METABOLIC MECHANISMS INVOLVED IN THE SWELLING OF ISOLATED MITOCHONDRIA AND IN ITS PREVENTION*

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SUMMARY

1. Rat-liver mitochondria shaken at 28° in the presence of all components necessary to oxidative phosphorylation (oxidizable substrate, AMP, Mg^{++} ions and inorganic phosphate) are largely protected from swelling when the oxidizable substrate is succinate, glutamate or α-ketoglutarate but are much less protected when other substrates are used.

2. With succinate, glutamate or α-ketoglutarate as substrates, DNP 10^{-4} *M* promotes the swelling of the mitochondria, but with all other substrates tested DNP 10^{-4} *M* actually helps to maintain the mitochondrial structure.

3. Malonate abolishes the protection found in presence of AMP + succinate, glutamate, or α-ketoglutarate.

4. It is suggested that under the test conditions phosphorylation coupled to oxidation of succinate is the main mechanism involved in the prevention of mitochondrial swelling.

INTRODUCTION

During the past few years several investigators have studied the changes in water content (or swelling) of isolated mitochondria incubated under various conditions. The morphology of these organelles was found to be affected *in vitro* by many factors, *e.g.* temperature, osmolarity, ionic environment, pH, metal ion complexing agents, nucleotides, metabolic intermediates, surface active agents, uncoupling agents, enzymic inhibitors, etc.

Much interest has been aroused by the reciprocal relationship between swelling and oxidative phosphorylation (or availability of high-energy phosphate bonds) in mitochondria. Using the phase-contrast microscope to assess the morphological changes, HARMAN AND FEIGELSON[2] concluded that the mitochondrial form not only influenced but was influenced by the rate of oxidative phosphorylation. They also observed that uncoupling agents, such as DNP, Janus green and usnic acid caused

The following abbrevations will be used: DNP = 2,4-dinitrophenol; AMP = adenosine-5′-monophosphate; ATP = adenosine triphosphate; TRIS = tris(hydroxymethyl)aminomethane.

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swelling of mitochondria incubated in the presence of all the constituents necessary to oxidative phosphorylation. The swelling also occurred when the high-energy phosphate bonds were formed but "filtered off irreversibly into an efficient trapping system" (glucose + hexokinase) (*cf.* also ERNSTER AND LÖW[3]). Furthermore, it has been found that ATP inhibits the swelling of mitochondria caused by succinate, inorganic phosphate and by aging and that the swelling by aging is accompanied by a drop in intramitochondrial ATP[4–7].

That mitochondria can maintain a low water content if incubated under conditions favourable to oxidative phosphorylation[2,8–12] and that ATP may help to preserve the organized structure of these organelles[4–6,11–17] has been repeatedly reported. However, MACFARLANE AND SPENCER[10] found that the addition of DNP to actively respiring and phosphorylating mitochondria in concentrations which uncoupled oxidative phosphorylation ($5 \cdot 10^{-5}$ *M*) did not affect appreciably the water content of the particles. On the other hand, PRICE *et al.*[11,12] showed that liver mitochondria shaken with succinate in the presence of phosphate and TRIS buffer, Mg^{++} ions and AMP maintained their initially low water content, but that the simultaneous addition of 10^{-4} *M* DNP caused a rapid swelling whilst $3 \cdot 10^{-5}$ *M* DNP had no effect on the water content. In a subsequent study FONNESU AND DAVIES[13,18] found that the swelling of rat liver mitochondria during incubation at 28° in isotonic media can be markedly prevented by ATP or AMP in the presence or absence of substrate, and that Mn^{++} or Mg^{++} ions plus AMP can largely protect non-respiring liver mitochondria from swelling even in solutions as dilute as 0.04 *M* sucrose. It has been observed that DNP, which causes swelling of respiring mitochondria in the presence of glutamate, succinate and α-ketoglutarate[2,11,12], actually protects the mitochondria incubated in the absence of substrate[13–15,18–20]. Furthermore, either anaerobiosis[13,18,21–23] or cyanide[13,18,23] protects the mitochondria. It is of interest in this regard that malonate prevents the swelling induced by succinate[5]. Recently, CHAPPELL AND GREVILLE[24] showed that the swelling is largely dependent on the presence of an oxidizable substrate.

To summarize, some data in the literature favour the hypothesis that a relationship exists between swelling of the mitochondria and ATP or oxidative phosphorylation, whilst other findings refute this. The results presented in this paper furnish an explanation for the apparent discrepancy. They show that the prevention of swelling observed when the mitochondria are incubated under conditions favourable to oxidative phosphorylation is dependent on the type of oxidizable substrate used. Support has also been found for the view that, under some conditions, the phosphorylation associated with the oxidation of succinate is the main mechanism involved in the prevention of mitochondrial swelling.

MATERIALS AND METHODS

Adult albino rats of either sex, weighing 200 to 250 g, were used throughout these expts. The rats received a standard diet until 12–14 h before the expt. when they were killed by decapitation. The livers were quickly removed and cooled for 3 min in partially frozen 0.25 *M* sucrose.

All operations for the preparation of the mitochondria were carried out in a cold room at 0°. The chilled tissue was blotted with filter paper and passed through a

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Fisher mincer. The resulting liver pulp was weighed and homogenized with 9 vols. of ice-cold 0.25 *M* sucrose in the all-glass apparatus of POTTER AND ELVEHJEM[25]. The homogenate was centrifuged in a Servall angle centrifuge for 15 min at 600 × *g* and the sediment discarded. The supernatant liquid was centrifuged for 30 min at 6000 × *g* to sediment the mitochondria; the supernatant liquid and the free-flowing layer of the deposit were poured off and the well-packed mitochondria washed twice with a vol. of 0.25 *M* sucrose equal to that of the original homogenate. The washed mitochondria were finally resuspended in 0.25 *M* sucrose for use.

Mitochondrial swelling was studied by following the O.D. at 520 mμ of the suspension in cuvettes of 10 mm light path in a Beckman spectrophotometer model DU (*cf.* CLELAND[26]). Evidence has been accumulated (see PRICE *et al.*[12]) that the decrease in the light-scattering and hence in the O.D. corresponds to swelling owing to the entrance of water.

5 ml of the mitochondria suspended in 0.25 *M* sucrose (giving an O.D. of approx. 0.40 when diluted 30 times with cold 0.25 *M* sucrose) were pipetted into 100 ml conical flasks containing the following constituents in a 10 ml vol. (final concns. after the addition of the mitochondria in parentheses): 75 μmoles of magnesium sulphate (0.005 *M*); 150 μmoles of potassium phosphate buffer pH 7.2 (0.01 *M*); 1200 μmoles of sucrose (0.163 *M*) and 300 μmoles of substrate (0.02 *M*) adjusted to pH 7.2 with sodium hydroxide. In the expts. without substrate, 300 μmoles of sodium chloride (0.02 *M*) were added. Where indicated, the incubation system contained 45 μmoles of potassium AMP (0.003 *M*). Unless otherwise stated, the concn. of DNP, adjusted to pH 7.2 with potassium hydroxide, was 10^{-4} *M*. In some expts. the medium contained 300 μmoles of potassium malonate (0.02 *M*) and this was replaced in the controls by 300 μmoles of potassium chloride (0.02 *M*); in these cases sufficient sucrose was removed to keep the osmolarity of the medium constant.

Soon after the addition of the mitochondria, a sample (1.0 ml) was removed and diluted with 9 vols. of 0.25 *M* sucrose at room temp., and its O.D. was determined. The value of this first reading was taken as the zero time or initial O.D. The open flasks were shaken horizontally in a bath at 28°. At various intervals 1.0 ml samples were removed, diluted and read in the spectrophotometer as indicated above.

Special chemicals

Oxaloacetic acid was prepared according to BARTLEY AND AVIDOR[27]. The following compounds were commercial preparations: α-ketoglutaric acid and AMP (Sigma Chemical Co., St. Louis, U.S.A.); sodium succinate, DL-malic, fumaric and malonic acids (E. Merck, Darmstadt, Germany); citric acid (Bayer, Leverkusen, Germany); isocitrate (trisodium salt, DL and allo, California Foundation for Biochem. Res., Los Angeles, U.S.A., lot 4111); sodium pyruvate (Hoffmann-La Roche Co., Basel, Switzerland); barium oxalosuccinate, *cis*-aconitic and L-glutamic acids (Ditta A. Binda, Milan, Italy).

RESULTS

Effects of AMP and DNP on the swelling of mitochondria in the absence of added substrate

In preliminary expts., the effects on the mitochondrial swelling of DNP, AMP and AMP plus DNP in the absence of any added substrate were studied. The results are shown in Fig. 1.

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It is apparent that AMP and DNP, either separately or in admixture, inhibit the swelling of mitochondria which occurs during incubation at 28° in the presence of sucrose, NaCl, phosphate buffer and Mg^{++} ions. During the first 30 min of incubation DNP was as effective as AMP plus DNP, and both were much more effective than AMP alone. As the incubation proceeded the protection by DNP decreased rapidly and after 60 min the mitochondria incubated in the presence of AMP, with or without DNP, were less swollen than those incubated in the presence of DNP alone. The above findings are substantially in agreement with previous observations[13,14,18,19] made under conditions significantly different from ours.

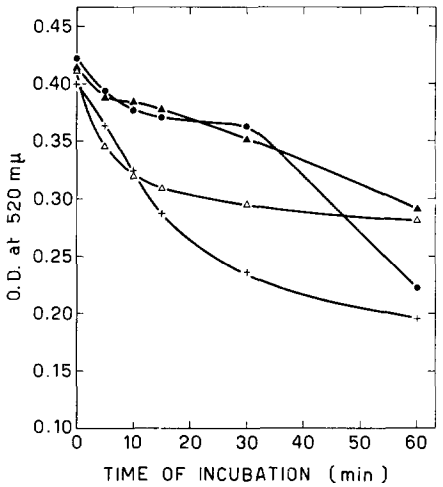

Fig. 1. Effects of AMP, DNP and AMP + DNP on the swelling of mitochondria in the absence of substrate. +—+ no additions; △—△ AMP; ●—● DNP; ▲—▲ AMP + DNP.

Effects of DNP on the swelling of respiring and phosphorylating mitochondria

The substrates tested were tricarboxylic acid cycle intermediates, pyruvate and glutamate. Each substrate was tested alone and in the presence of DNP, AMP and AMP plus DNP. Simultaneous controls were carried out with NaCl in place of the substrate and no further addition; the swelling in the control is referred to as the "spontaneous" swelling of the mitochondria.

Fig. 2 illustrates the results obtained using succinate as substrate. Succinate enhanced the spontaneous swelling of the mitochondria, as already observed under other exptl. conditions[4,5,14,15,24]. The degree of swelling was still greater with the simultaneous addition of succinate and DNP, although DNP *per se* effectively inhibited swelling (*cf.* Fig. 1). In the presence of succinate plus AMP no significant change in O.D. was observed in 60 min, *i.e.*, spontaneous swelling was completely prevented. However, if the incubation medium also contained DNP, the rapid swelling which occurred was more pronounced than the spontaneous swelling and of the same magnitude as that observed with succinate plus DNP. These findings agree with those of PRICE *et al.*[11,12].

Results with glutamate as substrate (Table I) closely resembled those obtained with succinate except in one respect; the addition of DNP to the medium containing glutamate and AMP caused a swelling which was almost the same as the spontaneous

swelling. The results of expts. carried out with α-ketoglutarate are also reported in Table I. α-Ketoglutarate alone or plus DNP did not appreciably affect the spontaneous swelling of the mitochondria. As in the case of succinate and glutamate, spontaneous swelling was largely prevented in the presence of α-ketoglutarate plus AMP but when the system also included DNP, the swelling was much less pronounced than the spontaneous swelling.

With malate the results (Fig. 3) were quite different. Swelling was only slightly prevented when the mitochondria were incubated in the presence of malate plus AMP. Furthermore, the addition of DNP to the medium containing malate, with or without AMP, markedly stabilized the mitochondria instead of promoting their swelling. It is apparent from Fig. 3 that the protection under these conditions is similar to that observed in the absence of oxidizable substrate (Fig. 1).

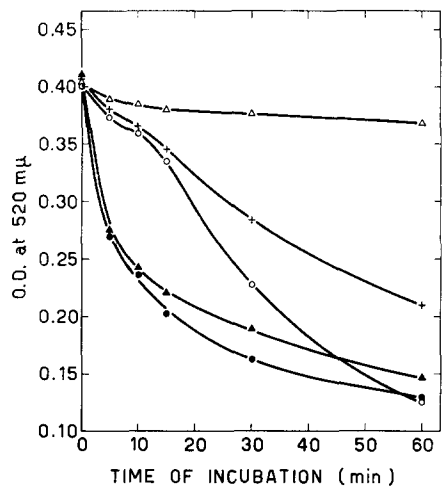

Fig. 2. Course of swelling of mitochondria with succinate as substrate. +—+ no substrate; ○—○ succinate; ●—● succinate + DNP; △—△ succinate + AMP; ▲—▲ succinate + AMP + DNP.

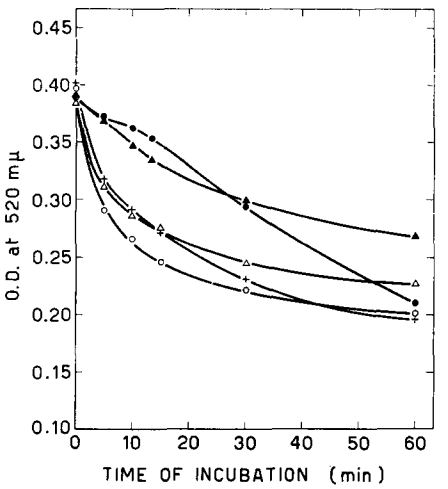

Fig. 3. Course of swelling of mitochondria with malate as substrate. +—+ no substrate; ○—○ malate; ●—● malate + DNP; △—△ malate + AMP; ▲—▲ malate + AMP + DNP.

The results obtained with fumarate, oxaloacetate or pyruvate (Table I) were similar to those with malate; the small differences were mainly observed with the substrate alone. Citrate, *cis*-aconitate, iso-citrate or oxalosuccinate have also been used as substrates (Table I). There were some differences depending on the substrate used, but in all cases the addition of DNP to the medium containing the substrate and AMP enhanced the protection from swelling. In considering the results, it should be kept in mind that citrate, apart from its activity as an oxidizable substrate, effectively protects mitochondria from swelling because it can bind Ca^{++} ions[5,13–15].

The results (see Figs. 2 and 3; Table I) clearly show that, in the presence of AMP and an oxidizable substrate, 10^{-4} *M* DNP may be either advantageous or disadvantageous in protecting mitochondria from swelling, the effect depending on the type of substrate. Moreover the conditions favourable to oxidative phosphorylation help to maintain the mitochondria in the contracted state only when the oxidizable substrate is succinate, glutamate or α-ketoglutarate.

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TABLE I

EFFECTS OF VARIOUS SUBSTRATES EITHER ALONE OR WITH ADDED DNP, AMP AND AMP + DNP ON THE SWELLING OF LIVER MITOCHONDRIA

For details on conditions, see text.

Conditions	*O.D. after incubation at 28° for*					
	0 min	*5 min*	*10 min*	*15 min*	*30 min*	*60 min*
Control without substrate	0.400	0.350	0.305	0.290	0.255	0.200
Glutamate	0.405	0.320	0.280	0.245	0.190	0.175
Glutamate + DNP	0.420	0.230	0.210	0.195	0.160	0.155
Glutamate + AMP	0.410	0.380	0.380	0.375	0.365	0.330
Glutamate + AMP + DNP	0.430	0.320	0.280	0.265	0.245	0.230
Control without substrate	0.400	0.365	0.320	0.275	0.225	0.195
α-ketoglutarate	0.405	0.355	0.320	0.280	0.235	0.185
α-ketoglutarate + DNP	0.425	0.365	0.280	0.270	0.245	0.190
α-ketoglutarate + AMP	0.420	0.390	0.380	0.380	0.375	0.350
α-ketoglutarate + AMP + DNP	0.405	0.330	0.320	0.315	0.310	0.305
Control without substrate	0.400	0.350	0.305	0.290	0.255	0.200
Fumarate	0.395	0.330	0.270	0.250	0.220	0.195
Fumarate + DNP	0.415	0.395	0.375	0.370	0.310	0.210
Fumarate + AMP	0.410	0.340	0.315	0.305	0.295	0.290
Fumarate + AMP + DNP	0.415	0.395	0.390	0.375	0.345	0.315
Control without substrate	0.400	0.335	0.310	0.280	0.230	0.190
Oxaloacetate	0.395	0.330	0.315	0.290	0.230	0.210
Oxaloacetate + DNP	0.390	0.380	0.380	0.370	0.345	0.200
Oxaloacetate + AMP	0.395	0.330	0.305	0.280	0.275	0.265
Oxaloacetate + AMP + DNP	0.405	0.390	0.385	0.385	0.340	0.230
Control without substrate	0.400	0.330	0.305	0.290	0.245	0.210
Pyruvate	0.405	0.375	0.340	0.325	0.275	0.245
Pyruvate + DNP	0.425	0.400	0.390	0.390	0.375	0.285
Pyruvate + AMP	0.420	0.380	0.350	0.340	0.335	0.310
Pyruvate + AMP + DNP	0.435	0.410	0.410	0.405	0.390	0.355
Control without substrate	0.400	0.340	0.300	0.270	0.220	0.195
Citrate	0.385	0.350	0.315	0.285	0.265	0.250
Citrate + DNP	0.375	0.365	0.365	0.365	0.350	0.290
Citrate + AMP	0.405	0.370	0.360	0.350	0.335	0.325
Citrate + AMP + DNP	0.370	0.365	0.365	0.360	0.360	0.350
Control without substrate	0.400	0.350	0.325	0.315	0.285	0.225
cis-aconitate	0.425	0.350	0.305	0.285	0.240	0.215
cis-aconitate + DNP	0.420	0.415	0.415	0.410	0.400	0.380
cis-aconitate + AMP	0.425	0.380	0.365	0.350	0.310	0.285
cis-aconitate + AMP + DNP	0.435	0.425	0.420	0.415	0.405	0.390
Control without substrate	0.400	0.320	0.295	0.270	0.240	0.210
iso-citrate	0.390	0.310	0.290	0.270	0.235	0.210
iso-citrate + DNP	0.390	0.375	0.360	0.345	0.300	0.235
iso-citrate + AMP	0.395	0.340	0.320	0.305	0.285	0.265
iso-citrate + AMP + DNP	0.395	0.380	0.375	0.375	0.360	0.330
Control without substrate	0.400	0.315	0.270	0.250	0.230	0.190
Oxalosuccinate	0.395	0.265	0.255	0.240	0.220	0.200
Oxalosuccinate + DNP	0.400	0.325	0.250	0.225	0.205	0.180
Oxalosuccinate + AMP	0.395	0.280	0.260	0.255	0.250	0.225
Oxalosuccinate + AMP + DNP	0.405	0.325	0.305	0.300	0.290	0.260

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Since the action of DNP on oxidation and phosphorylation depends on its concn. and on the nature of the substrate[28], it seemed of interest to study the effects of different concns. in presence of succinate, glutamate and α-ketoglutarate. 10^{-5} *M* and 10^{-3} *M* DNP concns. were tested as well as that used in the previous expts. (10^{-4} *M*). 10^{-5} *M* DNP was as effective as 10^{-4} *M* DNP in causing the swelling of the mitochondria incubated in the presence of the oxidizable substrate plus AMP. However, 10^{-3} *M* DNP produced only a small swelling in the presence of either glutamate plus AMP or α-ketoglutarate plus AMP. In a system containing succinate plus AMP, 10^{-3} *M* DNP caused a marked swelling which, however, was smaller than that produced by 10^{-4} *M* DNP.

Experiments in the presence of malonate

In the search for a common mechanism to explain the above observations, expts. were carried out in which succinic dehydrogenase was completely inhibited by malonate. The substrates used were succinate, glutamate and α-ketoglutarate, where a relationship seemed to exist between oxidative phosphorylation and mitochondrial swelling, and malate, where no relationship was apparent.

Fig. 4a shows the rate of swelling of mitochondria incubated, with or without malonate, in the medium without substrate and in that supplemented with succinate and AMP. Figs. 4b, c and d show the results of identical expts. carried out with glutamate, α-ketoglutarate and malate. Fig. 4a shows that malonate gives some protection against spontaneous swelling, as already noted[5,14,24] under other conditions. However, mitochondria incubated with AMP and succinate, glutamate or α-ketoglutarate swelled much more in the presence of malonate than in its absence (Figs. 4a, b and c). The results obtained with succinate as substrate (Fig. 4a) clearly show that the protection from swelling observed with a medium containing succinate plus AMP is a "metabolic effect" and is dependent on the oxidative metabolisme of succinate. Figs. 4b and c show that the protection observed with α-ketoglutarate or glutamate plus AMP is also dependent on the activity of succinic dehydrogenase and disappears if the oxidation of such substrates is prevented from proceeding beyond the succinate step. In other words, the phosphorylation associated with the one-step oxidation of α-ketoglutarate to succinate and with the two-step oxidation of glutamate to succinate does not protect the mitochondria. With a substrate such as malate, which in these conditions cannot yield succinate, the addition of AMP confers no protection and the further addition of malonate is without effect (Fig. 4d).

DISCUSSION

As already mentioned, previous work has indicated that although DNP promoted the swelling of mitochondria respiring in the presence of substrate[2,11,12], it was actually a potent protective agent against swelling of non-respiring mitochondria in the absence of substrate[13–15,18,19].

The results presented in this paper confirm that DNP protects non-respiring mitochondria, and show that in the presence of all components necessary to oxidative phosphorylation (oxidizable substrate, AMP, Mg^{++} ions and inorganic phosphate) DNP may either enhance or inhibit the swelling, depending upon the nature of the substrate. Under such conditions, 10^{-4} *M* DNP, a concn. which is known to uncouple

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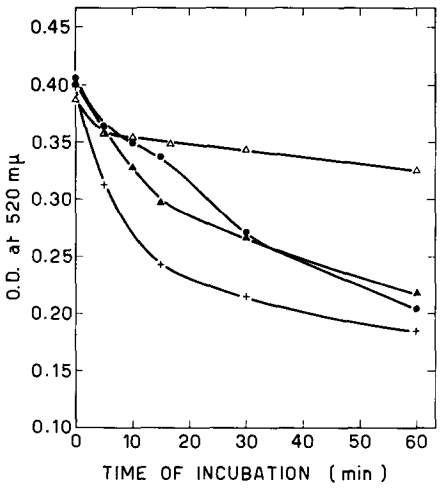

Fig. 4a.

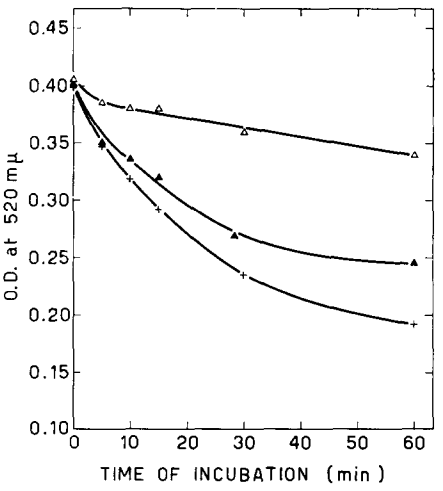

Fig. 4b.

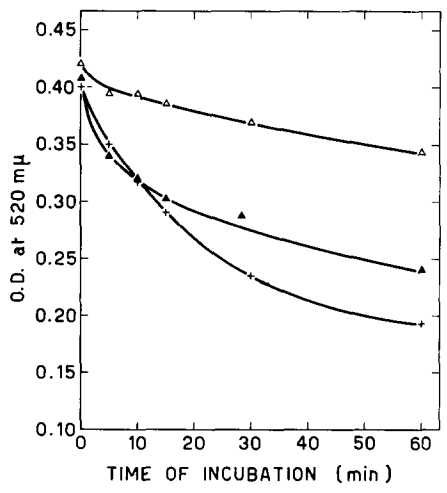

Fig. 4c.

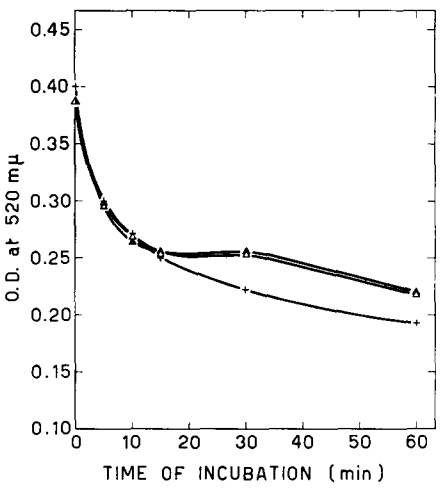

Fig. 4d.

Fig. 4. Effects of malonate on the swelling of mitochondria with succinate (a), glutamate (b), α-ketoglutarate (c) and malate (d) as substrates. +—+ no substrate; ●—● malonate; △—△ substrate + AMP; ▲—▲ substrate + AMP + malonate.

oxidative phosphorylation, promoted swelling only when the oxidizable substrate was succinate, glutamate or α-ketoglutarate. In previous studies of the swelling of mitochondria after the addition of DNP to a medium containing all components for oxidative phosphorylation, the oxidizable substrate was always succinate, glutamate or α-ketoglutarate[2,11,12].

The results obtained when succinic dehydrogenase was specifically inhibited with malonate indicate that most probably α-ketoglutarate and glutamate act like succinate in so far as they are converted into succinate during the incubation. In fact, when succinic dehydrogenase is blocked by malonate, neither α-ketoglutarate plus AMP nor glutamate plus AMP protects the mitochondria from swelling.

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A role of succinic dehydrogenase in the non-osmotic swelling of mitochondria was first postulated by RAAFLAUB[5] who found that succinate promoted the swelling of mitochondria incubated in the absence of adenosine phosphates and that malonate prevented such a swelling. TAPLEY[14] confirmed this antagonism between succinate and malonate but stated that its significance was limited since in his system malonate also prevented the swelling caused by fumarate, malate, orthophosphate or Ca^{++} ions. The present results support RAAFLAUB's concept and also indicate that phosphorylation coupled to the oxidation of succinate may be of utmost importance in the maintenance of the mitochondrial structure. The evidence for this hypothesis may be summarized as follows: (1) The mitochondria incubated in isotonic media in the presence of all components necessary to oxidative phosphorylation are largely protected from swelling when the oxidizable substrate is succinate, glutamate or α-ketoglutarate but are much less protected with other substrates (malate, fumarate, oxalacetate, pyruvate, *cis*-aconitate, iso-citrate, oxalosuccinate). The small protection observed with the latter substrates plus AMP may be attributed mainly to the protective action of AMP (Fig. 1). The great protection observed with citrate plus AMP is most probably due to the capacity of citrate to bind Ca^{++} ions (see below). (2) The addition of 10^{-4} *M* DNP to protecting systems (AMP plus succinate, glutamate, or α-ketoglutarate) abolishes the protection; but its addition to systems containing the other substrates plus AMP actually helps to maintain the mitochondrial structure. Since DNP enhances instead of inhibiting the marked protection by citrate plus AMP, in this case the protection is not dependent upon oxidative phosphorylation. (3) 0.02 *M* malonate, which completely inhibits succinic dehydrogenase without affecting oxidative phosphorylation of glutamate and α-ketoglutarate[29,30], reduces not only the protection conferred by AMP plus succinate, but also that observed with glutamate plus AMP or α-ketoglutarate plus AMP.

The fact that 10^{-3} *M* DNP is less effective than 10^{-4} *M* DNP in causing the swelling of respiring mitochondria is not inconsistent with the above hypothesis. The oxidation of succinate, which promotes swelling when uncoupled from phosphorylation, is in fact stimulated by 10^{-4} *M* DNP but inhibited by 10^{-3} *M* DNP[28].

It is impossible at present to say how the phosphorylation coupled to the oxidation of succinate can maintain the mitochondrial structure. It is of interest, however, that succino–oxidase system has been found associated with mitochondrial membranes[31].

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FORMATION OF 5-AMINOIMIDAZOLE RIBOSIDE BY ESCHERICHIA COLI: EVIDENCE FOR ITS STRUCTURE AND METABOLIC RELATIONSHIP TO THE PURINES

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SUMMARY

The arylamine accumulated by the mutant of *Escherichia coli* K-12, strain W-11, has been identified as 5-aminoimidazole riboside. The compound, isolated by an improved method, yielded upon chemical degradation glycine, formate and pentose in the expected proportions. The product contained no phosphorus. The arylamine was formed in microorganisms from [^{14}C]labeled formate or glycine and could be converted into inosinic acid by an enzyme system of pigeon liver. Chemical conversion of radioactive arylamine to the ureido derivative, hydrolysis to the free base and addition of carrier 5-ureidomidazole, yielded a picrate of 5-ureidoimidazole which could be crystallized to a constant specific activity.

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