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THE ABSENCE OF ENERGY CONSERVATION COUPLED WITH ELECTRON TRANSFER VIA THE ALTERNATIVE PATHWAY IN CYANIDE-RESISTANT YEAST MITOCHONDRIA

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Summary

Electron transfer via the alternative pathway in cyanide-resistant mitochondria of the yeast *Candida lipolytica* is not coupled with ATP synthesis, generation of membrane potential or energy-dependent reverse electron transport in the main respiratory chain. We conclude that during transfer via the alternative pathway no accumulation of energy in the form of high-energy compounds or membrane potential occurs.

The phenomenon of cyanide-resistant respiration has been observed in many organisms [1]. Insensitivity of respiration to cyanide in eucaryotic organisms is due mostly to the appearance of the alternative pathway of electron transport in mitochondria, with a branching-off point at the coenzyme Q level [2—3].

Most investigators studying energy-dependent reverse electron transfer [4], generation of proton-motive force [5] and oxidative phosphorylation [6—10] consider that there is no energy accumulation in the alternative pathway. Some investigators, however, do not exclude the possibility of phosphorylation in the alternative pathway [11—16].

We have attempted to obtain proof that there is no accumulation of energy in the alternative pathway.

Cultivation of the yeast, *Candida lipolytica*, and isolation of mitochondria are described elsewhere [17]. The cells were harvested in the early stationary phase, characterized by the appearance of cyanide-resistant respiration.

The phosphorylation coefficient, P/O ratio, was determined as the ratio of ATP formed to the amount of oxygen consumed during phosphorylation. ATP was measured fluorimetrically using hexokinase and glucose-6-phosphate dehydrogenase. Oxygen was measured polarographically using a Clark-type oxygen electrode.

Reverse electron transport was followed by oxidation of cytochrome *c* and also be reduction of endogenous pyridine nucleotides.

The mitochondrial membrane potential was determined using the positively-charged dye, safranine, as a probe [18]. The absorption at 510 nm compared to 530 nm was recorded by a spectrophotometer DSD-2 (Central Bureau for Designing of Medical Equipment, Moscow).

Protein concentration was determined by the biuret method.

Table I shows the phosphorylation efficiencies of oxidation of various substrates via alternative pathway. 1 mM cyanide inhibited the electron transport via the main respiratory chain.

Oxidation of pyruvate + malate in the presence of cyanide gave a P/O ratio somewhat lower than 1.0. Since the alternative pathway branches off the main respiratory chain after the first phosphorylation site, the P/O ratio obtained is, evidently, due to ATP production at site 1. Under the same conditions the P/O ratio during succinate oxidation is equal to 0.14. The site 1 phosphorylation can be assumed to contribute to this P/O ratio, due to oxidation of malate formed from succinate via fumarate. Indeed, the P/O ratio during succinate oxidation (with rotenone inhibiting site 1 phosphorylation) equals 0.03.

The P/O ratio during oxidation of α-glycerophosphate (via the alternative pathway in the presence of cyanide) is equal to 0.05. The above data suggest the absence of phosphorylation in the alternative pathway.

Fig. 1 shows the degree of cytochrome *c* reduction. There is almost complete reduction of this cytochrome after the addition of cyanide to cyanide-resistant mitochondria (the electrons come from endogenous substrates). Further addition of pyruvate + malate caused an unexpected effect, namely, oxidation of cytochrome *c* (Fig. 1, curve A). Haddock and Garland [19] were the first to suggest that this oxidation occurs due to electron transport from cytochrome *c* to coenzyme Q and, further, through alternative oxidase to oxygen. Indeed, if benzhydroxamic acid inhibiting the alternative oxidase was added after pyruvate + malate, cytochrome *c* was again reduced (Fig. 1, curve A). Antimycin A prevented the oxidation of cytochrome *c* upon the addition of pyruvate + malate (Fig. 1, curve B).

Electron transport from cytochrome *c* to cytochrome *b* against the gradient of redox potentials is known to be energy-dependent. Therefore, oxidation of

TABLE I

P/0 RATIOS FOR THE OXIDATION OF SUBSTRATES IN PRESENCE OF CYANIDE BY THE CYANIDE-RESISTANT MITOCHONDRIA OF THE YEAST *C. LIPOLYTICA*

Medium: 0.6 M mannitol, 10 mM Tris-phosphate (pH 7.0), 0.5 mM EDTA, 0.05% bovine serum albumin, 1 mM cyanide. Additions: 2.1 mg mitochondrial protein/ml, 5 mM pyruvate, 5 mM malate, 25 mM succinate, 25 mM α-glycerophosphate, 60 μM rotenone.

Substrate	P/0 ratio
Pyruvate + malate	0.69 *
Succinate	0.14
Succinate + rotenone	0.03
α-Glycerophosphate	0.05

* ADP/0 ratio measured polarographically upon oxidation of pyruvate + malate without cyanide equals 2.8.

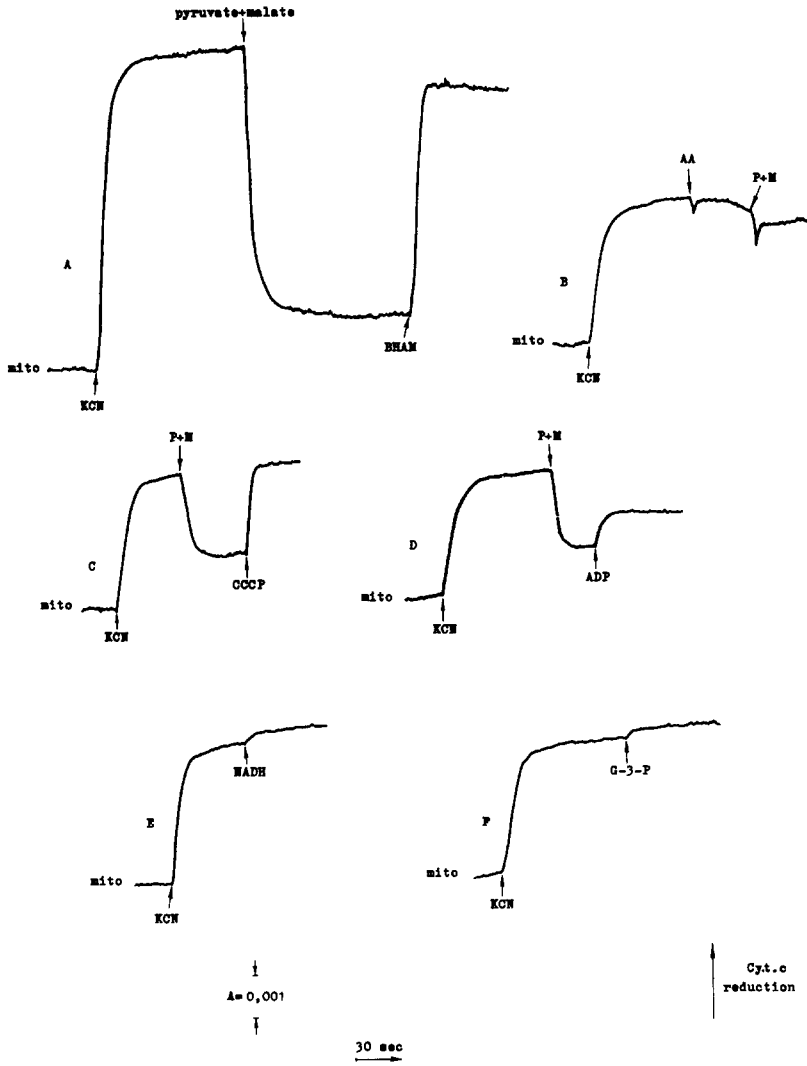

Fig. 1. The degree of cytochrome *c* reduction in cyanide-resistant mitochondria of *Candida lipolytica*. The medium: 0.6 M mannite, 10 mM Tris-phosphate buffer (pH 7.0), 0.5 mM EDTA, 0.05% bovine serum albumin; mitochondrial concentration, 2.5 mg protein/ml on recording the curve A and 1 mg protein/ml on recording other curves. Additions: 10 mM pyruvate + malate (P + M), 1 mM NADH, 20 mM α-glycerophosphate (α-G-P), 2 mM cyanide, 5 mM benzhydroxamic acid (BHAM), 2 μg/ml antimycin A (AA), 1 μM carbonyl-cyanide-*m*-chloromethoxyphenylhydrazone (CCCP), 1 mM ADP. Mito, mitochondria.

cytochrome *c* in the presence of cyanide should be inhibited by uncouplers or ADP (Fig. 1, curves C and D).

During oxidation of pyruvate + malate in the presence of cyanide the energy for the reverse electron transport from cytochrome *c* is supplied evidently by the phosphorylation site 1.

To check the probability of energy production for reverse electron transport in the alternative pathway itself, NADH and α-glycerophosphate substrates were used. The electrons of these substrates enter the main respiratory chain

passing site 1; thus, their oxidation via the alternative pathway in the presence of cyanide misses the known phosphorylation sites.

The addition of both NADH (Fig. 1, curve E) and α-glycerophosphate (Fig. 1, curve F) after cyanide did not appear to cause oxidation of cytochrome *c*.

Fig. 2 shows that reduction of pyridine nucleotides (which are reversibly oxidized with ADP) occurred during α-glycerophosphate oxidation (curve A). Similar results were obtained with addition to mitochondria of benzhydroxamic acid, which inhibited electron transfer via the alternative pathway (Fig. 2, curve B). There is, however, no reduction of pyridine nucleotides in the presence of α-glycerophosphate, if cyanide is added to the suspension of mitochondria (Fig. 1, curves C and D). Thus, electron transfer via the alternative

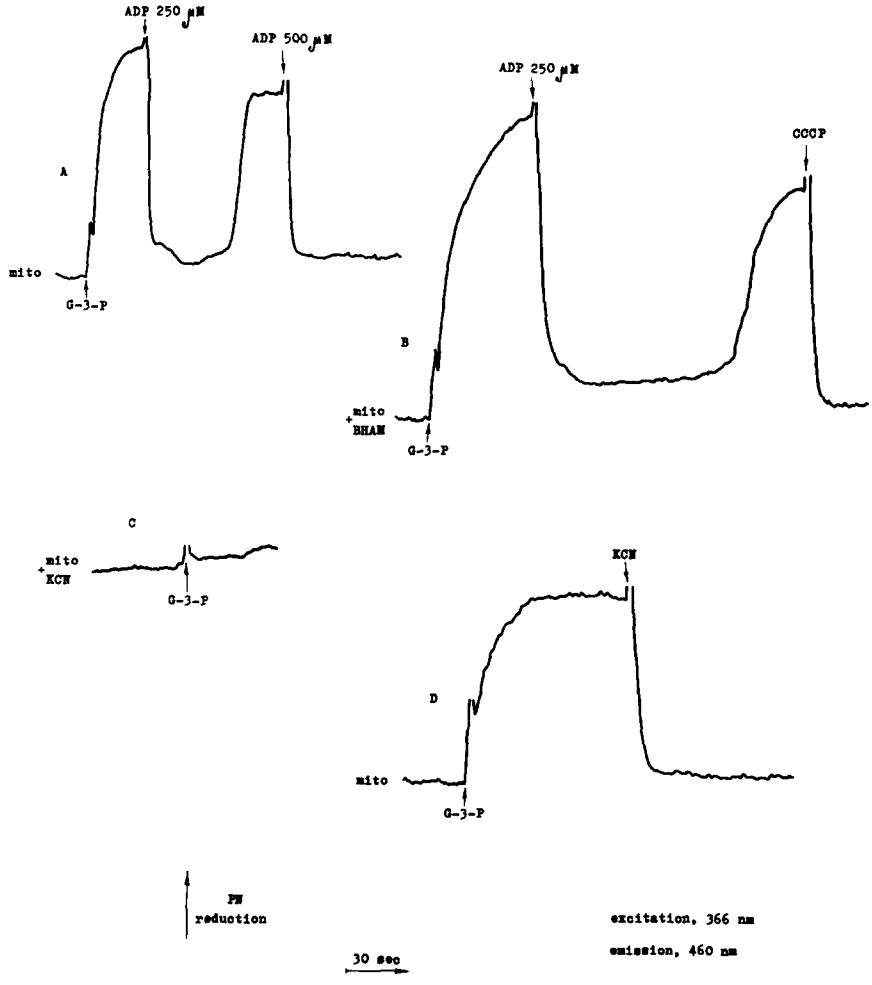

Fig. 2. The degree of pyridine nucleotide reduction in cyanide-resistant mitochondria of *C. lipolytica*. The medium and additions are the same as in the legend to Fig. 1; mitochondrial concentrations, 2 mg protein/ml on recording the curves A and C, 1 mg protein/ml on recording the curve B and 1.5 mg protein/ml on recording the curve D. Abbreviations as in Fig. 1; PN, pyridine nucleotide.

pathway does not provide energy for the reverse transport in the main respiratory chain.

Fig. 3 shows changes in safranine absorption due to the generation of membrane potential upon oxidation of various substrates. Addition of pyruvate + malate to mitochondria (curve A) resulted in an increase in the absorption of safranine at 510 nm, indicating energization of the mitochondrial membrane. Further addition of cyanide produced no detectable decrease in the absorption of the dye at this wavelength. The membrane potential, in this case, is probably generated at phosphorylation site 1. The addition of benzhydroxamic acid after cyanide caused a sharp drop in safranine absorption (Fig. 3, curve A), indicating that there was a decrease in the membrane potential, probably to zero. On oxidation of α-glycerophosphate and NADH by cyanide-resistant mitochondria, a similar disappearance of the membrane potential was observed

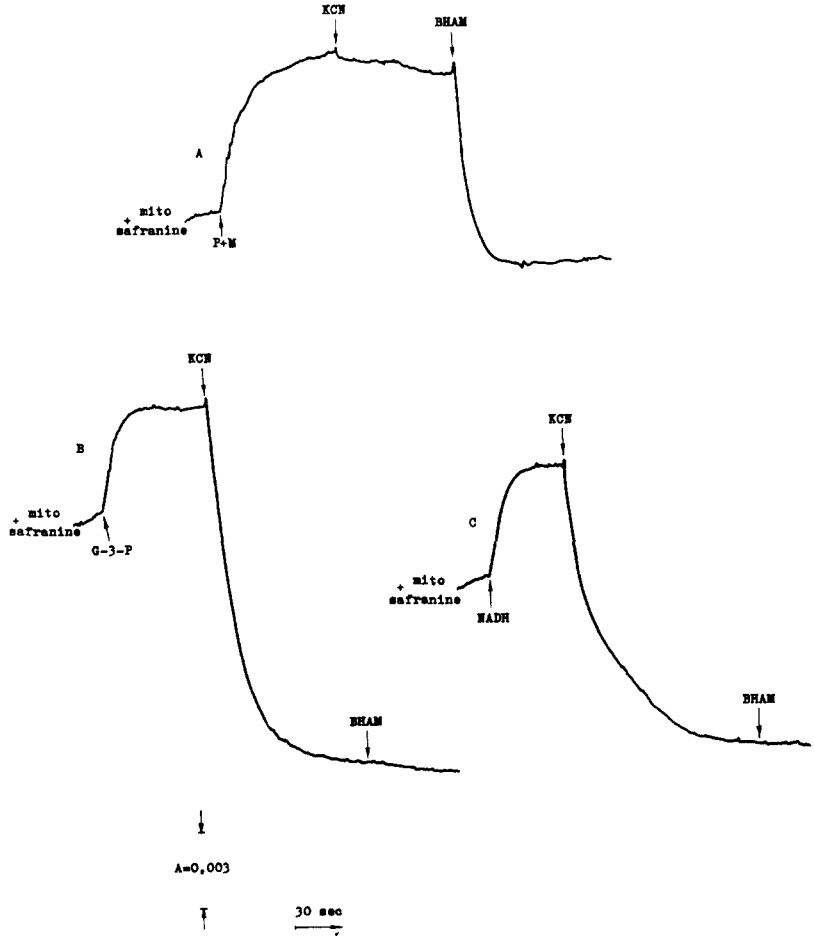

Fig. 3. The changes of safranine absorption according to the energetic state of cyanide-resistant mitochondria of *C. lipolytica*. The medium was the same as quoted in the legend to Fig. 1, with 15 μM safranine added; mitochondrial concentration 1.3 mg protein/ml. Additions: 1 mM NADH, 5 mM pyruvate + malate, 5 mM α-glycerophosphate, 2 mM cyanide, 5 mM benzhydroxamic acid. Abbreivations as in Fig. 1.

with the addition of cyanide only (Fig. 3, curves B and C).

All of the above data unambiguously indicate that electron transfer via the alternative pathway of cyanide-resistant yeast mitochondria is not coupled with the energy accumulation.

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