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Interaction between NADH and succinate during simultaneous oxidation by non-phosphorylating submitochondrial particles from bovine heart

KREBS *et al.*[1] and KULKA *et al.*[2] reported a marked stimulation by succinate of the aerobic reduction of acetoacetate to β-hydroxybutyrate catalysed by homogenates prepared from several types of tissues. These workers suggested that succinate can "dominate" the respiratory chain, and that it is oxidised preferentially over NAD-linked substrates. KREBS AND EGGLESTON[3] have interpreted their results as showing that this effect of succinate is not due to energy-dependent reduction of NAD^+ (ref. 4). On the other hand, ERNSTER and co-workers (see *e.g.*, refs. 5 and 6) in studies of the succinate-linked reduction of acetoacetate by liver mitochondria, concluded that the major part of this reaction proceeds by an endergonic mechanism which proceeds by way of succinate dehydrogenase, the NADH dehydrogenase flavoprotein, NAD^+, and β-hydroxybutyrate dehydrogenase.

Our studies were initiated to gain additional information on the relative involvement of energy coupling and of substrate concentration on the interactions between the succinate- and NADH-oxidase systems. This report describes the results of experiments designed to show interaction between NADH and succinate in submitochondrial particles which carry out electron transport without the involvement of energy-linked functions. An NADH-generating system was used to maintain a relatively constant concentration of NADH throughout the reactions. This system was particularly useful in obtaining data on interactions between succinate and NADH at low concentrations of NADH (*i.e.*, at concentrations which are rate limiting for oxidation by the oxidase system), since these conditions may more closely mimic those 'seen' by the respiratory chain enzymes *in situ*.

Submitochondrial particles were isolated from heavy beef heart mitochondria[7] after sonic irradiation in 0.15 M KCl, washed once in 1 M KCl and twice in 0.5 M potassium phosphate buffer (pH 7.4), and finally suspended in the latter to a final

concentration of approx. 50 mg protein per ml. These particles did not carry out oxidative phosphorylation, the ATP-dependent reduction of NAD^+ by succinate[4], or the energy-linked transhydrogenase reaction supported by ATP or by ATP *plus* succinate[8]. There was no detectible lag-time for oxidation of succinate, apparently as a result of suspension in high phosphate. Added cytochrome *c* stimulated the rate of oxidation of NADH 30–60%.

NADH was generated using glutamate, crystalline glutamate dehydrogenase (EC 1.4.1.2) and NAD^+. The steady-state concentration of NADH was manipulated by varying the amount of added NAD^+, while all other additions were held constant. The rate of NADH oxidation, employing the NADH-generating system, never reached that attained when NADH was added. This was shown to be due to inhibition of NADH oxidase by NAD^+ (Table I). NADH oxidation was inhibited 50% when the ratio NAD^+/NADH was approx. 30. NAD^+ had no effect on the oxidation of succinate. MINAKAMI *et al.*[9] demonstrated earlier that NAD^+ inhibited the reduction of ferricyanide by NADH competitively with respect to ferricyanide.

TABLE I

THE EFFECT OF NAD^+ ON NADH-OXIDASE AND SUCCINATE-OXIDASE ACTIVITIES

Oxidation rates were estimated polarographically in a Gilson Medical Electronics "Oxygraph", in a medium containing 25 mM potassium phosphate, 0.1 mM EDTA, 50 mM potassium L-glutamate, 4.5 μM cytochrome *c*, the concentration of added NAD^+ indicated, and 0.25 mg particle protein at a final pH of 7.4 and temperature of 25°. Glutamate dehydrogenase was not present in this experiment. Reactions were initiated by addition of substrate. Rates were obtained from initial slopes.

[NADH] (mM)	*[NAD^+] (mM)*	*[Succinate] (mM)*	*Respiratory rate (μatoms oxygen consumed per mg protein per min)*
1.0	—	—	1.55
—	—	10	0.75
1.0	—	10	1.57
1.0	10	—	1.28
1.0	25	—	0.95
1.0	50	—	0.44
5.0	50	—	1.25
—	50	10	0.73

The concentration of NADH was followed continuously during the reactions either spectrophotometrically, by observing the increase in absorbance at 340 nm on addition of particles *plus* glutamate dehydrogenase, or fluorimetrically, using a Turner model 111 fluorimeter. In each case, a cuvette containing all additions except NAD^+ and succinate served as a blank. α-Oxoglutarate production was taken as a measure of NADH oxidase, and fumarate production as a measure of succinate oxidase. Both products were estimated spectrophotometrically: α-oxoglutarate was determined in the presence of NADH, a large excess of NH_4Cl and glutamate dehydrogenase, and fumarate by the appearance of NADH in the presence of fumarase (EC 4.2.1.2), malate dehydrogenase (EC 1.1.1.37), citrate synthase (EC 4.1.3.7) and excess amounts of NAD^+ and acetyl-CoA. Parallel incubations in which polarographic determinations of oxygen consumption were carried out confirmed that the amounts of α-oxoglutarate

and fumarate produced were a valid measure of respiration. All purified enzymes and coenzymes were obtained from Sigma Chemical Co.

Added NADH was oxidised at 25° by various preparations (in presence of added cytochrome *c*) at rates of 1.3–1.7 μmoles/mg protein per min, and succinate at rates of 0.5–0.8 μmole/mg per protein min (Table I). In agreement with the results of WU AND TSOU[10], when both substrates were present, the rate of respiration was always that of NADH alone. Fig. 1 shows the effect of varying the concentration of NADH on this interaction. The steady-state concentration of NADH, at the highest concentration of NAD^+, is increased by succinate due to competitive inhibition of NADH oxidation (Fig. 1A).

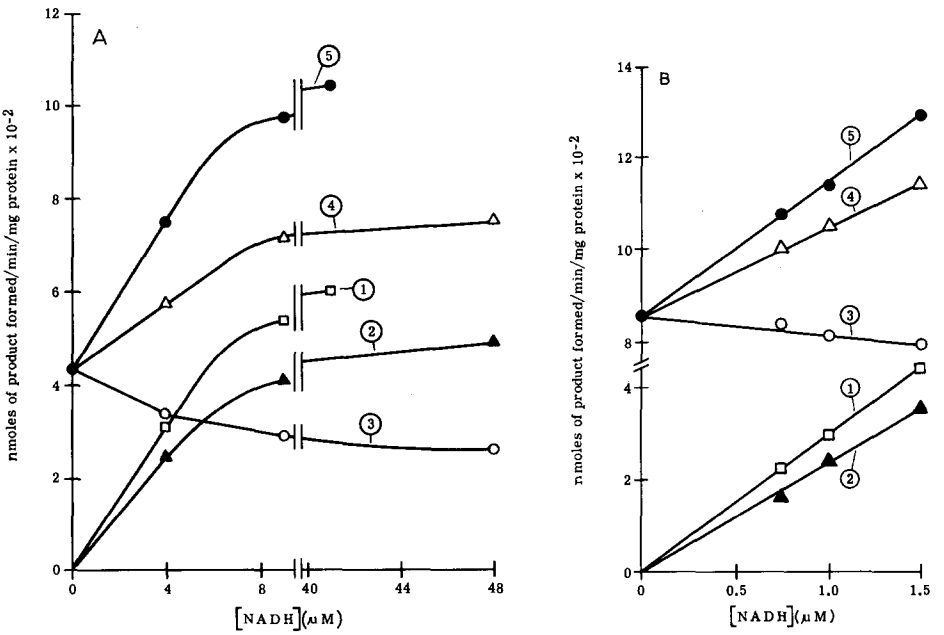

Fig. 1. Interaction between NADH and succinate oxidases measured as a function of [NADH]. The standard incubation mixture for simultaneous oxidation of NADH and succinate contained 25 mM potassium phosphate, 0.1 mM EDTA, 50 mM potassium L-glutamate, 0.02–2.0 mM NAD^+ and 10 mM succinate, to a final pH of 7.4. When succinate alone was being oxidised, NAD^+ was deleted from the mixture. Reactions were initiated by the simultaneous addition of submitochondrial particles and glutamate dehydrogenase (0.55 enzyme unit) and terminated with $HClO_4$. Incubations contained the standard medium *plus* 0.35 mg (A) or 0.25 mg (B) of particle protein in a volume of 3.0 ml (A) or 4.0 ml (B). [NADH] was followed spectrophotometrically (A) or fluorimetrically (B). Reactions were carried out at room temperature (A), or 35° (B) for 5 min. Curve 1, NADH oxidase; 2, NADH oxidase in presence of succinate; 3, succinate oxidase in presence of varying concentrations of NADH; 4, sum of Curves 2 and 3; 5, sum of NADH and succinate oxidases when incubated separately. Each point is taken from duplicate incubations.

It was found that both NADH and succinate inhibit the rate of oxidation of the other, even when the sum of their rates of oxidation is much less than the maximum capacity of the respiratory chain to reduce oxygen (Fig. 1). This result was obtained both with cytochrome *c*-depleted particles, and with particles to which excess cytochrome *c* was added. These data confirm the results of WU AND TSOU[10] that succinate and NAD-linked substrates compete for a respiratory assembly which is

common to both systems. These results further demonstrate that NADH and succinate compete for oxidation in particles which have no apparent energy-linked function, even at concentrations less than 1 μM of the former. Under no conditions was there a clear 'preference' for succinate. Subsequent studies of simultaneous oxidation of NADH and succinate in particles capable of oxidative phosphorylation should yield information concerning the role of energy-linked functions on respiratory-chain interactions.

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