LIVER MICROSOMAL ELECTRON TRANSPORT SYSTEMS.

II. The Involvement of Cytochrome b_5 in the NADH-Dependent Hydroxylation of 3,4-Benzpyrene by a Reconstituted Cytochrome P-448-Containing System.

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SUMMARY: An enzyme system in rat liver microsomes which catalyzes the NADH-dependent hydroxylation of 3,4-benzpyrene has been reconstituted. The essential components of this NADH-mediated electron transport chain are cytochrome b_5, NADH-cytochrome b_5 reductase, lipid, and cytochrome P-448.

It is well-established that NADPH is the most efficient electron donor for the liver microsomal hydroxylation system. NADH can also serve as an electron donor for this cytochrome P-450-containing system, although the rate of hydroxylation is slower (1). It is generally accepted that liver microsomes contain two electron transport systems, and it is not certain which of these chains NADH utilizes to transfer its reducing equivalents to cytochrome P-450 (see scheme).

Recently, however, several laboratories have reported that cytochrome P-450 can be reduced by NADH via some of the electron carriers in the cytochrome b_5 chain (2-6). There is some controversy as to whether the electrons from NADH are passed directly to cytochrome P-450 from cytochrome b_5 reductase or whether they pass from cytochrome b_5 reductase to cytochrome b_5 and then to cytochrome P-450.

In the present communication we report on the reconstitution of an enzyme system from rat liver microsomes which catalyzes the NADH-dependent hydroxylation of 3,4-benzpyrene at a rate similar to the rate in microsomes from 3-methylcholanthrene-treated rats. This NADH-

dependent, cytochrome P-448-mediated hydroxylation of 3,4-benzpyrene requires both cytochrome b_5 and cytochrome b_5 reductase.

Cytochrome b_5 has also been postulated to have a role in NADPH-dependent hydroxylation (2). As recently reported (7,8), we have prepared cytochromes P-450, P-448, and NADPH-cytochrome c reductase free of cytochrome b_5 and have studied the hydroxylation of 3,4-benzpyrene and the N-demethylation of benzphetamine with this reconstituted system in the presence and absence of cytochrome b_5. We have found that cytochrome b_5 is not an obligatory component for the NADPH-dependent metabolism of benzphetamine and 3,4-benzpyrene (7,8).

METHODS: Cytochrome P-448 from liver microsomes of 3-methylcholanthrene-treated-rats (male, Long-Evans, 50-60 g) was solubilized and partially purified (specific content, 10 nmoles/mg) by the method of Levin et al (9). This preparation was contaminated with a small amount of NADH-cytochrome b_5 reductase, but was free of cytochrome b_5, NADPH-cytochrome c reductase, and lipid. Liver microsomes from phenobarbital-treated rats were used as starting material for the solubilization and purification of the following components: cytochrome b_5 and NADH-cytochrome b_5 reductase by the method of Spatz and Strittmatter (10,11), lipid fraction (12), and NADPH-cytochrome c reductase by a recently published method (8).

RESULTS: Since the rate of NADH-dependent hydroxylation of drugs and carcinogens is slow, it is necessary to select a substrate that is hydroxylated to a product that can be assayed by a sensitive analytical method. 3,4-Benzpyrene was chosen for this study because picomole amounts of the hydroxylated products can be assayed by a fluorometric method (13). As shown in Table I, the NADH-dependent hydroxylation of 3,4-benzpyrene required cytochrome b_5, NADH-cytochrome b_5 reductase, lipid, and cytochrome P-448. Activity was abolished or greatly decreased when any one of these components was omitted from the reaction mixture.

TABLE I

Required Components for NADH-Dependent 3,4-Benzpyrene Hydroxylation by the Reconstituted System

Conditions	8-Hydroxy-3,4-benzpyrene Formed	
	NADH	NADPH
	nmole/5 minutes	
1. Complete (lipid + P-448 + b_5 + b_5 reductase)	0.611	--
-b_5	0.005	--
-P-448	0.000	--
-b_5 reductase	0.127	--
-lipid	0.005	--
2. Complete + NADPH-cytochrome c reductase	0.594	--
3. Lipid + P-448 + NADPH-cytochrome c reductase	0.024	2.40

The reaction mixture, in a final volume of 1.0 ml, contained 100 µmoles of potassium phosphate buffer (pH 6.8), 3 µmoles of $MgCl_2$, 0.2 µmoles of NADH (or 0.4 µmole of NADPH when indicated), 0.6 mg of bovine serum albumin, 80 nmoles of 3,4-benzpyrene (in 0.04 ml acetone) and the following microsomal components when indicated: cytochrome P-448 (0.31 nmole, 0.034 mg), cytochrome b_5 (0.29 nmole, 0.017 mg), NADH-cytochrome b_5 reductase (2 units, 0.030 mg), NADPH-cytochrome c reductase (50 units, 0.010 mg) and lipid (0.1 mg). The reaction mixture was incubated at 37° for 5 minutes. 8-Hydroxy-3,4-benzpyrene was measured by the method of Nebert and Gelboin (13). 1 unit of NADH-cytochrome b_5 reductase = 1.0 µmole ferricyanide reduced per minute assayed by the method of Mihara and Sato (14). One unit of NADPH-cytochrome c reductase = 1.0 nmole cytochrome c reduced per minute assayed by the method of Phillips and Langdon (15).

Because of the contamination of the cytochrome P-448 preparation with a small amount of NADH-cytochrome b_5 reductase, a complete dependence on cytochrome b_5 reductase was not observed. Under the assay conditions described, NADPH-cytochrome c reductase had no effect on the cytochrome b_5 pathway although in combination with cytochrome P-448 and lipid it supported a low but measurable rate of NADH-dependent hydroxylation. Table II clearly shows that the NADH-mediated hydroxylation pathway was dependent on the concentration of cytochrome b_5 when the other components were present in excess.

Since the cyanide-sensitive factor is also an essential component

TABLE II

Effect of Cytochrome b_5 Concentration on NADH-Dependent 3,4-Benzpyrene Hydroxylation

Cytochrome b_5 Added nmole	8-Hydroxy-3,4-benzpyrene Formed nmole/5 minutes
0	0.004
0.018	0.071
0.036	0.165
0.072	0.278
0.144	0.392
0.292	0.511
0.440	0.505

Assay conditions were similar to those described in Table I. The microsomal components used were: cytochrome P-448 (0.31 nmole, 0.034 mg), NADH-cytochrome b_5 reductase (2 units, 0.030 mg), lipid (0.1 mg) and the indicated amounts of cytochrome b_5.

of the NADH-dependent fatty acid desaturase pathway in liver microsomes (16,17), its possible role in the reconstituted, NADH-dependent hydroxylation of 3,4-benzpyrene was tested by studying the sensitivity of this reaction to cyanide. Table III shows that the NADH-mediated hydroxylation of 3,4-benzpyrene, both in microsomes and in the reconstituted system, was not inhibited by cyanide at concentrations known to inhibit the fatty acid desaturase system (16,17). Thus, the cyanide-sensitive factor does not appear to be involved in the NADH-dependent hydroxylation of 3,4-benzpyrene.

DISCUSSION: We have recently reported (7,8) that cytochrome b_5 is not an obligatory component of the NADPH-dependent benzphetamine and 3,4-benzpyrene hydroxylation systems whose components are lipid, NADPH-cytochrome c reductase, and cytochrome P-450 or P-448. In this report, we present evidence to show

TABLE III

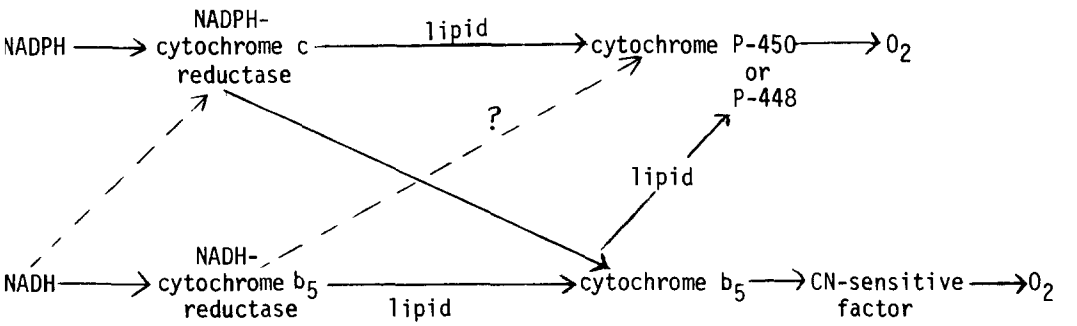
Effect of Cyanide on NADH-Dependent 3,4-Benzpyrene Hydroxylation in the Microsomal and Reconstituted Systems

Final Concentration Cyanide	Microsomal System	Reconstituted System
(mM)	%	%
0	100	100
0.01	102	--
0.1	104	112
0.5	108	--
1.0	106	107
5.0	98	--

Assay conditions were similar to those described in Table I except that 1.0 μmole of NADH was used. Microsomes from 3-methylcholanthrene-treated rats (0.050 mg microsomal protein) had a control activity (100%) of 0.101 nmole 8-OH-3,4-benzpyrene formed. The microsomal components used were: cytochrome P-448 (0.11 nmole, 0.012 mg), cytochrome b_5 (0.033 nmole, 0.002 mg), NADH-cytochrome b_5 reductase (1 unit, 0.015 mg) and lipid (0.1 mg) and had a control activity (100%) of 0.092 nmole 8-OH-3,4-benzpyrene formed.

that cytochrome b_5, NADH-cytochrome b_5 reductase, lipid, and cytochrome P-448 are required for the NADH-dependent hydroxylation of 3,4-benzpyrene. Cytochrome P-450 in the presence of cytochrome b_5, NADH-cytochrome b_5 reductase, and lipid will also support the NADH-dependent hydroxylation of 3,4-benzpyrene. The rate of hydroxylation supported by cytochrome P-450 is considerably slower than the rate of hydroxylation supported by cytochrome P-448 which is consistent with the idea that the specificity for hydroxylation of 3,4-benzpyrene supported either by NADPH or NADH resides in the cytochrome P-450 or P-448 fraction.

The involvement of cytochrome b_5 in the NADH-dependent hydroxylation pathway as presented in this paper is supported by the findings of Sasame et al



(5) that the reduction of cytochrome P-450 by NADH in liver microsomes is blocked by an antibody against cytochrome b_5. We have recently found that the NADH dependent peroxidation of cumene hydroperoxide also requires cytochrome b_5 and is supported by either cytochrome P-450 or cytochrome P-448 (Hrycay, E.G., Jonen, H.G., Lu, A. Y. H., and Levin, W., manuscript in preparation). Our preliminary experiments also indicated that cytochrome P-450 or P-448 is not reduced by NADH and cytochrome b_5 reductase unless cytochrome b_5 is present. On the other hand, Ichikawa and Loehr (3) have reported reduction of cytochrome P-450 by NADH in subparticles containing no cytochrome b_5 obtained from trypsin-treated rabbit liver microsomes. Caution should be used, however, for the interpretation of studies with enzymes treated with protease since such treatment has been shown to alter the properties of certain electron transport systems. Shimakata et al (17) have shown that when combined with the cyanide-sensitive factor, protease-solubilized cytochrome b_5 reductase catalyzed the desaturation reaction even in the absence of cytochrome b_5. In contrast, cytochrome b_5 is absolutely required for desaturation in the presence of the cyanide-sensitive factor and detergent-solubilized cytochrome b_5 reductase. Since protease treatment of microsomal enzymes may change the ability of microsomal electron carriers to interact with one another, all enzymes used in the present study were solubilized and purified in the presence of detergents.

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