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Dealkylation of Pentoxyresorufin: A Rapid and Sensitive Assay for Measuring Induction of Cytochrome(s) *P*-450 by Phenobarbital and Other Xenobiotics in the Rat

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The O-dealkylation of pentoxyresorufin (7-pentoxyphenoxazone) by rat liver microsomes was examined. The reaction appeared highly specific for certain phenobarbital inducible forms of cytochrome *P*-450 and was increased 95- to 140-fold by animal pretreatment with phenobarbital (75 mg/kg/day, four ip injections) and ~50-fold by Aroclor 1254 (500 mg/kg, one ip injection) while animal pretreatment with 3-methylcholanthrene (50 mg/kg/day, three ip injections) resulted in less than a 2-fold increase over the rate detected in control microsomes. It was observed that this activity, in microsomes for Aroclor-pretreated rats, was dependent on O_2 and was inhibited by metyrapone and SKF 525-A, indicative of cytochrome(s) *P*-450 mediation in the reaction. When antibodies directed against purified cytochrome(s) *P*-450s were employed to inhibit the pentoxyresorufin O-dealkylation reaction, antibodies to $P\text{-}450_{PB\text{-}B}$ greatly inhibited the reaction (>90%), while antibodies to $P\text{-}450_{PB\text{-}C}$ or $P\text{-}450_{PB/PCN\text{-}E}$ had minimal effects. Assay of hepatic microsomes from rats which were pretreated with varying doses of phenobarbital (0.9–75 mg/kg/day, four ip injections) indicated that while aminopyrine-*N*-demethylase activity was induced only 2-fold at the maximum dose (75 mg/kg/day), pentoxyresorufin O-dealkylase activity was induced ~140-fold at this dose and ~4-fold by a dose of phenobarbital as low as 0.9 mg/kg.

The cytochrome(s) *P*-450 are a group of hemoproteins associated with the microsomal membranes, which together with NADPH cytochrome *P*-450 reductase and reducing equivalents, mediate the primary (Phase I) oxidative metabolism of various xenobiotics. These hemoproteins are readily inducible by a variety of substrates (1-3) resulting in specific cytochrome *P*-450 isozymes which can exhibit singular catalytic activities toward various substrates.

Since the biological efficacies of many substrates are dependent on the particular form(s) of cytochrome(s) *P*-450 being employed to activate them, enzymatic assays which indirectly measure the major types (i.e., phenobarbital or 3-methylcholanthrene) and degree of induction of these various forms of cytochrome(s) *P*-450 have gained considerable importance. Previ-

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ously reported findings of Burke, Mayer, and co-workers (4–9) have demonstrated that dealkylation of specific alkoxyphenoxazone compounds may be mediated preferentially by specific forms of cytochrome *P*-450. Thus, dealkylation of ethoxyresorufin seems to be specific for 3-MC[3] inducible forms of cytochrome *P*-450 in a variety of species (3, 6–9), whereas the dealkylation of pentoxyresorufin is preferentially induced by phenobarbital in the mouse (8) and in the rat (9).

In this report we have utilized pentoxyresorufin (7-pentoxyphenoxazone) as a substrate with which to assay the specific PB-inducible forms of cytochrome *P*-450 in rat hepatic microsomal preparations. This assay appears to be a simple, rapid, and sensitive method for measurement of the major phenobarbital-inducible form of cytochrome *P*-450 ($P\text{-}450_{PB\text{-}B}$).

MATERIALS AND METHODS

Chemicals. Pentoxy- and ethoxyresorufin were synthesized from resorufin as described previously (7). Resorufin, 7,8-benzoflavone (7,8-BF), and 4-dimethylaminoantipyrine (aminopyrine) were purchased from Aldrich Chemical Company, Milwaukee, Wisconsin, 2-methyl-1,2-di-3-pyridyl-1-propanone (metyrapone) and semicarbazide were obtained from the Sigma Chemical Company, St. Louis, Missouri. Phenobarbital sodium (PB) was obtained from Mallinckrodt Chemical Works, St. Louis, Missouri, and Aroclor-1254 was from Analabs, North Haven, Connecticut. 3-Methylcholanthrene (3-MC) was obtained from Eastman-Kodak, Rochester, New York. SKF 525A was a gift from Smith, Kline, and French, Philadelphia, Pennsylvania. Fluorescamine was purchased from Roche Diagnostics, Nutley, New Jersey.

Preparation of samples. Male Fischer rats were obtained from Charles River Breeding Farms, Wilmington, Massachusetts, and were induced with Aroclor 1254 (one ip injection of 500 mg/kg body weight, in corn oil), 3-MC (three ip injections of 50 mg/kg body weight, in corn oil), PB (four daily ip injections of 75, 25, 8.3, 2.8, or 0.9 mg/kg body weight, in Hanks' balanced salt solution, BSS), corn oil (equivalent volume, one ip injection), or Hanks' BSS (equivalent volume, four daily ip injections). The animals were killed by CO_2 asphyxiation 1 day after the last 3-MC treatment, on day 5 of the PB regimen, and 5 days after Aroclor 1254 or corn oil treatment. Hepatic tissue from three rodents per treatment was assayed individually. Hepatic tissue was homogenized in 3 vol of 0.15 M KCl, clarified by centrifugation at 9000*g* and microsomal pellets obtained by recentrifugation at 105,000*g*. All steps were performed at 4°C. Microsomal pellets were washed in 0.15 M KCl and were then resuspended in a volume of 0.25 M sucrose, 0.05 M Tris buffer, pH 7.5, equivalent to that of the postmitochondrial (9000*g*) supernatant.

Pentoxyresorufin O-dealkylase assay. The dealkylation of 7-pentoxyresorufin was measured at 25°C in quartz cuvettes (1 cm path length) by monitoring resorufin formation using an Aminco-Bowman spectrophotofluorimeter fitted with 1.0 and 0.5 mm entrance and exit slit widths, respectively. The increase in fluorescence (excitation λ = 522 nm, emission λ = 586 nm) due to resorufin was recorded with an *X-Y* recorder. Reaction mixtures consisted of 2.0 ml of 0.05 M Tris buffer, pH 7.5, 0.025 M $MgCl_2$, 10 μM pentoxyresorufin, and 10–100 μl of enzyme sample, depending on the amount of enzyme activity. Reactions were initiated by addition of 125 μM NADPH to the cuvette. The rate of formation of resorufin was calculated by comparing the rate of increase in relative fluorescence to the fluorescence of known amounts of resorufin.

Ethoxyresorufin O-deethylase assay. The deethylation of 7-ethoxyresorufin was measured in exactly the same manner as described above for pentoxyresorufin O-dealkylation, except that the substrate (ethoxyresorufin) concentration utilized was 1.7 μM.

Aminopyrine N-demethylase assay. Sample (1 mg microsomal protein) was incubated with aminopyrine and appropriate cofactors for 30 min at 37°C as described by Mazel (10). Formaldehyde formed during the incubation was trapped with semicarbazide and detected using the Nash reagents. Absorbance at 415 nm was compared to that produced by a series of known concentrations of formaldehyde.

Protein determination. Hepatic microsomal preparations were assayed for protein concentration with fluorescamine (Fluoram) (11). The reaction of primary amine groups in proteins with fluorescamine results in a fluorescent complex (excitation λ = 395 nm; emission λ = 490 nm). Relative fluorescence values were compared to the values obtained with known amounts of bovine serum albumin.

Antibody inhibition of PTR O-dealkylase activity. Antibodies were raised to purified *P*-450s as previously described (3, 12). Evidence for specificity of

[3] Abbreviations used: aminopyrine, DMAP, 4-dimethylaminoantipyrine; 7,8-BF, 7,8-benzoflavone; 3-MC, 3-methylcholanthrene; metyrapone, 2-methyl-1,2-di-3-pyridyl-1-propanone; PB, phenobarbital; SKF 525-A, 2-diethylmanioethyl-2,2′-diphenylvalerate HCl; IgG, immunoglobulin G; PTR, pentoxyresorufin; ETR, ethoxyresorufin.

these preparations has been presented elsewhere (3). In the immunoinhibition studies, liver microsomes prepared from phenobarbital-treated rats (0.25–0.50 nmol *P*-450/ml) were incubated with varying levels of immunoglobulin G (IgG) fraction prepared from rabbit anti-P-450_{PB-B}, anti-P-450_{PB-C}, and anti-P-$450_{PB/PCN-E}$ preparations. Preimmune IgG, prepared from rabbits prior to immunization (12) was added to each mixture to obtain a total level (preimmune IgG *plus* anti-*P*-450 IgG) of 20 mg IgG per nmol *P*-450. The mixtures stood for 20 min at 23°C and were then stored on ice prior to fluorimetric assay.

RESULTS

Pentoxyresorufin O-dealkylase activities were measured in rat hepatic microsomal preparations following animal pretreatment with a variety of prototype inducing agents (Table I). PB and Aroclor 1254 pretreatment yielded microsomal PTR O-dealkylase activities which were induced ~100-fold relative to control values, whereas animal pretreatment with 3-MC resulted in microsomal PTR O-dealkylase activities which were not significantly different than that found in the controls. Similarly, aminopyrine *N*-demethylase (DMAP *N*-demethylase) activities, measured in these same microsomal preparations, were found to be induced by animal pretreatment with PB or Aroclor 1254, although the maximal induction was only two- to threefold.

The cofactor requirements and the effects of various specific inhibitors on the PTR O-dealkylase activity mediated by Aroclor-induced rat hepatic microsomes have been displayed in Table II. PTR O-dealkylase activity was found to be dependent on NADPH, and was inhibited by concentrations of metyrapone and SKF-525A which had little or no effect on ETR O-deethylase activity (mediated by 3-MC-inducible forms of cytochrome *P*-450) (5, 6). In contrast, 7,8-BF had little inhibitory effect on PTR O-dealkylase activity when employed at concentrations which greatly inhibited ETR O-deethylase activity.

The PTR O-dealkylation reaction was found to be linear with respect to time when the amounts of microsomal protein assayed were adjusted to yield rates of resorufin formation <1 nmol/min. Under these conditions the PTR O-dealkylation reaction also was linear with respect to the amount of microsomal protein added. Absolute reaction rates were decreased significantly in the absence of Mg^{2+} in the reaction mixture (data not shown).

The preceding data seemed compatible with the hypothesis that the pentoxyresorufin seemed to be a specific substrate for PB-induced forms of cytochrome(s) *P*-450. To determine which specific PB-induced form of cytochrome *P*-450 mediated this reaction we examined the ability of specific antibodies to inhibit this

TABLE I

INDUCTION OF MICROSOMAL PTR O-DEALKYLASE AND DMAP N-DEMETHYLASE ACTIVITIES IN HEPATIC PREPARATIONS BY VARIOUS INDUCERS

	PTR O-dealkylase[a]		DMAP N-demethylase[b]	
Inducing agent	Rate	Fold induction	Rate	Fold induction
Corn oil	55	1	2.7	1.0
Phenobarbital (75 mg/kg × 4)	5165	94	4.8	1.8
Aroclor-1254 (500 mg/kg × 1)	2870	52	7.4	2.7
3-Methylcholanthrene (50 mg/kg × 3)	75	1.4	2.6	1.0

[a] Picomoles resorufin formed per minute per milligram microsomal protein at 25°C.
[b] Nanomoles formaldehyde formed per minute per milligram microsomal protein at 37°C.

TABLE II

EFFECT OF VARIOUS INHIBITORS ON ALKOXYRESORUFIN-O-DEALKYLATION BY AROCLOR 1254-INDUCED RAT HEPATIC MICROSOMES[a]

Assay conditions	PTR O-dealkylase		ETR O-dealkylase	
	Activity[b]	% Inhibition	Activity[b]	% Inhibition
Enzyme only	<27		<27	
Enzyme + NADPH	1974	0	6634	0
Enzyme + NADPH + N_2	603	69	877	87
Enzyme + 10 μM metyrapone	439	78	5044	24
Enzyme + 1 μM metyrapone	987	50	6031	9
Enzyme + 50 μM SKF525A	603	69	5154	22
Enzyme + 5 μM SKF525A	1974	0	6031	9
Enzyme + 1 μM 7,8-BF	2138	0	713	89
Enzyme + 0.1 μM 7,8-BF	1809	8	2686	60

[a] Reaction rates were determined employing a reaction mixture containing 2 ml of Tris-$MgCl_2$ buffer, 50 μg Aroclor 1254-induced microsomal protein, ETR (1.7 μM) or PTR (10 μM), and inhibitors as indicated. Reactions were initiated by addition of NADPH.

[b] Picomoles resorufin formed per minute per milligram microsomal protein at 25°C, activity values given represent the mean value obtained from three determinations.

reaction in PB-induced microsomes (Fig. 1). There are at least four forms of cytochrome *P*-450 induced by phenobarbital (3), although the form designated $P\text{-}450_{PB\text{-}B}$ appears to be the major inducible form. As shown in Fig. 1, antibody to $P\text{-}450_{PB\text{-}C}$ or $P\text{-}450_{PB/PCN\text{-}E}$ did not inhibit the PTR O-dealkylation reaction, while antibody directed against $P\text{-}450_{PB\text{-}B}$ severely inhibited the reaction.

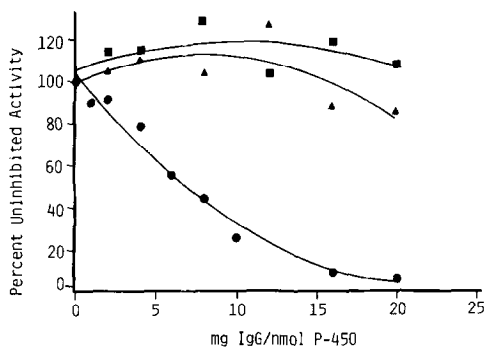

FIG. 1. Immunoinhibition of PTR O-dealkylase activity in PB-induced rat liver microsomes by antibodies raised to cytochromes *P*-450. The procedures are outlined under Materials and Methods. Preimmune IgG was added to each incubation to obtain a level of total IgG of 20 mg per nmol total *P*-450. Results are presented as means of two or three experiments with different antibodies: (●) anti-$P\text{-}450_{PB\text{-}B}$; (▲) anti-$P\text{-}450_{PB\text{-}C}$; (■) anti-$P\text{-}450_{PB/PCN\text{-}E}$.

The induction of PTR O-dealkylase and DMAP *N*-demethylase activities in rat hepatic microsomes by graded doses of PB was measured, in order to determine the relative sensitivity of each assay and to evaluate the capability of each methodology in the discrimination of varying degrees of induction. The results (Table III) indicate that DMAP *N*-demethylation induction was maximally only twofold and that rather similar levels of enzymatic activity were induced by a wide range of PB doses (75–8.3 mg/kg). In contrast, when PTR O-dealkylation was employed, a clear PB dose response was obtained with induction values ranging from 145- to 4-fold for the 75 and 0.9 mg/kg doses, respectively.

DISCUSSION

Methods for measuring the induction of PB-inducible forms of cytochrome(s) *P*-450 are of significant interest. While relatively sophisticated immunological antibody techniques or mRNA probes (3, 13–

TABLE III

INDUCTION OF *P*-450 ACTIVITIES IN RAT HEPATIC MICROSOMES BY VARYING DOSES OF PHENOBARBITAL

Phenobarbital dose (mg/kg)[a]	PTR O-dealkylase, $\bar{x} \pm$ SD[b]		DMAP N-demethylase, $\bar{x} \pm$ SD[c]	
	Rate	Fold induction	Rate	Fold induction
75	6504 ± 1274	145	8.1 ± 1.3	1.8
25	4097 ± 560	91	7.3 ± 1.2	1.6
8.3	3039 ± 802	68	7.9 ± 1.0	1.8
2.8	1241 ± 1223	28	5.5 ± 1.4	1.2
0.9	181 ± 52	4	4.0 ± 0.5	0.9
0	45 ± 8	1	4.5 ± 0.5	1.0

[a] Four daily injections (ip) at the indicated dose. Food was withheld after the fourth injection.
[b] Picomoles resorufin formed per minute per milligram microsomal protein at 25°C, n = 3 rats.
[c] Nanomoles formaldehyde formed per minute per milligram microsomal protein at 37°C, n = 3 rats.

16) can be used to study induction and synthesis of PB-inducible cytochrome(s) *P*-450, these questions may also be addressed utilizing enzymatic assays. These enzymatic methods can rapidly and inexpensively be employed to examine both the degree of PB cytochrome(s) specific induction and the ability of specific xenobiotics to induce PB-inducible cytochrome(s) *P*-450. There are a variety of *in vitro* assays which indirectly measure PB-induced forms of cytochrome(s) *P*-450, however each of these has certain disadvantages. The assays most commonly utilized, which employ aminopyrine or ethylmorphine as substrates, yield induced rates only 2.0- to 3.5-fold higher than control (uninduced) activity (2, 3) and are not highly specific for any particular form of cytochrome *P*-450 (3). While there are substrates (e.g., aldrin) which display greater specificity for PB-specific forms of cytochrome(s) *P*-450, the assays which employ these substrates require significant extraction or relatively time-consuming analysis utilizing sophisticated equipment, i.e., GC mass spectroscopy (17). The assay for dealkylation of pentoxyresorufin, in contrast, is rapid, sensitive, and apparently highly specific for PB-induced forms of cytochrome *P*-450. Although the assay described herein was performed with washed microsomes, we have recently revised this assay by the addition of dicumarol to the reaction mixture to allow measurement of the O-dealkylation of the alkoxyresorufins directly in 9000*g* supernatants or hepatocyte homogenates (18).

The PTR O-dealkylation assay appears highly specific for PB-induced forms of cytochrome *P*-450 based both on induction studies (PB, Aroclor) and on effect of inhibitors (metyrapone, SKF 525A, 7,8-benzoflavone). The inhibition studies described in this paper employed as the source of enzymatic activity microsomes prepared from the hepatic tissue of rats pretreated with Aroclor 1254, a mixed inducer. The ability of the various inhibitors to inhibit preferentially the PTR- or ETR O-dealkylation reactions even in this highly induced mixed milieu of PB- and MC-inducible forms of cytochrome *P*-450 is strong evidence for the primary role of the PB- and MC-inducible isozymes in the PTR- and ETR O-dealkylation reactions, respectively. The enhancement of PTR O-dealkylation rates by 25 mM Mg^{2+} which we observed is similar to the effect noted by Pohl and Fouts (19) using ETR O-deethylation activity as the endpoint.

Based on the inhibitor studies and the antibody results, pentoxyresorufin would appear to be a highly specific substrate for cytochrome *P*-450 PB-B. It is possible, however, that it might also reflect metabolism by the closely related and immunologically cross reactive cytochrome $P\text{-}450_{PB\text{-}D}$. We were unable to differentiate these two possibilities employing

purified cytochrome *P*-450 because purified NADPH-cytochrome *P*-450 reductase (0.1 μM) directly and quickly reduced the substrate (PTR) itself (unpublished data). The high level of induction of PTR O-dealkylase activity observed is not surprising if metabolism is highly specific for induced forms of cytochrome *P*-450 (e.g., PB-B), since both immunologic and mRNA investigations have implied 40- to 200-fold induction of this major form of PB-specific cytochrome (3, 13–16).

In summary, it appears that the pentoxyresorufin O-dealkylase reaction constitutes a rapid and sensitive assay for use in measuring the induction of PB-inducible forms of cytochrome(s) *P*-450 in the rat.

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