On the mitochondrial oxidation of isocitrate

It has been shown previously[1,2] that addition of DPN restores the aerobic oxidation of isocitrate in preparations of rat-liver mitochondria that have been aged at 30° in a 0.25 *M* sucrose–0.05 *M* phosphate medium for a period of 15 to 30 min. Since this type of treatment is known to lead to a release and destruction of mitochondrial nucleotides in general[3,4] and of the mitochondrial DPN in particular[5], it was anticipated that the preparations so-treated were devoid of endogenous TPN as well. This assumption was also supported by the finding[2] that these mitochondria did not decolorize 2,6-dichlorophenolindophenol in the presence of isocitrate unless a catalytic amount of TPN was added. The restoring effect of added DPN on the oxidation of isocitrate has therefore been interpreted[1,2] to mean that rat-liver mitochondria possess a DPN-specific pathway of isocitrate oxidation which is potent enough to account for the rate at which the aerobic oxidation of isocitrate proceeds in the intact mitochondria. A similar conclusion has recently been reached by GRANT AND MONGKOLKUL[6], concerning mitochondria from ox adrenal cortex.

Meanwhile, however, KAPLAN *et al.*[7] pointed out that mitochondria treated in the way described above still may contain traces of endogenous TPN, and more recently PURVIS[8], on the basis of accurate enzymic and fluorometric measurements, concluded that 1-h aging under the above conditions is necessary for a complete destruction of the liver mitochondrial pyridine nucleotides. From measurements of the reaction rates obtained in aged mitochondria for the reduction of 2,6-dichlorophenolindophenol by isocitrate in the presence of DPN, TPN and DPN + TPN, and for the aerobic oxidation of DPNH and TPNH, the latter with and without DPN, PURVIS[8] also concluded that the reaction sequence, TPN-linked isocitric dehydrogenase → transhydrogenase → DPNH-oxidase, constitutes virtually the only pathway sufficiently active to account for the observed rate of isocitrate-oxidation in rat liver (and heart) mitochondria.

In view of these conclusions, it was of interest to test, in the first place, the effect of DPN on the aerobic oxidation of isocitrate in rat-liver mitochondria which have been aged for 1 h or longer. Table I summarizes some typical results. As shown in Expt. 1, added DPN was able to restore isocitrate oxidation equally well after 0.5, 1, 1.5, 3 and 8 h aging. Added TPN had, just as previously found[2] with mitochondria aged for 0.5 h, relatively little effect by itself, and no appreciable additional effect when combined with DPN. Beyond 8-h aging, the level of endogenous substrate, which usually appears upon prolonged aging (and part of which can apparently be oxidized without added pyridine nucleotides) became too high to allow any further evaluation of the DPN effect. Citrate (Expt. 2) which, for reasons now being investigated, was oxidized considerably faster than isocitrate under the present conditions, gave a similar picture to that obtained with isocitrate, in that its oxidation could be restored almost completely by the addition of DPN alone after 1-h aging. Upon prolonged aging, the citrate oxidation could no longer be restored to any larger extent by either DPN or TPN, alone or in combination, probably because of a loss of the aconitase activity. Expt. 3 shows that the DPN effect in restoring isocitrate oxidation in mitochondria aged for 1 h was present even when the mitochondria were recentri-

Abbreviations: DPN, diphosphopyridine nucleotide; TPN, triphosphopyridine nucleotide; ATP, adenosine triphosphate.

TABLE I

EFFECTS OF ADDED PYRIDINE NUCLEOTIDES ON THE AEROBIC OXIDATION OF ISOCITRATE AND CITRATE IN AGED PREPARATIONS OF RAT-LIVER MITOCHONDRIA

The mitochondria were prepared[11] and aged[2] as described previously. The test incubations contained per Warburg vessel: mitochondria from 0.5 g wet wt. liver (about 10 mg biuret protein), 20 μmoles Na citrate or Na DL-isocitrate, 50 μmoles K phosphate (pH 7.5), 3 μmoles Na ATP, 8 μmoles $MgCl_2$, 1 μmole $MnCl_2$, 50 μmoles glucose, 50 μl of a stock solution[10] of yeast hexokinase and 125 μmoles sucrose, in a final volume of 2 ml. When indicated DPN and TPN were added in amounts of 1.5 μmoles each. The vessels were incubated at 30° with air as the gas phase and with 0.2 ml 2 *M* KOH in the center wells. The values refer to the O_2 consumption recorded between 5 and 20 min of incubation.

Expt.	*Time of aging h*	*Substrate*	*Added pryidine nucleotide*				*Notes*
			None	*DPN*	*TPN*	*DPN+TPN*	
			O_2 consumption (μatoms)				
1	0	Isocitrate	5.2	—	—	—	
	0.5	Isocitrate	0.1	4.7	1.5	4.8	
	1	Isocitrate	0.0	4.9	1.5	5.4	
	1.5	Isocitrate	0.3	4.7	2.2	4.9	
	3	Isocitrate	0.1	4.7	0.6	5.2	
	8	Isocitrate	1.9	4.9	1.9	3.6	
2	0	Citrate	12.5	—	—	—	
	1	Citrate	0.8	13.8	2.5	—	
	2	Citrate	1.0	2.3	1.7	2.6	
3	1	Isocitrate	0.7	7.7	—	—	
	1	Isocitrate	0.0	6.0	—	—	Recentrifuged after aging
	1	Isocitrate	0.0	5.8	—	—	Aging with dialysis
4	1	Citrate	1.2	9.9	2.7	10.7	
	1	Citrate	—	9'8	—	—	No hexokinase-glucose in test
	1	Citrate	—	9.4	—	—	No hexokinase-glucose-ATP in test
	1	Citrate	—	8.2	—	—	As above, 10^{-4} *M* dinitrophenol added

fuged after aging and resuspended in a new incubation medium, or when the aging was performed with simultaneous dialysis against 20 vol. of sucrose–phosphate medium or 0.05 *M* phosphate. These treatments, which efficiently remove the endogenous substrates appearing during aging, conceivably also promote the removal of the endogenous pyridine nucleotides. Finally, as shown in Expt. 4, the DPN effect was present also when hexokinase, glucose and ATP were omitted from the incubation mixture (the aged mitochondria, though still capable of oxidative phosphorylation[2,5,7,9], apparently no longer possess the obligatory link between respiration and phosphorylation, characteristic of intact mitochondria[10]) or when a phosphorylation-uncoupling concentration of 2,4-dinitrophenol was added to the system. These findings seem to eliminate the possibility that part of the added DPN was converted to TPN by means of ATP, added or generated during respiration.

The data presented above seem to substantiate the validity of the previous postulate[1,2] that rat-liver mitochondria possess a DPN-catalyzed, TPN-independent pathway by which isocitrate (or citrate) can be oxidized aerobically at a rate approaching the observed rate of aerobic oxidation of isocitrate (or citrate) in the intact mitochondria.

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Transhydrogenase and TPNH oxidase activities of rat-liver mitochondria

Because of recent controversies[1–5] as to the transhydrogenase and TPNH oxidase activities of rat-liver mitochondria, and as to the role played by these pathways in the aerobic oxidation of isocitrate, it was of interest to re-examine previous data published from this laboratory[2–3] on this subject. As a test system, that employed by PURVIS[4] was chosen, consisting in a measurement of the rate of aerobic oxidation of TPNH and its increment ensuing the addition of DPN. Two types of assay were performed in parallel: (a) the decrease in the concentration of TPNH was followed at 340 mμ in a recording DK2 Beckman spectrophotometer, and (b) the oxygen consumption was recorded polarographically, be means of the rotating platinum electrode method[6*]. The mitochondria were depleted of endogenous pyridine nucleotides by aging for 1 h[4] in a sucrose–phosphate medium at 30°. The values in Table I show a good agreement between the two types of assay. The mean values, expressed in terms of μmoles TPNH/min/g liver, are 0.05 for the TPNH oxidase and 0.12 for the transhydrogenase. Previous values[2–3], obtained with a slightly different test system, were 0.05 and 0.08, respectively.

The mean values in Table I, converted to μmoles TPNH/min/mg mitochondrial protein (assuming that mitochondria from 1 g liver contain 20 mg protein[3,8]), are 0.0025 for the TPNH oxidase and 0.006 for the transhydrogenase, which markedly differ from those recently reported by PURVIS[4] 0.000 for TPNH oxidase, 0.026 for the transhydrogenase. Although the explanation of these discrepancies must await the publication of the experimental details underlying these values in PURVIS's paper[4], the following comments may be helpful in clarifying the situation.

The TPNH oxidase activity reported above was fairly consistent from one experiment to another. It could be increased considerably, however, by increasing the concentration of TPNH. Similar observations were made when cytochrome *c* rather than oxygen was used as a terminal oxidant. A 20-fold increase of the TPNH

Abbreviations: DPN, DPNH, oxidized and reduced diphosphopyridine nucleotide; TPN, TPNH, oxidized and reduced triphosphopyridine nucleotide; PNH, reduced pyridine nucleotide.

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