breakdown of ATP that is normally masked by a concomitant synthesis due to respiratory chain-linked phosphorylation.

The delayed increase in ATP observed after the rapid phase of ATP disappearance suggests that a synthesis of ATP is still occurring even in the presence of oligomycin, possibly *via* the oligomycin-insensitive substrate-level phosphorylation linked to endogenous α-ketoglutarate oxidation. The delayed increase of ATP concentration is in fact reduced by arsenite, which is known to inhibit the substrate-level oxidative phosphorylation[11–13]. Moreover, with added α-ketoglutarate as substrate, no decrease, but actually an increase of endogenous ATP was observed (see Fig. 1).

Effect of inhibitors and succinate on added ATP

The results reported in Fig. 2 show that exogenous ATP added to the mitochondria also underwent a decrease upon incubation in the presence of oligomycin. This ATP disappearance was accompanied by a roughly proportional increase of P_i. Thus added extramitochondrial ATP is influenced by oligomycin in a manner that is similar to endogenous ATP. Fig. 2 also shows that addition of succinate enhances the decrease of added ATP and the increase of P_i. However, the stimulation of P_i formation is such that the amount of P_i formed is greater than the amount of ATP that disappeared.

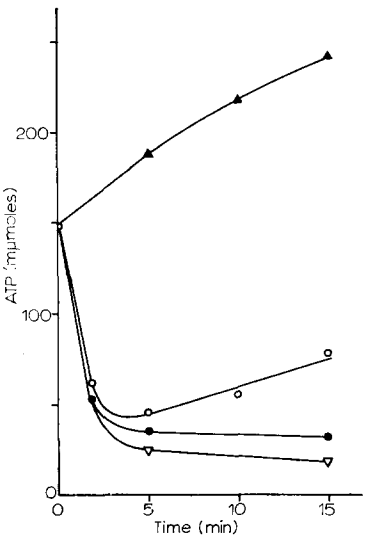


Fig. 1. Level of mitochondrial ATP in the presence of oligomycin and different oxidizable substrates. The incubation mixtures contained, in a final volume of 3 ml: 1 ml mitochondria (43 mg protein) suspended in 0.25 M sucrose, 10 μmoles $MgCl_2$, 100 μmoles Tris–HCl buffer (pH 7.4), 22 μg oligomycin and 30 μmoles substrate, where indicated. Variable amounts of KCl and sucrose were added in order to maintain a constant concentration of K^+ and osmolarity of the medium. Incubation was carried out at 25° and stopped by addition of 0.1 ml of 70 % $HClO_4$. The neutralized $HClO_4$ supernatants were used for the determinations of ATP. ○—○, none; ●—●, succinate; ▽—▽, arsenite (6 μmoles); ▲—▲, α-ketoglutarate.

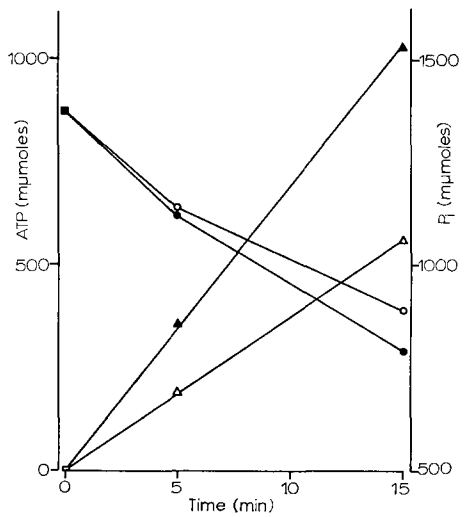


Fig. 2. Disappearance of added ATP and formation of P_i during incubation of mitochondria in the presence of oligomycin. Effect of succinate. Incubation medium and experimental conditions as described in Fig. 1: 20 μg oligomycin and approx. 0.8 μmole ATP were added. Mitochondrial protein: 38 mg. After incubation, the neutralized $HClO_4$ supernatants were used for the determination of ATP and P_i. ○—○, ATP, no substrate; ●—●, ATP, in the presence of 30 μmoles of potassium succinate; △—△, P_i, no substrate; ▲—▲, P_i, in the presence of 30 μmoles of potassium succinate.

ATPase activity of oxidative phosphorylation cannot account for the above results because oligomycin was present and because upon addition of succinate the P_i formation always exceeds the ATP disappearance on a molar basis, suggesting that ATP is not only simply hydrolyzed to ADP and P_i, but perhaps to AMP and 2 P_i. Therefore experiments were carried out in which the levels of different adenine nucleotides in the system were measured.

From the results of typical experiments reported in Table I, it can be seen that,

TABLE I

VARIATIONS OF ADENINE NUCLEOTIDES AND P_i IN MITOCHONDRIA INCUBATED IN THE PRESENCE OF OLIGOMYCIN. EFFECT OF ARSENITE AND SUCCINATE

Incubation medium and experimental conditions as described in Fig. 1; 35 μg oligomycin and approx. 0.8 μmole ATP were added. Mitochondrial protein: 70 mg. Adenine nucleotides and P_i were determined in the neutralized $HClO_4$ supernatants before and after incubation.

	ATP (*mμmoles*)	*ADP* (*mμmoles*)	*AMP* (*mμmoles*)	P_i (*mμmoles*)
Before incubation	897	403	333	1088
12-min incubation	321	964	327	1651
12-min incubation *plus* arsenite (6 μmoles)	230	494	767	2249
12-min incubation *plus* succinate (30 μmoles)	153	540	913	2637

in the presence of succinate, the decrease of ATP was accompanied by parallel increase of ADP and P_i, whereas the level of AMP was not significantly changed. However, when arsenite was present, the ratio of P_i formed to ATP disappeared approached 2.0 and there was a large increase of AMP but only a small increase of ADP. These findings indicate that arsenite makes more evident the breakdown of ATP into AMP and PP_i with a subsequent hydrolysis of PP_i into P_i.

Moreover in the presence of fluoride, which is a well-known inhibitor of pyrophosphatase activity[14,*], ATP breakdown and P_i formation are reduced while PP_i is accumulated (see Table II). It is known that fluoride up to a concentration of 10 mM does not inhibit mitochondrial adenylate kinase[15].

These results are consistent with the hypothesis that, in the presence of oligomycin, the main reactions responsible for ATP disappearance are the following:

$$ATP \longrightarrow AMP + PP_i$$
$$PP_i \longrightarrow 2\ P_i$$

The assumption that AMP is the primary product of the ATP breakdown is strongly supported by the great increase of AMP and P_i in the presence of arsenite,

TABLE II

VARIATIONS OF ADENINE NUCLEOTIDES, PP_i AND P_i UPON INCUBATION OF MITOCHONDRIA IN THE PRESENCE OF OLIGOMYCIN. EFFECT OF FLUORIDE

Incubation medium and experimental conditions as described in Fig. 1; 20 μg oligomycin and approx. 0.7 μmole ATP were added. Mitochondrial protein: 40 mg.

	ATP (*mμmoles*)	*ADP* (*mμmoles*)	*AMP* (*mμmoles*)	P_i (*mμmoles*)	PP_i (*mμmoles*)
Before incubation	789	192	189	595	12
15-min incubation	468	462	198	884	32
15-min incubation *plus* fluoride (20 μmoles)	556	421	173	574	115

* In control experiments it has been found that fluoride, even at concentrations less than 2.0 mM inhibited more than 80% of the hydrolysis of added PP_i by liver mitochondria.

which inhibits the substrate-level phosphorylation and the subsequent phosphorylation of AMP by GTP (ref. 16).

Effect of succinate on the substrate-level phosphorylation

The results in Table I also show that in the presence of oligomycin, succinate acts very much like arsenite: it stimulates ATP breakdown and AMP formation and causes the appearance of about 2 moles of P_i per mole of ATP disappeared.

The most reasonable explanation for this effect of succinate is that it has the same end effect as arsenite: it prevents the formation of ATP *via* substrate-linked phosphorylation. This hypothesis is also supported by the results reported in Table III.

It can be seen that in the presence of oligomycin and α-ketoglutarate, ATP was formed at the expense of added ADP and P_i. A simultaneous decrease of AMP also occurred. Addition of succinate strongly inhibited the formation of ATP and the decrease of AMP and P_i. More direct evidence for the inhibitory effect of succinate on the substrate-level phosphorylation is provided by the results reported in Table IV.

TABLE III

VARIATIONS OF ADENINE NUCLEOTIDES AND P_i SUPPORTED BY α-KETOGLUTARATE IN MITOCHONDRIA INCUBATED IN THE PRESENCE OF OLIGOMYCIN. EFFECT OF SUCCINATE AND ARSENITE

Incubation medium and general conditions as described in Fig. 1; 30 μmoles α-ketoglutarate, 1 μmole ADP, 1 μmole P_i and 35 μg oligomycin were added. Mitochondrial protein: 70 mg.

	ATP (*mμmoles*)	*ADP* (*mμmoles*)	*AMP* (*mμmoles*)	*P_i* (*mμmoles*)
Before incubation	192	1285	403	1985
15-min incubation	679	777	215	1072
15-min incubation *plus* succinate (30 μmoles)	266	656	814	2405
15-min incubation *plus* arsenite (6 μmoles)	290	604	830	2307

TABLE IV

EFFECT OF SUCCINATE AND ARSENITE ON THE $^{14}CO_2$ EVOLUTION FROM DL-[1-^{14}C]GLUTAMATE BY LIVER MITOCHONDRIA

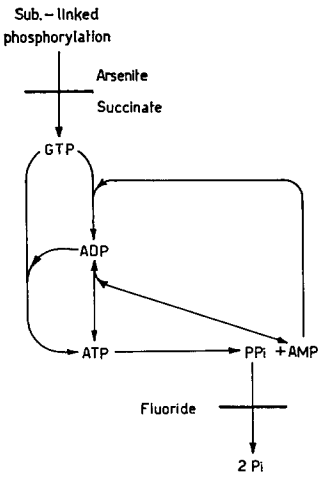
40 mg of liver mitochondria were incubated in Warburg vessels in a final volume of 3 ml containing: 100 μmoles Tris–HCl (pH 7.4), 12 μmoles $MgCl_2$, 20 μg oligomycin and the substrate indicated in the table, except DL-[1-^{14}C]glutamate (1 μC), carrier-free, which was in the side-arm. After thermal equilibration for 3 min at 25°, DL-[1-^{14}C]glutamate was poured into the flask containing reaction mixture and $^{14}CO_2$ was trapped by 0.2 ml of hyamine in the center well. Incubation time: 10 min at 25°.

	$^{14}CO_2$ (*counts/min*)	O_2 (*μl*)
DL-[1-^{14}C]Glutamate (carrier-free)	62 100	34
plus D-malate (30 μmoles)	65 300	31
plus L-malate (30 μmoles)	64 250	38
plus malonate (30 μmoles)	61 500	25
plus succinate (30 μmoles)	16 200	103
plus succinate (30 μmoles) *plus* malonate (60 μmoles)	50 300	44
plus arsenite (6 μmoles)	15 130	—

These results show that succinate, as well as arsenite, strongly inhibited the $^{14}CO_2$ evolution from added DL-[1-^{14}C]glutamate in the presence of oligomycin. No other dicarboxylic acid tested exerted such an inhibition of α-ketoglutarate oxidation.

DISCUSSION

The results reported above are consistent with the sequence of reactions indicated in Scheme I. These reactions are evident in the presence of oligomycin only; in the absence of oligomycin the continuous massive resynthesis of ATP through oxidative phosphorylation would of course mask the concomitant breakdown of ATP.



Scheme I.

Scheme I is supported by the following results:

(1) Oligomycin, in spite of its inhibitory effect on mitochondrial ATPase (refs. 1, 2), evokes a rapid disappearance of either endogenous or added ATP with a concomitant increase of P_i (see Fig. 1 and Table I).

(2) Fluoride, a well-known inhibitor of pyrophosphatase[14] inhibits the increase of P_i and allows appreciable amounts of PP_i to be accumulated (see Table II).

(3) Arsenite, which is known to inhibit the substrate-level phosphorylation associated with α-ketoglutarate oxidation[12,13], enhances the disappearance of ATP and the formation of P_i and AMP (see Table I).

The results obtained with arsenite, as well as those with fluoride, indicate that the process responsible for the disappearance of ATP in the presence of oligomycin would be a reaction leading to the accumulation of AMP and PP_i.

Disappearance of ATP in the presence of oligomycin probably occurs *via* PP_i-cleaving activation reactions occurring in mitochondria: *i.e.* activation of fatty acids and of amino acids. Thiol-ester deacylase activity present in mitochondria[17] can also account for a rapid utilization of ATP independently of a further oxidation of the activated fatty acids. AMP formed in such reactions can be phosphorylated to ADP by the GTP produced by the substrate-level phosphorylation, through the reaction described by HELDT AND SCHWALBACH[16]. ADP can in turn be transformed into ATP,

either through the adenylate kinase reaction or by the nucleoside diphosphokinase reaction.

This cycle can be inhibited either by fluoride, which prevents the breakdown of PP_i and, indirectly, the breakdown of ATP, or by arsenite, which inhibits the substrate-level phosphorylation associated with oxidation of α-ketoglutarate.

On the other hand it is known that fatty acids can be also activated through a GTP-dependent reaction in which GTP is split into GDP and P_i (ref. 18). The GTP-activating reaction, which is dependent on the substrate-linked phosphorylation, produces half as much P_i as the ATP-dependent activating reactions. Succinate, by inhibiting the substrate-level phosphorylation associated with α-ketoglutarate oxidation, would also inhibit the GTP-dependent reaction, so that the ATP-dependent reactions become more apparent.

This interpretation can account for the observed increase of P_i and AMP upon addition of succinate and for the molar ratio of P_i formed to ATP split, which approaches 2.0. Consequently, it appears likely that succinate by controlling the substrate-level phosphorylation, could also control the equilibrium between the GTP- and ATP-dependent metabolic processes.

The described effect of succinate appears to be rather specific, since none of the other dicarboxylic acids tested was effective. The mechanism by which succinate prevents ATP formation by the substrate-level phosphorylation associated with α-ketoglutarate oxidation is still obscure. It is possible that the inhibitory effect of succinate on glutamate or α-ketoglutarate oxidation, and then on the associated phosphorylation, is an expression of a kind of "feed-back" control of succinate on the α-ketoglutarate utilization in the Krebs cycle.

However, since malonate prevents this activity of succinate it is necessary to assume that the effect of succinate is exerted *via* succinyl dehydrogenase, which is sensitive to malonate, or *via* some other mechanism in which malonate can prevent the succinate effect.

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