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EVIDENCE FOR A PREDOMINANTLY NADH-DEPENDENT O-DEALKYLATING SYSTEM IN RAT HEPATIC MICROSOMES*

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Abstract—This study compared the NADH- and NADPH-supported *p*-nitrophenetole (NP) *O*-deethylase, ethylmorphine (EM) *O*-deethylase and EM *N*-demethylase activities of rat hepatic microsomes with respect to dioxygen requirement, inhibition by carbon monoxide, inhibition by classical inhibitors of cytochrome P-450 systems, and the involvement of NADH–cytochrome b_5, cytochrome b_5 reductase and NADPH–cytochrome P-450 reductase. The results generated the following conclusions and speculations:

(1) NADH- and NADPH-supported *O*-deethylations of NP involve different P-450 hemoproteins. This conclusion was based largely on the observations that (a) the NADPH-supported reaction was inhibited by carbon monoxide and cyanide (5 mM), whereas the NADH-supported reaction was not; (b) the NADH-supported reaction required a relatively high pO_2 for maximal activity, whereas the NADPH-supported reaction did not, and (c) the NADPH-supported reaction was depressed in microsomes from rats that had been administered Co^{2+}, Mn^{2+}, allylisopropylacetamide (AIA) or polyriboinosinic acid · polyribocytidylic acid (poly IC), whereas the NADH-supported reaction was not. However, the NADH- and NADPH-supported reactions shared some common features: both were strongly inhibited by α-naphthoflavone and weakly inhibited by 2-diethylaminoethyl 2,2-diphenyl valerate HCl (SKF 525-A), both were destroyed by linoleic acid hydroperoxide, and both were induced by 3-methylcholanthrene (MC) and phenobarbital. The use of antibodies against NADPH–cytochrome P-450 reductase, NADH–cytochrome b_5 reductase and cytochrome b_5 demonstrated that both the NADH- and the NADPH-supported reactions depend on established components of cytochrome P-450 systems.

(2) The P-450 hemoproteins involved primarily in both the NADH- and NADPH-supported deethylation of NP are the P_1-450 type, i.e. they are markedly induced by MC and inhibited by α-napthoflavone.

(3) The NADH- and NADPH-supported *O*-deethylations of NP involve separate electron transfer systems. This conclusion is based largely on the following observations: (a) the synergism of the reaction, observed under normal atmospheric conditions when both NADH and NADPH were present, was not seen when the pO_2 was restricted to 5%; (b) studies of the inhibitory effect of antibody against cytochrome b_5 showed that cytochrome b_5 was an absolute requirement of the NADH-supported reaction, but not of the NADPH-supported reaction; (c) studies that used antibody against NADPH–cytochrome P-450 reductase, the inhibitory effect of $NADP^+$, and the treatment of microsomes with trypsin showed that NADPH–cytochrome P-450 reductase was not involved in the NADH-supported reaction; (d) the use of antibody against cytochrome b_5 showed that cytochrome b_5 was required for a variety of NADH-supported monooxygenase reactions but not for the same NADPH-supported reactions. It is postulated from these observations that the cytochrome P-450(s) involved in the NADH-supported reaction is primarily NADH-dependent. The possibility is considered that this cytochrome P-450 may not exist as such normally but may be created from a more conventional cytochrome P-450 when it combines with NP to form a modified cytochrome P-450 with diminished affinities for carbon monoxide and dioxygen.

(4) NADH- and NADPH-supported O-deethylation of NP, O-deethylation of EM and N-demethylation of EM involve different cytochrome P-450 systems. This conclusion is based on the observation that none of the three reactions responded alike to all of a variety of conditions to which they were subjected.

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Shigematsu and coworkers [1–3] observed that NADH-supported O-dealkylation of *p*-nitrophenetole (NP§) by hepatic microsomes was not inhibited by carbon monoxide and concluded that the reaction is therefore probably not cytochrome P-450 dependent. However, Kamataki and associates [4, 5] observed that antibody against cytochrome P-450 inhibited the NADH-supported O-demethylation of *p*-nitroanisole; thus it would appear that the reaction is in fact mediated by a species of cytochrome P-450 even though it is atypical in not being inhibited by carbon monoxide.

The current study compares the NADH- and NADPH-supported NP *O*-deethylase, ethylmorphine (EM) *O*-deethylase and EM *N*-demethylase activities of rat hepatic microsomes with respect to dioxygen requirement, inhibition by carbon monoxide, inhibition by classical inhibitors of cytochrome P-450 systems, and the involvement of NADH–cytochrome b_5, cytochrome b_5 reductase and NADPH–cytochrome P-450 reductase.

MATERIALS AND METHODS

Materials. NADH, NADPH, $NADP^+$, glucose-6-phosphate, cytochrome *c* (horse heart, type VI), *p*-nitrophenol, polyriboinosinic acid · polyribocytidylic acid (poly IC), trypsin (type XI) and soybean trypsin inhibitor were purchased from the Sigma Chemical Co. (St. Louis, MO). *p*-Nitrophenetole and *p*-nitroanisole were obtained from the Fisher Scientific Co. (Chicago, IL) and purified by recrystallization. Glucose-6-phosphate dehydrogenase was obtained from Boehringer Mannheim Biochemicals (Indianapolis, IN). Carbon monoxide (99.5%) and oxygen (99.99%) were obtained from the Matheson Co. (Joliet, IL), 7-ethoxyresorufin from the Pierce Chemicals Co. (Rockford, IL), and resorufin and 3-methylcholanthrene (MC) from Eastman Organic Chemicals (Rochester, NY). Ethylmorphine HCl (EM), 7-hydroxycoumarin, α-naphthoflavone and benzo[*a*]pyrene were purchased from the Aldrich Chemical Co. (Milwaukee, WI), and sodium phenobarbital (PB) from Merck & Co. (Rahway, NJ). 2-Diethylaminoethyl 2,2-diphenyl valerate HCl (SKF 525-A) was supplied by Smith, Kline & French Laboratories (Philadelphia, PA). 7-Ethoxycoumarin and allylisopropylacetamide (AIA) were gifts from Wayne Levin, Hoffmann-LaRoche (Nutley, NJ). 3-*O*-[1′-^{14}C]ethylmorphine (4.25 mCi/mmol) was prepared by Dr. D. E. Nerland by the method of Baizer and Ellner [6]. All other chemicals were of the highest grade available commercially.

Antibodies to highly purified rat hepatic microsomal NADPH–cytochrome P-450 reductase (anti fpT IG), NADH–cytochrome b_5 reductase (anti fpD IG) and cytochrome b_5 (anti b_5 IG) were prepared from rabbit serum as described by Kuriyama *et al.* [7], Takesue and Omura [8] and Oshino and Omura [9] respectively. These antibody preparations were gifts from Dr. Tsuneo Omura, Department of Biology, Faculty of Science, Kyushu University, Fukuoka, Japan.

Animals. Male Simonson strain rats (175–200 g) were used. Some rats were injected intraperitoneally with sodium PB dissolved in saline, 40 mg/kg/day, for 4 days, or MC dissolved in corn oil, 20 mg/kg/day, for 4 days. Animals were killed 24 hr after the last injection. All animals were fed a stock diet until killed. Control rats received injections of saline or corn oil.

Microsomal preparations. Microsomes were prepared by differential centrifugation as described previously [10] from livers that had been removed from decapitated animals and perfused with 1.15% KCl solution. Microsomes were suspended in 1.15% KCl solution (10 mg protein/ml).

Suspensions of microsomes (10 mg protein/ml) in 0.1 M Tris–HCl buffer (pH 7.5) were incubated anaerobically at 4° for 16 hr with various amounts of trypsin (2–50 μg/mg microsomal protein). Soybean trypsin inhibitor (1 mg/mg microsomal protein) was added to terminate the reaction. The mixture was centrifuged at 105,000 *g* for 90 min; the pellets were diluted to the original volume with 0.1 M Tris–HCl buffer (pH 7.5).

Suspensions of microsomes (10 mg protein/ml) were treated with linoleic acid hydroperoxide (LAHP) to destroy cytochrome P-450 as described previously [11] except that 0.1 M Tris–HCl buffer (pH 7.5) was used instead of phosphate buffer.

Assays. O-Deethylation of NP was determined by a modified method of Shigematsu *et al.* [1]. The incubation mixture (final volume: 1 ml) consisted of hepatic microsomes (0.5 mg protein for NADPH-supported reactions; 1.0 mg protein for NADH-supported reactions), cofactor (0.25 mM NADH, 0.25 mM NADPH or 0.25 mM NADH + 0.25 mM NADPH) and 2 mM NP in 0.1 M Tris–HCl buffer, pH 7.5. The NP was added to the reaction mixture in 0.025 ml acetone. The reaction was started by adding cofactor to the mixture which had been preincubated for 3 min at 37° in open vessels or in closed vessels filled with various mixtures of O_2, N_2 and CO. The complete reaction mixture was incubated for 10 min. The protein content of the medium and the incubation time were shown in preliminary

§ Definitions and abbreviations: Cytochrome P-450 is one or more hemoproteins which exhibit a maximum absorption at or near 450 nm when reduced and complexed with carbon monoxide. Cytochrome P_1-450 is one or more cytochromes induced by the administration of 3-methylcholanthrene. NADPH–cytochrome P-450 reductase is the hepatic microsomal enzyme that transfers electrons from NADPH to cytochrome P-450; the term is used regardless of whether cytochrome P-450 or cytochrome *c* was used as the electron acceptor. NADH–cytochrome b_5 reductase is the hepatic microsomal enzyme that transfers electrons from NADH to cytochrome b_5 even though cytochrome *c* may have been used as the ultimate electron acceptor in its assay. EM = ethylmorphine; NP = *p*-nitrophenetole; MC = 3-methylcholanthrene; PB = phenobarbital; SKF 525-A = 2-diethylaminoethyl 2,2-diphenyl valerate HCl; LAHP = linoleic acid hydroperoxide; AIA = allylisopropylacetamide; TCA = trichloroacetic acid; poly IC = polyriboinosinic acid · polyribocytidylic acid; CIG = control immunoglobin; anti fpT IG = antibody against NADPH–cytochrome P-450 reductase; anti fpD IG = antibody against NADH–cytochrome b_5 reductase; anti b_5 IG = antibody against cytochrome b_5. U-microsomes, MC-microsomes and PB-microsomes are microsomes obtained from untreated, MC-treated and PB-treated animals respectively.

studies to provide optimal conditions for NADH- and NADPH-supported O-deethylation. The reaction was stopped by adding 0.3 ml of a 20% solution of TCA to the mixture, which was allowed to stand for 5 min at 37° before being centrifuged at 3000 rpm for 5 min. One milliliter of the supernatant fraction was transferred to a tube containing 0.25 ml of 4 N KOH solution in 0.5 ml of chloroform. After mixing with a vortex shaker for 20 sec and centrifuging for 5 min at 3000 rpm, the clear aqueous solution containing *p*-nitrophenol was transferred to a spectrophotometer where absorption at 400 nm was recorded. A standard curve was obtained by repeating the entire procedure with *p*-nitrophenol instead of NP. The same procedure was used for the determination of the O-demethylation of *p*-nitroanisole.

The fluorometric methods of Ullrich and Weber [12] and Burke and Mayer [13] were used for the determination of the O-deethylation of 7-ethoxycoumarin (1.0 mM) and ethoxyresorufin (0.25 μM) respectively (incubation time, 5 min; protein concentration, 0.5 or 1.0 mg/ml).

The O-deethylation of 3-*O*-[1′-^{14}C]ethylmorphine (2 mM) was determined by the method of Nerland and Mannering [14] (incubation time, 10 min; protein concentration, 0.5 or 1 mg/ml).

The N-demethylation of EM (2 mM) was determined as described previously [15] (incubation time, 10 min; protein concentration, 0.5 or 1 mg/ml).

The hydroxylation of benzo[*a*]pyrene (64 μM) was determined as described by Wattenberg and Leong [16] (incubation time, 10 min; protein concentration, 0.2 mg/ml).

Protein was determined by the method of Lowry *et al.* [17]; bovine serum albumin was used as the standard.

All monooxygenase assays were conducted at pH 7.5 and with substrate concentrations that provided maximal reaction rates throughout the incubation period.

RESULTS

Effects of partial pressures of dioxygen and carbon monoxide on NADH and NADPH-supported microsomal dealkylations. The effects of various partial pressures of O_2 or CO on NADH- and NADPH-supported O-deethylation of NP and the O- and N-dealkylation of EM in microsomes from untreated rats (U-microsomes) are illustrated in Fig. 1. The dioxygen requirements for NADPH- and NADH-supported O-deethylations of NP are seen to be quite different (Fig. 1A). Near maximal activity of the NADPH-supported reaction occurred when the atmosphere contained 5% O_2, but more than 20% O_2 was required for maximal activity of the NADH-supported reaction. Carbon monoxide had a profound inhibitory effect on NADPH-supported O-deethylation of NP, but had no effect on the NADH-supported reaction. The apparent inhibitory effect of CO on the NADH-supported reaction is due to the lowering of pO_2 that occurs when CO is substituted for O_2, i.e. no difference in activity was observed when CO was replaced with N_2. These observations are in agreement with those of Shigematsu *et al.* [1], who concluded that NADH-supported O-deethylation of NP is not inhibited by CO. It is to be noted in Fig. 1A that, when both NADH and NADPH were present, the reaction rate was greater than the sum of the reaction rates observed with NADH or NADPH alone (synergism) when the dioxygen content of the atmosphere was 10% or

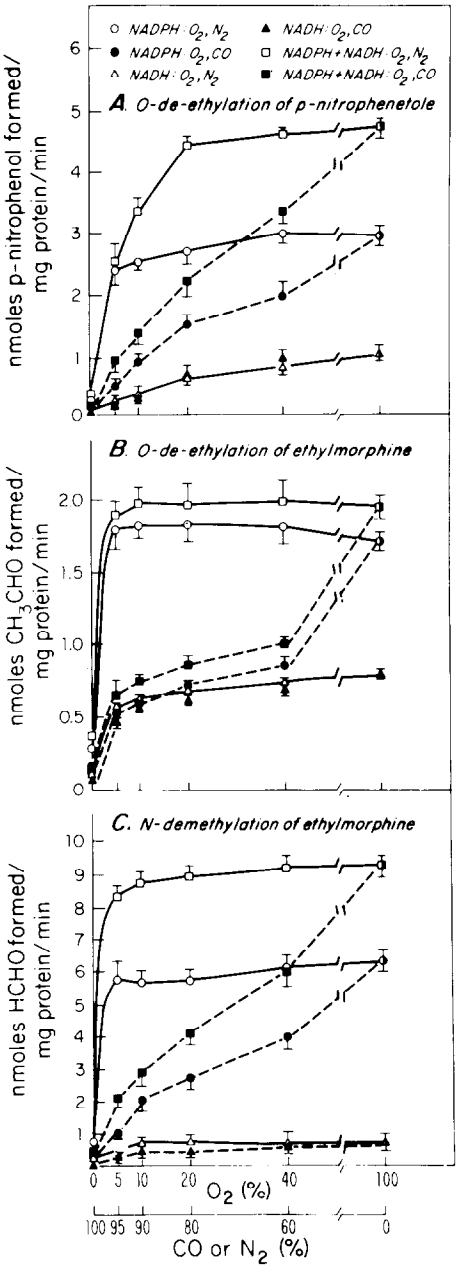


Fig. 1. Effects of partial pressure of dioxygen and carbon monoxide on O-deethylation of *p*-nitrophenetole (A), O-deethylation of ethylmorphine (B) and N-demethylation of ethylmorphine (C) by microsomes from untreated male rats. Reaction mixtures (1 ml) were incubated for 10 min at 37° in closed vessels containing atmospheres of different proportions of N_2 and O_2 or CO and O_2 as indicated. The mixtures contained 0.5 mg protein/ml for NADPH and NADPH + NADH-supported reactions and 1.0 mg microsomal protein for NADH-supported reactions. Each value is the mean ± S.E. of three experiments.

higher but that no synergism occurred when the dioxygen content was 5%. Maximal synergism was not seen unless a dioxygen content of 20% or higher was reached. Thus, both the NADH-supported reaction and synergism of the NADPH-supported reaction were depressed when the pO_2 was only 5%.

These studies were repeated using EM as the substrate for the O-deethylation reaction (Fig. 1B). As with NP, NADPH-supported O-deethylation of EM was inhibited markedly by CO, whereas the NADH-supported reaction was not. However, the NADH-supported O-dealkylation of ethylmorphine differed from that of NP in that it did not require a high pO_2. There was no synergism of the reaction when both NADH and NADPH were present.

The velocity of the NADPH-supported N-demethylation of EM was maximal when the atmosphere contained 5% O_2, and CO was markedly inhibitory (Fig. 1C). The apparent inhibition of the NADH-supported reaction did not prove to be real. Synergism was maximal at a pO_2 of 5%. It is to be noted that the degree of synergism was about the same at all levels of carbon monoxide.

A comparison of the values given in Fig. 1 shows that the O-dealkylation reactions were supported by NADH to about one third the extent supported by NADPH, whereas NADH supported the N-dealkylation reaction to only one-tenth that seen with NADPH.

Induction of NADH- and NADPH-supported microsomal O- and N-dealkylations with phenobarbital and 3-methylcholanthrene and the effects of dioxygen and carbon monoxide on the induced reactions. The effects of inducing agents, dioxygen con-

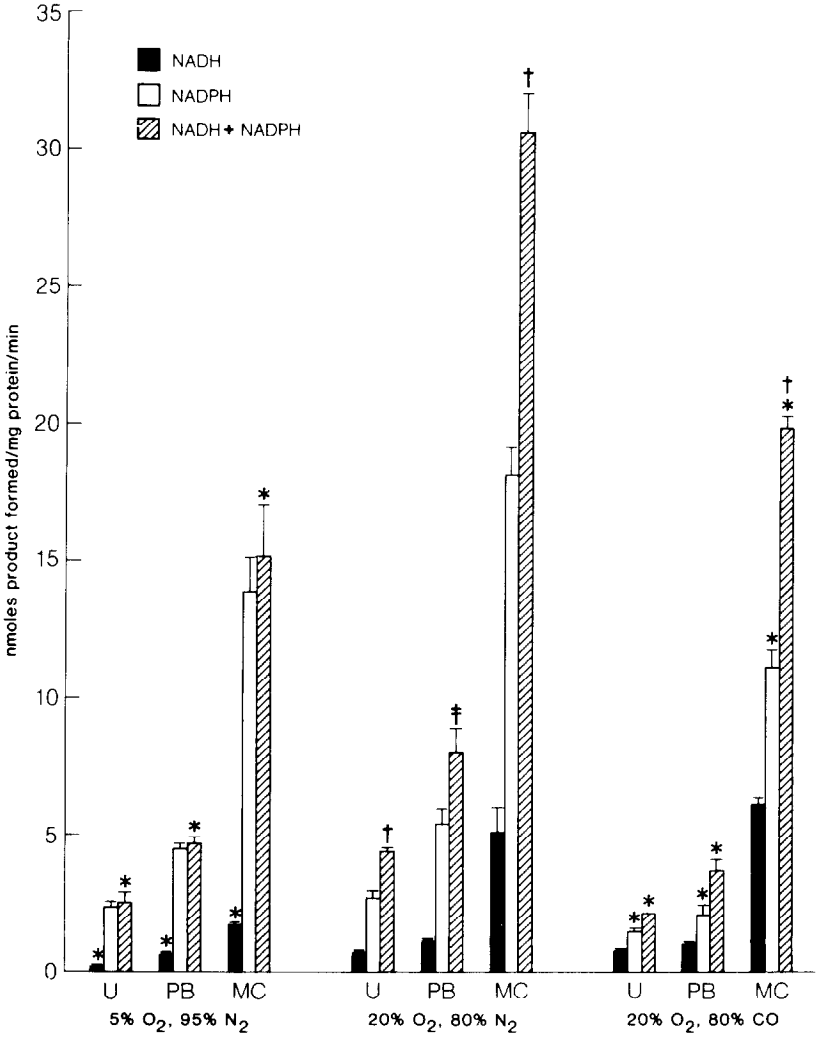


Fig. 2. Effects of partial pressure of dioxygen and carbon monoxide on NADH- and NADPH-supported O-deethylation of *p*-nitrophenetole by hepatic microsomes from untreated (U), phenobarbital (PB)-treated and 3-methylcholanthrene (MC)-treated rats. Experimental conditions were the same as those given in the legend of Fig. 1. Each value is the mean ± S.E. of three experiments. Key: (*) significantly ($P < 0.05$) lower than the corresponding value obtained with an atmosphere of 20% O_2, 80% N_2; (†) significant ($P < 0.05$) synergism, $([(A - B)/C] \times 100) - 100$, where A = the velocity when both NADH and NADPH were present, B = the velocity when only NADH was present, and C = the velocity when only NADPH was present; and (‡) as with (†) except $P < 0.01$.

centration, and carbon monoxide on NADH- and NADPH-supported NP and EM O-deethylations and EM N-demethylation are shown in Figs. 2–4. Under atmospheric conditions (20% O_2, 80% N_2) NADH- and NADPH-supported NP O-deethylation reactions were induced 1.5- to 2-fold by PB (Fig. 2). A similar induction of NADPH-supported O-deethylation of EM was observed but the NADH-supported reaction was not induced (Fig. 3). This suggests that different P-450 cytochromes may be involved in NADH- and NADPH-supported O-deethylations of EM. NADH- and NADPH-supported N-demethylations of EM were induced about 1.5- and 2.5-fold, respectively, by PB (Fig. 4).

Under normal atmospheric conditions, MC induced NADH- and NADPH-supported NP O-deethylation 8.0- and 6.6-fold, respectively (Fig. 2), but had no effect on the NADH- and NADPH-supported O- and N-dealkylations of EM (Figs. 3 and 4). These results suggest that the P-450 cytochromes involved in the NADH- and NADPH-supported O-deethylation of NP are not the same as those involved in the O- and N-dealkylations of EM.

Regardless of the source of microsomes, whether from untreated, PB-treated or MC-treated rats, an atmosphere of 5% O_2, 95% N_2 failed to support maximal NADH-supported O-deethylation of NP nor was the reaction synergized when both NADPH and NADH were present (Fig. 2). On the other hand, the NADPH-supported reaction proceeded equally well in 5 and 20% dioxygen atmospheres. This suggests that the cytochrome P-450(s) involved predominantly in the NADH-supported reaction in U-, PB- and MC-microsomes is not the same as that involved in the NADPH-supported reaction.

The rates of O- and N-dealkylation of EM were not depressed in the 5% dioxygen atmosphere regardless of the source of microsomes, the source of electrons or the substrate (Figs. 2–4). This suggests that the cytochrome P-450 involved in the NADH-supported O-deethylation of NP differs markedly from the P-450 cytochrome(s) involved in the other two reactions.

The substitution of carbon monoxide for nitrogen in a 20% O_2, 80% N_2 atmosphere depressed NADPH-supported O-deethylation of NP by about 50% in U-, PB- and MC-microsomes, but had no significant effect on the NADH-supported reactions in these microsomes (Fig. 2). The inhibitory effect of carbon monoxide on the O- and N-dealkylations of EM was very similar to that observed with NP except that carbon monoxide inhibited the O-deethylation of EM by about 30% in MC-microsomes (Figs. 3 and 4). These studies show that an atmosphere containing 80% carbon monoxide has little or no inhibitory effect on the NADH-supported N- and O-dealkylation reactions in hepatic microsomes from all three sources. These studies suggest that, when carbon monoxide is present, all three dealkylation reactions are mediated by NADH-dependent P-450 cytochromes that are different from those involved in the same NADPH-supported reactions in the absence of carbon monoxide.

From the preceding series of experiments it becomes evident that an NADH-supported NP *O*-deethylase is present in hepatic microsomes that (a) can be highly induced by MC, (b) requires a relatively high pO_2 for maximal activity, and (c) is not inhibited measurably by carbon monoxide. The failure of carbon monoxide to inhibit the reaction may raise some doubts that it is, in fact, mediated by cytochrome P-450. Criteria other than the inhibition by carbon monoxide can be used to support the involvement of cytochrome P-450 in oxidative microsomal reactions.

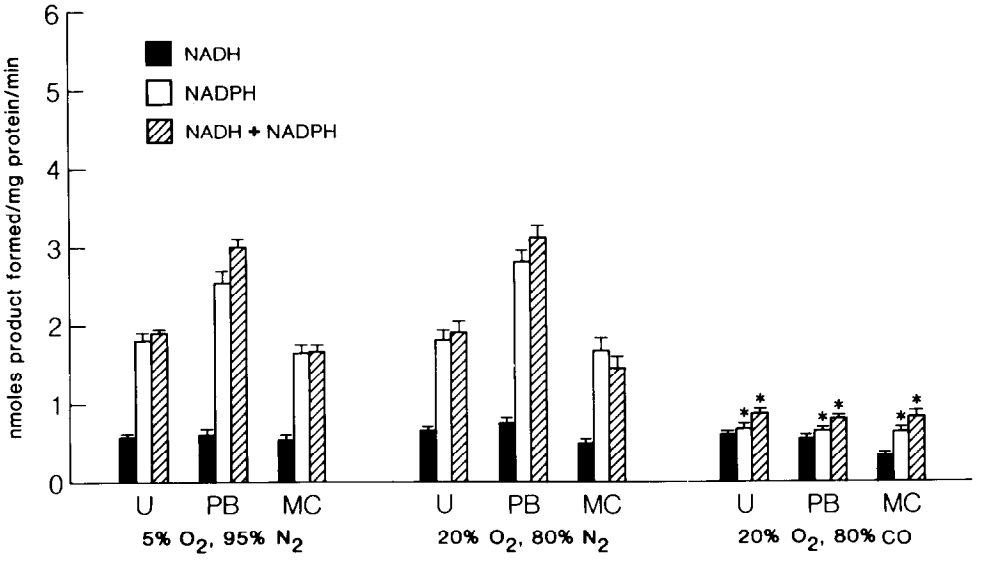


Fig. 3. Effects of partial pressure of dioxygen and carbon monoxide on NADH- and NADPH-supported O-deethylation of ethylmorphine by hepatic microsomes from untreated (U), phenobarbital (PB)-treated and 3-methylcholanthrene (MC)-treated rats. Experimental conditions were the same as those given in the legend of Fig. 1. Each value is the mean ± S.E. of three experiments. Key: (*) significantly ($P < 0.05$) lower than the corresponding value obtained with an atmosphere of 20% O_2, 80% N_2.

The experiments described in the next sections were initiated with this in mind.

Inhibition of p-*nitrophenetole O-deethylation by SKF* 525-*A and* α-*naphthoflavone.* SKF 525-A, the classic inhibitor of P-450-type hemoproteins induced by PB, and α-naphthoflavone, the classic inhibitor of P_1-450-type hemoproteins induced by MC [see Ref. 18 for review], were used to determine which of these two classes of cytochrome P-450 are involved in NADH- and NADPH-supported O-deethylation of NP. I_{50} values were determined from plots of enzyme activity against the logarithm of four concentrations of inhibitor. The NP concentration was 2 mM. I_{50} values (mM, means of two experiments) for the inhibition of NADH-supported dealkylation by SKF 525-A in MC- and PB-microsomes were 3.7 and 0.23 respectively; corresponding values for the NADPH-supported reaction in MC- and PB-microsomes were 1.0 and 0.16; I_{50} values for the inhibition of the NADH- and NADPH-supported reactions by α-naphthoflavone in MC- and PB-microsomes were 0.002 and >1 respectively. These results demonstrate that a P_1-450-type cytochrome is involved predominantly in both NADH- and NADPH-supported O-deethylations of NP in MC-microsomes and that a non-P_1-450 cytochrome is involved predominantly in these reactions in PB-microsomes. However, the P_1-450-type cytochrome principally responsible for the NADPH-supported reaction in MC-microsomes cannot be the same as that involved in the NADH-supported reaction because the former was inhibited by carbon monoxide and the latter was not (Fig. 2).

The observation that SKF 525-A was a more effective inhibitor of both NADH- and NADPH-supported reactions in PB-microsomes than in MC-microsomes, and that the reverse was the case for α-naphthoflavone, suggests that the cytochrome P-450 involved in the NP O-deethylation in PB-microsomes may resemble the P-450 rather than the P_1-450 type. If this is the case, it can be said that both P-450 and P_1-450-type cytochromes exist which are resistant to inhibition by carbon monoxide and require a relatively high pO_2 for maximal activity.

Effects of AIA, Co^{2+}, Mn^{2+}, *and poly IC on the O-deethylation of* p-*nitrophenetole.* Hepatic cytochrome P-450 levels can be lowered by the administration of Co^{2+} [19], Mn^{2+} [20], AIA [21] or polyriboinosinic acid · polyribocytidylic acid (poly IC) [22] to rats. Co^{2+}, Mn^{2+}, AIA or poly IC caused losses of microsomal cytochrome P-450 ranging between 35 and 50% (Table 1). Losses of NADPH- or NADPH + NADH-supported O-deethylation roughly paralleled losses of cytochrome P-450. On the other hand, the NADH-supported reaction was

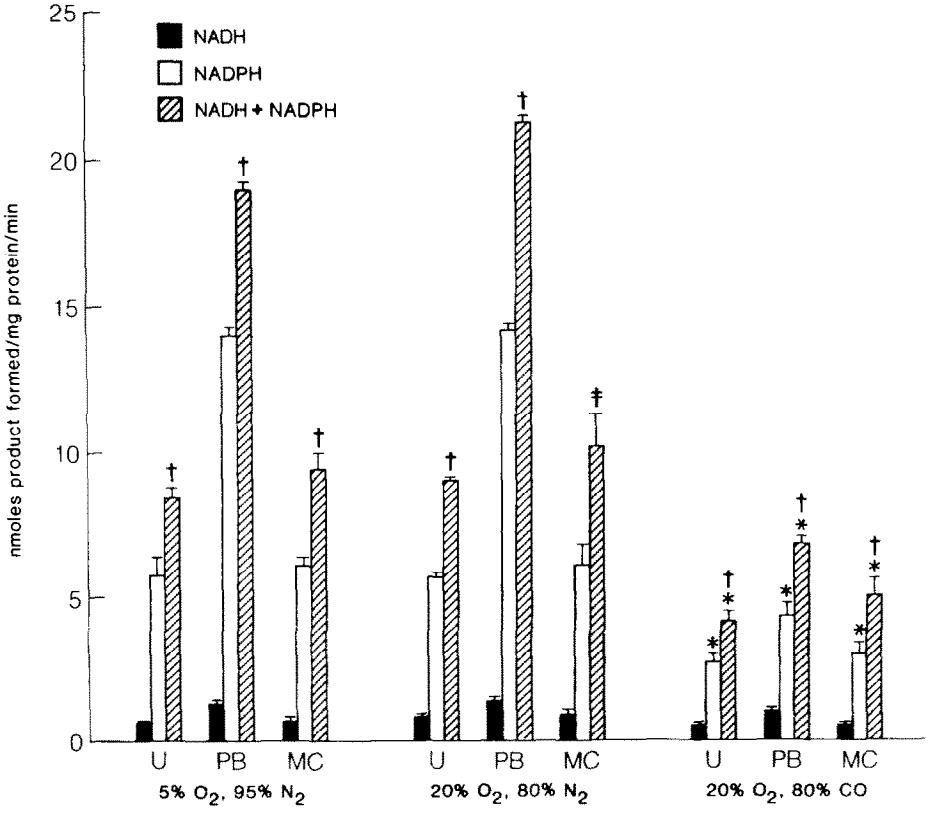


Fig. 4. Effects of partial pressure of dioxygen and carbon monoxide on NADH- and NADPH-supported N-demethylation of ethylmorphine by hepatic microsomes from untreated (U), phenobarbital (PB)-treated and 3-methylcholanthrene (MC)-treated rats. Experimental conditions were the same as those given in the legend of Fig. 1. Each value is the mean ± S.E. of three experiments. Key: (*) significantly ($P < 0.05$) lower than the corresponding value obtained with an atmosphere of 20% O_2, 80% N_2; (†) significant ($P < 0.05$) synergism (see legend of Fig. 2); and (‡) as with (†) except $P < 0.01$.

Table 1. Effects of administered Co^{2+}, Mn^{2+}, AIA and poly IC on microsomal O-deethylation of *p*-nitrophenetole

	% of Control*				
			O-Deethylation		
Agent	Cyt. b_5	Cyt. P_{450}	NADH	NADPH	NADH + NADPH
$CoCl_2 \cdot 6H_2O$†	101.3 ± 4.3	59.3 ± 2.3	94.2 ± 3.2	65.3 ± 1.2	62.7 ± 1.3
$MnCl_2 \cdot 4H_2O$‡	95.0 ± 8.7	65.0 ± 5.3	102.0 ± 4.4	69.0 ± 2.2	67.6 ± 1.5
poly IC§	92.7 ± 5.2	60.3 ± 4.2	120.1 ± 5.8	65.3 ± 0.9	71.0 ± 5.6
AIA‖	98.0 ± 3.8	50.0 ± 5.2	103.3 ± 3.7	70.7 ± 2.9	66.3 ± 1.9

Rats were killed 24 hr after injection of the agents except when AIA was used, in which case the animals were killed 5 hr after the injection. Each value is the mean ± S.E. of three experiments.

* Percent of values obtained with untreated rats: 100% values for cyt. b_5 and cyt. P-450 were 0.46 and 0.75 nmoles/mg protein, respectively, and for NADH- NADPH- and NADPH + NADH-supported reactions, 0.68, 2.95 and 4.22 nmoles *p*-nitrophenol formed/mg protein/min, respectively.

† Fifty mg/kg, s.c., single injection.

‡ Ten mg/kg, s.c., single injection.

§ Ten mg/kg, i.p., single injection.

‖ Four hundred mg/kg, i.p., single injection.

not affected by any of these agents. The results obtained with Co^{2+} are similar to those reported by Shigematsu and coworkers [2], who used rabbits rather than rats.

Effect of treatment of microsomes with linoleic acid hydroperoxide (LAHP) on the O-deethylation of p-*nitrophenetole.* Additions of increasing amounts of LAHP to microsomes cause increasing losses of cytochrome P-450 without losses of cytochrome b_5, NADH–cytochrome b_5 reductase or NADPH–cytochrome P-450 reductase [11]. The loss of cytochrome P-450 is biphasic; low concentrations of LAHP remove about half of the cytochrome P-450, but much higher concentrations are required to remove that which remains. The effects of LAHP on electron transfer components and monooxygenase activities of microsomes from untreated, PB-treated and MC-treated rats are shown in Fig. 5. LAHP-induced losses of NADH-, NADPH- and NADH + NADPH-supported NP O-deethylation equaled losses of cytochrome P-450 in U- and PB-microsomes (Fig. 5A, and B). In MC-microsomes, the losses of NADH-supported O-deethylation equalled losses of cytochrome P-450 in U- and PB-

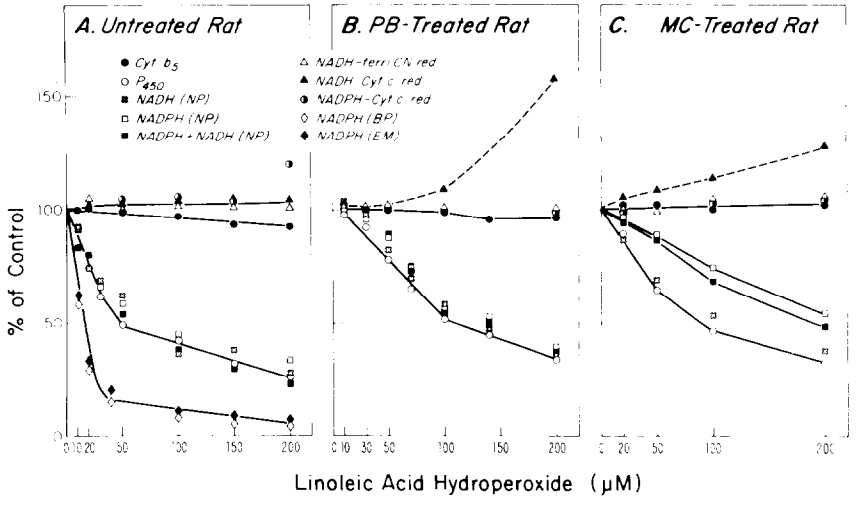


Fig. 5. Effects of linoleic acid hydroperoxide (LAHP) on the NADH- and NADPH-supported O-deethylation of *p*-nitrophenetole (NP) and components of cytochrome P-450 systems in microsomes from untreated (U), phenobarbital (PB)- and 3-methylcholanthrene (MC)-treated rats. Microsomes were treated with linoleic acid hydroperoxide (LAHP) as described previously [11]. Reaction mixtures were incubated in open vessels for 10 min at 37°. When NADH-supported reactions were evaluated, the concentration of protein in the mixture was 1.0 mg/ml; when NADPH- or NADPH + NADH-supported reactions were measured, the concentration of protein was 0.5 mg/ml. NADH–ferri CN red· and NADH cytochrome *c* red· are NADH–cytochrome b_5 reductase activities which were determined with ferricyanide and cytochrome *c* as the electron acceptors respectively; NADPH–cytochrome *c* red· is NADPH–cytochrome P-450 reductase activity determined with cytochrome *c* as the electron acceptor. Each value is the mean value from two experiments.

of NADPH- or NADPH + NADH-supported O-dealkylation paralleled, but were not as great as, those of cytochrome P-450 (Fig. 5C).

Effect of cyanide on the O-deethylation of p-*nitrophenetole.* Cyanide in relatively high concentrations combines with cytochrome P-450 [23]. NADPH-supported O-deethylation of NP in U-microsomes was inhibited 10, 12 and 30% by concentrations of KCN of 0.5, 1 and 5 mM, respectively, but no inhibition of the NADH-supported reaction was observed at any of these concentrations of cyanide. When hepatic microsomes from the rabbit were used, both NADH- and NADPH-supported NP O-demethylation were inhibited about 35% by 5 mM KCN [2].

NADPH–cytochrome P-450 reductase, NADH–cytochrome b_5 reductase and cytochrome b_5 are established electron transport components of cytochrome P-450 systems [see Ref. 24 for review]. The following studies were designed to determine the extent of involvement of these components in the NADH- and NADPH-supported reactions under investigation.

Effect of inhibition of NADPH–cytochrome P-450 reductase with NADP$^+$ on NADH-supported p-*nitrophenetole O-deethylation.* $NADP^+$ is an inhibitor of NADPH–cytochrome P-450 reductase but not of cytochrome b_5 reductase [25, 26]. In Fig. 6 it can be seen that $NADP^+$ is not an inhibitor of NADH-supported NP O-deethylation in U-, PB- or MC-microsomes: thus NADPH–cytochrome P-450 reductase appears not to be involved in the reaction. $NADP^+$ inhibited NADPH-supported O-deethylation, thus implicating the involvement of NADPH–cytochrome P-450 reductase in that reaction.

Effect of enzymic removal of microsomal NADPH–cytochrome P-450 reductase on NADH- and NADPH-supported O-deethylation of p-*nitrophenetole.* Mild digestion of microsomes with trypsin removes NADPH–cytochrome P-450 reductase without appreciable solubilization or destruction of NADH–cytochrome b_5 reductase, cytochrome P-450 or cytochrome b_5 [27–29]. Figure 7 shows the effects of trypsin digestion of PB-microsomes on the O-deethylation of NP. At a concentration of trypsin of 10 μg/mg of microsomal protein, losses of cytochrome b_5, NADH–cytochrome b_5 reductase or NADH-supported *O*-deethylase were 15% or less whereas the loss of cytochrome P-450 was 45% (Fig. 7A). At a concentration of 20 μg/mg, losses of cytochrome b_5, NAPH–cytochrome b_5 reductase and NADH-supported O-deethylation were increased to about 50% and the loss of P-450 was about 65% (Fig. 7B). The loss of the NADPH-supported reaction differed markedly from that of the NADH-supported reaction; concomitant and equal losses of NADPH–cytochrome P-450 reductase and NADPH- and NADPH + NADH-supported O-deethylation with increasing concentrations of trypsin were observed until only 25% of the original activities remained. This illustrates the requirement of NADPH–cytochrome P-450 reductase for the NADPH-supported reaction and the non-essentiality of this enzyme for the NADH-supported reaction. When microsomes were digested with the higher concentrations of trypsin (10–50 μg/mg microsomal protein), as much as 95% of the NADPH–cytochrome P-450 reductase activity and NADPH- and NADPH + NADH-supported O-deethylation were destroyed (Fig. 7B). These higher levels of trypsin also caused concomitant and equivalent loss of cytochrome P-450. The close correlation of losses of cytochrome b_5,

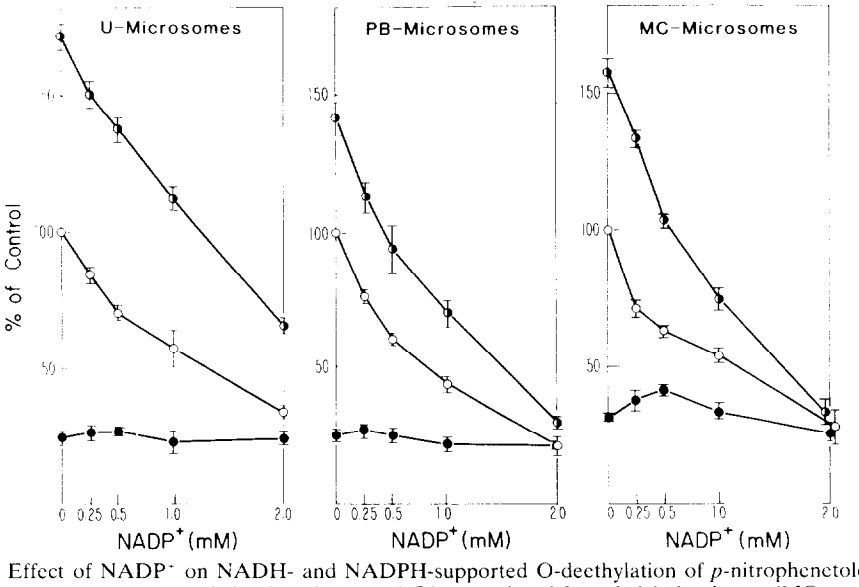


Fig. 6. Effect of $NADP^+$ on NADH- and NADPH-supported O-deethylation of *p*-nitrophenetole by microsomes from untreated (U), phenobarbital (PB)-treated and 3-methylcholanthrene (MC)-treated rats. Reaction mixtures were incubated in open vessels for 10 min at 37°. Mixtures contained 0.5 or 1.0 mg microsomal protein/ml, respectively, when NADPH- and NADH-supported reactions were evaluated. Values are percentages of the NADPH-supported reactions in the absence of $NADP^+$ (2.94, 5.14 and 18.9 nmoles *p*-nitrophenol formed/mg protein/min for U-, PB- and MC-microsomes respectively). Key: NADPH, (—○—); NADH, (—●—); NADPH + NADH: (—◑—). Each value is the mean ± S.E. of three experiments.

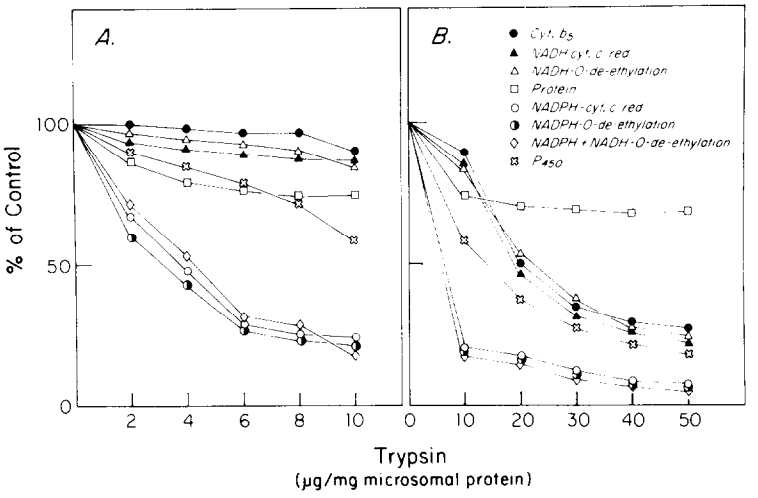


Fig. 7. Effect of trypsin treatment of microsomes from phenobarbital-treated rats on NADH- and NADPH-supported O-deethylation of *p*-nitrophenetole and components of cytochrome P-450 systems. Microsomes were incubated with the indicated amounts of trypsin as described in Materials and Methods. A mixture containing an amount of the trypsin-digested microsomes equivalent to 1.0 mg of microsomal protein was incubated for 10 min in open vessels at 37°. 100% control values: cytochromes P-450 and b_5, 1.68 and 0.57 nmoles/mg protein, respectively; NADPH–cytochrome P-450 and NADH–cytochrome b_5 reductases, 312 and 960 nmoles cytochrome *c* reduced/mg protein/min, respectively; NADH-, NADPH- and NADH + NADPH-supported O-deethylation, 1.52, 6.08 and 8.25 nmoles *p*-nitrophenol formed/mg protein/min; protein, 1.0 mg. Each value is the mean of two experiments.

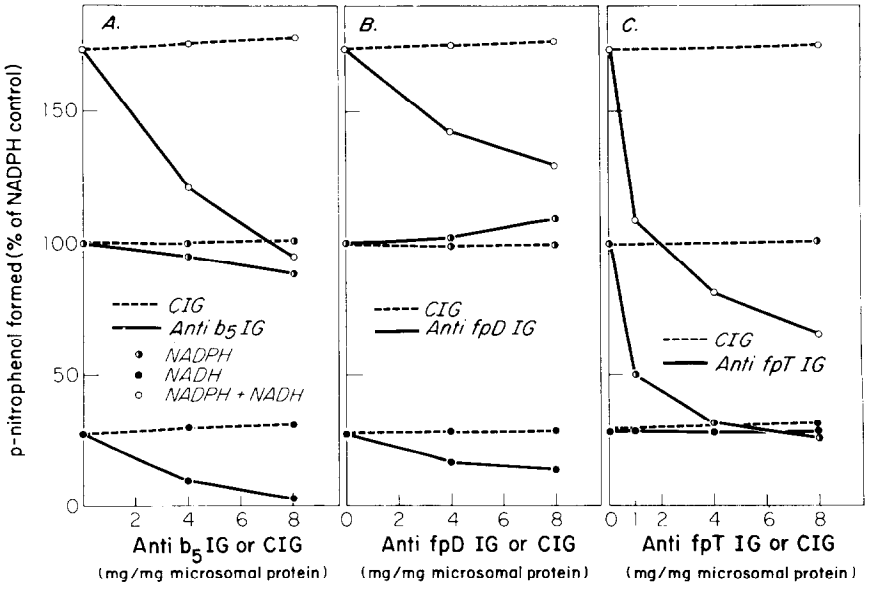


Fig. 8. Inhibitory effects of antibodies against (A) cytochrome b_5 (anti b_5 IG), (B) NADH–cytochrome b_5 reductase (anti f_pD IG) and (C) NADPH–cytochrome P-450 reductase (anti f_pT IG) on the NADH- and NADPH-supported O-deethylation of *p*-nitrophenetole by microsomes from 3-methylcholanthrene-treated male rats. Mixtures of microsomes (0.1 mg/ml for NADPH- or NADPH + NADH-supported reactions; 0.2 mg for NADH-supported reactions) were incubated with the indicated amounts of anti b_5 IG, anti f_pD IG or anti f_pT IG in 0.1 M Tris–HCl buffer (pH 7.5) at 37° for 10 min. NP (2 mM) was then added, and the mixtures were incubated for another 10 min. The rates of the NADPH-supported reactions (NADPH controls) were 19.5, 17.2 and 18.5 nmoles *p*-nitrophenol formed/mg protein/min in A, B and C respectively. Each value is the mean of three experiments.

NADH–cytochrome b_5 reductase, and NADH-supported O-deethylation suggests roles for both cytochrome b_5 and its reductase in the reaction.

Shigematsu and associates [3] obtained similar results with trypsin when rabbit microsomes were used.

Effects of antibodies to cytochrome b_5, *NADH–cytochrome* b_5 *reductase and NADH–cytochrome P-450 reductase.* The inhibitory effects of antibodies to cytochrome b_5 (anti b_5 IG), NADH–cytochrome b_5 reductase (anti f_pD IG) and NADPH–cytochrome P-450 reductase (anti f_pT IG) on NADH- and NADPH-supported O-deethylation of NP in MC-microsomes are illustratgd in Fig. 8. At the highest concentration of anti b_5 IG (Fig. 8A), both the NADH-supported O-dealkylation reaction and the synergism of the NADPH-supported reaction by NADH were inhibited almost completely. Anti b_5 IG had little or no effect on the NADPH-supported reaction. This reinforces the view that cytochrome b_5 is required for both the NADH-supported O-dealkylation of NP and the NADH synergism of the NADH-supported reaction, but not for the NADPH-supported reaction.

When anti f_pD IG was substituted for anti b_5 IG, results were similar to those seen in Fig. 8A except that anti f_pD IG had less of an inhibitory effect on the NADH-supported O-deethylation reaction and its synergism by NADH (Fig. 8B). This supports the view that NADH–cytochrome b_5 reductase is required for NADH-supported O-dealkylation of NP and the NADH-synergized NADPH-supported reaction, but that it is not required for the NADPH-supported reaction.

At the highest level used, anti f_pT IG had no effect on NADH-supported O-dealkylation, but it markedly inhibited the NADPH-supported reaction (Fig. 8C). This reaffirms the requirement of NADPH–cytochrome P-450 reductase for the NADPH-supported deethylation of NP and its non-involvement in the NADH-supported reaction.

Figure 9 illustrates the effects of anti b_5 IG and anti f_pT IG on NADH- and NADPH-supported N-demethylation of EM in PB-microsomes. Anti b_5 IG had no effect on NADPH-supported N-demethylation, but inhibited NADH-synergism of the NADPH-supported reaction almost completely (Fig. 9A), thus confirming previous studies by Sasame *et al.* [30] and Mannering *et al.* [31]. Anti b_5 IG inhibited NADH-supported N-demethylation almost completely. Anti f_pT IG inhibited NADPH-supported N-demethylation, but had no effect on the NADH-supported reaction (Fig. 9B). These experiments demonstrate the involvement of cytochrome b_5 in NADH-supported N-demethylation of EM, the non-involvement of cytochrome b_5 in the NADPH-supported reaction, and the involvement of NADPH–cytochrome P-450 reductase in the NADPH-supported reaction.

The antibody studies were extended to include four additional substrates: *p*-nitroanisole, 7-ethoxycoumarin, ethoxyresorufin, and benzo[*a*]pyrene (Table 2). In all cases, NADH-supported reactions were inhibited by anti b_5 IG, but not by anti f_pT IG; NADH synergism of NADPH-supported reactions were inhibited by anti b_5 IG. For the substrates examined, it appears that cytochrome b_5 is the common intermediate for NADH-supported reactions

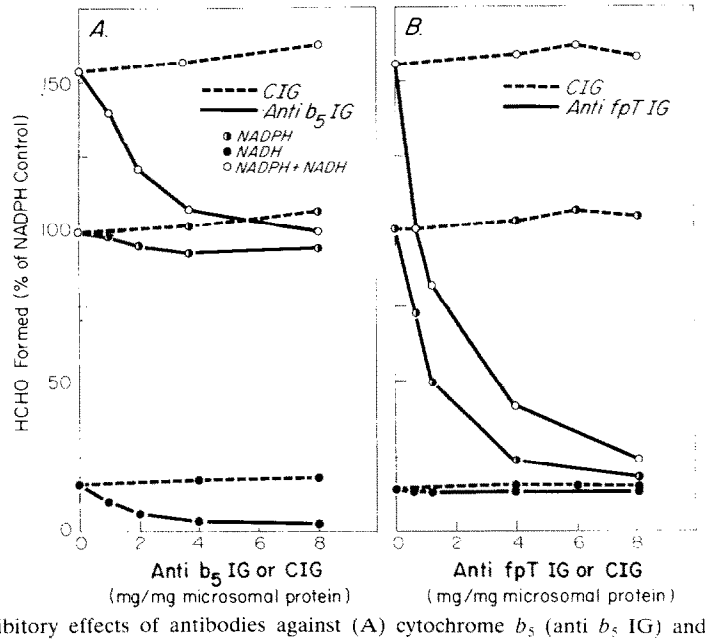


Fig. 9. Inhibitory effects of antibodies against (A) cytochrome b_5 (anti b_5 IG) and (B) NADPH–cytochrome P-450 reductase (anti f_pT IG) on NADH- and NADPH-supported N-demethylation of ethylmorphine by microsomes from phenobarbital-treated male rats. Incubation conditions were the same as those given in the legend of Fig. 8 except that 0.5 or 1.0 mg protein/ml of incubation mixture was used instead of 0.1 or 0.2 mg. The rate of the NADPH-supported reaction (NADPH control) was 14.4 and 15.8 nmoles HCHO formed/mg protein/min in A and B respectively. Each value is the mean of three experiments.

Table 2. Inhibition of microsomal monooxygenase reactions by antibodies against cytochrome b_5 (anti b_5 IG) and NADPH–cytochrome *c* reductase (anti f_pT IG)

Substrate (reactions)	Source of electrons	Product formation* (% of NADPH control)					
		Control†	CIG 8 mg/kg	anti b_5 IG 4 mg/kg	anti b_5 IG 8 mg/kg	anti f_pT IG 4 mg/kg	anti f_pT IG 8 mg/kg
p-Nitrophenetole (O-deethylation)	NADH	28	31	10	2	28	30
	NADPH	100	100	95	89	30	29
	NADH + NADPH	175	178	121	95	80	65
p-Nitroanisole (O-deethylation)	NADH	21	23	14	5	19	20
	NADPH	100	105	98	82	32	27
	NADH + NADPH	164	172	128	100	95	52
7-Ethoxycoumarin (O-deethylation)	NADH	12	11	9	3	11	12
	NADPH	100	110	100	95	35	20
	NADH + NADPH	130	133	105	99	92	62
Ethoxyresorufin (O-deethylation)	NADH	21	23	11	6	22	18
	NADPH	100	92	94	86	22	18
	NADH + NADPH	147	151	108	89	82	58
Ethylmorphine (O-deethylation)	NADH	32	34	10	8	31	33
	NADPH	100	105	95	82	30	22
	NADH + NADPH	105	107	110	102	39	32
Ethylmorphine (N-demethylation)	NADH	14	18	2	1	16	15
	NADPH	100	104	92	93	24	18
	NADH + NADPH	152	156	106	100	38	24
Benzo[*a*]pyrene (hydroxylation)	NADH	15	16	5	3	16	14
	NADPH	100	105	92	88	28	13
	NADH + NADPH	105	105	92	89	30	15

MC-microsomes were used except when EM was the substrate, in which case PB-microsomes were used. Experimental conditions were the same as those given in Figs. 8 and 9. Values are the means of two experiments except when the O-deethylation of NP and the N-demethylation of EM were measured, in which cases values are the means of three experiments.

* Values for NADPH-supported reactions using U-microsomes (nmoles product formed/mg protein/min): NP, 18.5; *p*-nitroanisole, 4.8; 7-ethoxycoumarin, 6.2; ethoxyresorufin, 1.5; EM (O-deethylation), 2.5; EM (N-demethylation), 14.4; and benzo[*a*]pyrene, 1.9.

† Values obtained in the absence of CIG, anti b_5 IG or anti f_pT IG.

and that NADPH–cytochrome P-450 reductase and cytochrome b_5 are involved in the NADH synergism of NADPH-supported reactions.

DISCUSSION

To facilitate discussion, pertinent results are summarized in Table 3. These results permit the following conclusions and speculations regarding the individuality of the cytochrome P-450 systems involved in the NADH- and NADPH-supported O-deethylations of NP and EM and the N-demethylation of EM.

(1) *NADH- and NADPH-supported O-deethylations of NP involve different cytochrome P-450s.* The NADH-supported reaction was not inhibited by carbon monoxide, it required a high pO_2 for maximum activity, and it was not inhibited by high concentrations of cyanide. The NADPH-supported reaction was inhibited by carbon monoxide, it did not require a high pO_2, and it was inhibited by cyanide. In addition, the NADPH-supported reaction was depressed in hepatic microsomes from rats that had been administered Co^{2+}, Mn^{2+}, AIA or poly IC; the NADH-supported reaction was not. However, NADH- and NADPH-supported reactions shared some common features: both were strongly inhibited by α-naphthoflavone and weakly inhibited by SKF 525-A, both were destroyed by LAHP, and both were induced by MC and PB. The antibody studies demonstrated that both reactions depended on established components of microsomal electron transfer systems involved in cytochrome P-450-linked monooxygenase reactions. Thus, it is apparent that, whereas the NADH-supported system differs from the NADPH-supported system in many of its features, and from cytochrome P-450 systems in general in its requirement for a high pO_2 and its insensitivity to carbon monoxide, it satisfies many other criteria of cytochrome P-450 systems.

The cytochrome P-450s involved in both the NADH- and the NADPH-supported reactions were highly induced by MC; both reactions were inhibited strongly by α-naphthoflavone. These two observations argue strongly that P_1-450 type cytochromes are involved primarily in both reactions even though they differ in their affinities for carbon monoxide and dioxygen. The observation that cytochrome P-450s can differ markedly in their affinities for carbon monoxide and dioxygen is not too surprising in view of the reports by Cooper and associates [32] that treatment of rats with 2,3,7,8-tetrachlorobenzo-*p*-dioxin causes a 6- to 9-fold increase in the ratio of carbon monoxide to dioxygen required to inhibit NADPH-supported benzo[*a*]pyrene hydroxylation by 50%, and by Gray [33], who distinguished three types of cytochrome P-450 in rat PB-microsomes by their second-order rate constants of carbon monoxide binding. The three cyanide binding constants

Table 3. Abbreviated summary of results

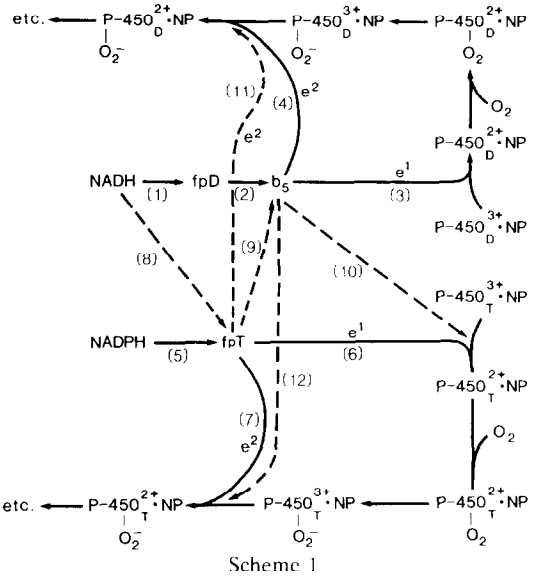
Observation	Type of microsomes			Monooxygenase activity					
				NP O-deethylation		EM O-deethylation		EM N-demethylation	
	U	PB	MC	NADH	NADPH	NADH	NADPH	NADH	NADPH
In vitro									
Inhibition by CO	×			−	+	+*	+	−	+
		×		−	+	+*	+	−	+
			×	−	+	+	+	−	+
High O_2 requirement	×	×	×	+	−	−	−	−	−
Inhibition by SKF 525-A		×		+*	+*				
			×	−	−				
Inhibition of α-naphthoflavone		×		−	−				
			×	+	+				
Inhibition by CN^-	×			−	+				
Destruction by LAHP	×	×	×	+	+				
Inhibition by $NADP^+$	×	×	×	−	+				
Inhibition by anti b_5 IG		×						+	
			×	+	−				
Inhibition by anti f_pT IG		×						−	+
			×	−	+				
Inhibition by anti f_pD IG			×	+	−				
In Vivo									
Induced by PB				2×	2×	NS†	1.5×	1.5×	2.5×
Induced by MC				8×	6.5×	<1×	NS	NS	NS
Depressed by Co^{2+}				−	+				
Depressed by Mn^{2+}				−	+				
Depressed by AIA				−	+				
Depressed by polyIC				−	+				

* Statistically meaningful at the $P < 0.1$ level; all other designations were statistically meaningful at the $P < 0.05$ level.
† NS = not significant statistically.

of microsomal cytochrome P-450 described by Comai and Gaynor [23] are relevant to the observation of Gray and to the current studies (including the different effects of cyanide on the two reactions) because dioxygen, carbon monoxide and cyanide are believed to compete for the same binding site on cytochrome P-450. Mori and associates [34] have recently described a cytochrome P-450 system in rat liver that demethylates 3′-methyl-*N,N*-dimethyl-4-aminoazobenzene, is not inhibited by carbon monoxide, and is inhibited by α-naphthoflavone.

The failure of the administration of Co^{2+}, Mn^{2+}, AIA or poly IC to depress the NADH-supported reaction does not in itself exclude the role of a cytochrome P-450 in the reaction because these agents can depress individual cytochrome P-450s selectively, e.g. AIA destroys only those species of P-450 with which it reacts suicidally [see Ref. 35 for review] and species of cytochrome P-450 that are turning over most rapidly are more readily depressed by poly IC [36]. Rather, the use of these agents serves to further distinguish the cytochrome P-450 systems responsible for the NADH- and NADPH-supported reactions.

(2) *The NADH- and NADPH-supported O-deethylations of NP involve separate electron transfer systems.* The two electron transfer pathways for NADH- and NADPH-supported NP O-deethylation are diagramed in Scheme 1.



Scheme 1

In the NADH-supported reaction, the first electron is transferred from NADH via steps 1, 2 and 3; the second electron is introduced via step 4. Step 3 or 4 (or both steps) is inhibited completely by anti b_5 IG. Step 2 is only partially inhibited by anti f_pD IG. f_pD is believed to be embedded more deeply in the membrane than either cytochrome b_5 or f_pT as evidenced by its greater resistance to enzymic removal [8]; this could account for its incomplete inhibition by anti f_pD IG. Because the reaction was inhibited completely by anti f_pT IG when NADPH was the sole source of electrons and because anti f_pT IG had no effect on its NADH-supported reaction (Fig. 8), electrons from NADH cannot be introduced to this electron pathway ($1 \rightarrow 2 \rightarrow 3$) via steps 8 and 9. This also means that electrons from NADPH cannot be transferred to b_5 via steps 5 and 9. In the NADPH-supported reaction, the first electron is transferred via steps 5 and 6, the second electron, via step 7. First or second electrons cannot be transferred via pathways involving steps 9, 10 and 12 because the NADPH-supported reaction is not inhibited by anti b_5 IG.

In summary, cytochrome b_5 is an absolute requirement for the transfer of both electrons 1 and 2 in the NADH-supported reaction, and cytochrome b_5 is not involved in the transfer of either the first or the second electron in the NADPH-supported reaction.

Kuwahara and Omura [37] purified cytochrome P-450_{MC} and P-450_{PB} from MC- and PB-treated rats respectively. In a reconstituted system, cytochrome b_5 was required for NADPH-supported O-deethylation of NP when P-450_{PB} was used, but not when P-450_{MC} was used. The results presented in Fig. 8 suggest that cytochrome P-450_{MC} may be the cytochrome P-450 isozyme predominantly involved in the NADPH-supported reaction in microsomes.

The question is now raised as to how these two electron transfer pathways may interact when both NADH and NADPH are present. Although the antibody studies eliminate the possibility of electrons being transferred from NADPH to the NADH electron transfer pathway, the possibility that electrons from NADH may be transferred to the NADPH electron transfer systems must still be considered. In fact, with certain substrates, the transfer of the second electron via steps 1, 2 and 12 is believed to account for the NADH synergism of many NADPH-supported reactions [see Ref. 24 for review]. Evidence for this concept is repeated in Fig. 9A where it can be seen that anti b_5 IG inhibits the synergism of NADPH-supported EM N-demethylation but has no effect on the NP reaction when NADPH is the only source of electrons. Synergism must be mediated via P-450_D (Scheme 1) because it occurred only after a pO_2 of 5% was exceeded. If it had been mediated via P-450_T via steps 1, 2 and 10 or 1, 2 and 12, synergism should have occurred even though the pO_2 was only 5% because P-450_T activity is not depressed at that low pO_2 when NADPH is the source of electrons. This being the case, one can speculate that when both NADPH and NADH were present it was NADPH synergism of the NADH-supported reaction rather than NADH synergism of the NADPH-supported reaction that occurred. This conclusion implies that P-450_D is primarily an NADH-dependent, not an NADPH-dependent, cytochrome P-450, whereas the reverse is the case for P-450_T.

NADPH synergism of NADH-supported NP O-deethylation can be visualized as follows. When NADH is the only source of electrons, $P\text{-}450_D^{2+} \cdot ND$ competes with $P\text{-}450_D^{3}(O_2) \cdot NP$ for electrons from cytochrome b_5. NADPH electrons transferred directly from f_pT to $P\text{-}450_D^{3+}(O_2) \cdot NP$ via steps 5 and 11 would relieve this competition; electrons transferred via steps 5, 9 and 4 would not. The low pO_2 of 5% prevents synergism because the formation of $P\text{-}450_D^{3+}(O_2) \cdot NP$ becomes rate-limiting for both the synergized and the non-synergized reactions. Carbon monoxide did not prevent synergism (Fig. 2) because it did not compete successfully with dioxygen for $P\text{-}450_D^{2+} \cdot NP$ and therefore did not limit the formation of $P\text{-}450_D^{2+}(O_2) \cdot NP$. The overall NADH + NADPH-supported reaction was inhibited by carbon monoxide because carbon monoxide inhibited $P\text{-}450_T^{2+}(O_2) \cdot NP$ formation.

The experimental findings are compatible with an alternative concept that would implicate the complexing of NP or a metabolite of NP with a conventional cytochrome P-450 to form an atypical but functional cytochrome P-450. Kuwahara and Omura [37] have described a purified rat cytochrome P-450 (P-450_{MC}) that in a reconstituted system requires cytochrome b_5 for the O-deethylation of NP but not for the N-demethylation of EM or the hydroxylation of aniline. Sugiyama and associates [38–40] have isolated a rabbit cytochrome P-450 (P-450_B) which has a high affinity for cytochrome b_5. In a reconstituted system containing P-450_{B1}, cytochrome b_5 is an absolute requirement for the O-demethylation of *p*-nitroanisole but not for the oxidation of benzphetamine, aminopyrine or aniline. These studies suggest that certain substrates (e.g. NP and *p*-nitroanisole) may produce conformational changes in certain P-450s such that they can accept second electrons only via NADH and cytochrome b_5, which is highly bound to this cytochrome P-450, whereas other substrates (e.g. EM, benzphetamine, aminopyrine and aniline) do not. In effect, substrates like NP and *p*-nitroanisole may have created a functionally new "species" of cytochrome P-450. In the context of the current experiments, this substrate-created cytochrome P-450 may have a low affinity for dioxygen and carbon monoxide as well as the other atypical properties that have been described in the current experiments. This concept does not contradict Scheme 1 if the substrate-created cytochrome P-450 is substituted for P-450_D.

(3) *Cytochrome* b_5 *is required for a variety of NADH-supported monooxygenase reactions but appears not to be involved when the same reactions are supported by NADPH.* Anti b_5 IG inhibited the following NADH-supported reactions markedly (Table 2): NP O-deethylation, *p*-nitroanisole O-demethylation, 7-ethoxycoumarin O-deethylation, ethoxyresorufin O-deethylation, EM O-deethylation, EM N-demethylation and benzo[*a*]-pyrene hydroxylation; when supported by NADPH, none of these reactions was inhibited by anti b_5 IG by more than 18%.

(4) *NADH- and NADPH-supported O-deethylation of NP, O-deethylation of EM and N-demethylation of EM involve different cytochrome P-450 systems.* The three dealkylation reactions differ in one or more of several of the categories listed in Table 3. NADH-supported O-deethylation of both NP and EM were not inhibited by carbon monoxide; all three NADPH-supported reactions were inhibited by carbon monoxide. Of the three NADH- and NADPH-supported reactions, only NADH-supported NP O-deethylation required a pO_2 greater than 5% for maximal activity. NADH-supported NP O-deethylation and EM N-demethylation were induced by PB; the O-deethylation of EM was not. All three NADPH-supported reactions were induced by PB. Both NADH- and NADPH-supported NP O-deethylation were highly induced by MC, NADH-supported EM O-deethylation was induced slightly by MC but the NADPH-supported reaction was not, nor was either the NADH- or NADPH-supported N-demethylation of EM induced by MC.

Most cytochrome P-450 monooxygenase reactions are supported by NADH at only 10–20% of the rate supported by NADPH. This has usually been interpreted to mean that NADH can supply an electron to a given ferricytochrome P-450 · substrate complex at a rate only 10–20% of that supported by NADPH. This has been visualized as occurring by transfer of electrons via steps 1, 2 and 10 with the same cytochrome P-450 ($P\text{-}450_T$) being involved in both the NADPH- and NADH-supported reactions. As discussed above, it seems more likely in the case of NADH- and NADPH-supported NP O-deethylation that different cytochrome P-450s ($P\text{-}450_D$ and $P\text{-}450_T$) may be involved in the two reactions. The possibility should therefore be considered that other monooxygenase reactions, including those considered in Table 2, may also employ different cytochrome P-450 isozymes for NADH- and NADPH-supported reactions.

These studies may provoke some interest in the isolation of a cytochrome P-450 that does not react readily with carbon monoxide. However, since by definition, the detection and measurement of cytochrome P-450 in crude preparations depend on the formation of a reduced cytochrome P-450 complex, this might require a different experimental approach than has been used for the visualization of cytochrome P-450s that form carbon monoxide complexes readily. The possibility should also be considered that solubilization and purification of the atypical cytochrome P-450 may increase its affinity for carbon monoxide. This could occur if the cytochrome P-450 in question was complexed with a membrane component that interfered with the carbon monoxide binding site.

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