Role of the α-Glycerol Phosphate Cycle in the Transfer of Hydrogen from Extramitochondrial Reduced Nicotinamide Adenine Dinucleotide into Mitochondria

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Cytoplasmic nicotinamide adenine dinucleotide (NAD) penetrates the intact mitochondrial membrane with great difficulty.[6] However, enzyme systems in the cell produce cytoplasmic reducing equivalents that are eliminated in the respiratory chain of the mitochondria. Loading the cell with oxidizable substrates (*e.g.* ethanol), which are primarily dehydrogenated in the cytoplasm, shifts the redox equilibrium strongly towards the reduced side. The situation is compensated by transfer of hydrogen into the respiratory chain, a process generally considered to be mediated by some carrier systems composed of redox substrate pairs.

Several carrier systems have been proposed to function in the elimination of cytoplasmic hydrogen. Their existence presupposes that there are both mitochondrial and cytoplasmic enzymes affecting the redox substrate pair in question. Hydrogen transfer systems considered to function in intact cells include the α-glycerol phosphate cycle,[3] the β-hydroxybutyric acid cycle,[4] and the malate cycle proposed by Borst.[1] The α-glycerol phosphate cycle has been the subject of intense investigation and is known to function as the main source of energy in the flight muscle of insects, but its role in mammalian metabolism has remained obscure.

The present investigation was undertaken as an extension to work in the field of the metabolism of ethanol and is based on the observations that ethanol greatly increases the hepatic α-glycerol phosphate content. A new approach was made by using tritium as a hydrogen marker and the mitochondrial energy-linked dismutation between α-glycerol phosphate and acetoacetate as an indicator of the penetration of the hydrogen into the mitochondria.

Experimental. The experiments were carried on *in vitro*, using isolated rat liver mitochondria and purified enzyme preparations. The mitochondria were suspended in 0.25 M sucrose containing 1 mM EDTA and 5 mM tris chloride, pH 7.4. The incubations were performed in a conventional Warburg constant volume respirometer, using air as the gas phase, 0.1 ml of 20 % KOH in the center well and 0.2 ml of 0.5 M semicarbazide hydrochloride in 0.05 M potassium phosphate buffer pH 7.4 in the side arm. Samples drawn from the incubation mixture were deproteinized with perchloric acid and the organic acids extracted from the filtrate with ether. From the ether phase the organic acids were extracted with 0.25 M $KHCO_3$ and the ether evaporated under a stream of nitrogen. The CO_2 was eliminated by acidification, the pH adjusted to 7.0 and the organic acids separated on Dowex-1-formate columns.[2] Dowex-1-formate columns were also used in the separation of the organic phosphates in the aqueous phase. Radioactivity in the collected fractions was measured by liquid scintillation counting, using Bray's scintillator and a Packard Tri-Carb liquid scintillation spectrometer. For assessment of the radioactivity in the acetaldehyde formed from ethanol-1,2-^{3}H, the semicarbazide was rinsed from the side arm of the Warburg bottle into a scintillation vessel and the contaminating ethanol eliminated by evaporation to dryness, after which the scintillation solution was added and the radioactivity in the semicarbazone of acetaldehyde counted. Corrections for quenching were calculated from the observed pulse-height shift.

The enzymes were commercial preparations obtained from Boehringer & Soehne GmbH, Mannheim. The ethanol-1,2-^{3}H was prepared by Centre d'Etudes Nucleaires de Saelay, France, and purchased from the California Corporation for Biochemical Research. Dihydroxyacetone phosphate was also a commercial product available from the California Corporation for Biochemical Research. Acetoacetate was prepared from commercial ethyl acetoacetate (The British Drug Houses Ltd)., which was saponified and the acid crystallized as the lithium salt and converted to the sodium salt in an ion exchange column.

Results and discussion. When ethanol-1,2-^{3}H was incubated with α-glycerol phosphate dehydrogenase, alcohol dehydrogenase, NAD, ATP, and isolated mitochondria in the presence of acetoacetate, tritium was found in β-hydroxy-

Table 1. Reverse mitochondrial electron flow and the elimination of ethanol in rat liver mitochondria

Components: In the main vessel of a Warburg flask: μmoles of potassium phosphate, pH 7.4, 150 μmoles of KCl, 10 μmoles of $MgCl_2$, 2 μmoles of NAD, 2 μmoles of ADP, 20 μmoles of sodium acetoacetate, 11.7 μmoles of dihydroxyacetone phosphate, 0.3 mg of yeast alcohol dehydrogenase, 0.02 mg of α-glycerol phosphate dehydrogenase, 200 μmoles of sucrose, 0.8 μmoles of EDTA, 34.3 μmoles of ethanol-1,2-^{3}H, specific activity 2.2 × 10^6 dpm/μmole, mitochondria from 0.8 g of rat liver (20 mg of protein). 0.1 ml of 20 % KOH in the center well and 0.2 ml of 0.5 M semicarbazide in the side arm. Incubation time 60 min.

Vessel *	oxygen consumption μatoms	β-hydroxybutyr. 10^6 dpm	acetaldehyde 10^6 dpm	α-glycerol phosphate 10^6 dpm
1	12.2	7.15	1.26	0.98
2	10.8	4.52	0.20	—
3	9.2	2.74	0.20	—

* 1 = complete
2 = without dihydroxyacetone phosphate
3 = without acetoacetate and dihydroxyacetone phosphate

butyric acid. The reduction of acetoacetate with tritium was greatly enhanced by dihydroxyacetone phosphate. The oxygen consumption of the mitochondria and the elimination rate of ethanol, measured as the radioactivity of the acetaldehyde formed, were concomitantly increased (Table 1). The distribution of the tritium label in the ethanol used was not checked, and hence the stoichiometry of the reaction cannot be calculated.

The α-glycerol phosphate cycle has been considered to play an important role as a link between the compartments of anaerobic and aerobic energy metabolism, but direct evidence concerning the process has been slight. The only report of a functioning α-glycerol phosphate cycle has been made by Borst,[1] who worked with the mitochondria of Ehrlich ascites tumor cells. It is also noteworthy that the enzymes of the α-glycerol phosphate cycle are not uniformly distributed in different tissues in contradistinction to the enzymes of the tricarboxylic acid cycle, which are present in constant proportions.[8] Obviously, there must also be other hydrogen carrier systems. The foregoing results, although they argue in favour of the functioning of the α-glycerol phosphate cycle, do not rule out the possibility of a β-hydroxybutyric acid cycle. Different results with respect to $NADH_2$ oxidation have been obtained with large external $NADH_2$ concentrations and $NADH_2$ regenerated enzymically in the presence of catalytic amounts of the nucleotide.[1]

In recent years several authors have reported that ethanol increases the α-glycerol phosphate content of the liver, and a potentiating effect of fructose on the increase of glycerol phosphate has been observed.[7] The irregularly observed increase in the elimination rate of ethanol has been attributed to the activation of the α-glycerol phosphate cycle.

The foregoing results show an increase in the oxidation of ethanol by alcohol dehydrogenase and isolated mitochondria in the presence of a reconstructed α-glycerol phosphate cycle. The reversal of the oxidative phosphorylation and the trapping of hydrogen by acetoacetate made possible the demonstration of the penetration of hydrogen into the mitochondria. Studies designed to elucidate the significance of the observed mechanism *in vivo* and mapping of the pathways of cytoplasmic hydrogen are in progress.

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