Biochemical Pharmacology, Vol. 29, pp. 1375–1383

0006-2952/80/0515-1375 $02.00/0

INDUCTION OF DIMETHYLNITROSAMINE DEMETHYLASE ACTIVITY IN MOUSE LIVER BY POLYCHLORINATED BIPHENYLS AND 3-METHYLCHOLANTHRENE

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(*Received* 31 *July* 1979; *accepted* 5 *November* 1979)

Abstract—Hepatic dimethylnitrosamine (DMN) demethylase activity in male C57BL/6 mice, assayed with 1 or 5 mM DMN and expressed per g liver, increased after i.p. treatment with Aroclor 1254 [polychlorinated biphenyls (PCBS), 500 mg/kg, 96 hr before assay], compared with parallel oil-injected controls. This increase was of statistical significance when either the postmitochondrial supernatant fraction (sup) or isolated microsomes were assayed. The induction appeared larger with 5 mM DMN as substrate (about 180 per cent) than with 1 mM DMN (about 65 per cent). PCBs also induced sup DMN (5 mM) demethylase activity in (C57BL/6 × BALB/c)F_1 and Swiss–Webster mice. 3-Methylcholanthrene exposure (80 mg/kg, 48 hr) resulted in a small but significant increase in sup hepatic DMN (5 mM) demethylase activity in C57BL/6 and F_1 mice. Isolated microsomes from C57BL/6 livers exhibited only 40–60 per cent of the DMN (1 or 5 mM) demethylase activity present in the corresponding sup; such preparations may not give an accurate indication of *in vivo* activity. Inclusion of the lipid layer with the sup resulted in a significant increase in DMN (5 mM) demethylase activity in Aroclor-induced, but not in control, C57BL/6 mouse livers. The number of cells per unit area of liver, determined microscopically after treatment of C57BL/6 mice with PCBs, decreased slightly (15 per cent) but significantly compared with controls. Thus, enzyme activity per g liver represents a conservative approximation of activity per cell, which is the parameter that should be measured for demonstration of induction or repression and for evaluation of potential toxic or carcinogenic effects.

The carcinogen dimethylnitrosamine (DMN) is sometimes found in foods, air and smoke [1]. It is also widely used in studies of carcinogen-target cell interaction. The biological effects of DMN are believed to be caused by reactive intermediates formed during metabolism [2]. Evidence is still being gathered as to the nature of the enzymes and intermediates involved in the metabolism and biological action of DMN [3–35]. It is nevertheless clear that DMN undergoes oxidative demethylation by the mixed-function oxygenase system of the endoplasmic reticulum and that formaldehyde is a major metabolic product [2]. Release of formaldehyde, which is easily quantified colorimetrically by the Nash reaction [36], has served as a measure of DMN demethylase activity.

Polycyclic hydrocarbons, polychlorinated biphenyls (PCBs) and other compounds, which normally cause an increase in microsomal mixed-function oxygenase enzymes, were found also to induce DMN demethylase in rat, mouse, hamster and guinea pig liver when 25–100 mM DMN was used as substrate in the assay [5, 12, 15, 20, 24, 32]. However, when 2–4 mM DMN was used as substrate, pretreatment with these xenobiotics resulted in an apparent repression of DMN demethylase activity in rat and hamster liver microsomes [4, 5, 24, 34]. No change in this activity was observed in the liver microsomes of Swiss–Webster mice treated with PCBs [6], but in a subsequent study these same investigators reported repression by PCBs of hepatic DMN demethylase activity in both Swiss–Webster and various inbred mice [5]. Sipes *et al.* [32] found that liver microsomal DMN demethylase in C57BL/6 mice, assayed with 1 mM DMN, was repressed by pretreatment with PCBs but induced by acetone.

On the basis of these findings, the existence of two DMN demethylase isoenzymes responding differently to xenobiotics was postulated [5]. It must be noted, however, that most of the experiments cited above were carried out with isolated microsomes. Separation of microsomes from the supernatant fraction of liver results in a lowering of oxidative activity toward some substrates [37–40], including DMN [21, 22]. Use of microsomes for studies in which quantitative comparisons are to be made thus seems questionable. Furthermore, in the studies cited above, activity was, in most cases, expressed per mg microsomal protein, a quantity which sometimes increased after treatment with xenobiotics. It has been argued that this mode of expression is necessary to demonstrate changes in the specific composition of the endoplasmic reticulum [4, 5]. However, changes in enzyme activity expressed on a per genome or per cell basis are also of interest, since induction and repression are believed by all experts to reflect changes in genome expression through alterations in rates of enzyme protein synthesis [41–45]. McLean and Day [46] pointed out that a decrease in product formation per mg microsomal protein does not necessarily reflect a "true fall in enzyme

* Supported by Grants CA 08748, 17085 and 22509 and Contract CP 75912-50 from the National Cancer Institute and by the Fund for Advanced Study of Cancer.

activity". This fact was also recognized by Hutton *et al.* [47], who expressed their DMN demethylase assay data on a per mg DNA basis.

In the study reported here, we have investigated whether expression of enzyme activity per gram tissue is a reasonable approximation of activity per cell, and have measured DMN demethylase activity in hepatic postmitochondrial supernatant fractions and isolated microsomes from control and PCB-treated mice. We have concluded that DMN demethylase, even when assayed with 1 or 5 mM substrate, is inducible by PCBs. Repression did not occur under the conditions we employed.

MATERIALS AND METHODS

Animals. C57BL/6, BALB/c (Jackson Laboratories, Bar Harbor, ME) and Swiss–Webster (Camm Research Institute, Wayne, NJ) mice, 2 to 4-months-old, were housed with hardwood shavings as bedding at 25 ± 2° under a 14/10 hr fluorescent light/dark cycle and were fed Purina Mouse Chow. (C57BL/6 × BALB/c)F_1 mice were bred in our colony. Male mice were injected i.p. and killed nonfasted by decapitation for assay between 9:00 and 10:00 a.m.

Chemicals. Organic chemicals and their sources were: NADP, glucose-6-phosphate, nicotinic acid, glucose-6-phosphate dehydrogenase (Type XII), 3-methylcholanthrene and bovine serum albumin from the Sigma Chemical Co. (St. Louis, MO); nicotinamide from the Eastman Kodak Co. (Rochester, NY); semicarbazide–HCl from the Sigma Chemical Co. or the Aldrich Chemical Co. (Milwaukee, WI); and DMN from the Schuchardt Co., Munich, F.R.G. Aroclor 1254 was provided by Mr. William Papageorge of the Monsanto Co., St. Louis, MO.

Preparation of tissue fractions. Livers were perfused through the hepatic vein with cold 0.1 M potassium phosphate buffer, pH 7.3, according to the procedure of Rao and Vesselinovitch [48]. Whole individual mouse livers were used for 20% (w/v) homogenates in phosphate buffer, prepared by ten strokes of a glass–Teflon homogenizer. Postmitochondrial supernatant fractions were obtained by centrifugation at 9000 *g* for 30 min at 4°. The layer of lipoidal material accumulating at the top of the centrifuge tube was included with the supernatant fraction in some experiments, as indicated. Microsomes were centrifuged from the post mitochondrial supernatant fraction at 100,000 *g* for 60 min. The surface of the microsomal pellet was washed once and microsomes from 1 g liver were resuspended in 1 ml phosphate buffer by five strokes of a glass–Teflon homogenizer.

DMN demethylase assay. Although it would have been desirable to carry out the assays with postmitochondrial supernatant fractions and isolated microsomes under identical conditions, this was not possible, since the composition of the supernatant fraction, as a complex cellular extract, was not definable, and since the unusually high concentration of phosphate buffer required for maximal activity with isolated microsomes resulted in the formation of a precipitate in the Nash reaction when the postmitochondrial supernatant fraction was used. For the assays presented in Table 4, using the supernatant fraction, a 2-ml reaction mixture in 0.1 M potassium phosphate buffer (pH 7.3) contained 0.5 μmole NADP, 10 μmoles glucose-6-phosphate, 10 μmoles $MgCl_2$, 14 μmoles nicotinamide, 20 μmoles semicarbazide–HCl and 5 mM DMN. For supernatant fraction assays presented in Tables 2 and 3, where microsomal and supernatant fraction activities were directly compared, the concentration of phosphate buffer was elevated to 0.15 M and 8 μmoles nicotinic acid were substituted for the nicotinamide. These changes, intended to give a closer approximation to the microsomal incubation mixture, resulted in a slight increase in supernatant fraction activity. For the assay with microsomes, the reaction mixture, identical to that employed by Venkatesan *et al.* [34], contained 0.8 μmole NADP, 4 μmoles $MgCl_2$, 8 μmoles nicotinic acid, 15 μmoles semicarbazide–HCl, 8 μmoles glucose-6-phosphate, 0.8 units glucose-6-phosphate dehydrogenase, 0.6 mmole phosphate buffer (pH 7.3), 1 or 5 mM DMN, and 1.15% KCl to bring the volume to 2 ml. Systematic variation of the concentrations of individual reactants in both the supernatant fraction and microsomal assays indicated that reaction rates were optimized in both cases.

The reactions were started by the addition of supernatant fraction or microsomal suspension. The amount of enzyme source and the time of incubation were chosen to ensure linearity of reaction with time and protein concentration: 0.25 or 0.5 ml of liver supernatant fractions and 0.1 ml of microsome suspension for 30 min. The incubations were carried out at 37° with shaking at 90 strokes/min, in an atmosphere of air. The reactions were terminated by addition of 1.5 ml of cold 2.5% $ZnCl_2$, followed by 0.5 ml of cold 0.5 N NaOH. The precipitate was removed by centrifugation (2200 r.p.m., 20 min), and the supernatant fraction was mixed with an equal volume of Nash reagent [36]. After incubation in a water bath at 58° for 12 min, the color was measured at 413 nm. Formaldehyde standards were included with each colorimetric determination. All assays were carried out in duplicate. Formaldehyde added to the reaction mixture in the absence of DMN was recovered in yields of 93 ± 0.6 per cent (three determinations), and experimental values were corrected accordingly. The blanks used for determination of background absorbancy consisted of all reaction components except the NADPH-generating system, incubated for the same period as the experimental samples. Protein content was estimated by the method of Lowry *et al.* [49] with bovine serum albumin as the standard.

In one series of determinations, 800 *g* (10 min) supernatant fractions were derived from 10% homogenates. The reaction flasks contained 5 mM DMN, 0.1 M phosphate buffer, and the other ingredients used for assaying the 9000 *g* supernatant fraction including nicotinic acid. Reaction rates were proportional to time and protein concentration when 0.2-ml aliquots of the 800 *g* supernatant fraction were incubated for 30 min.

Nuclei counts. Three C57BL/6 mice were injected i.p. with Aroclor 1254 (500 mg/kg) or olive oil. After 96 hr, four pieces were cut at random from each liver

and fixed in Bouin's solution. Histological slides were made of 7 μm sections and stained with hematoxylin and eosin. Photomicrographs were made at a magnification of 350 times, and counts were taken of the number of hepatocyte nuclei in a field of 0.150 mm^2. The counts from all treated or control liver sections were averaged.

Statistical analysis. Control and experimental animals were always assayed in parallel with equal numbers of animals in each group. Day-to-day variation was greater than variation among triplicate animals assayed on a given day, as has been often observed for microsomal enzyme activities [50]. Nevertheless, the differences between treated and control mice, when observed, were consistent from day to day. We, therefore, applied the *F*-test for significance of difference between treated and control mice, using 'within treatment, within day' as the measure of variation [51]. Experiments involving data gathered on a single day were analyzed by Student's *t*-test.

RESULTS

Change in the amount of DMN demethylase per cell is a relevant parameter in the assessment of the effect of other xenobiotics on the cellular actions of DMN. In order to ascertain whether activity expressed, for convenience, on a per mg liver basis provides an accurate indication of changes in activity per cell, we determined the effect of Aroclor treatment on the number of cells per unit area of liver. The livers of PCB-treated mice had slightly, but significantly, fewer nuclei per 0.15 mm^2 area than control livers (Table 1), with very little variation among triplicate animals. Aroclor-caused changes in enzyme activity, therefore, would be somewhat less when expressed per mg liver than when expressed per cell, and the per mg liver mode of data presentation gives a conservative estimation of inductive effects.

DMN demethylase activity in the livers of Aroclor-treated and control C57BL/6 mice was assayed with 5 mM (Table 2) and 1 mM (Table 3) DMN. With both concentrations of substrate, the isolated microsomes from livers of control and treated mice had 40–60 per cent less DMN demethylase activity, compared with that measured in the postmitochondrial supernatant fractions of the same livers. The lower activity in the isolated microsomes was not due to the difference in assay reagents, since use of the supernatant fraction assay reagents with isolated microsomes resulted in a large decrease in activity (data not shown). A concentration of 0.3 M phosphate buffer was found to be required for maximal activity in the microsomes.

In the assays employing 5 mM DMN as substrate (Table 2), Aroclor treatment resulted in a 3-fold increase in DMN demethylase activity in the postmitochondrial supernatant fractions and in the microsomes, when expressed per 100 mg liver. The activity was also significantly greater after PCB treatment when expressed per mg microsomal protein, but, because of the Aroclor-induced increase in microsomal protein, the difference between control and experimental mice was less than this difference when results were expressed per 100 mg liver.

With 1 mM DMN as substrate (Table 3), Aroclor induction of DMN demethylase was also demonstrated, when activity was expressed per 100 mg liver. The apparent per cent increase was less than that observed with 5 mM DMN, and there was no difference between control- and Aroclor-treated liver activities in either supernatant fractions or microsomes, when expressed per mg microsomal protein.

The study of the effects of xenobiotics on hepatic supernatant DMN demethylase assayed with 5 mM DMN was extended to several other strains and to another commonly used inducer, 3-methylcholanthrene (Table 4). The enzyme was induced by the PCBs in the livers of (C57BL/6 × BALB/c)F_1 and Swiss–Webster mice, as expressed per 100 mg liver or per mg supernatant protein. Activities calculated per mg microsomal protein were not significantly different in control and treated mice. 3-Methylcholanthrene treatment resulted in increased DMN demethylase in the livers of the F_1 hybrids and increased activity per mg supernatant protein in the livers of the C57BL/6 mice, but affected only liver weight in the Swiss–Webster mice.

Another variable examined was the inclusion of the top lipid layer with the supernatant fraction. The effects of this material were of interest in the light of possible loss of enzymatic activity due to lipid peroxidation. Inclusion of the lipid resulted in significantly higher DMN demethylase activity (per 100 mg liver or per mg supernatant protein) in Aroclor-treated, but not control, C57BL/6 livers (Table 4).

In order to rule out the possibility that the difference between DMN demethylase activities in the 9000 *g* supernatant fractions of control vs treated mice was due to greater loss of microsomes from the control supernatant fraction during centrifugation, assays were carried out with supernatant fraction after centrifugation at 800 *g*, which would be expected to remove little microsomal material. DMN (5 mM) demethylase activity in the 800 *g* supernatant fractions from livers of Aroclor-treated C57BL/6 mice was 269.7 ± 49.5 (N = 3) nmoles formaldehyde/100 mg liver/30 min, compared with 99.8 ± 27.5

Table 1. Effect of treatment with Aroclor 1254 on hepatic nuclei count in mice

Treatment group*	Mean No. nuclei ± S.D. per 0.15 mm^2†
A	214 ± 7‡(12)§
C	251 ± 20(10)

* Animals were injected i.p. with Aroclor 1254 (A, 500 mg/kg) or olive oil (C) 96 hr before being killed.

† Adult C57BL/6 male mice (three treated and three control) were used. Four pieces taken at random from each liver were fixed in Bouin's solution and 7 μm sections prepared (H & E staining). Photographs were made of 0.15 mm^2 areas and the number of hepatocyte nuclei was counted.

‡ Significantly lower than control value ($P < 0.001$, Student's *t*-test).

§ Number of counts made.

Table 2. Aroclor 1254 induction of DMN demethylase, assayed with 5 mM DMN, in postmitochondrial supernatant fractions and microsomes of C57BL/6 mouse liver*

					DMN demethylase activity (nmoles formaldehyde/30 min)				
					9000 *g* Supernatant†			Microsomes	
		Liver wet wt (g)	9000 *g* Sup. protein (mg/100 mg liver)	Microsomal protein (mg/100 mg liver)	Per 100 mg liver	Per mg 9000 *g* sup. protein	Per mg microsomal protein	Per 100 mg liver	Per mg microsomal protein
Treatment‡	(A) Aroclor 1254	2.3 ± 0.1	7.6 ± 0.4	1.6 ± 0.1	280.8 ± 10.3	37.2 ± 2.3	180.0 ± 6.3	117.0 ± 8.9 (42%)§	74.7 ± 5.1 (42%)§
	(C) Olive oil	1.4 ± 0.04	6.1 ± 0.3	0.7 ± 0.02	98.7 ± 4.6	16.4 ± 1.1	147.3 ± 9.1	41.5 ± 3.2 (42%)§	61.9 ± 5.4 (42%)§
	%Change (A−C/C × 100)	64.3	24.6	128.6	184.5	126.8	22.2	181.9	20.7
F test		$P < 0.001$	$P < 0.001$	$P < 0.001$	$P < 0.001$	$P < 0.001$	$P < 0.05$	$P < 0.001$	$P < 0.01$

* All values are means ± S.E.

† Supernatant fraction after centrifugation of 20% homogenate at 9000 *g* for 30 min, including lipid layer.

‡ Mice were injected i.p. with Aroclor 1254 (500 mg/kg) or vehicle 96 hr before being killed; there were six mice in each treatment group (three experimental and three control mice) on each of 2 days.

§ Per cent of activity in 9000 *g* supernatant fraction.

Table 3. Aroclor 1254 induction of DMN demethylase, assayed with 1 mM DMN, in postmitochondrial supernatant fractions and microsomes of C57BL/6 mouse liver*

					DMN demethylase activity (nmoles formaldehyde/30 min)				
					9000 *g* Supernatant fraction†			Microsomes	
		Liver wet wt (g)	9000 *g* Sup. protein (mg/100 mg liver)	Microsomal protein (mg/100 mg liver)	Per 100 mg liver	Per mg 9000 *g* sup. protein	Per mg microsomal protein	Per 100 mg liver	Per mg microsomal protein
Treatment‡	(A) Aroclor 1254	1.99 ± 0.05	7.8 ± 0.04	1.8 ± 0.1	186.8 ± 12.0	24.9 ± 2.6	110.5 ± 8.1	103.9 ± 11.0 (56%)§	57.5 ± 4.0 (52%)§
	(C) Olive oil	1.46 ± 0.05	7.7 ± 0.2	1.0 ± 0.1	111.6 ± 7.9	14.6 ± 0.9	116.1 ± 12.6	64.7 ± 5.6 (61%)§	63.1 ± 2.4 (54%)§
	% Change (A−C/C × 100)	36.3	1.0	80.0	67.4	70.5	−4.8	60.6	−8.8
F test		P < 0.001	NS‖	P < 0.001	P < 0.001	P < 0.01	NS	P < 0.001	NS

* All values are means ± S.E.

† Supernatant fraction after centrifugation of 20% homogenate at 9000 *g* for 30 min, including lipid layer.

‡ Mice were injected i.p. with Aroclor 1254 (500 mg/kg) or vehicle 96 hr before being killed; there were nine mice in each treatment group (three treated and three control mice) on each of 3 days, except that only eight values were available for control supernatant fraction activity (one sample lost).

§ Per cent of activity in 9000 *g* supernatant fraction.

‖ Not significant.

Table 4. Liver postmitochondrial supernatant DMN demethylase activity assayed with 5 mM DMN after treatment of various mouse strains with Aroclor 1254 or 3-methylcholanthrene (3-MC)*

Mouse strain	N	Treatment†	Liver wet wt (g)	Lipid‡,§	9000 *g* Sup. Protein (mg/100 mg liver)	Microsomal Protein (mg/100 mg liver)	DMN demethylase activity‡ (nmoles formaldehyde/30 min) Per 100 mg liver	Per mg 9000 *g* sup. protein	Per mg microsomal protein
C57BL/6	9	Aroclor, 96 hr	2.0 ± 0.1‖	+	8.6 ± 0.2¶	1.6 ± 0.1‖	228 ± 8.1‖****	26.7 ± 0.8‖**	139.5 ± 5.2¶
		Olive oil	1.4 ± 0.1	+	7.8 ± 0.2	0.9 ± 0.1	99.4 ± 7.4	12.8 ± 1.0	112.9 ± 10.0
	8	Aroclor, 96 hr	1.8 ± 0.1‖	−	9.2 ± 0.8	1.3 ± 0.1††	154.6 ± 8.1**††	17.1 ± 1.0‖**	121.1 ± 8.1
		Olive oil	1.3 ± 0.1	−	9.0 ± 0.6	1.0 ± 0.1	98.2 ± 5.1	11.2 ± 0.8	103.0 ± 11.2
	7	3-MC, 48 hr	1.5 ± 0.1	+	7.4 ± 0.4	1.2 ± 0.1	125.2 ± 11.8	16.8 ± 0.8¶	106.5 ± 5.4
		Olive oil	1.4 ± 0.1	+	7.2 ± 0.3	1.0 ± 0.1	105.6 ± 11.1	14.4 ± 1.1	103.7 ± 8.1
(C57BL/6 × BALB/c)F_1	3	Aroclor, 96 hr	1.8 ± 0.1	−	8.8 ± 0.5	1.7 ± 0.1‖	191.9 ± 6.6††	22.0 ± 1.8¶	112.6 ± 6.7
		olive oil	1.5 ± 0.1	−	7.2 ± 0.3	0.9 ± 0.1	120.7 ± 6.9	16.6 ± 0.4	134.7 ± 8.0
	9	3-MC, 48 hr	1.5 ± 0.1	−	7.2 ± 0.1	1.3 ± 0.1††	145.3 ± 7.5††	20.2 ± 0.9††	116.8 ± 6.6
		olive oil	1.3 ± 0.1	−	7.2 ± 0.2	0.9 ± 0.2	115.8 ± 8.7	16.1 ± 1.2	129.8 ± 9.8
Swiss–Webster	9	Aroclor, 96 hr	3.5 ± 0.2	−	7.4 ± 0.3	1.2 ± 0.2¶	154.5 ± 5.8††	21.0 ± 0.7††	125.2 ± 8.3
		Olive oil	2.5 ± 0.1	+	7.2 ± 0.2	0.9 ± 0.1	112.9 ± 10.2	15.5 ± 1.3	119.6 ± 10.0
	6	3-MC, 48 hr	2.8 ± 0.1¶	+	7.2 ± 0.5	0.9 ± 0.1	113.4 ± 3.2	16.1 ± 1.4	126.9 ± 7.0
		Olive oil	2.5 ± 0.1	+	7.4 ± 0.5	0.9 ± 0.1	109.2 ± 7.9	15.0 ± 1.2	118.3 ± 8.3

* All values are means ± S.E.

† Mice were injected i.p. with Aroclor 1254 (500 mg/kg), 3-methylcholanthrene (80 mg/kg), or vehicle 2 or 4 days before being killed. Mice were tested, usually in triplicate, in two or three separate experiments.

‡ In supernatant fraction after centrifugation of 20% homogenate at 9000 *g* for 30 min.

§ The symbol (+) indicates inclusion of lipid layer with supernatant fraction.

‖ $P < 0.001$, *F*-test, values from treated mice are significantly higher than corresponding control value.

¶ $P < 0.05$, *F*-test, values from treated mice are significantly higher than corresponding control value.

** Activity in the presence of supernatant lipid is significantly greater than in its absence ($P < 0.001$, *t*-test).

†† $P < 0.01$, *F*-test, values from treated mice are significantly higher than corresponding control value.

(N = 3) for controls ($P < 0.001$). These values are similar to those listed in Table 2.

DISCUSSION

The results of our experiments lead to the conclusion that DMN demethylase, assayed with 1 or 5 mM DMN, is induced in mouse livers by Aroclor 1254. This conclusion is at variance with that of several other investigators who have reported either repression by or no effect of PCBs on mouse liver DMN demethylase, assayed with 1–10 mM DMN [5, 6, 32, 34]. There are at least two reasons for this discrepancy: (1) most of these experiments were done with isolated microsomes, and (2) results were usually expressed in terms of amount of product per mg microsomal protein.

Limitations of isolated microsomes for DMN demethylase assay. Although isolated microsomes represent a purified source of endoplasmic reticulum enzymes, some of these enzymes, including DMN demethylase, experience a large reduction in apparent activity upon separation from the cytosol [21, 22, 38]. Since the endoplasmic reticulum is ordinarily in contact with the cytosol, the activity in isolated microsomes must be regarded as abnormally low, and not a true measure of *in vivo* activity. There are several possible reasons for the lower apparent activity in the microsomes, including destruction or denaturation of the enzyme, alteration of the active site, absence of an essential soluble cofactor, and/or reduced effective substrate concentration due to nonspecific binding on the microsomes. There is considerable evidence [52–56] that reduced enzymatic activity in isolated microsomes is due, in part, to lipid peroxidation. The soluble fraction may contain substances that inhibit, and/or provide alternative substrates for, lipid peroxidation. The fact that in our experiments inclusion of the lipid layer of the supernatant fraction resulted in higher DMN demethylase activity in induced preparations suggests that this supernatant fraction component may spare microsomal lipids by providing alternative substrates for peroxidation.

Sipes *et al.* [32], measuring DMN demethylase in isolated microsomes from control and Aroclor 1254-treated C57BL/6 male mice, observed that Aroclor treatment reduced activity with 1 mM DMN and had no effect with 10 mM DMN; activity was expressed per mg microsomal protein. In our experiments involving Aroclor treatment of the same type of mouse, we found that DMN demethylase (per mg microsomal protein) in isolated microsomes from Aroclor-treated mouse liver did not change with 1 mM DMN and increased significantly with 5 mM DMN, compared with controls. This discrepancy may be due to the use of Tris buffer by Sipes *et al.* Hutton *et al.* [47] have confirmed our observation that DMN demethylase activity is higher when assayed with phosphate buffer than Tris buffer.

Mode of expression of enzyme activity. A second source of confusion in interpretation of the effects of xenobiotic inducers relates to the mode of expression of enzyme activity. A significant increase in microsomal protein content of mouse liver occurs after treatment with inducers such as Aroclor 1254 [5, 8, 47]. Therefore, presentation of activity on a per mg microsomal protein basis may not give an accurate indication of changes in the absolute amount in the cell of the particular enzyme protein in question, which is what induction and repression are presumed to entail [41–45]. Expression of activity per mg microsomal protein, furthermore, does not show changes in amounts or concentrations of enzyme products in the cell, an important parameter in analysis of toxic and/or carcinogenic effects. Enzyme activity should be expressed on a per cell basis, if induction or repression is to be demonstrated conclusively, and if the cellular significance of changes in enzyme activity is to be appreciated. Changes in activity per mg microsomal protein are also of interest in certain contexts and perhaps should be given a special terminology, such as 'specific increase' and 'specific reduction'.

We have demonstrated that expression of enzyme activity per g tissue is a conservative representation of activity per cell. Treatment of mice with Aroclor 1254 resulted in a slight but significant reduction in the number of cells per unit area of liver. A comparable decrease in DNA content per g occurs in the livers of Sprague–Dawley male rats 96 hr after treatment with 500 mg/kg Aroclor 1254.* Probably the liver cells grow larger after PCB treatment, as occurs after exposure to phenobarbital [57]. Thus, induced activity expressed on a per cell basis would be even greater than that calculated per g liver.

Hutton *et al.* [47] reported no change in DNA per g liver in mice following Aroclor 1254 treatment; their practice of pooling data from eight inducible and noninducible strains may have obscured a small but significant effect such as we have observed. These workers detected no inductive or repressive effect of Aroclor 1254 on DMN demethylase per mg DNA in C57BL/6 mouse liver; they employed the postmitochondrial supernatant fraction and 1 mM DMN as substrate. Their experiments were done with female mice, which might be less responsive to inducers than males. It might also be noted that their mouse livers were frozen at −70° before use; frozen storage has been shown to result in a greater loss of DMN demethylase activity from Aroclor-induced microsomes than from control preparations [58].

In conclusion, investigators should consider carefully the type of tissue preparation used for assay of DMN demethylase and perhaps other microsomal enzymes, in the light of the lowered activity in isolated microsomes. The mode of expression of the results should also be thoughtfully chosen, depending on the purpose of the experiment. Where changes in the composition of the endoplasmic reticulum are of primary interest, enzyme activity should be expressed per mg microsomal protein. Where the results are relevant to toxicity or carcinogenicity, data should be expressed per cell, per g DNA, or per g tissue. Our findings, analyzed in accordance with these considerations, indicate induction, not repression, of DMN demethylase in mouse liver by PCBs, even when assayed with a low concentration

* L. M. Anderson, unpublished observation.

of DMN. Data relevant to the effect of inducers on the carcinogenicity of DMN (e.g. Refs. 7, 8 and 59), mutagenicity (e.g. Refs. 12 and 15) and toxicity (e.g. Refs. 20 and 33) should be evaluated with these considerations in mind.

The inductive effect of Aroclor in our experiments was greater when the DMN demethylase was assayed with 5 mM, compared with 1 mM, substrate. While this difference may reflect the operation of two isoenzymes of DMN demethylase of differing inducibility, as postulated by Arcos *et al.* [5], other explanations are possible. Induced microsomal preparations might be differentially sensitive, compared to controls, to loss of activity in a process influenced by substrate concentration, for example, lipid peroxidation. Lipid peroxidation, which increases in liver after phenobarbital treatment [60, 61], is reduced by substrates for NADPH-dependent oxidation [62, 63]. The proposed isoenzymes of DMN demethylase must be isolated for their existence to be demonstrated conclusively.

Acknowledgements—We would like to thank Dr. Isabel M. Mountain for statistical analysis.

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