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Comparison of hepatic microsomal enzyme induction by methadone, phenobarbital and 3-methylcholanthrene in the mouse*

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In earlier reports, we described an elevation in a variety of hepatic microsomal enzyme activities following oral administation of methadone to the mouse [1–3]. Maximal induction was observed after 6 days of treatment (50 mg/kg/day) for a number of mouse strains [2,3]. Although this type of induction shares some properties with phenobarbital induction, such as an elevation of both type I and type II enzyme activities, increased hepatic microsomal protein, and hepatomegaly [4], it is important to compare methadone to two classic inducers of hepatic microsomal activity: phenobarbital and 3-MC†, which differ from each other in their mechanisms of enzyme induction [4]. Dose–response curves were generated for a family of microsomal parameters and the similarities and differences for the three agents in this study were compared.

Materials and methods

Animals. Male ICR mice (Harlan Industries, Cumberland, IN), with an initial weight of 25–30 g, were housed in clear plastic cages (five to six cage) on San-I-Cel bedding (Paxton Processing Co., White House Station, NJ). The bedding was changed every 2 days to avoid ammonia accumulation, a phenomenon which inhibits microsomal enzyme activity [5]. The mice received Purina Lab Chow (Ralston-Purina Co., St. Louis, MO) and water *ad lib.* The animal quarters were maintained on a 12-hr light/dark cycle at an ambient temperature of 21 ± 1°.

Chemicals and dosing. dl-Methadone hydrochloride was obtained from Mallinckrodt Chemical Works, St. Louis, MO. Sodium phenobarbital and sodium pentobarbital were purchased from Abbott Laboratories, North Chicago, IL; 3-MC was obtained from the Sigma Chemical Co., St. Louis, MO. Methadone was dissolved in water for oral administration, whereas sodium phenobarbital and sodium pentobarbital were dissolved in saline for intraperitoneal administration. 3-MC was administered intraperitoneally dissolved in warm corn oil (Mazola Corn Oil, Best Foods, Englewood Cliffs, NJ). All doses were calculated on the basis of the formula weight of the respective chemical. The volumes of solutions administered via the oral and intraperitoneal routes were 0.02 and 0.01 ml/g body wt, respectively. In the control groups, the appropriate vehicle was substituted for drug administration. The animals were dosed between 2:00 p.m. and 3:00 p.m. daily. Phenobarbital, methadone and 3-MC were given daily for 3, 6 and 5 days, respectively. Phenobarbital and methadone were administered at doses of 3.13, 6.25, 12.5, 25, 35 and 50 mg/kg/day. 3-MC-treated mice received doses of 0.02, 0.20, 2.0, 20 and 200 mg/kg/day.

Tissue preparation and assays. Mice were killed 24 hr after the last dose of chemical or vehicle by cervical dislocation followed by decapitation and exsanguination. The gall bladder was excised, and the liver removed, weighed and immediately homogenized in 3 vol of ice-cold 0.05 M Tris–HCl–0.15 M KCl buffer, pH 7.4. The 12,000 *g* supernatant fraction was used for all enzyme assays as described previously [2]. The procedure and cofactors for each assay were listed by Fouts [6]. Twenty μmoles methadone hydrochloride or 20 μmoles aminopyrine, and 10 μmoles aniline hydrochloride were used as substrates for the *N*-demethylase and hydroxylase assays respectively. Reactions were carried out for 30 min during which time product information in each assay occurred at a constant rate for induced and non-induced animals. Formaldehyde generation in the *N*-demethlase assays was determined according to the method of Nash [7], and PAP formation in the hydroxylase assay was monitored as outlined by Mazel [8]. The microsomal fraction was obtained by centrifuging 0.5-ml aliquots of the 12,000 *g* supernatant fraction for 1 hr at 105,000 *g*. Protein was quantitated according to the method of Lowry *et al.* [9], utilizing bovine serum albumin (Fraction V; Nutritional Biochemical Corp., Cleveland, OH) as the protein standard.

Pentobarbital sleeping times. Animals were challenged 24 hr after the last dose of drug or vehicle with sodium pentobarbital (70 mg/kg) injected intraperitoneally. The time that elapsed between the injection and the loss of righting reflex was noted in addition to the duration of hypnosis (sleeping time), which terminated upon regaining of the righting reflex.

Expression of microsomal parameters. The activities of the three *in vitro* microsomal enzyme systems which were examined are expressed two different ways in the Results. The first expression describes enzyme activity per kg of body weight. This is referred to as relative enzyme activity. The second expression, specific enzyme activity, more conventionally is calculated with respect to enzyme activity per mg of liver microsomal protein per kg of body weight. The rationale for using both expressions has been described previously [10] utilizing two basic concepts.

Statistical analysis. All calculations, statistics and correlations were performed with a DEC-10 computer utilizing SPSS programs [11]. These included calculation of Student's '*t*'-values as well as correlation coefficients (*r*). Dif-

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† Abbreviations: 3-MC, 3-methylcholanthrene; Tris, Tris (hydroxymethyl) aminomethane; NADP, nicotine adeninedinuleotide phosphate; PAP *p*-aminophenol; and *r* correlation coefficient.

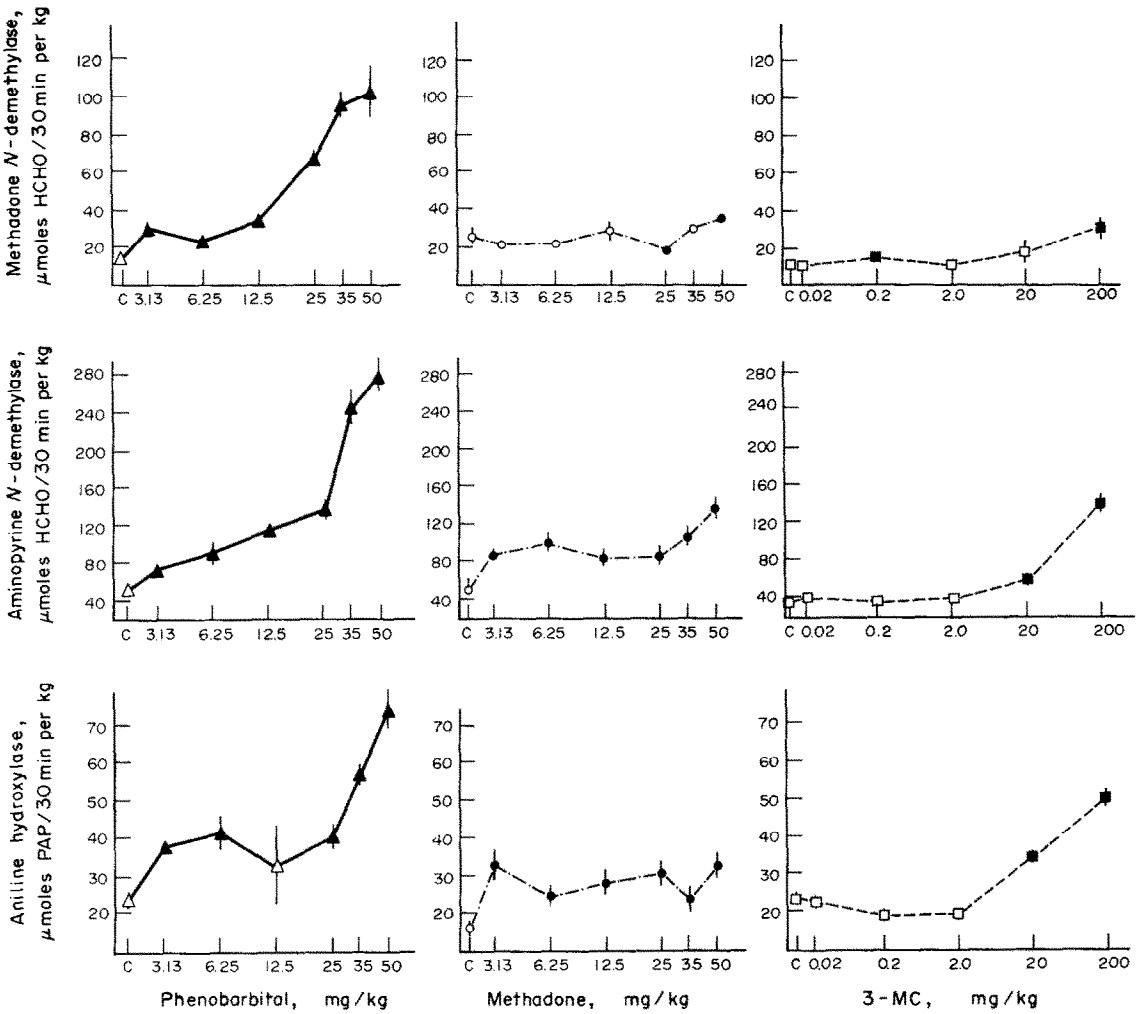

Fig. 1. Dose-related effect of phenobarbital (i.p., three daily doses), methadone (p.o., six daily doses) and 3-MC (i.p. five daily doses) treatment on relative enzyme activity (μmoles product/30 min/kg body wt) of methadone *N*-demethylase, aminopyrine *N*-demethylase and aniline hydroxylase. Each point represents the mean activity of eight to twelve mice per group, and the vertical bars represent the standard error of the mean. The solid symbols indicate a significant difference ($P < 0.05$) from the respective control values.

ferences were considered statistically significant if $P < 0.05$.

Results

Methadone N-*demethylase.* Relative methadone *N*-demethylase activity (μmoles formaldehyde/30 min/kg body wt) for methadone-treated animals was significantly different from controls at the 25 and 50 mg/kg dose levels. While 25 mg/kg elicited a significantly lower activity, there was an approximate 2-fold elevation in enzyme activity at 50 mg/kg (Fig. 1). Phenobarbital-treated animals exhibited significant increases in relative methadone *N*-demethylase activity with all doses employed; the highest dose level (50 mg/kg) produced the greatest increase in activity (5-fold above control values). In contrast, the relative activity for 3-MC-treated animals was significantly elevated only at the 0.2 and 200 mg/kg dose levels, attaining a 3-fold increase at the highest dose. Since body weights were lower in these groups in contrast to the remaining mice (see "body weights"), the relevance of these activities is minimized.

Specific methadone *N*-demethylase activity (μmoles formaldehyde/30 min/mg of microsomal protein/kg body wt) for methadone-dosed animals was not significantly different ($p > 0.05$) from control at any dose level. Both phenobarbital and 3-MC treatments resulted in significant elevations in specific enzyme activities for the majority of doses employed. The greatest increases in activities were observed at the highest respective doses: 2-fold for 3-MC and 3-fold for phenobarbital.

Aminopyrine N-*demethylase.* In contrast to methadone *N*-demethylase activity, the relative aminopyrine *N*-demethylase activity (Fig. 1) of the methadone-treated animals was significantly greater than the control at all doses employed, with a maximal 3-fold elevation observed at the highest dose. The phenobarbital-treated animals demonstrated a similar pattern with the relative enzyme activity also significantly elevated over controls at all dose levels and an 8-fold elevation at the highest treatment level. On the other hand, the 3-MC-treated animals exhibited significantly elevated relative activities only at the 20 and 200 mg/kg dose levels, with the latter dose level giving the greatest activity (a 3-fold elevation). Moreover, there was good statistical correlation between the specific and the relative enzyme activities of this enzyme for the three test compounds employed in this study.

Aniline hydroxylase activity. Relative aniline hydroxyl-

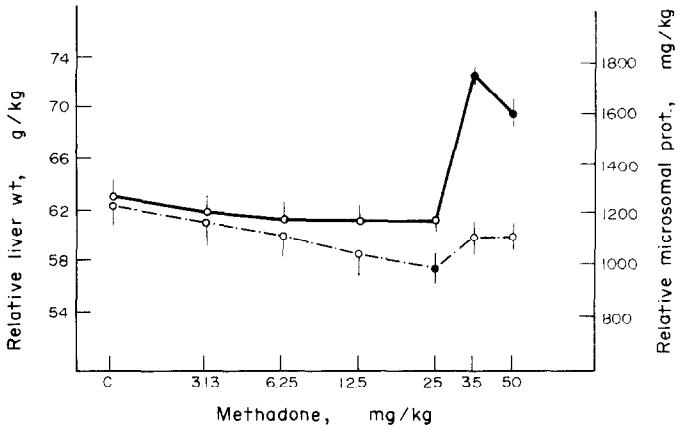

Fig. 2. Dose-related effect of methadone administration (p.o., six daily doses) on relative liver weights (g liver/kg body wt) (——) and relative microsomal protein (mg microsomal protein/kg body wt) (–·–·–). Each point represents the mean activity of eight to twelve mice per group, and the vertical bars represent the standard error of the mean. The solid symbols indicate a significant difference ($P < 0.05$) from the respective control values.

ase activities of methadone-treated animals (Fig. 1) were significantly and almost equally increased for all doses, with maximal activities of 2-fold over control values. PAP production in the phenobarbital-treated mice was significantly elevated above control level at all doses employed except 12.5 mg/kg; maximal activity is seen with the highest dose (3-fold over control value). In contrast, 3-MC-treated animals exhibited significant enzyme induction only at 20 and 200 mg/kg, the latter dose showing the greatest elevation of 2-fold.

Specific aniline hydroxylase activity following all three treatments showed essentially the same pattern of induction as the relative enzyme activity.

Relative microsomal protein and relative liver weight. The relative microsomal protein (mg protein/liver/kg body wt) (Fig. 2) was significantly increased in methadone-treated mice only at the two highest doses of 35 and 50 mg/kg, although there was essentially no dose-related change in liver weights. Phenobarbital-treated mice (Fig. 3) exhibited an increase in microsomal protein at all dose levels, which appeared to parallel a similar increase in liver weights. On the other hand, mice treated with 3-MC (Fig. 4) demonstrated microsomal protein increases only with the 20 and 200 mg/kg doses. Relative liver weights tended to decline with dose, although the livers from the highest dose group were elevated significantly.

Pentobarbital sleeping time. Methadone-treated animals demonstrated significantly depressed pentobarbital sleeping times (Fig. 5) at the 12.5, 25, 35 and 50 mg/kg levels which were in the range of 72–60 per cent of control values. Phenobarbital-treated mice likewise exhibited a decrease in sleeping times at all but the lowest dose employed, with sleeping times depressed to 30 per cent of control for the three highest doses. Mice pretreated with 3-MC did not exhibit sleeping times significantly different from the respective control value.

Body weights. The body weights of the methadone-treated mice were decreased significantly only at the highest dose (50 mg/kg) to 93 per cent of control level. The phenobarbital-treated mice did not exhibit any significant deviation from control body weights. In contrast, the body weights of the 3-MC-treated animals were depressed significantly at the 0.02 and 200 mg/kg dose levels to 95 and 91 per cent of control respectively.

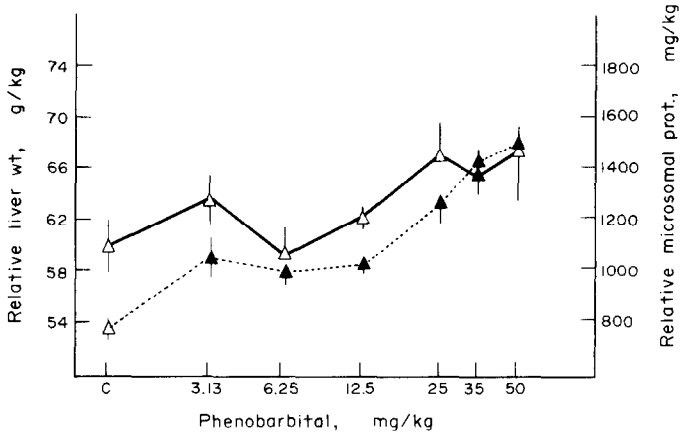

Fig. 3. Dose-related effect of phenobarbital administration (i.p., three daily doses) on relative liver weights (g liver/kg body wt) (——) and relative microsomal protein (mg microsomal protein/kg body wt) (------). Each point represents the mean activity of eight to twelve mice per group, and the vertical bars represent the standard error of the mean. The solid symbols indicate a significant difference ($P < 0.05$) from the respective control values.

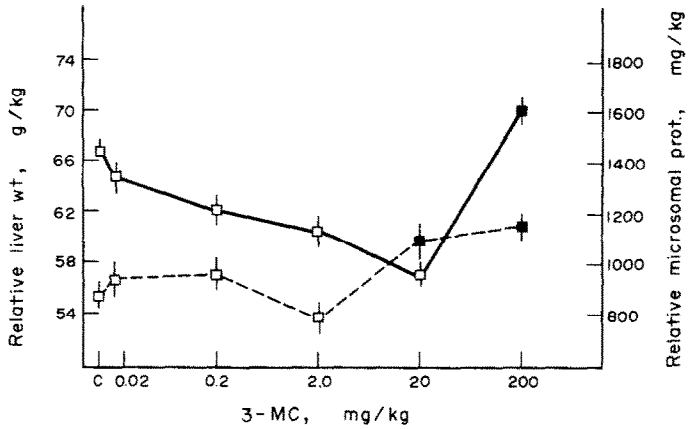

Fig. 4. Dose-related effect of 3-MC administration (i.p., five daily doses) on relative liver weights (g liver/kg body wt) (——) and relative microsomal protein (mg microsomal protein/kg body wt) (------). Each point represents the mean activity of eight to twelve mice per group, and the vertical bars represent the standard error of the mean. The solid symbols indicate a significant difference ($P < 0.05$) from the respective control values.

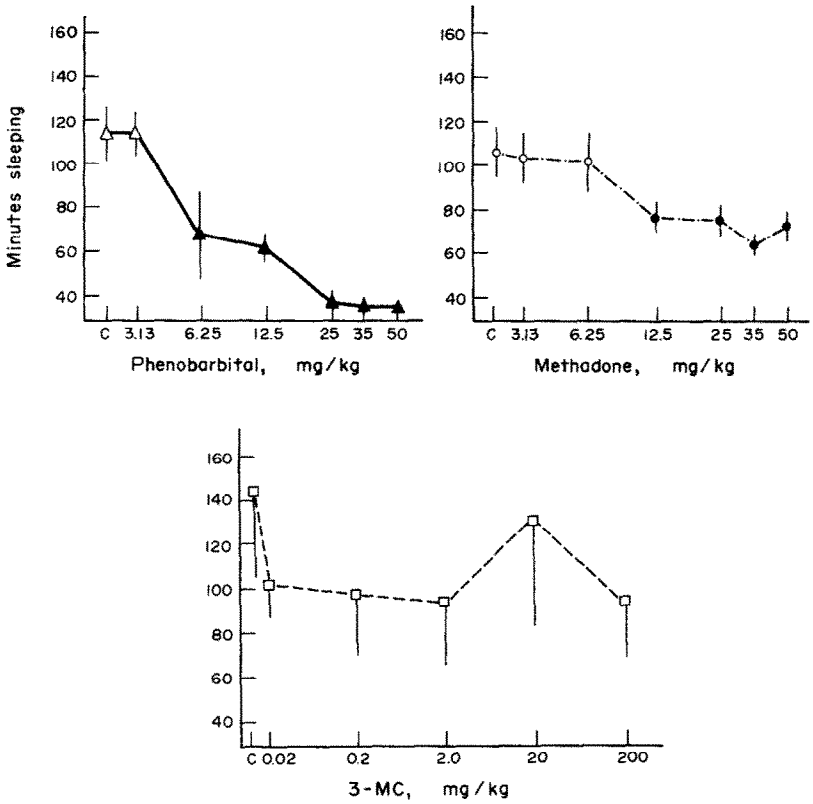

Fig. 5. Dose-related effect of phenobarbital (i.p., three daily doses), methadone (p.o., six daily doses) and 3-MC (i.p., five daily doses) treatment on pentobarbital (i.p., 70 mg/kg)-induced sleeping times. Each point represents the mean sleeping time in minutes of eight to twelve per group, and the vertical bars represent the standard error of the mean. The solid symbols indicate a significant difference ($P < 0.05$) from the respective control values.

Table 1. Correlation coefficients for the log dose–response relationship of methadone, phenobarbital or 3-MC treatment on both *in vitro* and *in vivo* microsomal parameters

Treatment	Methadone *N*-demethylase		Aminopyrine *N*-demethylase		Aniline hydroxylase		Microsomal protein‡	Liver wt§	Pentobarbital sleeping time
	Relative*	Specific†	Relative*	Specific†	Relative*	Specific†			
Methadone	0.401‖¶	0.164	0.469¶	0.121	0.653¶	0.226	0.442¶	−0.047	−0.481¶
Phenobarbital	0.804¶	0.754¶	0.800¶	0.766¶	0.450¶	0.196	0.560¶	0.218	−0.717¶
3-MC	0.175	0.073	0.484¶	0.377**	0.352**	0.250	0.084	0.289††	0.003

* μmoles expressed in product formed/30 min/kg body wt.
† μmoles expressed in product formed/30 min/mg of microsomal protein/kg body wt.
‡ Relative microsomal protein = mg microsomal protein/kg body wt.
§ Relative liver weight = g liver/kg body wt.
‖ Correlation coefficient, *r*.
†† $0.05 > P > 0.01$.
** $0.01 > P > 0.001$.
¶ $P < 0.001$.

Correlation of microsomal parameters and log dose of treatment. For methadone and phenobarbital, the degree of correlation for both *N*-demethylases with the log dose of the respective agent was high and nearly identical for each relative enzyme activity following the respective treatments (Table 1).

Also, with both treatments there was a significant correlation between the log dose of the respective agent and the pentobarbital sleeping times (Table 1). The log dose of the respective agent were equally well correlated with the relative microsomal protein values, although neither treatment generated a significant log dose–response relationship with the relative liver weights (Table 1). This finding helps explain the correlation between relative microsomal protein and relative activities of both *N*-demethylases and aniline hydroxylase following either methadone or phenobarbital treatment. These correlations are much more pronounced in barbiturate-treated mice.

The major difference in the effects observed after treatment with methadone or phenobarbital was the correlation of the specific activities for the two *N*-demethylases and the log dose. While there was a high correlation in the case of phenobarbital, a non-significant relationship was seen for methadone treatment.

Methadone enzyme induction appears to be dissimilar to the 3-MC type of induction based on log dose–response correlations (Table 1). Only the relative enzyme activities of aminopyrine *N*-demethylase were similar for both agents. In contrast, most other microsomal enzyme parameters exhibited marked differences between the two treatments in the correlation with the log dose.

Discussion

The primary objective of this study was to characterize and compare the microsomal enzyme induction resulting from oral methadone administration with that elicited by phenobarbital and 3-MC treatment in the mouse. Previous comparisons have been made in the rat for phenobarbital and 3-MC not for methadone [4]. However, information comparing all three compounds is currently not available for the mouse. Parameters which often are examined in such studies include changes in a number of microsomal enzyme activities, in hepatic microsomal protein content and in liver weights. Moreover, differentiation between the two inducers has been made on the basis of the dose and time required for the induction of an enzyme activity [4,12].

In the present study, all but the last two parameters listed above have been employed. Since we were interested in obtaining maximal changes in the microsomal parameters monitored, a wide range of doses was administered over a relatively extended period of time, making a comparison of the rate of induction impossible to measure. However, in addition to those parameters just listed, the shape of the dose–response curves and the correlation of the log dose–response for each parameter were examined for each treatment. It is important to note that the highest dose employed in these studies was limited by solubility for 3-MC and by toxicity for methadone.

Phenobarbital administration, previously reported in the rat to increase a variety of microsomal enzyme activities, as well as to increase liver microsomal protein and liver weights [4], did likewise in our study. Nearly every dose employed was responsible for a significant increase of activity in each system monitored, although a steep elevation was observed at the two or three highest dose in each dose–response curve. Furthermore, phenobarbital administration was responsible for a dose-related lowering of pentobarbital sleeping times to approximately one-third of control values at the highest doses (Fig. 3), also indicating a dose-related microsomal induction [13]. These results are also comparable to the findings in the rat [4]. Similar findings were noted in the mouse following phenobarbital administration [13–15].

The results of a pilot study with our mice indicated that no significant induction occurred following one set of 3-MC doses even at the highest dose (20 mg/kg). As a consequence, a longer period of dosing was initiated (5 days), at the end of which time significant elevations of microsomal activity were noted. In contrast to phenobarbital, 3-MC treatment was responsible for significantly elevated enzyme activities only at the highest one or two doses (20 mg/kg and 200 mg/kg) (Fig. 1). Not suprisingly, there were no significant decreases in pentobarbital sleeping times with this treatment (Fig. 3). Thus, it appears that the inductive effect of 3-MC on these mice was minimal even though the doses administered were as much as 100-fold greater and administered five times longer than the time shown to elicit this effect in the rat [4].

As with phenobarbital, studies of 3-MC effects on microsomal enzyme activity are limited in the mouse. However, the work published is similar to our findings with respect to qualitative changes [16,17]. The major difference that we noted was that the strain of mouse used in our study appears to be more refractory to 3-MC-induced effects than were the mice in the studies just cited.

Methadone, as an inducer of hepatic microsomal activity, shares a number of properties in common with the phenobarbital type of enzyme induction [2,4]. Earlier, we had reported that, in addition to inducing methadone *N*-demethylase and aniline hydroxylase activity in the mouse, often the liver weights and hepatic microsomal protein content were also elevated [2,4]. However, the elevation of microsomal protein following oral methadone administration appears to occur only at the highest doses administered, while phenobarbital caused significant increases over a wide range of doses.

In the present study, mice induced with methadone had relative microsomal activities much lower than their phenobarbital-treated counterparts even at the highest methadone doses employed (Fig. 1). The greatest increases in the methadone animals were seen for aminopyrine *N*-demethylase, followed by aniline hydroxylase and methadone *N*-demethylase activities. For the most part, each of these activities appeared almost to plateau with increasing dose. Only aminopyrine *N*-demethylase activity demonstrated a dose–response curve similar in appearance to that of the phenobarbital-treated mice. Even more striking was the absence of an elevation for the specific enzyme activity of methadone *N*-demethylase. The other two specific enzyme activities, in contrast, demonstrated dose–response curves similar in appearance to their respective relative activity dose–response curves. Pentobarbital sleeping times were depressed significantly for most doses of methadone. Moreover, these values declined linearly with increasing dose, yielding a response similar to that of the phenobarbital-treated mice. However, the decrease in sleeping times was not as great as that observed in the barbiturate-treated group.

In summary, it can be seen that methadone microsomal enzyme induction is similar to the phenobarbital-induced phenomenon. Both induce all three enzyme activities, monitored at nearly every dose administered, as well as increasing hepatic microsomal protein content and depressing pentobarbital sleeping times. However, significant differences are also evident, including the superior efficacy of induction for both relative and specific activities by phenobarbital, as indicated by the greater correlation coefficients generated for log dose treatment compared to the respective microsomal enzyme activity. In addition, the failure of methadone to increase liver weights in this study contrasts with phenobarbital hepatomegaly. Finally, the appearances of the dose–response curves for each of these agents are only remotely comparable.

In contrast to phenobarbital, 3-MC induction is markedly different from that of methadone. Although the percent increase of microsomal enzyme activity and elevation of

microsomal protein for both agents at the highest respective dose is nearly equivalent, 3-MC failed to elicit a significant elevation for any enzyme activity below a dose of 20 mg/kg. On the other hand, methadone administration was associated with an elevation of activity for almost every dosage regimen employed. Moreover, the respective dose–response curves for each agent are markedly dissimilar. Finally, other differences between the two treatments include a lack of effect on pentobarbital sleeping time and a decline in the relative liver weights following 3-MC dosing, all of which were not observed in the methadone-treated animals.

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