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Demonstration *in vitro* of competition between added NADH and glutamate for oxidation by liver mitochondria

Since the observation of LEHNINGER[1] in 1951 that intact liver mitochondria do not oxidise added NADH, it has become generally recognised that hydrogen equivalents in the form of NADH do not directly traverse the mitochondrial inner membrane. However, the cell possesses efficient systems for the transfer of hydrogen equivalents from one intracellular compartment to another. Some striking examples of this are (a) heart muscle oxidising glucose under conditions in which there is little or no net lactate formation, or when lactate itself is the major substrate being oxidised (see *e.g.* ref. 2); (b) the "glucogenic" liver which requires the rapid efflux of hydrogen equivalents from the mitochondria into the cytosol for the reductive synthesis of glucose; and (c) mitochondrial oxidation in the liver of NADH generated in the cytosol resulting from the oxidation of ethanol (*cf.* ref. 3).

In order to understand the integration of glycolysis and mitochondrial respiration (the Pasteur effect), it is important to evaluate the mechanism of transfer and compartmentalization of hydrogen equivalents, and to understand how these parameters are controlled by the cell. A number of substrate "shuttle" systems have been proposed to explain the known physiological transfer of hydrogen equivalents to and from the intramitochondrial space. Each of these proposals incorporates an NAD-linked redox couple. The appropriate dehydrogenase is of necessity available on both sides of the restricting membrane, and the redox couple of substrates must be permeable to it. L-Malate has often been considered as a carrier for hydrogen equivalents (*cf.* refs. 4–6). The reversible flow of hydrogen equivalents by the oxaloacetate–malate system requires that the restricting membrane be permeable to both oxaloacetate and malate. LARDY *et al.*[6] and more recently, HASLAM AND KREBS[7] have presented evidence that the translocation of oxaloacetate is too restrictive to support a rapid, reversible shuttle. However, SHRAGO AND LARDY[8] have pointed out that, under certain conditions in liver, transfer of hydrogen equivalents from the mitochondria into the cytosol would be quantitatively of greater importance than transfer in the reverse direction. Under these conditions, there would be no specific requirement for rapid entry of oxaloacetate into the mitochondria.

BORST[9] suggested a shuttle which included the malate–oxaloacetate system, coupled with transamination catalysed by aspartate aminotransferase on both sides of the mitochondrial membranes SHRAGO AND LARDY[8] obtained data supporting the physiological function of such a cycle.

We have recently obtained data[10,11] which strongly support the suggestion[3] that the antiglucogenic effect of ethanol in fasted animals is due to a competition between cytoplasmically generated NADH, and NADH produced during oxidation of glucogenic amino acids in the citric acid cycle, with the result that the net flow of precursors to oxaloacetate is diminished. It was therefore of considerable importance directly to demonstrate competition between added NADH and a glucogenic substrate for oxidations mediated by the respiratory chain. The demonstration of this competition is reported in the present paper

Rabbit liver mitochondria were prepared as described by JOHNSON AND LARDY[12].

Incubation media containing 50 mM KCl, 25 mM Tris–HCl (pH 7.4), 5 mM $MgCl_2$, 5 mM potassium phosphate (pH 7.4), 20 μM 2,4-dinitrophenol and other additions as listed in Tables I and II, in a volume of 1.0 ml. Reactions were begun by addition of mitochondria, and stopped with $HClO_4$, or when NADH was measured, with KOH. Respiration was followed at 30° in a Gilson differential respirometer. All substrate assays were enzymatic[13]. Added NADH was determined as described by KLINGENBERG[14]. When purified aspartate aminotransferase or malate dehydrogenase was included, 4.0 international units of dialyzed enzyme were added Enzymes and coenzymes were obtained from Sigma Chemical Co.

Hepatic glucose synthesis is largely derived from the breakdown of amino acids which funnel into the citric acid cycle and, hence, undergo oxidations between α-oxoglutarate and oxaloacetate. It appears likely that the rate of hepatic respiration is limited by the energy demand, and not by the capacity of primary dehydrogenases or by the respiratory-chain enzymes. We have therefore studied the metabolism of glutamate, with 20 μM dinitrophenol present to maintain a constant, but less than maximal rate of respiration. If hydrogen equivalents from extramitochondrial NADH were being transported to the respiratory chain under these conditions, competition for oxidation between the intra- and extramitochondrially derived NADH would be expected to result, with a decrease in the amount of glucogenic substrate oxidised. Little or no change in the rate of respiration due to NADH oxidation would be expected, since its addition would have no obvious effect on work requirements.

Added NADH was not oxidised by liver mitochondria when added alone or when glutamate and aspartate were also present (Table I). However, when aspartate aminotransferase and malate dehydrogenase were added, NADH was rapidly oxidised, and glutamate metabolism was markedly reduced. NADH was not oxidised in the

TABLE I

CONDITIONS FOR OXIDATION OF ADDED NADH AND INHIBITION OF GLUTAMATE METABOLISM BY RABBIT LIVER MITOCHONDRIA

Reaction vessels contained the basic medium, 5 6 mg mitochondrial protein and, where indicated, 0 93 μmole aspartate, 4.0 international enzyme units of aspartate aminotransferase and malate dehydrogenase, 10 μmoles potassium L-glutamate, and 7.3 μmoles NADH, in a total volume of 1 0 ml Incubation time, 40 min Respiration values are the extra oxygen consumed due to added substrates

Additions					$-\Delta NADH$ (μmoles)	$+\Delta Aspartate$ (μmoles)	$-\Delta Oxygen$ (μatoms)
Glutamate	*Aspartate*	*NADH*	*Aspartate aminotransferase*	*Malate dehydrogenase*			
−	−	+	−	−	0	0	0
+	−	−	−	−		2 07	8 8
+	+	−	−	−		1 96	8 7
+	+	+	−	−	0	1 63	8 2
+	+	−	+	+		2 15	9 8
+	+	+	+	+	3 22	0 16	8 4
+	+	−	+	−		2 05	8 5
+	+	+	+	−	2 00	0 84	8 3
+	+	−	−	+		2 05	8 7
+	+	+	−	+	0	1 75	8 7

TABLE II

THE EFFECT OF ASPARTATE CONCENTRATION ON OXIDATION OF ADDED NADH AND INHIBITION OF GLUTAMATE METABOLISM BY RABBIT LIVER MITOCHONDRIA

Incubations were for 45 min in the basic medium, 10 μmoles of potassium L-glutamate, 3 7 mg mitochondrial protein and other additions as listed Where indicated, 6 7 μmoles of NADH were present

Additions				$-\Delta NADH$	*Phospho-enol-pyruvate*	Δ*Aspartate*	$-\Delta$*Oxygen*
Aspartate	*Aspartate amino-transferase*	*Malate dehydrogenase*	*NADH*	(*μmoles*)	(*μmole*)	(*μmole*)	(*μatoms*)
0	—	—	—		0 17	0 80	3 2
0 86	—	—	—		0 24	0 98	4 0
0	+	+	—		0 14	0 77	3 7
0	+	+	+	0 71	0 07	0 01	4 2
0 07	+	+	—		0 12	0 85	3 4
0 07	+	+	+	1 30	0 06	0 05	3 4
0 19	+	+	—		0 12	0 88	3 6
0 19	+	+	+	1 82	0 03	−0 05	3 4
0 86	+	+	—		0 15	0 86	4 5
0 86	+	+	+	1 97	0 03	−0 16	3 9

absence of added aspartate aminotransferase. However, the requirement for malate dehydrogenase was only partial since there was appreciable (but not optimal) oxidation of NADH and inhibition of aspartate production when malate dehydrogenase was deleted. The minimum requirements for NADH oxidation by mitochondria oxidising glutamate were added aspartate aminotransferase *plus* malate dehydrogenase (Table II). However, increasing amounts of aspartate stimulated oxidation of NADH, and increased the inhibition of glutamate metabolism. In no case was there appreciable effect by NADH on the rate of respiration

These data are interpreted as showing the function of a substrate shuttle system for transfer of hydrogen equivalents involving intra- and extramitochondrial aspartate aminotransferase and malate dehydrogenase; *i.e* essentially the system suggested earlier by BORST[9] This scheme (Eqns. 1–10, where $_o$ and $_i$ denote outside and inside the mitochondrial membrane, respectively) requires that aspartate aminotransferase and malate dehydrogenase be present on both sides of the restricting membrane, and that the latter can be traversed by malate, α-oxoglutarate, glutamate and aspartate.

$$\text{Oxaloacetate}_o + \text{NADH}_o + \text{H}_o^+ \rightleftharpoons \text{malate}_o + \text{NAD}_o^+ \quad (1)$$
$$\text{Malate}_o \rightleftharpoons \text{malate}_i \quad (2)$$
$$\text{Malate}_i + \text{NAD}_i^+ \leftrightharpoons \text{oxaloacetate}_i + \text{NADH}_i + \text{H}_i^+ \quad (3)$$
$$\text{Oxaloacetate}_i + \text{glutamate}_i \leftrightharpoons \alpha\text{-oxoglutarate}_i + \text{aspartate}_i \quad (4)$$
$$\text{Aspartate}_i \leftrightharpoons \text{aspartate}_o \quad (5)$$
$$\text{Aspartate}_o + \alpha\text{-oxoglutarate}_o \leftrightharpoons \text{oxaloacetate}_o + \text{glutamate}_o \quad (6)$$
$$\text{Glutamate}_o \leftrightharpoons \text{glutamate}_i \quad (7)$$
$$\alpha\text{-Oxoglutarate}_i \leftrightharpoons \alpha\text{-oxoglutarate}_o \quad (8)$$
$$\text{NADH}_i + \text{H}_i^+ + \tfrac{1}{2}\,\text{O}_2 \longrightarrow \text{NAD}_i^+ + \text{H}_2\text{O} \quad (9)$$

Sum: $$\text{NADH}_o + \text{H}_o^+ + \tfrac{1}{2}\,\text{O}_2 \longrightarrow \text{NAD}_o^+ + \text{H}_2\text{O} \quad (10)$$

There is no requirement for penetration by oxaloacetate, once a catalytic amount is present on both sides of the permeability barrier. The malate shuttle was not func-

tional under the present conditions, since added aspartate aminotransferase was absolutely required for oxidation of external NADH. Glutamate presumably furnished the dehydrogenation and transamination pairs through its own metabolism.

According to BORST's[9] calculations the malate–aspartate shuttle could not be operative in the transfer of reducing equivalents from a relatively oxidised to a relatively reduced environment without the input of energy. However, an asymmetric distribution of the substrate pairs (*e.g.* glutamate and aspartate) would be expected to have a marked influence on the equilibrium position of the sum reaction (Reaction 10). It is well known that permeability barriers in mitochondria exist for the entry of most, if not all, citric acid cycle intermediates, and that the degree of intramitochondrial accumulation of a given substrate is influenced by the other ionic species which may be present. There appear to be a number of relatively specific anion-exchange reactions which are facilitated by mitochondrial carrier systems (see *e.g.* refs. 15–18). Both the rate and direction of flow of reducing equivalents (Reaction 10) would be influenced by the relative and absolute concentrations of substrate pairs on opposing sides of the restricting membrane The nature of the transporters would, in turn, determine the degree of asymmetric distribution of substrate anions.

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Department of Biochemistry,
Indiana University, School of Medicine,
*Indianapolis, Ind 46202 (U.S.A.)***

LAWRENCE LUMENG*
E. JACK DAVIS**

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* Present address Walter Reed Institute of Research, Department of Nuclear Medicine, Washington, D C, U S A
** Inquiries should be sent to this address