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SHORT COMMUNICATION

Evidence Against Participation of Cytochrome b_5 in the Hepatic Microsomal Mixed-Function Oxidase Reaction

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SUMMARY

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The addition of increasing concentrations of a detergent (Triton X-100)-solubilized cytochrome b_5 from liver microsomes to liver microsomes of untreated or phenobarbital-treated animals produced correspondingly greater inhibition of aminopyrine dealkylation. Even addition of NADH to the NADPH-supported reaction did not prevent this inhibition, although the added cytochrome b_5 functioned as an endogenous hemoprotein; i.e., it was reduced by NADH and stimulated the reduction of exogenous cytochrome *c*. These observations suggest that cytochrome b_5 does not function in hepatic microsomal mixed-function oxidase reactions.

Since the report by Estabrook and his co-workers (1) that cytochrome P-450 is the terminal oxidase in the adrenal cortex C-21 mixed-function steroid hydroxylase reaction, and the subsequent observation by these investigators (2) that cytochrome P-450 plays a similar role in the liver microsomal mixed-function oxidase, many investigators have studied the mechanism of action of the latter enzyme system. The sequence of events in the reaction seems to be initiated by a reversible binding of the drug substrate to the oxidized form (Fe^{3+}) of the terminal oxidase, usually causing formation of a type I spectral change (3). Formation of this enzyme-substrate complex facilitates electron flow to cytochrome P-450 (4).

The mixed-function oxidase reaction in liver microsomes requires NADPH. Two electrons are supplied by the pyridine nucleotide (5), ostensibly for activation of molecular oxygen (6), probably sequentially, since the reductase has been shown to function between the fully reduced and half-reduced forms (7). Capture of the oxygenated intermediate of a bacterial cytochrome P-450-containing monooxygenase (8), with subsequent discharge of this complex, suggested that a 1-electron reduction occurs, followed by binding of oxygen. The oxygen-bound form is discharged by a second electron. An oxygenated intermediate

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has been observed in liver microsomes during drug oxidation (9).

Although NADH itself poorly supports hepatic microsomal drug oxidations, it has a synergistic effect when added with adequate NADPH (10, 11). NADH, however, reduces cytochrome b_5 to a greater extent than NADPH. Based upon a decrease in the level of reduction of cytochrome b_5 by NADPH or by NADH plus NADPH in the presence of substrates of the microsomal mixed-function oxidase, Hildebrandt and Estabrook (11) suggested that cytochrome b_5 functions in the mixed-function oxidase reaction by supplying the second electron to the oxygenated, substrate-bound hemoprotein.

In light of the recent observation of Ichikawa and Loehr (12) that reducing equivalents could still reach cytochrome P-450 from NADH in the absence of microsomal cytochrome b_5, we have investigated the possible role of this latter hemoprotein in the mixed-function oxidase reaction. Our results cast doubt on its involvement.

Liver microsomes were prepared from adult (200–300-g) male Sprague-Dawley rats by calcium ion sedimentation (13) and were washed in 0.15 M KCl to remove the calcium. Cytochrome b_5 was prepared by a modified method of Spatz and Strittmatter (14). In our studies a 45-min incubation of rat liver microsomal acetone powder in 1.5% Triton X-100 (Rohm and Haas) at 37° was sufficient for extraction of virtually all of the cytochrome b_5. The microsomes were not sonicated with NaCl before forming an acetone powder, as this caused a poor yield. The preparations used here were purified to 17 nmoles of cytochrome b_5 per milligram of protein and were detergent-free. Binding of cytochrome b_5 to liver microsomes was accomplished as described by Strittmatter *et al.* (15), at 37° for 20 min, which is sufficient time for complete binding at this temperature (16). Phenobarbital-induced animals received 80 mg/kg of drug for 4 days prior to use. Aminopyrine demethylase activity was measured from the formaldehyde produced, as described earlier (13).

In agreement with the report of Strittmatter *et al.* (15) and Enomoto and Sato (16), added cytochrome b_5 bound to liver microsomes functioned as native hemoprotein. The addition of NADH or NADPH to these microsomes rssulted in essentially complete reduction of the total b_5 hemoprotein in the microsomes. Figure 1 shows the NADH-reduced minus oxidized spectra of a washed microsomal preparation which had been incubated with different amounts of cytochrome b_5. The hemoprotein b_5 content was increased over 4-fold, from 0.31 to 1.49 nmoles/mg of protein. The binding characteristics of microsomes from phenobarbital-treated rats were similar to those of untreated rats (Fig. 2). The extent of cytochrome b_5 incorporation into the microsomes increased in roughly linear fashion with increasing concentrations of the hemoprotein in the medium (Fig. 2). The incor-

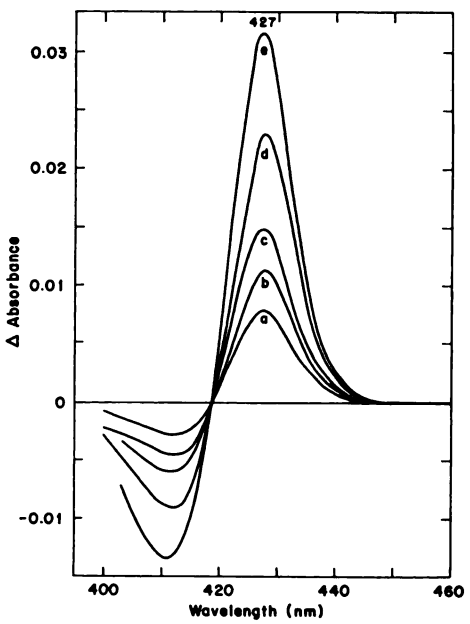

FIG. 1. *NADH reduction of cytochrome b_5-fortified liver microsomes of phenobarbital-treated rats*

Microsomes, treated as described for Fig. 2, were suspended to 1.7 mg of protein per milliliter in 0.1 M Tris-HCl buffer, pH 7.5. Difference spectra were recorded 1 min after addition of about 1 mg of dry NADH to the sample cuvette. The cytochrome P-450 content of microsomes was 1.35 nmoles/mg. The cytochrome b_5 content was 0.31 nmole/mg (*a*), 0.51 nmole/mg (*b*), 0.69 nmole/mg (*c*), 1.11 nmoles/mg (*d*), and 1.49 nmoles/mg (*e*).

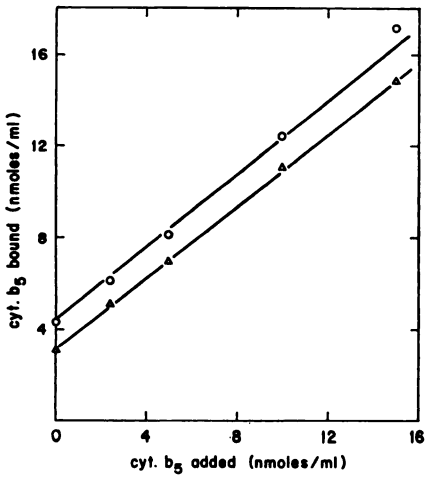

FIG. 2. *Binding of detergent-solubilized cytochrome b_5 to liver microsomes of untreated* (△) *and phenobarbital-treated* (○) *rats*

Binding of cytochrome b_5 was determined as described in the text. Microsomes were suspended to 10 mg of protein per milliliter. After incubation the microsomes were sedimented by centrifugation at 148,000 × *g* for 20 min, resuspended to their original volume in 0.15 M KCl, and resedimented by centrifugation. The washed microsomes were resuspended to their original volume for measurement of the NADH-reducible bound cytochrome b_5 ; all the hemoprotein appeared to be enzymatically reducible.

poration was determined after the microsomes had been sedimented at 148,000 × *g* for 15 min and washed once with 0.15 M KCl. The washed microsomes were used in the experiments described below. NADH–cytochrome *c* reductase activity of the microsomes was also considerably enhanced by increased cytochrome b_5 concentrations (Fig. 3), as observed by Okuda *et al.* (17) and first reported by Strittmatter and Velick (18).

The effect of increased cytochrome b_5 levels in the microsomes of untreated and phenobarbital-treated rats is shown in Fig. 4. The rate of NADPH-supported demethylation of aminopyrine decreased with increasing cytochrome b_5 content in the microsomes of untreated (Fig. 4A) and phenobarbital-treated rats (Fig. 4B). Although our experiments confirm a previous report that addition of NADH stimulates the rate of NADPH-supported aminopyrine demethylation (13), the stimulated rates of demethylation declined with the increase in cytochrome b_5 roughly in parallel with the non-NADH-stimulated rates. Inhibition did not reach 50% even when ratios of total cytochrome b_5 to the endogenous b_5 level (or the endogenous cytochrome P-450 level) were increased 5-fold. The data in Fig. 4A exhibited a biphasic plot on semilogarithmic paper, with a sensitive phase showing 50% inhibition at a concentration of cytochrome b_5 about twice the native level, in both the presence and absence of NADH. The less sensitive phase showed 50% inhibition at 5.5 times the native cytochrome b_5 level in both the presence and absence of NADH. Of interest was the failure to observe a biphasic plot with microsomes from phenobarbital-treated animals; parallel lines were obtained in both the presence and absence of NADH, with 50% inhibition occurring at 5.5 times the native level of cytochrome b_5 .

Our results tend to rule out the possibility that cytochrome b_5 is involved in the microsomal mixed-function oxidase reaction. The

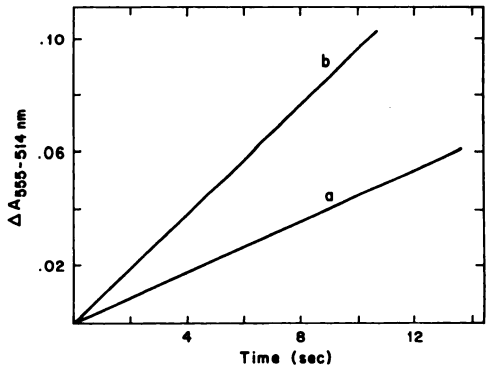

FIG. 3. *NADH–cytochrome c reductase activity of liver microsomes fortified with extra cytochrome b_5*

Liver microsomes of untreated rats, containing 0.475 nmole of cytochrome P-450 and 0.314 nmole of cytochrome b_5 per milligram, and the same microsomes containing 0.5 nmole of cytochrome b_5 , were examined at 22° in an Aminco-Chance spectrophotometer at 555 nm minus 541 nm. Either 0.1 mg of unfortified (*a*) or fortified (*b*) microsomes was added to a medium containing 0.1 M Tris-HCl buffer, pH 7.5, and 60 nmoles of horse heart cytochrome *c* in 3 ml. The reaction was initiated by addition of dry NADH (approximately 1 mg).

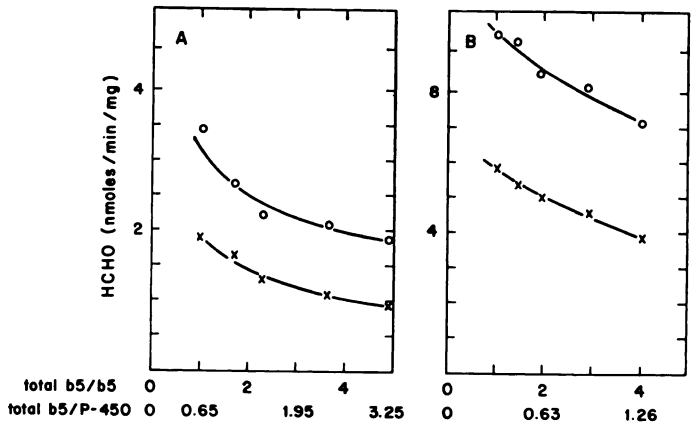

FIG. 4. *Effect of enhanced cytochrome b_5 content on aminopyrine demethylase activity in liver microsomes of untreated (A) and phenobarbital-treated (B) rats*

Liver microsomes were treated as described for Fig. 2. The enzyme assay was conducted at 37° for 7 min and contained 8 mM aminopyrine and 0.4 mM NADP (plus an NADP-generating system). The abscissa indicates the ratios of total amount of cytochrome b_5 to the initial amount of cytochrome b_5 and cytochrome P-450. ×, no added NADH; ○, 1 mM NADH. A. Initial concentrations of cytochrome b_5 and cytochrome P-450 were 0.31 and 0.43 nmole/mg, respectively. B. Initial concentrations of cytochrome b_5 and cytochrome P-450 were 0.43 and 1.35 nmoles/mg, respectively.

suggestion by Hildebrandt and Estabrook (11) that cytochrome b_5 is involved in the reaction was based upon the following observations. (a) NADH increased the over-all rate of NADPH-supported *N*-demethylation of ethylmorphine, but not the substrate requirement (K_m) for the reaction. (b) Increased cation concentrations stimulated ethylmorphine oxidation supported by NADPH but not by NADH, and also enhanced the NADH stimulation of the NADPH-supported reaction. (c) NADH had no effect upon the NADPH-supported rate of reduction of cytochrome P-450 in the presence or absence of ethylmorphine. (d) The addition of ethylmorphine to aerobic microsomes reduced by NADPH decreased the extent of reduction of cytochrome b_5. The addition of NADPH to microsomes reduced by NADH in the presence of ethyomorphine also decreased the level of reduction of cytochrome b_5, but to a lesser extent. (e) The addition of NADPH to aerobic microsomes containing NADH and ethylmorphine increased the rate of oxidation of NADH.

These observations suggested that the increased rate of drug metabolism involves a step after the interaction of substrate with the microsomes (a) and the initial reduction of cytochrome P-450 (c), and is not due to an influence of NADH on cytochrome *c* reductase (b), which is believed to be the rate-limiting step in drug metabolism. The enhanced rate of NADH oxidation (e) when NADPH was present along with ethylmorphine was taken to indicate that NADPH acts as a valve or control for input of electrons from NADH. The decline in the level of reduction of cytochrome b_5 by NADPH (d) when ethylmorphine was present, and its partial recovery when NADH was added, indicated that electron flow is mediated directly via cytochrome b_5 to an oxygen-bound substrate complex of cytochrome P-450. The change in oxidation-reduction state of cytochrome b_5 means that cytochrome b_5 may be capable of donating electrons faster than it accepts them, as long as drug metabolism proceeds, i.e., its reduction is rate-limiting.

Although the changes in the steady-state levels of reduced cytochrome b_5 explain the wavelength shift in spectral change with drugs and NADPH (19), they do not necessarily mean that cytochrome b_5 functions as an electron donor in the mixed-function oxidation of drugs. A change in the level of

reduced cytochrome b_5 could also mean a change in the availability of electrons for cytochrome b_5. Thus, if the rate of reduction of cytochrome b_5 is rate-limiting, increasing the amount of this electron acceptor available to the reductase should accelerate its reduction and increase the amount of reduced cytochrome b_5, thereby allowing an increase in the rate of electron flow from cytochrome b_5.

As shown in Fig. 3, electron flux from NADH to cytochrome *c* via cytochrome b_5 is about double in microsomes in which the cytochrome b_5 level was doubled, indicating that the added hemoprotein is functional in liver microsomes. However, all additions of cytochrome b_5 diminished aminopyrine demethylase activity. There seemed to be no relationship between the amount of cytochrome P-450 present and the binding of cytochrome b_5 to the microsomes. A 2.5-fold elevation of the microsomal content of cytochrome P-450 with only a minor (40%) increase in the endogenous content of cytochrome b_5 did not alter the relative stimulation (17%) afforded by NADH or the extent of inhibition caused by additional amounts of cytochrome b_5.

An alternative possibility could be that cytochrome b_5 drains electrons from the NADPH pathway, possibly to the lipid desaturase pathway, in which cytochrome b_5 has been shown to function (20). The more cytochrome b_5 present, the greater the drain, even when the NADPH pathway is subsidized by added NADH. The positive modifier action of type I substrates like aminopyrine (4) and ethylmorphine (21, 22), facilitating and speeding electron flow through the mixed-function oxidase pathway, probably diminishes the availability of electrons to cytochrome b_5, thereby lowering its steady-state reduced level.

The data of Hildebrandt and Estabrook (11) and Cohen and Estabrook (23, 24), while not necessarily demonstrating the involvement of cytochrome b_5, do indicate the involvement of another pathway which can feed electrons of NADH into a step after the initial reduction of cytochrome P-450 in the mixed-function oxidase reaction; this step, since it is stimulated by NADH, is probably rate-limiting in the absnce of NADH. From the observation by Ichikawa and Loehr (12) that even in the absence of cytochrome b_5 a route exists for input of NADH reducing equivalents, as well as our present findings, it is highly unlikely that cytochrome b_5 serves as the link between NADH and cytochrome P-450.

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