PERMEABILITY FACTORS IN THE ASSAY OF MITOCHONDRIAL DEHYDROGENASES

Thomas P. Singer* and Carol J. Lusty

Edsel B. Ford Institute for Medical Research, Henry Ford Hospital, Detroit, Mich.

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While in mitochondrial fragments and other types of particulate preparations phenazine methosulfate is the only electron acceptor known to measure the full activity of succinic dehydrogenase (Giuditta and Singer, 1959), in intact mitochondria the phenazine assay registers a small and variable fraction of the succinic dehydrogenase activity. Thus we have noted that liver, heart, and brain mitochondria, isolated in 0.25 M sucrose, show a considerably lower Q_{O_2} for succinic dehydrogenase than mechanically fragmented, bile salt-treated, or acetone-dried preparations derived therefrom, when phenazine methosulfate serves as the electron acceptor (Table I). Among the various methods tested, the addition of low (0.75 mM) concentrations of Ca^{++} proved to be the most convenient one for increasing the rate of the succinate—phenazine methosulfate reaction to a level which equals or approximates that found in damaged mitochondria. It is the purpose of this note to show that a major cause of the depressed rate of the succinate—phenazine methosulfate reaction in undamaged mitochondria is a hitherto unrecognized permeability barrier toward this dye.

Before arriving at this interpretation the possibility was explored that the apparently low succinic dehydrogenase activity in mitochondria was the result of oxaloacetate (OAA) inhibition, since despite the fact that the brief

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TABLE I

SUCCINIC DEHYDROGENASE ACTIVITY IN INTACT AND DAMAGED MAMMALIAN MITOCHONDRIA

Components	Succinic dehydrogenase activity (Q_{O_2} or Q_{H_2})	
	Phenazine methosulfate assay	Fumarate - $FMNH_2$ assay
Rat liver mitochondria	62.4	22.0
Same + 0.75 m$\underline{M}$ Ca^{++}	512	30.0
Acetone powder of rat liver mitochondria	395	38.1
Beef heart mitochondria	856	62.4
Same + 0.75 m$\underline{M}$ Ca^{++}	1938	55.4
Acetone powder of beef heart mitochondria	1540	
Same + 0.75 m$\underline{M}$ Ca^{++}	1540	
Beef liver mitochondria	205	20.4
Same + 0.75 m$\underline{M}$ Ca^{++}	713	28.8
Acetone powder of beef liver mitochondria	536	
Same + 0.75 m$\underline{M}$ Ca^{++}	454	
Pig brain mitochondria	173	9.0
Same + 0.75 m$\underline{M}$ Ca^{++}	196	11.8
Acetone powder of pig brain mitochondria	402	35.8

Mitochondria isolated in 0.25 $\underline{M}$ sucrose were used immediately after preparation. All assays were performed at 38° in 0.05 $\underline{M}$ phosphate, pH 7.6. The conditions of the phenazine assay were as described by Singer and Kearney (1957); the fumarate—$FMNH_2$ reaction was assayed by the method of Warringa $\underline{et\ al.}$ (1958). All values in the table are based on V_{max} data (infinite dye concentration). Q_{O_2} and Q_{H_2} values refer to protein measured by the biuret reaction. Moles of H_2 uptake = moles of fumarate reduced.

(5 minute) phenazine assay measures essentially initial rates and is performed in the presence of 1 m$\underline{M}$ cyanide, the possibility existed that a sufficient concentration of OAA was rapidly established to cause extensive inhibition of the dehydrogenase. Further, chemical damage of the mitochondria, such as treatment with Ca^{++} or deoxycholate, would lead to the loss of intramitochondrial DPN,

with consequent arrest of malic dehydrogenase action, as would desiccation by acetone. The apparent activation of succinic dehydrogenase by Ca^{++} was, in fact, many years ago suggested to be the indirect result of its inhibitory effect on the formation of OAA (Swingle et al., 1942).

This point was tested by adding cysteinesulfinic acid (CSA) to the usual assay medium (Singer and Kearney, 1957), since this amino acid is considered to be the most effective agent known for the rapid and stoichiometric removal of OAA from mitochondrial systems (Singer and Kearney, 1955). (CSA undergoes a very rapid transamination with OAA, yielding β-sulfinylpyruvate, which spontaneously decomposes to pyruvate and sulfite.)

As shown in Table II, the succinoxidase activity of rat liver mitochondria

TABLE II

EFFECT OF Ca^{++} ON PERMEABILITY OF MITOCHONDRIA TO PHENAZINE METHOSULFATE

Conditions	μMoles succinate oxidized /min./mg. protein		
	Succinoxidase assay	Phenazine assay	Ferricyanide assay
Rat liver mitochondria	0.242	0.040	0.118
Same + CSA	0.424	0.159	0.206
Same + CSA + Ca^{++}	0.414	0.380	0.195

The phenazine assay was run at fixed dye concentration (0.67 mg./ml.). Succinoxidase was measured in the presence of 0.05 M phosphate, pH 7.6, 0.02 M succinate and 0.17 mg. added cytochrome c per ml. Succinoxidase activity was calculated from the linear rate obtained between 0 and 20 minutes. Ferricyanide reduction was measured manometrically (Singer and Kearney, 1957). Ca^{++}, where present, was 0.75 mM and CSA 9 mM. The rates were calculated by taking into consideration that in the succinoxidase assay the removal of 1 pair of electrons yields 1 atom O_2 uptake but in the phenazine assay 1 mole O_2 uptake. It was assumed that the O_2 uptake due to malate oxidation is negligible.

is almost doubled by the addition of 9 mM CSA. This increase is due to the removal of OAA by transamination. In the presence of CSA, the addition of Ca^{++} does not increase the activity further. In contrast, when succinic dehydrogenase activity is measured with phenazine methosulfate as acceptor, a very different picture emerges which suggests that the action of Ca^{++} in this instance cannot be fully

ascribed to the removal of OAA. First, it may be noted (Table II) that without added Ca^{++} or CSA the dehydrogenase activity measured is but a small fraction of the succinoxidase activity. The reason for this is that phenazine methosulfate catalyzes a more rapid cycling of the intramitochondrial DPN than does the DPNH oxidase operative under the conditions of the succinoxidase assay, and hence relatively more OAA accumulates in the dehydrogenase assay. As a result, the inclusion of CSA causes a more pronounced stimulation in the succinic dehydrogenase than in the succinoxidase assay. Nevertheless, when Ca^{++} is added along with CSA, the rate is more than doubled. Since it may be assumed that in the presence of CSA no OAA can accumulate, the stimulatory effect of Ca^{++} points to a different action of the metal.

The simplest interpretation of the effect of Ca^{++} is in terms of its known ability to destroy the permeability barrier of mitochondria (Cleland and Slater, 1953) and thereby permit free penetration of the reactants. The reactant with the limiting rate of penetration is phenazine methosulfate, rather than succinate, since the oxidation of succinate via the respiratory chain or with ferricyanide as acceptor (Table II) is not stimulated by Ca^{++} when provisions are made to prevent the accumulation of OAA. A similar permeability barrier apparently also exists toward $FMNH_2$ (Table I). The considerably lesser degree of stimulation of the fumarate--$FMNH_2$ reaction than of the succinate--phenazine methosulfate interaction by Ca^{++} or by desiccation with acetone may be explained by the slower rate of the former reaction, which would be less markedly influenced by the rate of penetration of the dye. The enhancement of the fumaric reductase activity by Ca^{++} or by acetone drying further shows that the stimulation afforded by these treatments cannot be entirely ascribed to the removal of OAA inhibition, since under the condition of the fumarate--$FMNH_2$ assay the formation of OAA is clearly precluded.

The proposed interpretation that the rate of penetration of phenazine methosulfate is the limiting factor in the assay of succinic dehydrogenase in mitochondria is also supported by experiments involving the use of low concentrations of deoxycholate. Like Ca^{++}, deoxycholate enhances the rate of the succinate--phena-

zine methosulfate reaction even when no OAA can accumulate.

The stimulating effect of Ca^{++} is observed at relatively low concentrations: 0.25 mM Ca^{++} enhances the succinic dehydrogenase activity of rat liver mitochondria over 4-fold and maximum stimulation is reached with 0.8 to 1 mM Ca^{++}, which other studies in this laboratory have shown to be also optimal for facilitating the entry of choline into rat liver mitochondria. In damaged mitochondria and in acetone powders Ca^{++} no longer stimulates and may be even slightly inhibitory (Table I).

The extent to which Ca^{++}, under the conditions tested, overcomes the permeability barrier to phenazine methosulfate seems to vary with the source of the mitochondria. In beef heart mitochondria the rates in the presence of 0.75 mM Ca^{++} are consistently high enough to suggest that the full activity of the dehydrogenase is being measured. In rat liver the rates with Ca^{++} (and CSA) present sometimes equal succinoxidase activity, sometimes only approximate it (Table II). In pig brain mitochondria Ca^{++} causes relatively little stimulation but desiccation with acetone doubles the activity of the dehydrogenase and quadruples that of the fumarate—$FMNH_2$ reaction (Table I). This may suggest that in brain mitochondria a strong permeability barrier exists toward $FMNH_2$ and phenazine methosulfate which is not completely abolished by Ca^{++} under the experimental conditions.

Since phenazine dyes do not appear to penetrate freely into mitochondria, whil other electron acceptors have indeterminate reaction sites in the respiratory chain and do not measure the full activity of the dehydrogenase, there would seem to be at present no reliable method for the assay of the full succinic dehydrogenase activity in mammalian mitochondria with an intact permeability barrier. For the same reason caution is indicated in employing phenazine methosulfate either as a termina electron acceptor or as a carrier to other dyes or O_2 also in the assay of other dehydrogenases in mitochondria. It is desirable to ascertain for each enzyme in each tissue that the rate of penetration of the dye is sufficient to keep up with the rate of the dehydrogenation of the substrate.

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