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INHIBITION BY ETHYLMORPHINE AND PENTOBARBITONE *IN VITRO* OF THE METABOLISM OF [UREYL-^{14}C]TOLBUTAMIDE BY HEPATIC MICROSOMAL PREPARATIONS FROM MALE AND FEMALE RATS TREATED WITH PHENOBARBITONE

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Abstract—Lineweaver–Burk plots for the hepatic microsomal metabolism *in vitro* of [ureyl-^{14}C]tolbutamide show that ethylmorphine and pentobarbitone act as inhibitors in a "mixed" fashion, irrespective of the sex or phenobarbitone-treatment of the rats. Apparent K_m values for the metabolism of [ureyl-^{14}C]tolbutamide are similar for microsomes from male and female animals; whether phenobarbitone-treated or not. V_{max} is lower for female rats and is increased by phenobarbitone-treatment of the animals to the same extent (2½ times) as for male rats. The metabolism of [ureyl-^{14}C]tolbutamide (0·4 mM) by hepatic microsomes from female animals is poorly inhibited by pentobarbitone and ethylmorphine *in vitro* compared with the metabolism by microsomes from male animals. Phenobarbitone-pretreatment of the animals *in vivo* increases the degree of inhibition of the microsomal metabolism by the higher concentrations relative to the lower concentrations of each inhibitor *in vitro*.

ALTHOUGH many compounds inhibit the hepatic microsomal metabolism of drugs *in vitro*,[1,2] relatively few studies have been carried out to determine the type of inhibition. In one of the earlier studies, Rubin, Tephly and Mannering,[3] showed that the inhibition of the *N*-demethylation of ethylmorphine by hexobarbitone, chlorpromazine, zoxazolamine, phenylbutazone and acetanilide was always fully competitive, even if the male rats used were treated with phenobarbitone for 5 days before preparation of microsomes. We have investigated the inhibition of the metabolism of the more slowly metabolized drug, tolbutamide (1-butyl-3-(*p*-tolylsulphonyl)urea) and we have employed [ureyl-^{14}C]tolbutamide as substrate because of the high sensitivity of the assay for its metabolite, 1-butyl-3-(*p*-hydroxymethylphenyl)sulphonyl-[^{14}C]-urea (hydroxy[ureyl-^{14}C]tolbutamide). Pentobarbitone and ethylmorphine have been used as inhibitors—the former itself is metabolized by hepatic microsomes *in vitro* by an aliphatic hydroxylation, while the latter is *N*-demethylated, and differences might be expected in the type of inhibition. The effect of phenobarbitone-pretreatment of the animals *in vivo* on the degree of inhibition of the hepatic microsomal metabolism of [ureyl-^{14}C]tolbutamide *in vitro* is shown to vary with the sex of the rats used. Few other studies have been reported concerning the effect of natural factors, such as sex, on the inhibition of drug metabolism by artificial agents, although the effect of such natural factors on microsomal drug metabolism *per se* is well documented.[4–7]

MATERIALS AND METHODS

Animals. Male (200–240g) and female (160–190g) rats of the Wistar strain were supplied by Scientific Products Farm Limited, Ash, Kent, England. Phenobarbitone was administered intra-peritoneally in aqueous solution at a dose of 50 mg/kg body weight once daily for 2 days, and the animals were killed 2 days after the second dose. Control animals were sham-injected similarly, using isotonic saline.

Chemicals. $NADP^+$ and glucose-6-phosphate (sodium salt) were bought from Boehringer Corporation, London W5, England. Glucose-6-phosphate dehydrogenase was obtained from Sigma (London) Limited, Lettice Street, London SW6, England. Ethylmorphine hydrochloride (Evans Medical Limited, Liverpool 24, England), and pentobarbitone sodium ("Nembutal", gift of Abbott Laboratories Limited, Queenborough, Kent, England) were used as supplied. [Ureyl-^{14}C]tolbutamide was a gift from Farbwerke Hoechst A/G., Frankfurt/Main, Germany and before use a trace contaminant with chromatographic properties similar to those of the hydroxylated metabolite was removed, as described elsewhere.[8]

Preparation of microsomes. Animals were killed by cervical dislocation and the livers placed after excision in ice-cold buffered 1·15% KCl, which was 0·005 M with respect to potassium phosphate pH 7·4. Homogenization and centrifugation procedures followed those of Darby.[9] The microsomal pellets were finally suspended in ice-cold buffered 1·15% KCl, so that 1 ml contained 500 mg wet liver equivalent.

Assay of [ureyl-^{14}C]tolbutamide metabolism. Incubations were carried out for 15 min, essentially as described elsewhere.[8] Incubation mixtures (2·5 ml, pH 7·4) contained, in addition to potassium phosphate (150 μmoles), $MgSO_4$ (10 μmoles), and NADPH-generating mixture ($NADP^+$ 0·5 μmoles: glucose-6-phosphate 8 μmoles; glucose-6-phosphate dehydrogenase 1·8 u); microsomal suspension (0·5 ml); and [ureyl-^{14}C]-tolbutamide (0·375–1·0 μmoles in the kinetic studies; 1·0 μmole in the other studies; 3·75 μc/μmole). Pentobarbitone sodium, or ethylmorphine hydrochloride were added as aqueous solutions so that final concentrations were 0·5 and 1·5 mM. Appropriate "recovery" mixtures were run simultaneously, the [ureyl-^{14}C]tolbutamide being added at the end of the incubation period. The reaction was stopped by the addition of 2·0 ml of 1·0 M sodium acetate solution, pH 5·0.

Measurement of the hydroxy[ureyl-^{14}C]tolbutamide formed was performed as described elsewhere.[8] using petroleum spirit to extract differentially the unchanged [ureyl-^{14}C]tolbutamide, and ethyl acetate to extract hydroxy[ureyl-^{14}C]tolbutamide. However, methanol, instead of ethyl acetate, was used to dissolve the radioactive residues after evaporation of the ethyl acetate extracts prior to liquid scintillation counting. Corrections were made for the recovery values. Microsomal protein was measured by the method of Lowry *et al.*,[10] bovine serum albumin being used as standard.

Michaelis constants. Experimental data were plotted as reciprocals for Lineweaver–Burk presentation of the relationship between the rate of metabolism of [ureyl-^{14}C]-tolbutamide and its concentration. Straight lines were fitted to the points by a standard least-squares regression analysis. As pointed out by Darby *et al.*,[8] although Riggs,[11] criticizes this method because the reciprocals of the lowest concentrations of substrates in a wide range may be subject to a large error, the narrow range of concentrations used here enables least-squares method to be applied.

RESULTS

Figure 1 shows typical inhibition patterns found when ethylmorphine was used as inhibitor. In no case was a completely clear-cut point of intersection observed. There is no question that all the plots are soundly statistically based, the *lowest* value of t being 5·93 (D.F. = 2) (r = 0·972), giving P < 0·05. It is clear from Figs. 1a and 1c, despite the imperfect intersections, that ethylmorphine inhibits the metabolism of [ureyl-^{14}C]tolbutamide by hepatic microsomes from male rats, phenobarbitone-treated or not, in a manner neither fully competitive nor fully non-competitive, while the metabolism of [ureyl-^{14}C]tolbutamide by hepatic microsomes from female rats, phenobarbitone-treated or not, is also inhibited in a mixed manner, but apparently more non-competitively at the higher inhibitor concentrations (Figs. 1b and 1d).

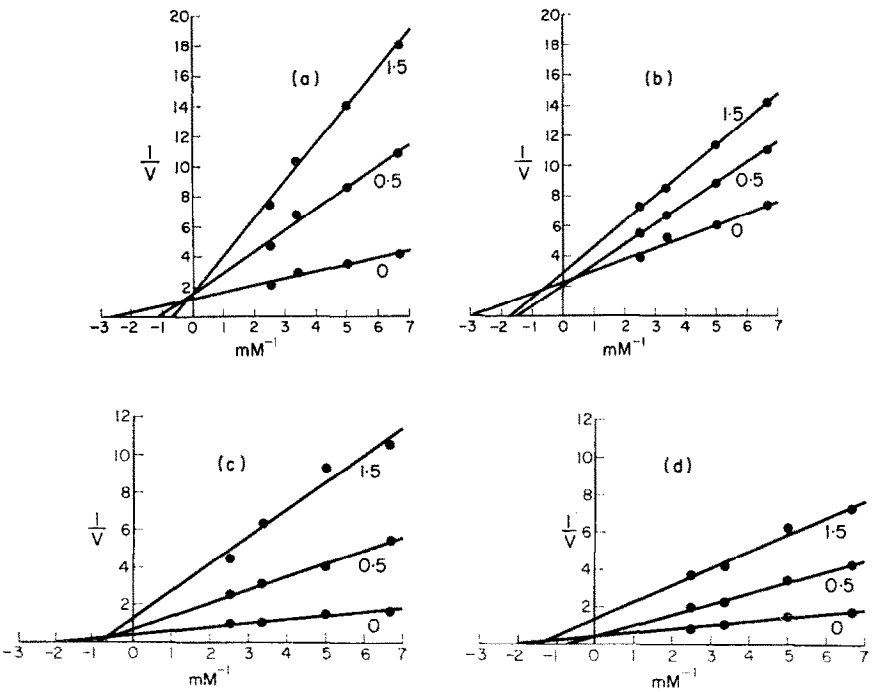

FIG. 1. Typical Lineweaver–Burk plots of the inhibition by ethylmorphine of the metabolism *in vitro* of [ureyl-^{14}C]tolbutamide by hepatic microsomal preparations from male and female rats treated with phenobarbitone or sham-injected.
(a) Male, control. (b) Female, control. (c) Male, phenobarbitone-treated. (d) Female, phenobarbitone-treated. V = nmoles hydroxy [ureyl-^{14}C]tolbutamide formed/min/mg microsomal protein. P < 0·05 for all lines. Inhibitor concentrations are in mM.

An almost identical pattern is apparent from the inhibition patterns found when pentobarbitone was used as inhibitor (Fig. 2). The lowest value of t was 5·2 (D.F. = 2) (r = 0·964), giving P < 0·05 (Fig. 2c). The activity of microsomes from male rats is inhibited in a mixed manner (Figs. 2a, 2c), while the activity of microsomes from female rats is again inhibited in a mixed manner and apparently more non-competitively at the higher inhibitor concentration. All the inhibition patterns shown in Figs. 1

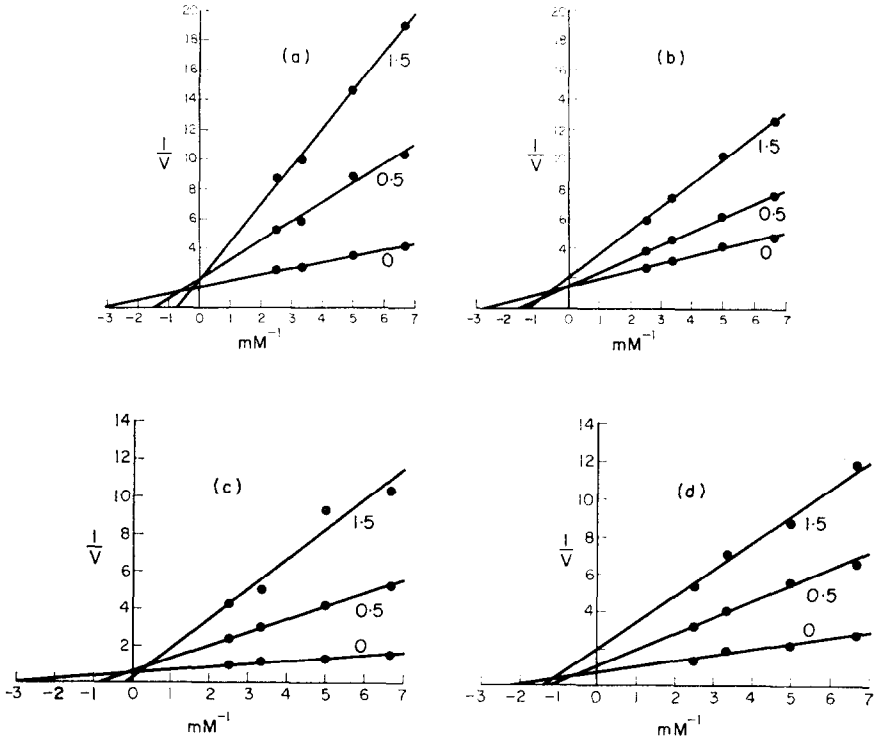

FIG. 2. Typical Lineweaver–Burk plots of the inhibition by pentobarbitone of the metabolism *in vitro* of [ureyl-^{14}C]tolbutamide by hepatic microsomal preparations from male and female rats treated with phenobarbitone or sham-injected.
(a) Male, control. (b) Female, control. (c) Male, phenobarbitone-treated. (d) Female, phenobarbitone-treated. V = nmoles hydroxy [ureyl-^{14}C]tolbutamide formed/min/mg microsomal protein. $P < 0{\cdot}05$ for all lines. Inhibitor concentrations are in mM.

and 2 could be duplicated or triplicated, essentially the same mixed inhibitions being observed, even the greater degree of non-competitive inhibition at the higher inhibitor concentrations with microsomes from female rats.

Table 1 sets out apparent Michaelis parameters calculated from the data used to prepare Figs. 1 and 2. Although the value of K_m (mM) is similar for male and female rats, both sham-injected and phenobarbitone-treated, V_{max} is lower for female animals. V_{max} is elevated to the same extent (2·5-fold) for female animals as for male animals (2·4-fold) following phenobarbitone treatment, i.e. the ratio V_{max} (male)/V_{max} (female) is the same (1·5) for treated and untreated animals.

Figure 3 compares the effects of the different concentrations of inhibitors *in vitro* on the rate of metabolism of a fixed concentration of [ureyl-^{14}C]tolbutamide by microsomal preparations from the livers of male and female, sham-injected or phenobarbitone-treated animals. The inhibitors affect microsomes from control female animals least and those from phenobarbitone-treated male rats most. Phenobarbitone pretreatment of the animals *in vivo* apparently causes the hepatic microsomal metabolism of [ureyl-^{14}C]tolbutamide to become more easily inhibited *in vitro* by pentobarbitone or ethylmorphine. This is seen from Table 2, which compares the ratios of the rates

TABLE 1. APPARENT MICHAELIS CONSTANTS FOR THE METABOLISM *in vitro* OF [UREYL-^{14}C]-TOLBUTAMIDE BY HEPATIC MICROSOMES PREPARED FROM MALE OR FEMALE RATS TREATED WITH PHENOBARBITONE, OR SHAM-INJECTED

Animals	Number of experiments	K_m (mM)	V_{max} (nmoles of hydroxy-[ureyl-^{14}C]tolbutamide formed/min/mg microsomal protein)
Male control	8	0·40 ± 0·04*	0·89 ± 0·06†
Male phenobarbitone-treated	5	0·46 ± 0·05*	2·13 ± 0·17†
Female control	7	0·48 ± 0·08*	0·59 ± 0·07†
Female phenobarbitone-treated	8	0·41 ± 0·05*	1·48 ± 0·12†

The data are expressed as means ± S.E.M.
* Not significantly different.
† Highly significantly different from each other ($P < 0·01$ or $P < 0·001$; Student's *t*-test).

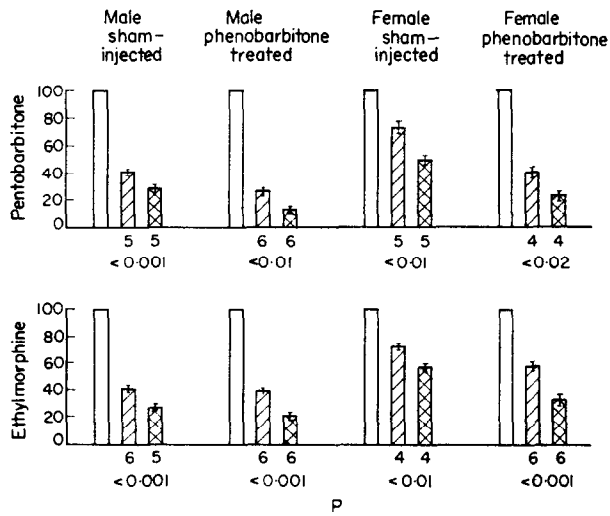

FIG. 3. Effect *in vitro* of pentobarbitone or ethylmorphine on the metabolism of [ureyl-^{14}C]tolbutamide by hepatic microsomes from male or female rats sham-injected or treated with phenobarbitone *in vivo*.

□ No inhibitor *in vitro*, expressed as 100 per cent.
▨ Inhibitor concentration *in vitro* 0·5 mM.
▩ Inhibitor concentration *in vitro* 1·5 mM.
The height of each vertical bar represents the rate of metabolism expressed as a percentage relative to the corresponding no-inhibitor value and calculated as a mean. The solid vertical lines at the top of each bar represents twice the S.E.M.
P values were derived by Student's *t*-test from the data for 0·5 and 1·5 mM inhibitor concentrations. The number under the vertical bars represent the number of animals used.

of metabolism of [ureyl-^{14}C]tolbutamide at the two different concentrations of each inhibitor. Phenobarbitone pre-treatment of the rats increases the ratios by approximately 30 per cent (pentobarbitone as inhibitor *in vitro*, male or female rats) or 20 and 40 per cent (ethylmorphine as inhibitor, male or female rats respectively). The effect of phenobarbitone is seen in Fig. 3 as a decrease in the rates of metabolism

in vitro associated with a particular inhibitor concentration. There is one apparent anomaly in that the inhibitory effect of ethylmorphine at 0·5 mM *in vitro* is not affected by the phenobarbitone pre-treatment of the male rats *in vivo*. The metabolism of [ureyl-^{14}C]tolbutamide by microsomes prepared from the livers of female rats is clearly less sensitive to ethylmorphine or pentobarbitone *in vitro* at either concentration than the metabolism by hepatic microsomes from male rats.

TABLE 2. EFFECT OF PHENOBARBITONE-TREATMENT OF MALE AND FEMALE RATS *in vivo* ON THE RATIO OF THE RATES OF METABOLISM OF [UREYL-^{14}C]TOLBUTAMIDE BY THEIR HEPATIC MICROSOMES IN THE PRESENCE OF 0·5 AND 1·5 mM PENTOBARBITONE OR ETHYLMORPHINE

Inhibitor	Male control	Male phenobarbitone-treated	Female control	Female phenobarbitone-treated
Pentobarbitone	1·48 ± 0·12 $n = 5$	1·98 ± 0·08 $n = 6$	1·49 ± 0·02 $n = 5$	1·87 ± 0·12 $n = 4$
Ethylmorphine	1·49 ± 0·06 $n = 5$	1·82 ± 0·03 $n = 6$	1·25 ± 0·04 $n = 4$	1·75 ± 0·10 $n = 6$

The values are calculated as:

$$\frac{\text{rate of metabolism of [ureyl-}^{14}\text{C]tolbutamide in the presence of 0·5 mM inhibitor}}{\text{equivalent rate of metabolism in the presence of 1·5 mM inhibitor}}$$

and expressed as mean ± S.E.M.

All pairs of values ("control" vs. "phenobarbitone-treated" for each inhibitor) are significantly ($P < 0·02$) or highly significantly different ($P < 0·01$ or $< 0·001$) from each other.

DISCUSSION

There are few published studies of the effects of phenobarbitone treatment of animals *in vivo* on the kinetic constants of their hepatic microsomal drug metabolism. No studies seem to have been made with tolbutamide as substrate, and so comparisons cannot easily be made between the present study and the literature. It is known that treatment of male rats with phenobarbitone does not change K_m for the 9000 *g* supernatant or microsomal metabolism of ethylmorphine[3,12] (K_m 0·3–0·6 mM), hexobarbitone[3,13] (K_m 0·5–1·2 mM), aminopyrine[14] (K_m 1·0 mM), and naphthalene[15] (K_m 0·6 mM). However, K_m for the metabolism of morphine and aniline may be increased 2-fold[12,16] (K_m (control values) 0·5 and 0·04 mM). Phenobarbitone-treatment of the animals increases V_{max} by 2- to 4-fold for all substrates except morphine, for which there is no change.[12] That K_m for the microsomal metabolism of [ureyl-^{14}C]-tolbutamide is unchanged while V_{max} increases by 2½-times following treatment of the rats with phenobarbitone (Table 1) is in agreement with the reports cited for ethylmorphine, hexobarbitone and aminopyrine. The rise in V_{max} and lack of change in K_m is good evidence that a true induction (i.e. increased synthesis) of the microsomal drug-metabolizing system has occurred, rather than changes in the kinetic properties of the enzyme system.[3,14]

Sex differences have been reported for untreated rats for the metabolism by hepatic microsomes of hexobarbitone and aminopyrine[17] (K_m higher in the female than the male; V_{max}, lower), and ethylmorphine[4,5] (K_m probably unchanged, V_{max} lower in the female), and the differences in Table 1 are in line with these. The actual values for K_m are in agreement with those quoted earlier for other drugs. The values for V_{max}

are, when male control animals are considered, generally five to ten times lower than those published for ethylmorphine, aminopyrine and hexobarbitone. V_{max} values for female animals are three to ten times lower. Interestingly, the V_{max} values are similar to those published for aniline,[16] although the K_m values are four to ten times higher than those for aniline.

Because of the imprecise intersections of the Lineweaver–Burk plots in Figs. 1 and 2, it is difficult to be more precise about the mode of inhibition of ethylmorphine and pentobarbitone other than to say it is neither fully competitive nor fully non-competitive. If the three drugs concerned were all metabolized at the same site on the same enzyme system, then a fully competitive inhibition would be expected. The major reason why this is not observed, although sometimes approached, as in Figs. 1a and 2c, is that the microsomal preparations used constitute very crude enzyme systems. It is noteworthy that while the reconstitutable submicrosomal systems obtained by Lu, Strobel and Coon[18] represent a partially purified enzyme system, and can be used to demonstrate a fully competitive inhibition of benzphetamine hydroxylation by aniline, there is still appreciable scatter of the points along the lines fitted to them (Lineweaver–Burk plots). With small numbers of points (3–5), a small experimental error in one can alter the slope of the line fitted to them by a considerable number of degrees, and intersection points may by missed, even if mathematical methods are used to fit straight lines to the data. Careful inspection of the literature on drug metabolism shows that this consideration may apply generally to the determination of K_m and V_{max} and inhibition patterns by Lineweaver–Burk graphical methods.

The origin of the sex differences noted in Figs. 1 and 2 is not obvious. The data in Table 2 do not support the idea of there being more than one microsomal enzyme system involved in the metabolism of [ureyl-^{14}C]tolbutamide. The relative effects of the two concentrations of their inhibitor *in vitro* on the rates of metabolism are, in general, the same whether the microsomes originated from male or female animals. Phenobarbitone pre-treatment of the rats may in general increase the susceptibility to pentobarbitone and ethylmorphine *in vitro* of the microsomal metabolism of [ureyl-^{14}C]-tolbutamide (Fig. 3 and Table 2), but the relative effects of the two concentrations of either inhibitor are the same whether the microsomes originated from male or female phenobarbitone-treated animals. The mechanism by which phenobarbitone increases the sensitivity to pentobarbitone and ethylmorphine of the microsomal system metabolizing [ureyl-^{14}C]tolbutamide can perhaps be explained in terms of microsome-bound phenobarbitone (or its metabolites) synergizing with the added inhibitor *in vitro*. Such binding has been shown to occur by Ernster and Orrenius,[19] although they showed maximal binding to occur in the 12 hr immediately after treatment, and the animals used here were not killed until 48 hr after the last dose. If phenobarbitone treatment differentially reduced the lipid content of the microsomes, then higher effective concentrations of inhibitor in the incubation mixtures might result. However, the phospholipid content of microsomes is known to increase as a result of phenobarbitone treatment,[19,20] and the changes in susceptibility *in vitro* cannot be explained in this way. Experiments with sexually-immature rats should clarify the lower susceptibility to the inhibitors of the metabolism of [ureyl-^{14}C]tolbutamide by the female rats.

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