Effects of Piericidin A and Amytal on the Energy-Linked Swelling of Rat Liver Mitochondria

We had suggested[1,2] that the first energy conservation site is on the oxygen side of the *O*-phenanthroline sensitive site associated with the non-heme iron of the NADH-dehydrogenase but on the substrate side of the rotenone-sensitive site. This was confirmed later[3,4] by SINGER et al. using optical and EPR studies. They have shown that site I is on the NADH dehydrogenase between one of the non-heme iron centres and the type 5-SH group. Experiments based on the energy linked swelling of mitochondria, on which we based our suggestion[1] regarding the rotenone sensitive site, also place the piericidin A and amytal sensitive site on the oxygen side of the energy conservation site.

Preparation of rat liver mitochondria and the conditions for assaying swelling mediated specifically through energy changes at site I as well as the parameters of mitochondrial swelling used have been detailed in earlier communications[1,2]. The results with β-hydroxybutyrate and pyruvate plus malate as substrate under conditions when only site I is operative are given in Figures 1 and 2, respectively. In order to prevent any contribution from substrate-level phosphorylation due to α-ketoglutarate resulting from oxidation of pyruvate plus malate, 1 m*M* arsenite was also included in this system. At this concentration arsenite completely eliminates the oxidation of α-ketoglutarate[5,6]. In both figures, curve 1 represents the swelling mediated by all the 3 energy conserving sites in untreated mitochondria. Treatment with 0.15 μM

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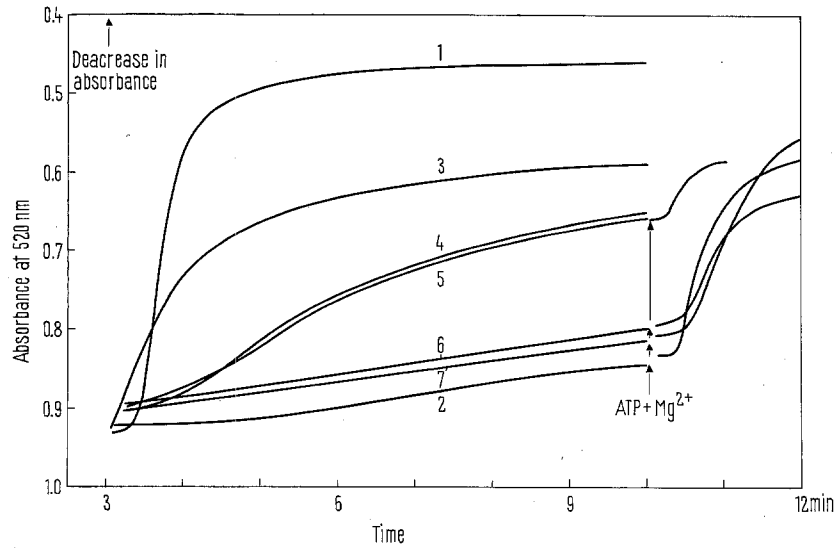

Fig. 1. Effect of amytal and piericidin A on induction of swelling by phosphate plus thyroxine in rat liver mitochondria using β-hydroxy-butyrate as substrate. Mitochondria (0.6 mg protein) were suspended in 3 ml of 0.15 *M* KCl containing 20 m*M* *Tris*-HCl (pH 7.4) at 28 °C. Swelling by phsophate (2 m*M*) plus thyroxine (20 μM) was started after a 3 min incubation with the following: 1, β-hydroxybutyrate, 5 m*M*; 2, antimycin A, 0.15 μM and KCN, 0.5 m*M* added to system 1; 3, PMS, 0.1 m*M* added to system 2; 4, amytal, 1.8 m*M* added to system 3; 5, piericidin A, 0.06 μM added to system 3; 6, amytal, 1.8 m*M* added to system 2; and 7, piericidin A, 0.06 μM added to system 2. In curves 2, 5, 6 and 7, ATP and Mg^{2+}, 60 μM of each was added at the end of 7 min after addition of phosphate plus thyroxine.

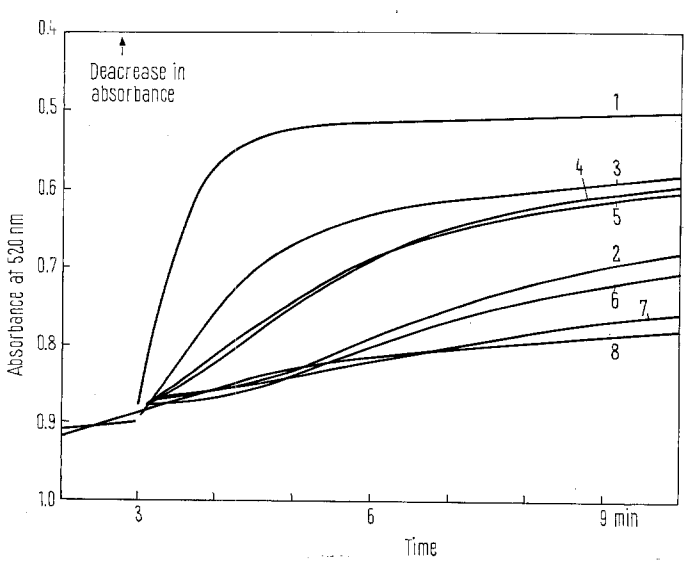

Fig. 2. Effect of amytal and piericidin A on induction of swelling by phosphate plus thyroxine in rat liver mitochondria using pyruvate plus malate as substrate. Mitochondria (0.6 mg protein) were suspended in 3 ml of 0.15 *M* KCl containing 20 m*M* *Tris*-HCl (pH 7.4) at 28 °C. Swelling by phosphate (2 m*M*) plus thyroxine (20 μM) was started after a 3 min incubation with the following: 1, Pyruvate, 5 m*M*, malate, 1 m*M*, and arsenite 1 m*M*; 2, antimycin A, 0.15 μM and KCN, 0.5 m*M* added to system 1; 3, PMS, 0.1 m*M* added to system 2; 4, amytal, 1.8 m*M* added to system 3; 5, piericidin A, 0.06 μM added to system 3; 6, amytal 1.8 m*M* added to system 2; 7, piericidin A, 0.06 μM added to system 2; 8, phosphate and thyroxine omitted from system 1.

antimycin A and 0.5 mM KCN blocked mitochondrial swelling (curve 2). If, however, 0.1 mM PMS were added to the above system swelling occurred (curve 3). Addition of 1.8 mM amytal or 0.06 μM piericidin A[7] did not eliminate the swelling supported by the bypass created with PMS (curves 4 and 5, respectively). Addition of amytal or piericidin A in the absence of PMS to the system represented by curve 2 did not induce swelling (curves 6 and 7, respectively). In the absence of PMS, swelling could be supported by addition of 60 μM each of ATP and Mg^{2+}. (Figure 1). Although not shown here, induction of swelling by APT and M^{2+} was also observed for the systems represented by Figure 2. Curve 8 in Figure 2 represents the swelling mediated by all the 3 energy conserving sites in mitochondria in the presence of 1 mM arsenite without the addition of phosphate and thyroxine.

It is generally accepted that large amplitude swelling of mitochondria is an energy-linked process[8]. In our experimental system energy changes were restricted to the first site specifically by using a combination of antimycin A and cyanide and channelling the electrons from the substrates to an artificial acceptor, namely PMS[1]. That sites II and III are not operative under these conditions was established. No swelling took place if PMS was not included (curve 2) in the system indicating that flux of electrons from substrates to PMS is necessary. Any possibility that PMS per se might induce swelling is ruled out by the fact that in the absence of swelling agents, phosphate plus thyroxine, there was no swelling. Addition of amytal and piericidin A did not eliminate the swelling (curves 4 and 5). Under conditions when substrate oxidation does not take place addition of ATP and Mg^{2+} induced swelling indicating the energy-requiring nature of the process (curves 2, 6, 7). These results indicate that site I energy conservation is on the substrate site of amytal and piericidin A sensitive sites associated with NADH-dehydrogenase. Our observations[9] are consistent with those of GUTMAN et al[3].

Zusammenfassung. Es gelingt, die Schwellung der Rattenlebermitochondrien durch Energiekonservierung an jedem der 3 Phosphorylierungsorte herbeizuführen. Bei Gegenwart von Piericidin A oder Amytal wird die energieabhängige Schwellung nicht unterdrückt, insofern Energieänderungen auf den ersten Phosphorylierungsort beschränkt bleiben, was dafür spricht, dass die Wirkungsorte von Piericidin A und Amytal auf der Sauerstoffseite der Energiekonservierung am Phosphorylierungsort I liegen.

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Identification of Alexandrin

Centaurea alexandrina a biennial or often perennial pubscent herb, occurs only in Egypt[1] and is common in the Mediterranean coastal strip. SALEH and GHARBO[2] reported the isolation of a crystalline principle named 'alexandrin' from the leaves of *C. alexandrina* and stated that the substance (m.p. 261–262), is neither alkaloidal nor glycosidal in nature.

Extraction of the dried powered leaves with ether[2], followed by concentration of the extract yielded a crystalline deposit, which after dissolving in alcohol and decolorising with activated charcoal afforded the substance corresponding to the so-called alexandrin. Thin-layer chromatography (adsorbent: silica gel G, solvents[3,4]: carbon tetrachloride-ethanol-water 10:8:2, hexane-ethyl acetate-ethanol-water 4:6:5:5, benzene-methanol 8:2) of the substance revealed, upon spraying with *p*-anisaldehyde[5], the presence of two components; the major one has the same Rf as β-sitosterol-β-D-glucoside, while the other is present in traces. Repeated recrystallization of the substance from methanol afforded the major constituent in pure form, m.p. 298–300° (undepressed), and proved to be β-sitosterol-β-D-glucoside. The identity was proven by chemical and physicochemical means (IR, NMR, MS, tetraacetate, hydrolysis).

The reportedly new constituent thymelol, also isolated by SALEH et al.[6] from *Thymelea hirsuta*, has been proved by RIZK and RIMPLER[7] to be a mixture of daphnoretin and β-sitosterol-β-D-glucoside.

Zusammenfassung. Die als Alexandrin beschriebene Substanz, die als Bestandteil von *Centaurea alexandrina* isoliert worden ist, wurde als β-sitosterol-β-D-glukosid identifiziert[8].

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