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Hepatic Organelle Interaction

II. Effect of Tricarboxylic Acid Cycle Intermediates on *N*-Demethylation and Hydroxylation Reactions in Rat Liver

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SUMMARY

Hepatocytes in rat liver slices accomplish the slow oxidative metabolism of drugs like aminopyrine and ethylmorphine. Although the enzyme system responsible for this metabolism resides in the endoplasmic reticulum, mitochondrial Krebs cycle intermediates also affect the process. Succinate and isocitrate each elevate the metabolism of aminopyrine and ethylmorphine about 2–3-fold; none of the other intermediates tested had any effect. NADPH elevated the rates of aminopyrine and ethylmorphine *N*-demethylation 18- and 28-fold, respectively; NADP was only 50% as effective as NADPH in supporting these oxidations. When NADPH and isocitrate were added together, an additive effect was obtained on aminopyrine and ethylmorphine oxidation; the combination of succinate and NADPH caused a synergistic effect, more marked on the oxidation of aminopyrine than of ethylmorphine. Fumarate, oxalacetate, or malate, when combined with NADPH, was antagonistic to the liver slice oxidation of these two drugs, while malonate, when added with succinate and NADPH, only removed the synergism. The same effects as obtained with liver slices could be demonstrated with whole liver homogenates, but not with isolated liver microsomes. None of the Krebs cycle intermediates had a stimulatory effect on aniline hydroxylation, although oxalacetate decreased the hydroxylation seen with aminopyrine and ethylmorphine. These observations indicate that drug metabolism in the hepatocyte endoplasmic reticulum is controlled by the mitochondria, not merely via a flow of reducing equivalents, since even in the presence of an excess of NADPH succinate exerts a synergistic effect, more than doubling the rate of aminopyrine oxidation and causing a 50% increase in ethylmorphine oxidation.

INTRODUCTION

In the past, studies on drug biotransformations have been performed *in vivo* or, in accord with current biochemical trends, in increasingly smaller subcellular fractions. Such studies, while providing information about metabolic routes and clearance of foreign compounds *in vivo*, on the one hand, and mechanism of action, on the other hand, do not provide insight into cellular control of drug metabolism. These approaches neglect the potential cell-cell and organelle-organelle interactions which undoubtedly occur in a tissue. In order to gain insight into the cellular control of drug metabolism, and

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the possible role of the mitochondria in this process, studies were performed on surviving hepatocytes in liver slices.

Studies on the adrenal cortex mitochondrial steroid 11β-hydroxylase have shown (1–7) that this cytochrome P-450-containing, mixed-function oxidase can be supported by Krebs cycle intermediates in the oxidation of deoxycorticosterone. Although hepatic mitochondria do not contain cytochrome P-450, Cinti and Schenkman (8) reported that tricarboxylic acid cycle intermediates, such as succinate and isocitrate, could provide reducing equivalents for reduction of cytochrome P-450 in the endoplasmic reticulum. Furthermore, it was observed (8) that such intermediates could support the reported (9, 10) enhanced rate of cytochrome P-450 reduction caused by the presence of drug substrates of the mixed-function oxidase. Hence it seemed reasonable that the hepatic cell mitochondria could support drug metabolism by supplying reducing equivalents to the endoplasmic reticulum. The results indicate a complex interaction.

MATERIALS AND METHODS

Young, male Sprague-Dawley rats (125–200 g) were obtained commercially and fed a standard laboratory diet. Livers were removed and treated as described previously (8). The livers were then transferred to a cold room, and slices were prepared using a Stadie-Riggs microtome. A section of each liver was set aside for homogenization. Each slice was placed on cold aluminum foil, blotted, and immediately weighed and transferred to a 25-ml Erlenmeyer flask containing the incubation mixture for measuring enzyme activities. Slices chosen for use were between 0.4 and 0.6 mm thick.

Livers were homogenized in 0.25 M sucrose–1 mM EDTA (1:1, w/v) with a Potter-Elvehjem tissue homogenizer, and 0.5 ml (250 mg of liver) was used in enzyme assays.

Enzyme activities were determined at 37° in a medium containing 50 mM Tris-HCl buffer, pH 7.4, and 1.0 mM NADPH or 1.0 mM NADP, with or without 5.0 mM glucose 6-phosphate and 7.5 μg/ml of glucose 6-phosphate dehydrogenase (Sigma, type XI, 3.5 μmoles of NADPH generated per minute per milligram at 25°).

Various substrates of the tricarboxylic acid cycle, when used, were added to the reaction vessel in final concentrations of 10 mM. These substrates included isocitrate, succinate, fumarate, malate, and oxalacetate, or 10 mM malonate was added as a competitive inhibitor of succinate oxidation.

Aminopyrine, ethylmorphine, and aniline were used as substrates for the determination of the different oxidative activities. Each 25-ml Erlenmeyer flask contained either 8 mM aminopyrine or ethylmorphine or 5 mM aniline in a total reaction volume of 3.0 ml. Incubation time in a Dubnoff shaker was 10 min for aminopyrine and ethylmorphine demethylation, and 15 min for aniline hydroxylation. Demethylase activity was determined by measuring formaldehyde produced, with the pH 6.0 Nash reagent (11, 12). Aniline hydroxylase activity was determined by measuring the formation of *p*-aminophenol (11).

All chemicals and biochemicals were of the highest purity available commercially and, with the exception of aniline, were not purified further; aniline was redistilled under vacuum to a colorless liquid.

RESULTS

Effect of oxidized and reduced nicotinamide adenine dinucleotide phasphate on N-demethylation in liver slices. When liver slices were incubated with 8 mM aminopyrine or ethylmorphine they were able to oxidize these drugs slowly, forming about 18 nmoles of formaldehyde per gram of liver in 10 min (Fig. 1). The addition of 1.0 mM NADP or NADPH markedly increased the rate of drug oxidation; in the presence of added NADPH, aminopyrine oxidation was increased about 16-fold and ethylmorphine oxidation was increased about 28-fold. Higher amounts of NADPH did not elevate activity further. NADP produced about 40–50% of the stimulation of oxidation of either drug caused by NADPH.

Since previous examination of the liver slices by electron microscopy had shown the plasma membranes of the hepatocytes to be structurally well preserved (8), these findings suggested that the hepatocyte membranes were permeable to reduced and oxidized triphosphopyridine nucleotides. This

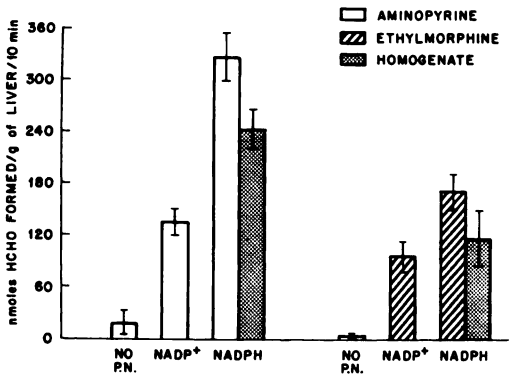

FIG. 1. *Effect of NADP and NADPH on metabolic transformation of aminopyrine and ethylmorphine in male rat liver slices*

The slices (250–300 mg) were incubated in 3.0 ml of 50 mM Tris-HCl buffer, pH 7.4, for 10 min in the presence and absence of oxidized and reduced pyridine nucleotides (P.N.). Each bar represents the mean and standard error of at least seven animals for the experiments using liver slices. The dotted bars represent the effect of NADPH (plus an NADPH-generating system) on aminopyrine and ethylmorphine *N*-demethylation in liver homogenates equivalent to 250 mg of liver in 3.0 ml of incubation mixture. The dotted bars represent the means and standard errors of two experiments.

was further tested by comparing the liver slice drug oxidase activities with those in liver homogenates. Approximately the same, or slightly lower, activities were obtained when NADPH was added to the whole homogenate assays (Fig. 1); addition of 20 mM nicotinamide to the medium did not improve the drug oxidase activities.

The apparent uptake of the pyridine nucleotides into hepatocytes could also be observed on administration of NADP *in vivo*. Approximately 0.6% of the label from 1 μCi of [^{14}C]NADP (54 μg) was found in the livers of rats 15 min after intraperitoneal injection; the amount rose to 4.5% in 30 min and was still at this level 1 hr after injection.

Effect of tricarboxylic acid cycle intermediates on biotransformation of aminopyrine and ethylmorphine in liver slices and homogenates. Table 1 shows the effect of substrates of the Krebs cycle on liver slice *N*-demethylation of aminopyrine. Although the incubation medium contained NADPH, isocitrate effected a 22% increase in aminopyrine metabolism, while the addition of 10 mM succinate resulted in a better than 2-fold increase in product formation: 647 nmoles of formaldehyde were generated per gram of liver in 10 min, compared with 320 nmoles in the absence of any mitochondrial substrates. To establish that the mitochondria were indeed participating in drug metabolism, malonate, a competitive inhibitor of mitochondrial succinate dehydrogenase, was added to the incubation medium containing succinate. Although this caused a 20% increase in activity compared with the control, malonate actually inhibited by 40% the *N*-demethylase activity supported by succinate alone (Table 1, 395 nmoles vs. 647 nmoles). Fumarate, malate, and oxalacetate each decreased the rate of oxidation of aminopyrine by the liver slices to approximately 50% of the control. In the absence of added pyridine nucleotide, the Krebs cycle intermediates succinate and isocitrate were capable of stimulating

TABLE 1

Effects of various tricarboxylic acid cycle intermediates on N-demethylation of aminopyrine in rat liver slices

Liver slices (250–300 mg) were incubated at 37° for 10 min with 3.0 ml of reaction mixture containing NADPH. The Krebs cycle substrate concentrations were 10 mM. The concentration of aminopyrine was 8 mM.

Treatment	Formaldehyde production[a]	Percentage of individual control[b]
	nmoles/g liver/10 min	
Control	320	100
Isocitrate	387	122 ± 3
Succinate	647	220 ± 56
Succinate + malonate	395	124 ± 10[c]
Fumarate	143	51 ± 15
Malate	133	47 ± 13
Oxalacetate	179	52 ± 5

[a] Values are means for at least seven animals.

[b] Since control values differed with different livers, each liver served as its own control. Values shown are means ± standard errors.

[c] The percentage activity relative to that observed with succinate alone was 62 ± 8, indicating approximately 40% inhibition by malonate.

the *N*-demethylation of aminopyrine 2–4-fold; isocitrate was somewhat more effective than succinate in this respect.

Qualitatively, the *N*-demethylations of ethylmorphine and aminopyrine were similarly affected by the tricarboxylic acid cycle intermediates (Table 2). Isocitrate and succinate increased the ethylmorphine demethylase activity by 37% and 50%, respectively. Malonate inhibited the succinate-stimulated reaction by 42%, as was observed with aminopyrine. Similarly, fumarate, malate, and oxalacetate exerted an inhibitory effect on the enzyme activity, ranging from 55 to 80% of the control (Table 2).

Table 3 shows the effects of isocitrate, succinate, and oxalacetate on the *N*-demethylation of both aminopyrine and ethylmorphine in rat liver homogenates. In liver slices isocitrate and succinate stimulate, and oxalacetate inhibits, the *N*-demethylase system. The Krebs cycle intermediates were without effect when added to the isolated microsomal fraction of liver.

TABLE 2

Effects of various tricarboxylic acid cycle intermediates on N-demethylation of ethylmorphine in rat liver slices

Liver slices (250–300 mg) were incubated at 37° for 10 min with 3.0 ml of reaction mixture containing NADPH. The Krebs cycle substrate concentrations were 10 mM. The concentration of ethylmorphine was 8 mM. Values represent the means and standard errors of at least five animals.

Treatment	Formaldehyde production	Percentage of individual control[a]
	nmoles/g liver/10 min	
Control	175	100
Isocitrate	239	137 ± 7
Succinate	346	150 ± 20
Succinate + malonate	208	119 ± 6[b]
Fumarate	18	31 ± 9
Malate	27	20 ± 18
Oxalacetate	98	44 ± 12

[a] Each rat liver served as its own control.

[b] The percentage activity relative to that observed with succinate alone was 58 ± 6, indicating 42% inhibition by malonate.

TABLE 3

Effects of succinate, isocitrate, and oxalacetate on N-demethylation of aminopyrine and ethylmorphine in rat liver homogenates

The homogenates (equivalent to 250 mg of liver) were incubated at 37° for 10 min with 3.0 ml of reaction mixture containing NADPH. Drug concentrations were 8.0 mM; Krebs cycle substrates were present at 10 mM concentrations. Values represent means and standard errors of six different preparations.

Treatment	Aminopyrine[a]	Ethylmorphine[b]
	% control	*% control*
Succinate	171 ± 24	229 ± 40
Isocitrate	146 ± 32	130 ± 14
Oxaloacetate	47 ± 12	49 ± 11

[a] The aminopyrine demethylase activity was 240 ± 54 nmoles/g of liver.

[b] The ethylmorphine demethylase activity was 112 ± 32 nmoles/g of liver.

Since aniline is a known substrate for the mixed-function oxidase system and yet forms a type II spectral change (10), it was of interest to study its metabolism as affected by the mitochondrial substrates. In contrast to *N*-demethylation, the *p*-hydroxylation of aniline in the presence of NADPH was not stimulated by either isocitrate or succinate. However, oxalacetate produced 50% inhibition, similar to its effect on aminopyrine and ethylmorphine metabolism. About 100 nmoles of aniline were hydroxylated per gram of liver in 15 min of incubation in the absence of oxalacetate, and 50 nmoles of *p*-aminophenol were formed when the reaction mixture contained oxalacetate.

DISCUSSION

The observation that maximal drug biotransformation in hepatocytes occurs in the presence of NADPH suggests a rate-limiting role for this cofactor. In the absence of exogenous oxidized or reduced pyridine nucleotide, the metabolism of aminopyrine or ethylmorphine by the liver slices was minimal, the activity observed possibly being supported by endogenous production of reducing equivalents; no metabolism of either drug could be demonstrated in liver homogenates

in the absence of NADPH. Although drug metabolism was observed in the presence of NADP, it was only about 50% as fast as that seen in the presence of NADPH. Two reasons could possibly account for this: (a) insufficient levels of endogenous substrates were present to provide reducing equivalents for the oxidized pyridine nucleotide, and/or (b) the oxidized form of the pyridine nucleotide may be less permeable to the hepatocyte plasma membrane because of its greater charge.

It has generally been considered that cellular membranes are impermeable to pyridine nucleotides, since the report by Lehninger (13) that suspensions of washed mitochondria could not oxidize extramitochondrial NADH. However, reduction with extramitochondrial NADH does occur, although at a much slower rate than with intramitochondrial NADH. Since the rate of electron flow along components of the endoplasmic reticulum mixed-function oxidase, such as in the presence of drug substrates, is relatively slow [7 nmoles of cytochrome P-450 reduced per minute per milligram of microsomal protein at 37° (14)], this permeability of the pyridine nucleotides may not limit the drug oxidase system. The similarity between the rates of aminopyrine and ethylmorphine metabolism in liver slices and homogenates also would suggest that permeability does not limit the reaction. Pyridine nucleotide permeability is not the result of loss of hepatocyte integrity, as light and electron microscopy indicate that cell membranes are morphologically well preserved (8). Furthermore, the livers of rats, representing about 5% of the body weight, were capable of taking up about 4.5% of an intraperitoneal injection of NADP in 1 hr *in vivo*.[1] Other investigators (15) have found that intraperitoneal injection of a large amount of NADH results in hepatic uptake without cleavage of the pyridine nucleotide. The uptake is maximal at about 6 hr. NAD injection resulted in slower uptake, reaching a peak at 12 hr, and a lower extent of incorporation, 7% vs. 22%. The extent of uptake did not appear to be dose-dependent (15).

[1] Unpublished observations, D. L. Cinti and J. B. Schenkman.

Various intermediates of the mitochondrial tricarboxylic acid cycle were found to be capable of affecting hepatocyte endoplasmic reticular drug oxidation. Two of these, succinate and isocitrate, exerted a 2–3-fold stimulatory effect on aminopyrine and ethylmorphine oxidative *N*-demethylation; succinate was somewhat more effective than isocitrate. The pyridine nucleotides NADP and NADPH were capable of exerting a greater stimulatory effect on the oxidation of these drugs; the stimulation of NADP was about twice that of isocitrate on aminopyrine demethylation, and that by NADPH was about 6 times greater. The effects on ethylmorphine *N*-demethylation were qualitatively the same. However, when both NADPH and the tricarboxylic acid cycle intermediate succinate were added to the liver slice incubation medium, a synergistic rather than an additive effect was observed; the rate of aminopyrine dealkylation, for example, was increased (Table 1) 2.2-fold above the rate in the presence of NADPH (rather than the expected 25% increase). The stimulation obtained with isocitrate, however, appeared to be an additive effect. When ethylmorphine was used as substrate for the mixed-function oxidase, qualitatively similar effects were again obtained; however, the magnitude of the stimulation by NADHP plus succinate was not as great as for aminopyrine *N*-dealkylation. Synergistic effects were also obtained when whole homogenates of liver were tested with succinate or isocitrate and NADPH; in the absence of NADPH there was no oxidation of aminopyrine or ethylmorphine, even in the presence of Krebs cycle intermediates.

The tricarboxylic acid intermediates fumarate, malate, and oxalacetate all had no effect on the endogenous rate of liver slice aminopyrine or ethylmorphine *N*-dealkylation; however, when combined with NADPH, all these intermediates exerted an antagonistic effect, diminishing the dealkylation reactions to rates below those supported by NADPH alone. The inhibitory effects diminished the rates of oxidation of aminopyrine by 50%, and that of ethylmorphine, by 20–40%. This agrees with the finding in the preceding paper (8) that intermediates at the end of the

Krebs cycle (malate, fumarate, oxalacetate) do not stimulate the reduction of cytochrome P-450, but inhibit the drug-stimulated magnitude of reduction.

These results support our hypothesis that the functioning of the endoplasmic reticular mixed-function oxidase (drug oxidase system) is only partially controlled by means of the flow of reducing equivalents to it. The nature of the control is at present not completely understood. It takes place not merely via the *amount* of reducing equivalents provided, since the Krebs cycle intermediates exert their synergistic or antagonistic effects on the drug oxidase system in the presence of an excess of NADPH. Nor is control exerted via a direct action of the intermediates on the endoplasmic reticulum, since the intermediates with NADPH have no effect on oxidation by isolated microsomes. Control probably occurs by a well-defined route, which provides a second electron required by the mixed-function oxidase for activation of oxygen. The route appears to be via the mitochondria, since the effects of the Krebs cycle intermediates can also be observed in whole liver homogenates, but not with isolated microsomes, and since the antagonistic action of the succinate dehydrogenase inhibitor malonate on succinate-stimulated metabolism only diminishes the rate of drug metabolism to a level approaching that in the presence of NADPH alone.

While the idea of a mitochondria-controlled endoplasmic reticulum function is a new concept with regard to cell organelle interactions, it is based upon observations of a cytochrome P-450-containing mixed-function oxidase in other tissues. In the adrenal cortex the mitochondria contain a cytochrome P-450-containing mixed-function oxidase, shown to be responsible for a steroid hydroxylation (16). This enzymatic reaction, the 11β-hydroxylation of deoxycorticosterone, was shown to be supported by Krebs cycle intermediates in the presence of NAD and ATP (2, 3, 17). However, it has been suggested (5) that support of 11β-hydroxylation is mediated via transhydrogenases, transferring reducing equivalents from NADH to NADP. The present study indicates that in hepatocytes, at least, control is exerted by means of a more complicated interaction between different hepatocyte organelles. A similar suggestion, implicating cytochrome b_5 as the source of the second electron, has been made by Hildebrandt and Estabrook (18), using NADH and NADPH with isolated microsomes.

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