THE ROLE OF THE MITOCHONDRIA IN RAT LIVER MIXED FUNCTION OXIDATION REACTIONS

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SUMMARY. Recombination experiments indicate a complex role by the mitochondria in controlling drug biotransformation reactions. The use of liver slices and homogenates demonstrates that the effect of Krebs cycle intermediates on aminopyrine N-demethylation is not via an increase in NADH, but probably by a well-defined route from the mitochondria to the endoplasmic reticulum.

During N-demethylation of aminopyrine, Orrenius (1) observed a 1:1:1 stoichiometric relationship between formaldehyde production, oxygen consumption and NADPH oxidation. It was later reported (2) that mixed function oxidation reactions catalyzed by cytochrome P-450 require the transfer of two reducing equivalents, although cytochrome P-450, the terminal oxidase, is an acceptor of only one electron (3). Since two electron equivalents are necessary for the overall reaction, several hypotheses have been postulated to explain the origin of the second electron and the ability of P-450 to accept it. One hypothesis (2) suggests a group, such as, sulfhydryl, as a component of the hemoprotein and capable of accepting electrons. Furthermore, Hildebrandt and Estabrook (4) postulated that NADH supports drug biotransformation reactions by transferring its electron equivalents to an "oxygenated intermediate" of P-450 via cytochrome $\underline{b}_5$. Evidence for this hypothesis was based upon the redox changes of cytochrome $\underline{b}_5$ in microsomes during drug hydroxylation. Recently, we (5) reported succinate to cause a 2 to 4-fold increase in the rate of reduction of cytochrome P-450 in rat liver slices when succinate was added to a superfusion medium containing aminopyrine or ethylmorphine. Certain Krebs cycle substrates, like succinate and isocitrate, were also found to stimulate N-demethylation reactions in liver

slices and homogenate (6). From these studies, it was postulated (6) that in the liver cell a route exists between the mitochondria and the endoplasmic reticulum for the transfer of the second electron to the mixed function oxidase.

The purpose of this investigation was to provide further evidence for a mitochondrial role in controlling drug biotransformation reactions and to demonstrate that in rat liver slices and homogenate the effect of the Krebs cycle intermediates, such as, succinate, on aminopyrine N-demethylation is not via an increase in the NADH concentration, as was suggested (7) by support of mixed function oxidation reactions in the presence of a fixed amount of NADPH.

METHODS

Tissue slices were prepared from livers of male, Sprague-Dawley rats (250-300g) as described previously (6). Livers were homogenized 1:1 (w/v) in 0.25 M sucrose, and an aliquot equivalent to 250 mg liver was used in the enzyme assays. Liver mitochondria were generally prepared in 0.25 M sucrose as described by Simpson et al. (8). Microsomal fractions were prepared at 0^{o} from the 0.25 M sucrose homogenates as described elsewhere (9). Rat liver nuclei were prepared (10) from 25% homogenates using 0.32 M sucrose containing 3.0 mM $MgCl_2$, and suspended in 0.25 M sucrose containing 1.0 mM $MgCl_2$.

The enzyme activity was determined at 37^{o} C in an iso-osmotic medium, pH 7.4, described previously (5); various concentrations of NADPH and/or NADH were used as indicated in the figures below. Substrates of the Krebs cycle and aminopyrine were added to the reaction flask in final concentrations of 10 mM and 8 mM, respectively. Incubation time in the Dubnoff shaker was for 5 minutes. Demethylase activity was determined from formaldehyde generation using the Nash reaction(11).

RESULTS

An earlier report (6) showed that liver slice drug oxidation was stimulated by Krebs cycle intermediates like succinate and isocitrate, and that the succinate stimulation was blocked by malonate. In order to demonstrate further the

TABLE I

Aminopyrine Demethylase Activity in Cellular Fractions from Rat Liver

	nmoles HCHO formed/mg protein/minute			nmoles HCHO formed/mg microsomal protein/minute				
Treatment	nuclei	mitochondria	soluble supernate	microsomes	mitochondria + microsomes	nuclei + microsomes	soluble supernate + microsomes	mitochondria + soluble supernate + microsomes
Control	0.6 ± .02	1.1 ± .06	0	9.3 ± 0.2	8.7 ± 0.3	9.0 ± 0.2	8.6 ± 0.4	9.0 ± 0.2
+ Succinate	0.8 ± .04	0.9 ± .06	0	9.1 ± 0.3	12.2 ± 0.3	8.9 ± 0.1	8.9 ± 0.5	12.7 ± 0.5

The cellular fractions were prepared as described in the Methods. The assay mixture consisted of a buffer medium, pH 7.4 (5), 1.0 mM NADPH, 2.0 mM ADP, 8.0 mM aminopyrine and 10 mM succinate when present; total volume was 3.0 ml; incubations were at 37° C for 5 minutes. When fractions were combined, equal amounts of protein from each fraction were used (except the soluble supernate); soluble supernatant fraction was added in protein concentrations of 1, 5 and 10 times greater than the other fractions, without any effect. Values are means ± S.E. from three separate experiments.

role of the mitochondria in drug oxidation reactions, recombination experiments were performed. Four cellular fractions were obtained from liver homogenates: 1) nuclei, 2) mitochondria, 3) microsomes and 4) soluble supernate. As seen in Table I, aminopyrine N-demethylation occurred only when microsomes were added to the incubation mixture; the slight activity observed in the mitochondrial fraction was attributed to contamination by microsomes, as evidenced by electron microscopy and glucose-6-phosphatase activity. Combination of the various cell fractions with the microsomes did not affect the aminopyrine N-demethylase activity, when the incubation mixture contained only the mixed function oxidase substrate, aminopyrine. However, when microsomes plus mitochondria were added to the incubation medium containing the Krebs cycle intermediate, succinate, a 25-30% increase in aminopyrine demethylation was noted above that observed in the absence of succinate. When only microsomes were used in the assay mixture, succinate and other Krebs cycle intermediates had no stimulatory effect on the biotransformation of aminopyrine. The addition of the supernatant fraction to the microsomal plus mitochondrial mixture produced no further increase in demethylase activity (Table I). As seen in Table II, three other Krebs cycle intermediates, citrate, isocitrate, and α-ketoglutarate also increased microsomal demethylase activity about 20-25% in the presence of mito-

TABLE II

Effect of Krebs Cycle Intermediates on Aminopyrine N-Demethylation In Reconstituted Cellular Fractions From Rat Liver

Treatment	% of Control*
Citrate	122%
Isocitrate	120%
α-Ketoglutarate	125%
Succinate	130%

*Control values were 8.7 nmoles HCHO formed per mg microsomal protein per minute, and this was taken as 100%; similar values were observed in two separate experiments. Equal amounts of mitochondrial and microsomal protein were added to the incubation mixture.

chondria. When liver slices were incubated with these Krebs cycle intermediates, stimulation of aminopyrine demethylation was much higher (40% to 200% above control values); a similar effect was observed with liver homogenates.

Estabrook and Cohen (7), and more recently, Cohen and Estabrook (12, 13) have reported NADH supported mixed function oxidation reactions. Since increased levels of NADH in mitochondria result from stimulation of the Krebs cycle the increased demethylase activity observed with Krebs cycle intermediates could conceivably be due to an increase in the concentration of NADH; the NADH would then provide the second electron to the enzyme-substrate complex, resulting in the oxidation of the substrate. To test this possibility, liver slices and homogenates were incubated with an assay mixture containing 2.0 mM NADPH, 8 mM aminopyrine, 10 mM succinate and varying concentrations of NADH. In the presence of this high concentration of NADPH, increasing concentrations of NADH caused a biphasic effect on the oxidation of aminopyrine (Figs. 1, 2). In both slices and homogenates, there was a decrease in demethylase activity at low concentrations of NADH, followed by an increase at higher concentrations. In the liver slice, an increase in aminopyrine demethylation did occur at high

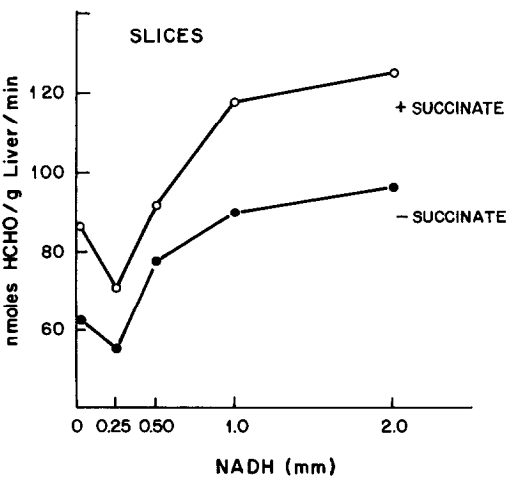

FIGURE 1. Effect of increasing concentrations of NADH on aminopyrine N-demethylation in the presence of a fixed concentration of NADPH (2.0 mM) and in the presence and absence of succinate in liver slices. Assay mixture and incubation period are indicated in Table I. Each slice weighed approximately 200 mg. Values are the means of three separate experiments.

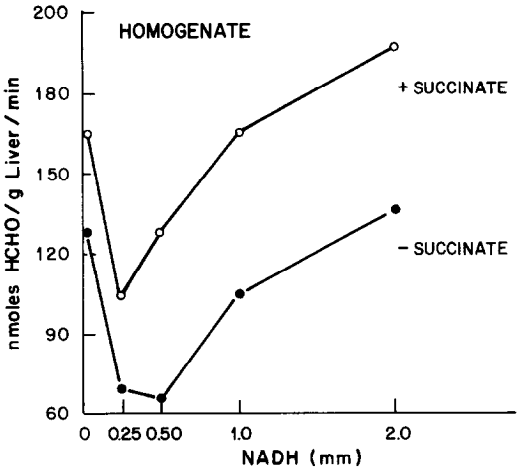

FIGURE 2. Effect of increasing concentrations of NADH on aminopyrine N-demethylation in the presence of a fixed concentration of NADPH (2.0 mM) and in the presence and absence of succinate in liver homogenates (50%). Assay mixture and incubation period are indicated in Table I; homogenate equivalent to 250 mg liver was used. Values are the means of three separate experiments.

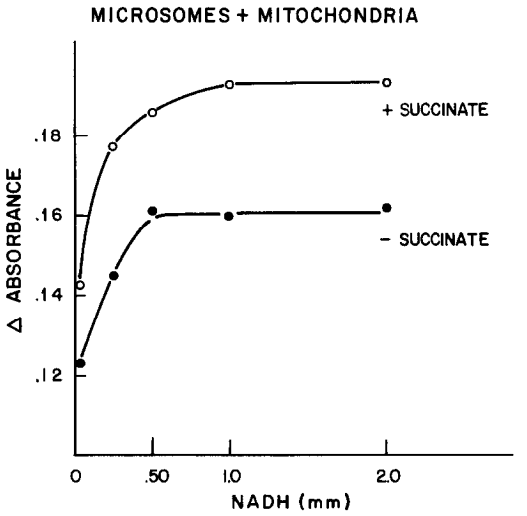

FIGURE 3. Effect of increasing concentrations of NADH on oxidation of aminopyrine in the presence of 1.0 mM NADPH and in the presence and absence of succinate in recombination of mitochondria and microsomes; equal amounts of mitochondrial and microsomal proteins were used. Assay is described in Table I. An absorbance of 0.10 corresponds to about 7.0 nmoles HCHO formed per mg microsomal protein per min. Values are the means of two separate experiments.

NADH concentrations (Fig. 1); however, when homogenate was used (Fig. 2), the activity observed with 1.0 and 2.0 mM NADH was no greater than that present in the absence of NADH. In the presence of succinate, the pattern was similar in both slices and homogenates; but even at 2.0 mM NADH, succinate stimulated a higher demethylase activity, suggesting that the Krebs cycle effect is not via NADH. Significant demethylation activity also occurred in both slices and homogenate in the absence of any pyridine nucleotides, with higher activity usually seen in the presence of succinate. When reconstituted fractions were employed, i.e., microsomes plus mitochondria, NADH did support the mixed function oxidation reaction (Fig. 3). Maximal activity was observed when the NADH: NADPH ratio was 0.5; in the presence of succinate, however, the activity was still higher.

DISCUSSION

The increase in drug metabolism in liver slices and homogenates observed with intermediates of the Krebs cycle, the inhibition of the succinate effect by malonate, and the recombination experiments with microsomes and mitochondria, strongly suggest that the mixed function oxidase system is significantly influenced by the mitochondria.

The precise role of the mitochondria in supporting drug oxidation is at present not clear. The mitochondria do not appear to be functioning simply by manufacturing NADH, since the latter did not stimulate aminopyrine demethylation in the homogenate in the presence of NADPH. Furthermore, since succinate increased demethylase activity even after addition of increasing concentrations of NADH, the effect produced by the Krebs cycle intermediates is not via NADH. The NADH supported oxidations reported by Estabrook and Cohen (7) were also observed with our recombination experiments; however, succinate in the presence of NADH still produced a greater effect. Recently, Ichikawa and Loehr (14) reported the reduction of cytochrome P-450 by NADH in the absence of cytochrome $\underline{b}_5$. Their data suggests the presence of a non-cytochrome $\underline{b}_5$ route via which reducing equivalents may reach cytochrome P-450. It is doubtful that this is the path-

way utilized by the mitochondria for support of drug metabolism, since the stimulation caused by succinate occurs even in the presence of NADH.

An alternative explanation for the mitochondrial role is that reducing equivalents may be transferred by way of a well-defined route from the mitochondria to the endoplasmic reticulum via a carrier. The relatively small (30%) increase caused by succinate addition to the recombined system (Table II) may reflect the absence of the carrier between the two cellular organelles; dilution of the homogenate decreases the 2-fold stimulation in drug metabolism obtained with succinate. Studies are currently in progress to determine if such a carrier can be isolated.

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