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EFFECTS OF PHENYLBUTAZONE ON LIVER MICROSOMAL DEMETHYLASE

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Abstract—The inhibitory action of phenylbutazone on the ethylmorphine demethylating enzyme was studied with microsomes from rats injected with phenylbutazone and with microsomes from untreated rats incubated with phenylbutazone. The enzymic activity in microsomes from treated animals was noncompetitively inhibited, while addition of phenylbutazone to the microsomal incubation mixtures caused competitive inhibition. The noncompetitive inhibition was shown to be irreversible by gel filtration experiments and associated with irreversible binding of phenylbutazone or a metabolite to microsomes.

PHENYLBUTAZONE (1,2-diphenyl-4-*n*-butyl-3,5-dioxopyrazolidine) is one of a number of drugs with a biphasic action on drug-metabolizing enzymes in the liver microsomes.[1] After the administration of a single dose, the activity of the enzymes is first depressed, and after 12 hr, the activity is enhanced.[2, 3] This report describes the inhibitory phase of phenylbutazone action on the demethylation of ethylmorphine by rat liver microsomes. The results indicate that the inhibition of microsomal demethylase produced by the drug *in vitro* is competitive, whereas that produced by administration of the drug to the living animal is noncompetitive and appears to be associated with irreversible binding of phenylbutazone to the microsomes.

MATERIALS AND METHODS

Tissue preparation. Male Sprague–Dawley rats weighing 80–100 g were killed by cervical dislocation. The livers were removed, washed in cold 0·5% NaCl, and homogenized with 3 vol. of 1·15% KCl–0·02 M Tris, pH 7·4 (Tris-KCl). The pooled homogenate from 4 animals was centrifuged at 9,000 *g* for 15 min and the supernatant fluid separated and centrifuged for 1 hr at 105,000 *g*. The microsomes were resuspended in a volume of Tris-KCl buffer equivalent in weight to the original liver. This suspension had a protein concentration of about 15 mg/ml.

In the washing procedures, the 105,000 *g* pellet above was resuspended in 30 ml of Tris-KCl buffer and again centrifuged at 105,000 *g* for 30 min. This process was repeated twice and the resulting pellet was resuspended in a volume of Tris-KCl buffer equivalent to one-half of the weight of liver used. The resulting suspension had a protein concentration of about 15 mg/ml.

* Portions of this work have been taken from a thesis submitted to the Department of Biochemistry, George Washington University, Washington, D.C., in partial fulfillment of the requirements for the Master of Science Degree.

For the assay of tissue levels of phenylbutazone, the animals were anesthetized with ether, and blood was collected by cardiac puncture. The livers were removed, homogenized as described above, and 1·5 ml of the homogenate extracted to assay phenylbutazone.[4]

Incubation mixtures. The complete reaction mixture was incubated at 37° (final volume, 3 ml in 25-ml Erlenmeyer flasks) and consisted of 1 ml of the microsomal suspension above, Tris, pH 7·4 (150 μmoles), NADP (1·2 μmoles), glucose 6-phosphate (15 μmoles), glucose 6-phosphate dehydrogenase (0·3 units/ml), ethylmorphine (1·8–6·0 μmoles), and phenylbutazone (10^{-3}–10^{-5} M) when indicated. The mixture was incubated for 10 min and the reaction stopped by successive additions of 1·5 ml saturated barium hydroxide, 0·5 ml zinc sulfate (17·8%), and 0·5 ml of saturated sodium borate. The suspension was centrifuged at 110 *g* for 10 min, and 3 ml of supernatant fluid was assayed for formaldehyde, the product of the demethylation reaction.

Chemical assays. Formaldehyde was assayed as described by Davies *et al.*,[5] and microsomal protein was determined by the method of Lowry *et al.*,[6] using crystalline serum albumin as a standard. Phenylbutazone-^{14}C in homogenates and plasma was extracted into heptane[4] and the radioactivity assayed by liquid scintillation spectrometry. Burns *et al.*[4] have shown that this extraction procedure removes unchanged phenylbutazone but none of its metabolites.

The radioactivity in microsomal suspensions was determined by counting 0·5 ml of the suspension added to 15 ml of scintillation phosphor consisting of 0·4% BBOT (2,5-bis-(5-*tert*-butylbenzoxazolyl) thiophene), 0·8% naphthalene, and 40% methylcellosolve in toluene. The dpm per mg protein was converted to phenylbutazone equivalent by dividing by the specific activity of the injected phenylbutazone.

Gel filtration of microsomes. A 4-ml vol. microsomal suspension containing about 30 mg/ml protein was prepared from the pooled livers of four rats and filtered through a 50-ml column (28 cm × 1·5 cm) of Sephadex G-50 (fine) prepared in the Tris-KCl buffer. The microsomes were eluted with Tris-KCl buffer and centrifuged at 80,000 *g* for 30 min and resuspended to a final concentration of about 15 mg/ml. This suspension was then assayed for demethylase activity or bound radioactivity as indicated in the text.

Chemicals. The phenylbutazone-^{14}C (sp. act. 3·09 μc/mg, uniformly labeled in the phenyl rings) was generously provided by Dr. Murray Weiner of Geigy Pharmaceuticals, Ardsley, N. Y.

RESULTS

Figure 1 shows the effects of phenylbutazone (100 mg/kg, i.p.) on the demethylation of ethylmorphine in rat liver microsomes. Six hr after administration of phenylbutazone, demethylation was inhibited by about 35 per cent. The activity was partly restored in 12 hr, was greater than the control value in 24 hr, and 24 hr after a second injection was 50 per cent greater than the control value.

Table 1 shows the inhibitory effects of phenylbutazone on ethylmorphine demethylation in microsomes from rats killed 2 hr after the administration of various doses of phenylbutazone. The per cent inhibition increases with dose but approached a limiting value of about 35 per cent. A reciprocal plot of the reaction velocity vs. the substrate

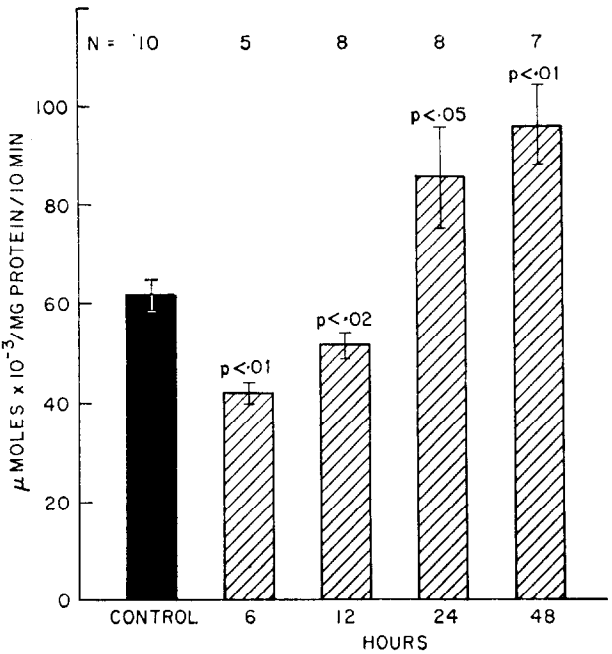

FIG. 1. Effect of phenylbutazone on demethylase activity *in vivo*. Animals were injected with 100 mg/kg phenylbutazone and killed at the times indicated. The microsomes were assayed for demethylase activity at a substrate concentration of 2·0 mM. The 48-hr animals were given a second injection at 24 hr. N = number of animals used, and the vertical lines are standard errors.

TABLE 1. COMPARISON OF THE INHIBITORY EFFECT OF PHENYLBUTAZONE ON MICROSOMAL DEMETHYLASE ELICITED *IN VIVO* AND *IN VITRO**

In vivo		*In vitro*	
Dose (mg/kg)	Demethylase activity (% Control)	Concentration of phenylbutazone (moles/l.)	Demethylase activity (% Control)
10	93 ± 0 (3)	10^{-5}	93·0 ± 4 (3)
50	75 ± 7 (4)	5×10^{-5}	70·0 ± 3 (3)
100	56 ± 8 (3)	10^{-4}	56·5 ± 8 (3)
175	64 ± 6 (3)	5×10^{-4}	32·0 ± 3 (3)
		10^{-3}	20·0 ± 4 (2)

The rate of ethylmorphine demethylation was determined in microsomes incubated for 10 min with 0·8 mM ethylmorphine. The effect *in vitro* is the enzyme activity in microsomes from untreated rats in the presence of various concentrations of phenylbutazone. The treated animals *in vivo* were killed 2 hr after injection. The activity is expressed in per cent of control ± S. D., i.e. of untreated microsomes. Numbers in parentheses refer to the number of separate experiments.

concentration showed that phenylbutazone inhibited the activity of the demethylase noncompetitively (Fig. 2 A). The apparent V_{max} of the enzymes was reduced.

In contrast, the demethylase activity was inhibited competitively when microsomes from untreated animals were incubated with phenylbutazone (Fig. 2 B). The apparent K_m was increased without changing the V_{max} of the enzyme. Moreover, the inhibition of the enzyme did not appear to reach a limiting value and was inhibited by about 85 per cent at 10^{-3} M phenylbutazone (Table 1).

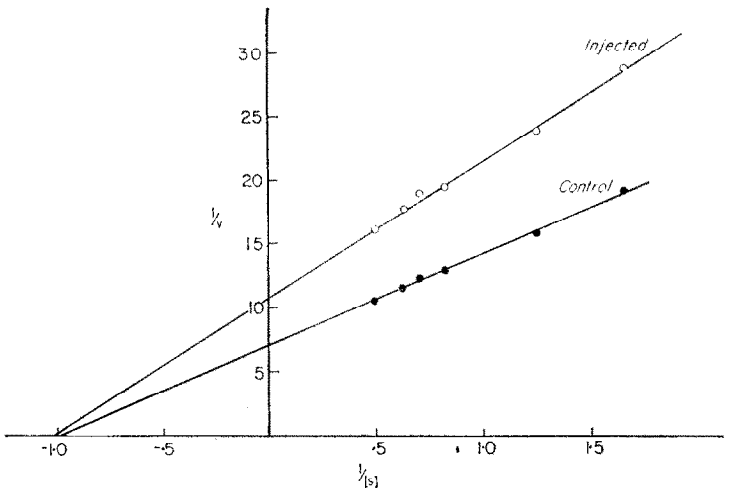

FIG. 2A

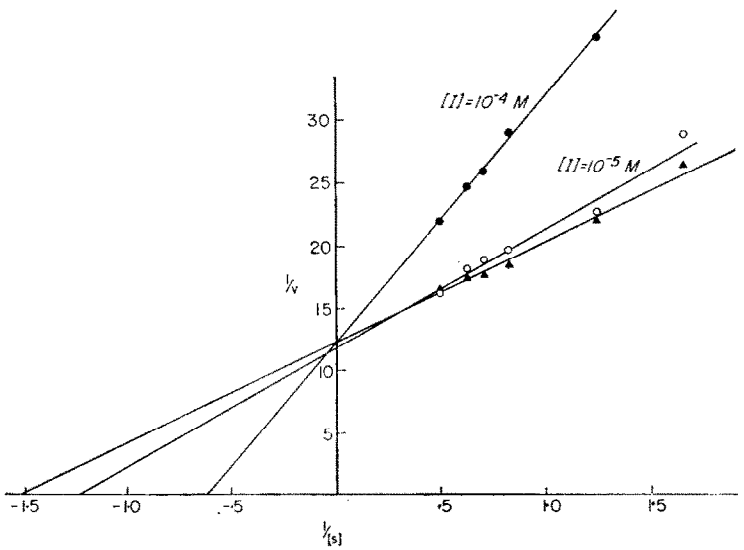

FIG. 2B

FIG. 2. A. Lineweaver–Burk plot of the effects of phenylbutazone *in vivo* on the demethylation of ethylmorphine by rat liver microsomes. Phenylbutazone (100 mg/kg) was given i.p. 2 hr. before killing the rats. S = concentration of ethylmorphine (mM). V = mμmole HCHO formed per 10 min per mg protein. B. Lineweaver–Burk plot of the effects of phenylbutazone *in vitro* on the demethylation of ethylmorphine by rat liver microsomes. [I] = concentration of phenylbutazone.

Table 2 shows the time course of plasma and liver phenylbutazone and the inhibition of demethylation at various times after the administration of phenylbutazone. The results show that the drug elicited only a small inhibitory effect in 30 min when the levels of the drug were high, but this effect increased while the plasma and tissue levels were decreasing.

TABLE 2. PLASMA AND LIVER LEVELS OF PHENYLBUTAZONE AND MICROSOMAL DEMETHYLASE ACTIVITY AT VARIOUS TIMES AFTER ADMINISTRATION OF PHENYLBUTAZONE (100 mg/kg, i.p.)*

Time (hr)	Plasma (mg/ml)	Liver (mg/g)	Demethylase activity (% Control V_{max})
0·5	0·38 ± 0·03	0·20 ± 0·02	103 ± 22 (4)
1	0·39 ± 0·06	0·23 ± 0·06	82 ± 5 (5)
3	0·32 ± 0·06	0·15 ± 0·03	70 ± 9 (5)
6	0·25 ± 0·05	0·11 ± 0·01	75 ± 7 (4)

* The plasma and liver levels are the mean ± S. D. of two animals per time point. The demethylase activity is the mean ± S. D. of the number of experiments in parentheses.

To determine whether free drug was required for inhibition of demethylation, the microsomes from treated animals were subjected to gel filtration and to the washing procedure described in Methods. Gel filtration produced a complete separation of protein and phenylbutazone when the labeled drug was added to the microsomal suspension just before filtration (Fig. 3). The washing procedure removed 95 per cent of the added radioactivity. Neither procedure restored the loss of demethylase activity, indicating that the inhibition was irreversible and did not require free drug (Table 3). However, when microsomes from animals injected with ^{14}C-labeled drug were

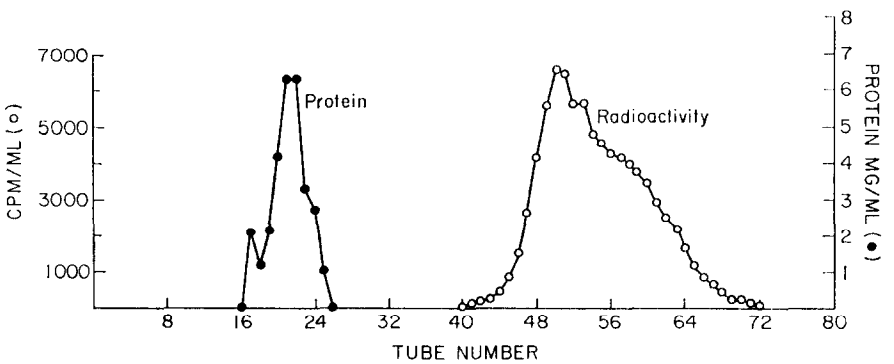

FIG. 3. Gel filtration of microsomal suspension from untreated animals to which phenylbutazone-^{14}C (1 μmole/ml) was added 15 min before filtration.

TABLE 3. EFFECT OF GEL FILTRATION AND WASHING ON DEMETHYLASE ACTIVITY*

Treatment	Control		Injected		% Control
	Km' (mM)	V_{max}' (mμmoles HCHO/mg protein/10 min)	Km' (mM)	V_{max}' (mμmoles HCHO/mg protein/10 min)	V_{max}
Untreated	0·65 ± 0·13	95 ± 8	0·71 ± 0·14	68 ± 5	71 (6)
Gel filtration	0·82 ± 0·11	133 ± 21	0·59 ± 0·12	91 ± 6	69 (2)
Washing	0·68 ± 0·09	181 ± 25	0·69 ± 0·05	106 ± 11	59 (2)

* Pooled microsomes from three control or three treated (100 mg/kg phenylbutazone, i.p., 3 hr before killing) animals were subjected to the washing or gel filtration procedures described in Methods. Values are means ± S. D. with the number of experiments in parentheses.

subjected to gel filtration, a considerable amount of radioactivity was associated with the microsomes (Fig. 4). This irreversibly bound radioactivity was examined with additional gel filtration experiments. As shown in Table 4, the amount of irreversibly bound material was approximately constant from 1 to 6 hr while the total radioactivity declined by about 30 per cent. Furthermore, the level of binding at 6 hr was dependent on the initial dose.

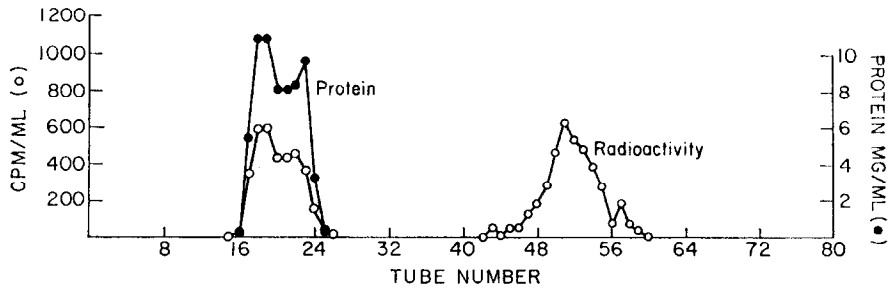

Fig. 4. Gel filtration of microsomal suspension from animals injected with phenylbutazone (100 mg/kg) 3 hr before sacrifice.

Table 4. Levels of radioactivity associated with microsomes before and after gel filtration*

Time after injection (hr)	Dose (mg/kg)	Drug equivalent before filtration (mμmoles/mg protein)	Drug equivalent after filtration (mμmoles/mg protein)
1	100	2·7 ± 0·07 (3)	1·20 (1)
3	100	2·5 ± 0·03 (4)	1·05, 0·8 (2)
6	100	1·8 ± 0·03 (4)	1·65, 1·0 (2)
6	50	1·0 (1)	0·88 (1)
6	10	0·3 (1)	0·23 (1)

* Rats were injected with phenylbutazone and microsomes isolated at the times indicated. Radioactivity and protein were determined in aliquots of the suspensions before and after gel filtration. The mμmole drug equivalent is a conversion from counts per min using the specific activity of the injection solution. Pooled livers from four rats were used for each value and the number of experiments is given in parentheses with standard deviations.

DISCUSSION

These experiments show that phenylbutazone can inhibit the demethylation of ethylmorphine by liver microsomes in two ways. The compound competitively inhibits demethylase activity when incubated with microsomes *in vitro* at 10^{-4} M, but noncompetitively inhibits this reaction when injected into animals. The level of drug in the microsomal suspension 3 hr after injection (Table 4) is such that the concentration of the drug in the microsomal incubation mixture is about 10^{-5} M, and this concentration of drug had no effect on demethylase activity when added *in vitro* (Table 1). The competitive inhibition *in vitro* at 10^{-4} M has been described by Rubin *et al.*[7] and results from the simultaneous metabolism of ethylmorphine and phenylbutazone. The extent of competitive inhibition would, therefore, decrease as the level of phenylbutazone in the liver decreased. The inhibitory effect *in vivo* differs from the effect *in vitro* in that the inhibition is not related to levels of drug in the liver but to the initial dose (Table 1). Consistent with this observation, removal of free drug by gel

filtration does not reverse the noncompetitive inhibition. The presence of radioactivity in the microsome fraction after gel filtration indicates that phenylbutazone or a derivative is irreversibly bound to high molecular weight material. While the chemical structure of the irreversibly bound radioactivity is unknown, it must represent a substantial part of the original drug as the compound was labeled in the benzene rings and not as a readily removable group, such as a methyl radical.

The irreversible binding of a compound that induces but does not inhibit microsomal demethylase has been described by Ernster and Orrenius.[8] These workers showed that microsomes from phenobarbital ^{14}C-treated animals contained irreversibly bound drug equivalent to about 1 mμmole/mg protein after a dose of 100 mg/kg, comparable to the 1 mμmole/mg protein of phenylbutazone bound in this work. As both of these compounds induce demethylase activity, the involvement of the binding in the induction phase was considered. Orrenius *et al.*[9] showed that bound radioactivity increased to a maximum at 3 hr and remained at that level for 6 hr. Phenylbutazone appears to be maximally bound at 1 hr (Table 4) and essentially constant for the next 5 hr. In a study of the time course of phenobarbital induction, microsomal enzyme activity was shown to increase in phenobarbital-treated animals between 3 and 6 hr[9] after injection, while phenylbutazone-treated animals do not show enzyme activity increases until 12–24 hr after injection. Thus, while both phenobarbital and phenylbutazone are irreversibly bound to microsomes after injection, the rate at which maximal binding occurs and the time course of the effect on the microsomal drug enzymes are different. The time course of the irreversible binding by phenylbutazone is more closely associated with the inhibitory phase of its action, because 1 hr after injection, when the binding is maximal, demethylase activity is inhibited. Furthermore, the extent of binding appears to be dose related as is the inhibition.

The prolonged noncompetitive inhibition could explain the dose-dependent differences in plasma half-life reported by Dayton *et al.*[10] In work with the plasma half-life of phenylbutazone in dogs, they reported a half-life of 12 hr after a dose of 50 mg/kg and 3 hr after a dose of 10 mg/kg. These differences in half-life remained even when the same plasma concentrations were reached. To account for this dose-dependent half-life, these workers postulated a prolonged decrease in enzyme activity as a result of a "tightly-bound" drug enzyme complex. The data reported in this paper are consistent with their postulate and suggest that the other compounds in their study might exert a similar action.

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