Phosphorylation coupled to α-ketoglutarate oxidation in Ehrlich-ascites-cell mitochondria

Ehrlich-ascites-cell mitochondria, like those of other tumours, exhibit a pronounced DPN requirement for maximal oxygen uptake[1,2]. However, little information is available concerning the phosphorylations coupled to oxidation of substrates of the tricarboxylic-acid cycle in these mitochondria. KUN, TALALAY AND WILLIAMS-ASHMAN[3] reported P/O ratios for Ehrlich mitochondria respiring with succinate in a medium without DPN or the DPNase inhibitor, nicotinamide. LINDBERG, LJUNGGREN, ERNSTER AND RĒVĒSZ[4] quote P/O ratios for pyruvate, citrate, α-ketoglutarate, succinate and malate in a medium again without DPN or nicotinamide. We have measured P/O ratios for succinate, ascorbate, α-ketoglutarate and L-glutamate in similarly unfortified media, and have reported preliminary observations concerning the influence of DPN and nicotinamide on α-ketoglutarate and L-glutamate oxidation[2]. This communication describes a further examination of the effect of DPN and nicotinamide additions on the phosphorylations associated with α-ketoglutarate oxidation.

Mitochondria were isolated in 0.25 *M* sucrose according to the method of HAWTREY AND SILK[2] and were allowed to stand at 0° for periods of 4–5.5 h, in order to deplete them of pyridine nucleotides under mild conditions and permit examination of the effect of adding back DPN. Such ageing of tumour mitochondria is reported by KIELLY[5] to result in a lowered capacity for phosphorylation which can be largely restored by subsequent addition of DPN. Mitochondria of normal cells do not, however, appear to undergo loss of phosphorylation activity on corresponding ageing.

TABLE I

EFFECT OF DPN AND NICOTINAMIDE ADDITIONS ON PHOSPHORYLATION ASSOCIATED WITH α-KETOGLUTARATE OXIDATION IN AGED EHRLICH-ASCITES-CELL MITOCHONDRIA

Flasks contained the following basic medium in a final vol. of 3.0 ml: – K, Na phosphate buffer (pH 7.4), 0.045 *M*; D-glucose, 0.034 *M*; KF, 0.015 *M*; $MgCl_2$, 0.008 *M*; ADP, 0.0025 *M*; AMP, 0.001 *M*; ethylenediaminetetra-acetic acid (pH 7.4), 0.002 *M*; KCl, 0.01 *M*; sucrose, 0.062 *M*; α-ketoglutarate, 0.0066 *M*; malonate, 0.02 *M*; cytochrome *c*, $9.8 \cdot 10^{-6}$ *M*; hexokinase (crystalline), 1–2 mg; mitochondrial suspension (aged 4–5.5 h in sucrose at 0°) in 0.25 *M* sucrose (0.4 ml) equivalent to 2.11 ± 0.29 mg protein determined according to CLELAND AND SLATER[6]. Temp. 30°. The reaction was stopped after 30–40 min by addition of 1.0 ml 30 % trichloroacetic acid. Phosphate uptake was determined according to FISKE AND SUBBAROW[7]. The values given are the means ± the average deviations of results obtained in a total of 11 determinations with different batches of mitochondria.

Special additions	O_2 *consumed* *μatoms/mg protein/h*	*P esterified* *μatoms/mg protein/h*	*P/O*
DPN ($2.4 \cdot 10^{-3}$ *M*) + nicotinamide (0.02 *M*)	5.40 ± 1.02	11.60 ± 2.78	2.17 ± 0.35
DPN ($2.4 \cdot 10^{-3}$ *M*)	5.51 ± 1.10	8.35 ± 3.14	1.49 ± 0.40
Nicotinamide (0.02 *M*)	3.43 ± 1.00	7.28 ± 2.07	2.17 ± 0.49
None	3.77 ± 1.10	6.45 ± 4.10	1.63 ± 0.92

In accord with previous results[2] and the findings of other workers concerning tumour mitochondria[1,5], we have shown that addition of DPN causes a significant increase in the oxygen uptake of aged Ehrlich mitochondria respiring with α-keto-

Abbreviations: DPN, DPNH, oxidised and reduced diphosphopyridine nucleotide; AMP, ADP, adenosine mono- and di-phosphate.

glutarate as substrate (Table I). Oxygen uptake was increased approximately 46 % by addition of $2.4 \cdot 10^{-3}$ *M* DPN to the medium. Nicotinamide (0.02 *M*), on the other hand, had no significant effect on the oxygen uptake in either DPN-fortified or unfortified media.

The phosphorylation activity of mitochondria aged 4–5.5 h was markedly influenced by addition of nicotinamide which raised the P/O ratio by approximately 46 % in media fortified with DPN and by approximately 33 % in media without DPN. The effect was greatest with low concentrations of mitochondrial protein.

TABLE II

OXIDATION OF DPNH BY AGED EHRLICH-ASCITES-CELL MITOCHONDRIA

Flasks contained the basic medium of Table I without malonate. Special additions as indicated. Temp. 30°. Inorganic phosphate was liberated instead of esterified.

Special additions	*Mitochondrial protein mg*	*O_2 consumed µatoms/mg protein/h*	*P esterified µatoms/mg protein/h*
DPN ($2.4 \cdot 10^{-3}$ *M*) + alcohol dehydrogenase (0.4 mg) + ethanol (0.04 *M*)	1.44	4.49	+ 2.40
DPN ($2.4 \cdot 10^{-3}$ *M*) + alcohol dehydrogenase (0.4 mg) + ethanol (0.04 *M*) + nicotinamide (0.02 *M*)	1.44	4.71	+ 1.42

In a medium without nicotinamide, DPN fortification seems to cause a lowering of approximately 9 % in P/O ratio. Although DPN addition raised the oxygen uptake, the esterification of inorganic phosphate was not correspondingly increased, with the result that lower P/O ratios were observed in the presence of DPN (Table I). Similar results were obtained by KIELLEY[5] confirming that externally added DPN does not completely restore the phosphorylation activity lost on ageing.

Mitochondria aged only 1 h showed a P/O ratio of approximately 1.56 (average of 1.49, 1.64) in the absence of DPN and nicotinamide, while mitochondria aged for 4–5.5 h show a P/O ratio of approximately 1.63 in the same medium (Table I).

Externally added DPNH is readily oxidised by aged Ehrlich mitochondria but the oxidation does not appear to be coupled to phosphorylation. In fact a small output of inorganic phosphate was observed in experiments in which the DPNH was generated *in situ* by addition of DPN, ethanol and alcohol dehydrogenase to the medium (Table II)

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[1] S. WEINHOUSE, *Advances in Cancer Research*, 3 (1955) 269.
[2] A. O. HAWTREY AND M. H. SILK, *Biochem. J.*, 74 (1960) 21.
[3] E. KUN, P. TALALAY AND H. G. WILLIAMS-ASHMAN, *Cancer Research*, 11 (1951) 855.
[4] O. LARSBERG, M. LJUNGGREN, L. ERNSTER AND L. RÉVÉSZ, *Exptl. Cell Research*, 4 (1953) 243.
[5] R. K. KIELLEY, *Cancer Research*, 12 (1952) 124.
[6] K. W. CLELAND AND E. C. SLATER, *Biochem. J.*, 53 (1953) 547.
[7] C. H. FISKE AND Y. SUBBA ROW, *J. Biol. Chem.*, 66 (1925) 375.

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