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Effect of the route of administration on microsomal enzyme induction following repeated administration of methadone in the mouse

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The metabolic inactivation of methadone is largely a result of the hepatic microsomal *N*-demethylating enzyme systems in both the rat [1, 2] and mouse [3], since the metabolic products of this pathway lack analgetic potency [4]. The induction of microsomal enzyme activity has been observed after repeated methadone administration following the oral route in mice [5] and rats [6–8] and intraperitoneal injection in mice [9]. On the other hand, at least one report found a lack of induction and a significant inhibition of microsomal enzyme activity in rats following as long as 60 days of repeated daily intubation with 50 mg/kg of this narcotic [10]. We suspect that differences in induction vary with the strain of rat employed. Thus far, this situation does not seem to be as extreme in mice [10].

Alvares and Kappas [1] reported that no increase in methadone *N*-demethylase activity could be demonstrated after intraperitoneal administration of 20 mg/kg of methadone daily in rats for 14 days. Peters [11] also reported that daily i.p. injections of 5 mg/kg of methadone to rats chronically did not significantly increase the activity of the methadone *N*-demethylase in liver microsomes. However, more recently the induction phenomenon was observed following intraperitoneal administration of methadone in mice, even with extremely low doses for long periods of time [9].

Although tolerance to the analgetic effect of methadone has been reported after prolonged subcutaneous administration in rats [12], Masten *et al.* [5] found only a slight increase in the activity of methadone *N*-demethylase in mice after 6 days of 20 mg/kg via this route. Thus, the development of CNS cellular tolerance to methadone is most likely the explanation of the former observation [13].

To date, no study has investigated the effects of the three routes of administration on the induction of microsomal metabolism in the same strain and species of rodent. Thus, we sought to investigate the effects of methadone administration upon its own *N*-demethylation by hepatic microsomal enzymes in mice receiving this narcotic by the three different routes (oral, intraperitoneal, and subcutaneous) employing identical dosage regimens for 6 days. We also wished to determine the effect of repeated methadone administration on aniline hydroxylation (a Type II substrate) and aminopyrine *N*-demethylation (a Type I substrate) *in vitro* [14], as well as on the metabolism of pentobarbital (a Type I substrate) *in vivo* [14], employing sleeping time.

Animals. Male albino ICR mice (25–30 g), obtained from Harlan Industries, Cumberland, IN, were used for both *in vivo* and *in vitro* phases of this study. These animals were housed in clear plastic mouse cages (five or six per cage) which were cleaned every other day to avoid the accumulation of ammonia, a potential inhibitor of microsomal metabolism [15]. A 12-hr light–dark cycle was maintained, while the ambient temperature was kept at 23 ± 1°. The mice were allowed free access to food (Purina Laboratory Chow, Ralston–Purina Co., St. Louis, MO) and water. SAN-I-CEL bedding (Paxton Processing Co., Whitehouse Station, NJ) was used throughout the study. After a 2-week acclimation period in our animal facilities, mice were employed in the experiments as described.

Drugs and dosage. Methadone hydrochloride and sodium pentobartbital were purchased from the Mallinckrodt Chemical Works, St. Louis, MO, and Abbott Laboratories, North Chicago, IL, respectively. Methadone, administered by oral intubation, was dissolved in water, whereas the parenteral solutions were dissolved in saline. The volumes of solution administered via the oral and the parenteral routes were 0.02 and 0.01 ml/g body weight, respectively. In the control groups, the appropriate vehicle was substituted for each drug administered.

The mice received either 3.13, 6.25, 12.5 or 25.0 mg/kg/day of methadone hydrochloride for 6 days by three different routes of administration: oral, intraperitoneal and subcutaneous. Two additional dosages 35.0 and 50.0 mg/kg/day were employed only via the oral route since these regimens were lethal to mice when administered repeatedly by either of the other two routes. All mice were taken from the same shipment of animals with the exception of the mice used for *in vitro* work for oral dosages of 25.0, 35.0 and 50.0 mg/kg. Each group consisted of eight to twelve mice.

Administration of drugs or vehicle took place between the hours of 1:00 and 2:00 p.m. each day; body weights were recorded at this time.

Tissue preparation and assays. For all *in vitro* work, mice were weighed and killed 24 hr after the sixth daily dose of methadone. This length of time was shown to be adequate for significant microsomal induction via the oral route with methadone [5]. Death via cervical dislocation was followed by decapitation.

The livers were removed (excising the gall bladder), blotted dry and weighed. The liver was then homogenized immediately with 3 vol. of ice-cold 0.05 M Tris–HCl–0.15 M KCl buffer, pH 7.4. After centrifugation, the 12,000 *g* supernatant fraction was used for the enzyme assays. The metabolic

procedures as outlined by Fouts [16] were followed. Each assay employed a portion of the 12,000 *g* supernatant fraction which was equivalent to approximately 5.0 mg of microsomal protein. Twenty μmoles of methadone HCl or of aminopyrine was used as substrate for the respective *N*-demethylase assays. Formaldehyde generation in these assays was determined by the method of Nash [17]. In the aniline hydroxylase assay, 10 μmoles of aniline hydrochloride was utilized as the substrate and *p*-aminophenol formation was monitored according to Mazel [18]. All reactions were run for 30 min during which time the product was generated at a constant rate. 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*. The amount of protein present in the resulting pellet was determined by the method of Lowry *et al.* [19] using bovine serum albumin (Fraction V; Nutritional Biochemical Corp., Cleveland, OH) as the standard.

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

Expression of microsomal parameters. The activities of the *in vitro* microsomal enzyme systems which were examined are expressed two different ways in Results. The first expression describes enzyme activity per kg of the body weight of the animal. An example would be μmoles of product formed/30 min/kg body weight. This is referred to as the relative activity of the enzyme. 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 [20].

Statistical analysis of results. Initially, analysis of variance was run on the data for each route to insure the homogeneity of the sample. Student's two-tailed *t*-test was run on all comparisons, the criterion for significance being $P < 0.05$. Calculations were made using the services of the University of Mississippi DEC-system 10 Computer and the Statistical Package for Social Sciences (SPSS) format [21].

Methadone N-*demethylase.* As seen in Fig. 1, the relative methadone *N*-demethylase activity (expressed as μmoles formaldehyde/30 min/kg body weight) was increased by methadone administered by both the oral and the intraperitoneal routes. There appeared to be an increase in the inductive effect after intraperitoneal treatment beginning with doses as low as 6.25 mg/kg, and reaching a 72 per cent increase in activity at the 25.0 mg/kg level. Following oral administration of methadone, enzyme activity was elevated with 6.25 mg/kg and all larger doses except 12.5 mg/kg. Each elevation via this route was statistically significant. Interestingly, the percentage increase was essentially the same with 25.0 mg/kg for the p.o. and i.p. routes. Doses above this level also demonstrated significant enzyme induction via the oral route. The lowest dose of methadone caused a slight decrement of *N*-demethylase activity by both routes. However, the depression observed was significant only for the i.p. route. In contrast, no increase in methadone *N*-demethylase activity was observed for any dose of this narcotic via the subcutaneous route. Following one dose (12.5 mg/kg), there was actually a significant decrease in activity.

The specific methadone *N*-demethylase activity in Fig. 1 (expressed as μmoles formaldehyde/30 min/mg of microsomal protein/kg body weight) does not show a pattern similar to that of the relative activity. Via the intraperitoneal route, only the highest dose was responsible for a significant elevation, whereas following oral administration the specific enzyme activity was elevated only after 6.25 mg/kg. The highest oral doses did not produce any change in this activity except for 50.0 mg/kg, after which, it was significantly depressed ($P < 0.05$). Finally, injection by the s.c. route was followed by a decrease in specific enzyme activity which was

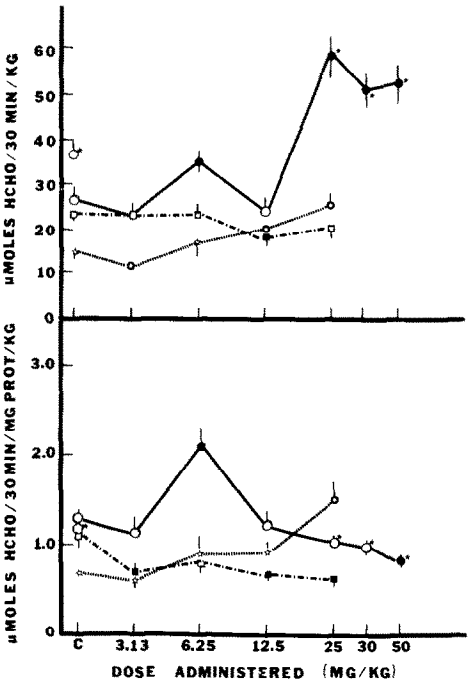

Fig. 1. Dose-related effects of repeated methadone administration by three different routes on the relative activity (top) and the specific activity (bottom) of methadone *N*-demethylase. Mice received methadone hydrochloride daily for 6 days by either the oral (○), intraperitoneal (*), or subcutaneous (□) route of administration. The points represent the mean activity of eight to twelve mice per group and the vertical bars represent the standard error of the mean. The solid symbols represent points significantly different ($P < 0.05$) from their respective control values. Oral doses of 25 mg/kg and higher were run at a later date and are starred, along with their respective control value.

statistically significant for three out of the four dosages employed. Since the expression of enzyme activity is related to microsomal protein, we will consider changes in this parameter below.

Aminopyrine N-*demethylase activity.* The relative aminopyrine *N*-demethylase activity (Fig. 2) exhibited the same general increases that were seen when methadone was used as an *N*-demethylase substrate. However, there appears to have been a leveling of activity using the i.p. route for regimens of 12.5 mg/kg and greater, with approximately a 2-fold increase over the controls. Significant increases in the relative *N*-demethylase activity were seen with both the 3.13 and 6.25 mg/kg doses when the s.c. route was employed. However, at higher doses using this route, there was actually a slight depression in activity, including a significantly decreased level after 12.5 mg/kg, paralleling that observed with methadone *N*-demethylase. The inductive effect of 25 mg/kg of methadone was demonstrated following i.p. and p.o. administration by 68 and 40 per cent increases, respectively, over control values. The induction of enzyme activity remained elevated at the highest oral doses. Once again, it should be pointed out that a separate control group was employed with doses of 25.0 mg/kg and greater. In the case of aminopyrine *N*-demethylase activity, an increased variability in the control values was noted for oral administration when compared to methadone *N*-demethylase activity. Thus, the validity of comparing the respective control groups to the appropriate treated animals was justified.

The effect of repeated doses of methadone on the specific activity of aminopyrine *N*-demethylase (Fig. 2) bears little

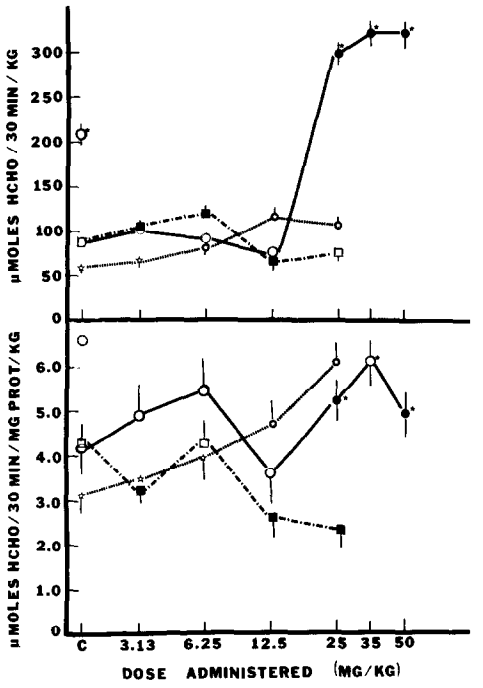

Fig. 2. Dose-related effects of repeated methadone administration by three different routes on the relative activity (top) and specific activity (bottom) of aminopyrine *N*-demethylase. See the legend of Fig. 1 for further details.

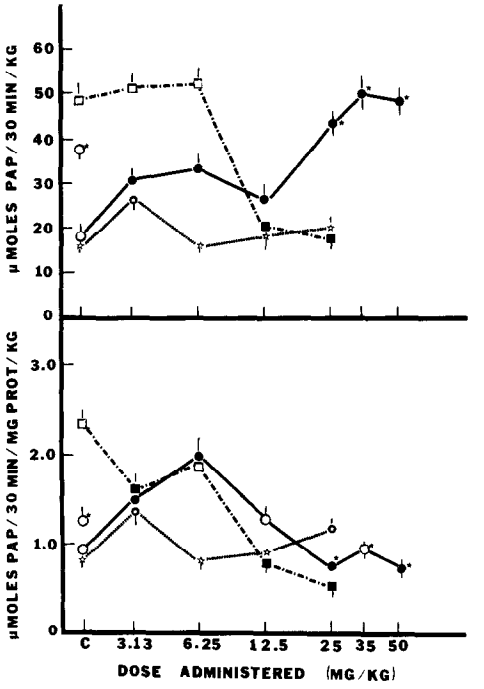

Fig. 3. Dose-related effects of repeated methadone administration by three different routes on the relative activity (top) and the specific activity (bottom) of aniline hydroxylase. See the legent of Fig. 1 for further details. PAP = *p*-aminophenol.

resemblance to the relative enzyme activity except that the i.p. route resulted in increased activity for higher doses of methadone. In contrast, both the p.o. and s.c. routes showed either no change or were depressed significantly. Overall, the pattern of activity for this microsomal enzyme is quite similar to the methadone *N*-demethylase activity previously mentioned.

Aniline hydroxylase activity. Repeated oral administration of methadone resulted in an elevation of the relative aniline hydroxylase activity (μmoles *p*-aminophenol/30 min/kg body weight) to significant levels for all doses tested (Fig. 3). The most pronounced elevation following oral administration occurred at the two lowest doses of methadone (a 71 and 80 per cent elevation for 3.13 and 6.25 mg/kg, respectively), although all doses employed via this route resulted in significant induction of activity. In contrast, the only dosage effective in inducing aniline hydroxylase activity via the i.p. route was 3.13 mg/kg. Other doses elicited no significant change in this parameter. Subcutaneously, methadone administration resulted in a significant depression at the two highest dose levels with no change in activity at lower doses.

The specific activity of aniline hydroxylase was elevated only at the two lowest doses orally, and was actually depressed at higher doses. Only the lowest and highest doses of the i.p. administered methadone elicited a significant induction of activity; however, no significant depression was noted. Nearly all doses of methadone were associated with a significant depression of aniline hydroxylase activity following s.c. administration.

Pentobarbital sleeping times. Figure 4 shows the difference between the routes of methadone administration with respect to pentobarbital-induced sleep. Mice slept significantly less after all doses of methadone administered intraperitoneally. There were also decreased sleeping times which correlated with increases in oral methadone administration, although only the increases after the two highest doses were statistically significant. In contrast, s.c. methadone treatment did not shorten the duration of sleep at any dose employed. If anything, there was a trend toward increase in this measure.

Relative liver weights and relative liver microsomal protein. Three distinct patterns were observed for these measures using the three routes of administration of methadone (Fig. 5). Following orally administered methadone, the only change noted by day 6 was a significant elevation in the relative liver microsomal protein (mg microsomal protein/kg body weight), and this effect was found only in animals receiving 25.0 mg/kg/day or more. Intraperitoneally, methadone did not affect either parameter significantly. In contrast, the subcutaneous route was associated with a dose-related diminution in the relative liver weight (g liver/kg body weight), and an elevation in the relative liver microsomal protein content.

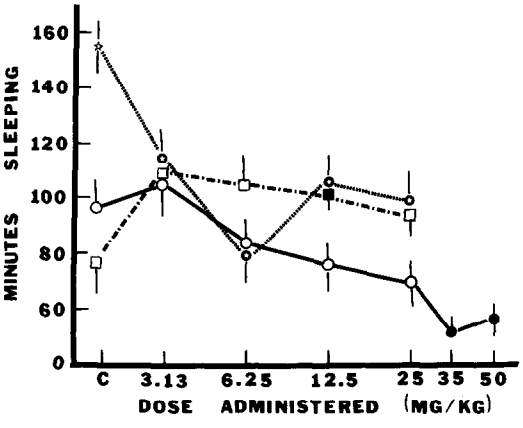

Fig. 4. Dose-related effects of repeated methadone administration by three different routes on pentobarbital-induced sleeping times. See the legend of Fig. 1 for further details.

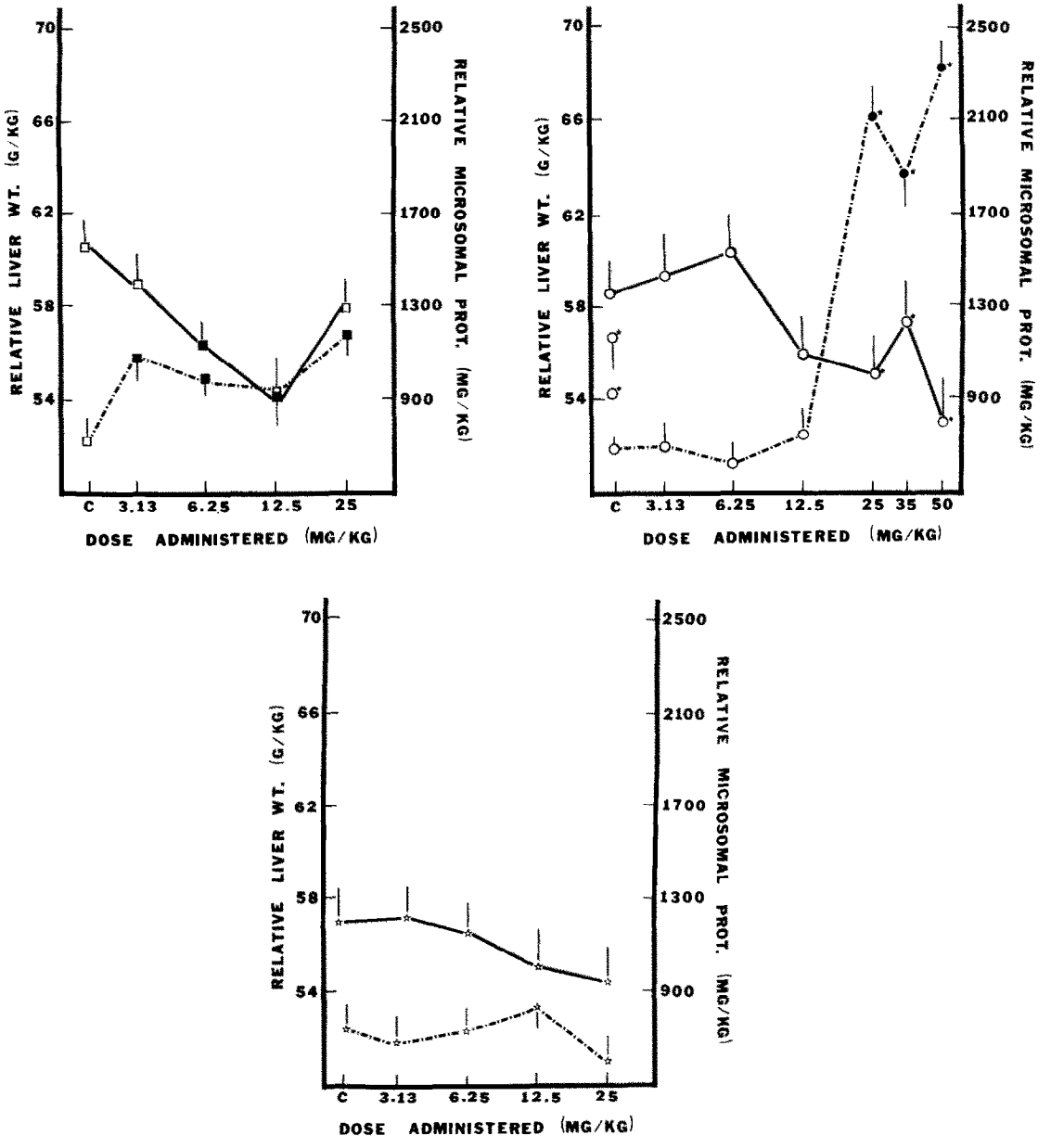

Fig. 5. Dose-related effects of repeated methadone administration, via the subcutaneous (left), oral (right) and the intraperitoneal (lower) routes, on relative liver weights (solid line) and relative microsomal protein (broken line). The diamonds and stars indicate the respective control values compared to the three highest dose groups of the orally administered methadone. See the legend of Fig. 1 for further details.

Body weights. There were no significant changes in the body weights of mice receiving any of the doses of methadone used in this study throughout the 6-day administration period. The means of the control body weights of mice on day 6 were 31.2 ± 0.7, 30.3 ± 0.6 and 29.9 ± 0.5 g for the oral, intraperitoneal and subcutaneous routes respectively.

Hepatic microsomal enzyme induction was marked after repeated administration of methadone, employing either the oral or the intraperitoneal route in mice. This inductive effect was prominent in both our *in vitro* and *in vivo* test systems. Methadone was capable of inducing microsomal enzyme activity for both a Type I (aminopyrine) [14] and a Type II (aniline) [14] binding substrate, in addition to inducing its own metabolism. It is interesting to note that only the oral route was associated with a major elevation of aniline hydroxylase activity, while a marginal increase in activity was observed following the i.p. administration of this narcotic. In contrast to these two routes, subcutaneous administration produced little or no induction of any microsomal activity monitored in this study. In fact, a substantial decrease in aniline hydroxylase activity was noted following higher doses of methadone.

Furthermore, the results obtained with pentobarbital sleeping times seemed to follow the same pattern of induction demonstrated for aminopyrine metabolism. A significant decrement in sleeping times was demonstrated with both oral and i.p. routes of administration, while use of the s.c. route was associated with a significant elevation in this measure for one dose of methadone. The apparent decrease in enzyme activity and increase of pentobarbital sleeping times following

subcutaneous administration of the highest dose of methadone may be explained by the fact that this narcotic can accumulate in the liver after repeated doses. A high endogenous level would be expected to inhibit *in vitro* microsomal assays due to competitive inhibition. Moreover, methadone (s.c.) is bound to plasma [22] and tissue protein [23, 24] to a greater extent when compared to the oral or i.p. route. The lungs have been reported to be the major binding site for methadone in rats [6, 25], dogs [26], and man [27]. Chi and Dixit [25] have shown that the lungs have a large capacity to bind methadone against an apparent concentration gradient after 20 mg/kg, s.c., in rats. In this regard, the cumulative binding of methadone to lung tissue seems likely after its repeated administration, especially when introduced peripherally to the liver. This phenomenon may also explain why chronic s.c. doses do not induce liver microsomal enzymes as readily as methadone given i.p. or orally.

In comparing the subcutaneous with the oral route, Misra *et al.* [6] found that the brain and plasma levels of methadone and its metabolites were consistently lower in rats after chronic subcutaneous administration than for controls dosed acutely. However, they found that plasma half-lives of methadone were reduced much more effectively after repeated oral doses of methadone, suggesting a superior inductive capacity of this route.

Additional circumstantial evidence for this difference in routes of administration can be inferred from significantly higher LD_{50} values in rats treated orally compared to those treated subcutaneously [28, 29] with methadone. Also, Stockhaus and Wick [30] reported a 2-fold increase in the LD_{50} of methadone in rats when given intraduodenally compared to the s.c. route. Thus, any route favoring the initial passage of methadone through the liver, and resulting in its *N*-demethylation to inactive metabolites, may emphasize the development of metabolic tolerance.

It appears quite likely that the disposition of methadone via the i.p. route favors a significant role for the liver in its metabolism and biliary excretion as has been reported previously for rats [6, 31–33]. Thus, it is not too surprising that our results appear to show a similarity between the oral and i.p. routes for repeated methadone administration, since both routes favor the liver in the primary disposition of this narcotic. At the present time, however, no explanation can be offered which will account for the differences observed, with respect to aniline hydroxylase activity, in methadone-treated mice when the methadone is administered via these two routes.

It is of interest that with all doses tested, via the three routes of administration, there was no increase in the relative liver weights. In fact, there was a decrease in liver weight when methadone was given repeatedly by the s.c. route. Moreover, our results show that repeated oral administration of methadone may elevate liver microsomal protein by as much as 100 per cent over controls at 50 mg/kg in 6 days. However, we found no change in liver microsomal protein after i.p. methadone treatment when microsomal enzyme activity was significantly elevated. Furthermore, the statistically significant rise of microsomal protein after the s.c. methadone treatment (in light of a depressed enzyme activity) tends to raise some doubt as to the conceptual relevance of expressing enzyme activity only per mg of microsomal protein.

In conclusion, we feel that the results of this study may explain differences in the pharmacologic effects of methadone following these three routes of administration. Furthermore, these findings may potentially aid investigators in the interpretation of apparent discrepancies in the existing literature employing various expressions of microsomal enzyme activity.

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