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METABOLISM OF DIAZEPAM *IN VITRO*

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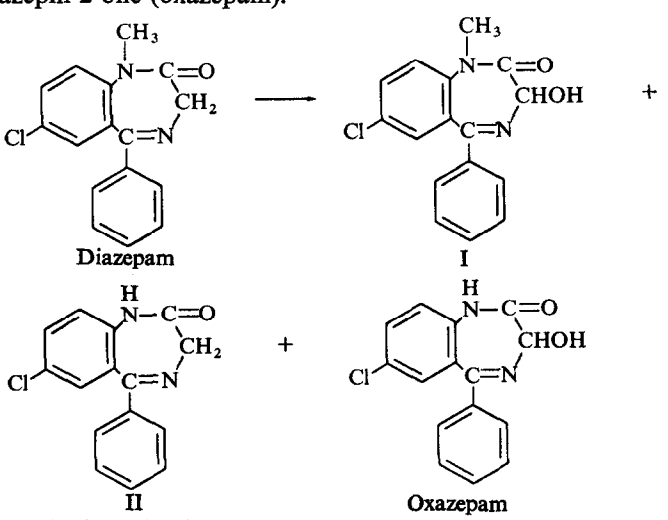
Abstract—^{3}H-diazepam (7-chloro-1,3-dihydro-1-methyl-5-phenyl-2H-1,4-benzodiazepin-2-one), labeled randomly in the 5-phenyl ring, was incubated for 1 hr at 37° with fortified 9000 *g* supernatants prepared from liver of control and phenobarbital-treated dogs and rats. Liver supernatants from 5 control dogs metabolized diazepam to roughly equal amounts of 7-chloro-1,3-dihydro-3-hydroxy-1-methyl-5-phenyl-2H-1,4-benzodiazepin-2-one (I) and 7-chloro-1,3-dihydro-5-phenyl-2H-1,4-benzodiazepin-2-one (II) and only minimal amounts of 7-chloro-1,3-dihydro-3-hydroxy-5-phenyl-2H-1,4-benzodiazepin-2-one (oxazepam). Pretreatment of 5 dogs with phenobarbital resulted in an increased conversion of diazepam to II and oxazepam. ^{3}H-labeled I and II produced *in vitro* were isolated and used as substrates in a 2-hr incubation with liver supernatant from a phenobarbital-treated dog; each was converted about equally well to oxazepam. No metabolism of diazepam was detected on incubation with dog brain homogenate or 9000 *g* supernatant. The metabolism of diazepam by a pooled 9000 *g* liver supernatant from 4 control rats was very similar to that seen with control dog liver supernatants. The pooled liver supernatants from 4 phenobarbital-treated rats yielded a much greater metabolism of diazepam with not only a marked increase in oxazepam, but also the production of polar unidentified metabolites.

THE METABOLITES *in vivo* of diazepam* (7-chloro-1,3-dihydro-1-methyl-5-phenyl-2H-1,4-benzodiazepin-2-one) in man and dog include: the 3-hydroxy analog, 7-chloro-1,3-dihydro-3-hydroxy-1-methyl-5-phenyl-2H-1,4-benzodiazepin-2-one (I); the *N*-desmethyl analog, 7-chloro-1,3-dihydro-5-phenyl-2H-1, 4-benzodiazepin-2-one (II); and the 3-hydroxylated *N*-desmethyl analog, 7-chloro-1,3-dihydro-3-hydroxy-5-phenyl-2H-1,4-benzodiazepin-2-one (oxazepam).[1, 2]



* Diazepam is the active ingredient in Valium, marketed by Hoffmann-La Roche Inc., Nutley, New Jersey.

In the rat, I was found in addition to three phenolic metabolites; these were the 5-phenyl *para*-hydroxylated derivatives of diazepam, I and II.[3] Oxazepam and II were not detected.

The present studies *in vitro* were undertaken to gain information concerning the formation of the above metabolites.

EXPERIMENTAL

^{3}H-diazepam. The synthesis of diazepam randomly labeled with ^{3}H in the 5-phenyl ring has been described.[2] The radiochemical purity of this material was confirmed by paper chromatography (ascending on Schleicher and Schuell acetylated paper, grade No. 2495, with ethyl acetate–dioxane–water, 20:45:45, as solvent system) before it was diluted with unlabeled diazepam and used as substrate in the studies *in vitro*.

Enzyme preparations. Ten dogs were used for the preparation of 9000 *g* liver supernatants. Five were killed (by being placed in a chamber of carbon dioxide) without prior treatment and 5 after treatment with phenobarbital, which was given daily in oral doses (by gelatin capsule) of 20 mg/kg for 2 days followed by 10 mg/kg for 8–10 days. Three of the treated dogs also received an oral 10 mg/kg dose of diazepam 2 days before the phenobarbital treatment was started and again 3–4 days prior to being killed. Blood samples drawn from these 3 dogs were analyzed for diazepam and II, but meaningful results were not obtained and they will not be reported.

The liver of each animal was rinsed in cold water and homogenized in 2 vol. of ice-cold 1·15% KCl solution in a Potter–Elvehjem glass homogenizer. Each homogenate was maintained at 0° while being centrifuged at 9000 *g* for 20 min; each of the resulting supernatants was stored at — 20° until used within a month as enzyme source. One frozen supernatant from a treated dog was used after 3 weeks and again after 3 months of storage. The disappearance of diazepam and formation of metabolites were essentially the same after both storage periods, indicating that these preparations were stable with respect to diazepam-metabolizing enzymes. The brains of 2 control dogs and of 1 phenobarbital-treated dog were also removed and 9000 *g* supernatants were prepared and stored exactly as described above. Also stored were the total homogenates of the brains of the 2 control dogs.

The rat liver enzyme preparations were obtained from 2 groups of 4 male Charles River rats, each rat weighing approximately 250 g. Rats of the control group were killed by decapitation and the 9000 *g* liver supernatants, prepared as described above, were pooled before storage at — 20°. The rats of the second group received 2 i.p. injections of 37·5 mg/kg of phenobarbital a day for 4 days before being killed on the fifth day. Again, the 9000 *g* liver supernatants were pooled before storage.

Incubation in vitro. The incubation mixture contained substrate, enzyme source, pyridine nucleotides and an NADPH-generating system.[4] The incubation volume of 3 ml consisted of 0·3 ml of 9000 *g* liver supernatant, 0·2 ml of 0·01 M ATP, 0·1 ml of 0·003 M NADP, 0·1 ml of 0·003 M NAD, 0·1 ml of 0·6 M nicotinamide, 0·2 ml of 0·03 M glucose 6-phosphate, 0·1 ml of 0·1 M $MgCl_2$, 0·1 ml of 2·0 M KCl, 0·6 ml H_2O, 1·0 ml of 0·1 M potassium phosphate buffer (pH 7·4) and 0·2 ml of 0·35 μmole (100 μg) ^{3}H-diazepam in aqueous solution. The aqueous substrate solution was freshly prepared for each experiment by dissolving 5 mg ^{3}H-diazepam in 0·4 ml of 0·1 N HCl and bringing the volume to 10 ml with water; 0·2 ml contained 100 μg and approximately 7×10^5 dpm of ^{3}H-diazepam. The ATP, NAD and NADP solutions were

neutralized before being added to the flasks. The flasks were incubated in a Dubnoff shaker at 37° for a period of 1 hr.

Analysis of ^{3}H-diazepam and metabolites. After the incubation, the entire contents of each flask was extracted twice with 6 ml ethyl acetate. Each combined extract was evaporated to dryness under nitrogen and each residue was dissolved in 5 ml ethanol. Aliquots of these ethanol solutions were counted and were analyzed by thin-layer chromatography (TLC). The primary solvent system pair used for 2-dimensional TLC was system AB′: heptane–chloroform–ethanol (10:10:1) followed by heptane - chloroform–acetic acid–ethanol (5:5:1:0·3). System AC, in which heptane–chloroform–ethanol (10:10:1) was followed by isopropanol–conc. ammonia (20:1), was also used. The details of the 2-dimensional TLC of an extract containing labeled diazepam and metabolites with internal and external reference compounds and of the liquid scintillation counting procedures have been reported.[2] The metabolism of ^{3}H-diazepam was quantitatively determined from the amount of ^{3}H which migrated with internal standards of authentic diazepam, I, II and oxazepam.

RESULTS

Metabolism by dog liver enzymes. Initial experiments in which ^{3}H-diazepam was incubated with liver supernatant from control and phenobarbital-treated dogs indicated that most of the incubated ^{3}H was distributed among 4 compounds: diazepam, I, II and oxazepam. The effect of phenobarbital treatment of the dog on this distribution is shown in Table 1. Incubation with control dog 9000 *g* liver super-

TABLE 1. METABOLISM *IN VITRO* OF ^{3}H-DIAZEPAM BY 9000 *g* LIVER SUPERNATANTS OF CONTROL AND PHENOBARBITAL-TREATED DOGS

Dog	Sex	Substrate and metabolites after 1-hr incubation*				
		Diazepam (μmole)	I (μmole)	II (μmole)	Oxazepam (μmole)	Unaccounted for (μmole)
Controls						
C-1	F	0·281	0·017	0·018	nil	0·039
C-2	F	0·195	0·058	0·058	nil	0·044
C-3	M	0·268	0·015	0·032	nil	0·040
C-4	M	0·078	0·112	0·078	0·016	0·071
C-5	M	0·173	0·045	0·102	0·007	0·028
		0·199 ± 0·037†	0·049 ± 0·018	0·058 ± 0·015	0·005 ± 0·003	0·044 ± 0·007
Phenobarb.-tr.						
T-1	M	0·027	0·077	0·140	0·033	0·078
T-2	M	0·100	0·054	0·134	0·013	0·054
T-3	F	0·185	0·058	0·078	0·005	0·029
T-4	M	0·006	0·021	0·258	0·025	0·045
T-5	M	nil	0·013	0·246	0·039	0·057
		0·064 ± 0·035	0·045 ± 0·012	0·171 ± 0·035	0·023 ± 0·006	0·053 ± 0·008
% Change from control		−68	−8	197	360	20
P‡		<0·05	N.S.	<0·05	<0·05	N.S.

* The amounts recovered as each compound after ethyl acetate extraction of the incubation medium (which, with few exceptions, removed at least 90 per cent of the incubated ^{3}H) and 2-dimensional TLC in system AB′. The amount of ^{3}H-diazepam added to each flask was 0·354 μmole. Nil signifies that less than 0·0002 μmole of compound was present, N.S. = not significant.

† Average value ± standard error.

‡ Probability calculated by Student's *t*-test.

natant led to the disappearance of 0·155 μmole (44 per cent) of the substrate. Roughly 15 per cent was metabolized to I and II, and only a very slight amount (measurable only in the C-4 and C-5 incubations) to oxazepam. Stimulation of the metabolism of diazepam *in vitro* is evident from the finding that 0·290 μmole (82 per cent) disappeared after incubation with liver supernatant from phenobarbital-treated dogs. This statistically significant increase in diazepam metabolism was accompanied by a significant increase in the formation of II (197 per cent increase) and oxazepam (360 per cent increase), while the amounts of I and of the unaccounted for ^3H did not significantly change. Of the additional 0·135 μmole diazepam metabolized by liver supernatants of the phenobarbital-treated group, 0·113 (0·171 — 0·058) μmole (86 per cent) was converted to II and 0·018 μmole (13 per cent) was converted to oxazepam (through the intermediate formation of I or II or both). It is evident, therefore, that phenobarbital treatment resulted in an increased *N*-demethylation of diazepam to form II and in only a very minor (if any) increased 3-hydroxylation to produce I.

In order to produce sufficient quantities of labeled I and II so that each could be used as substrate, 6 flasks each containing 0·354 μmole ^3H-diazepam and liver supernatant from phenobarbital-treated dog T-1 were incubated for 1 hr. After incubation, each incubation medium was extracted with ethyl acetate and the combined concentrated ethyl acetate extract was chromatographed (TLC) in chloroform–acetone (9:1). Under short-wave u.v. light, one band was seen to have migrated the same as did authentic I (R_f 0·58) and one band the same as did authentic II (R_f 0·39). Each labeled metabolite was eluted from the silica gel by 3 successive extractions with ether and each combined extract was evaporated to dryness and brought to 1 ml with ethanol. From the ^3H content of each, it was calculated that 0·24 μmole of I and 0·58 μmole of II had been isolated. The purity of each fraction was checked by 2-dimensional TLC in system AB′: 94 per cent of the ^3H of the metabolite I fraction was I, 3 per cent was II and none migrated as diazepam or oxazepam; 80 per cent of the ^3H of the metabolite II fraction was II, while none was found as diazepam, I or oxazepam. Both fractions were considered sufficiently pure to be used as labeled substrates.

Table 2 presents the results of a 2-hr incubation of labeled I and II with the liver supernatant of a phenobarbital-treated dog. Significant metabolism of I to oxazepam

TABLE 2. METABOLISM *IN VITRO* OF LABELED I AND II TO OXAZEPAM*

Expt. No.	Substrate†		% Distribution of extracted ^3H on TLC in system AB′			
	(μmole)	Compound	Diazepam	I	II	Oxazepam
1	0·164	I	nil	80	2	14
2	0·362	II	nil	nil	67	15
3	0·181	II	nil	nil	70	16
3	0·181	II (Control)‡	nil	nil	83	nil

* Each labeled isolated compound was incubated for 2 hr with 9000 *g* liver supernatant of a phenobarbital-treated dog.

† In the preparation of substrate, each of the labeled compounds was diluted 1:2 with unlabeled compound before being added to the incubation flask.

‡ No 9000 *g* liver supernatant was added to this flask so that the amount of nonenzymatic breakdown of II would be seen.

was seen; the slight amount of II found represented the II which was found above as a contaminant of the isolated I. Oxazepam was formed from II at both substrate concentrations and the results of the control incubation clearly show that the conversion of II to oxazepam was enzymatic. It is evident that both I and II are capable of being metabolized to oxazepam by the drug-metabolizing enzymes of the 9000 *g* supernatant of dog liver.

Metabolism by dog brain enzymes. ^{3}H-diazepam (0·36 μmole) was incubated for 1 hr with brain homogenate (0·3 ml) from 2 untreated dogs and brain 9000 *g* supernatant (0·6 ml) from 2 untreated dogs and 1 phenobarbital-treated dog. Each incubation yielded the same result; on TLC of the resulting extracts in system A, heptane–chloroform–ethanol (10:10:1), all of the ^{3}H was still present in diazepam, clearly showing that no metabolism of the substrate had occurred.

Metabolism by rat liver enzymes. Over 90 per cent of the ^{3}H was ethyl acetate-extractable after the 1-hr incubation of labeled diazepam (0·351 μmole) with 9000 *g* liver supernatant from control rats. This almost complete extractability had also been found after incubation with 9000 *g* liver supernatant from both control and phenobarbital-treated dogs (first footnote of Table 1). In contrast to these results, the ^{3}H derived from labeled diazepam incubated with 9000 *g* supernatant from phenobarbital-treated rats was only partially extractable; 48 per cent was ethyl acetate-extractable in one experiment and 71 per cent in another. It is therefore evident that pretreatment with phenobarbital caused an increased formation of polar (nonextractable) diazepam metabolite(s).

In Table 3 the consistent distribution of the ethyl acetate-extracted radioactivity between diazepam, I, II and oxazepam on TLC in systems AB′ and AC is shown.

TABLE 3. TLC OF THE RADIOACTIVITY REMOVED BY ETHYL ACETATE AFTER A 1-hr INCUBATION OF ^{3}H-DIAZEPAM (0·351 μMOLE) WITH 9000 *g* SUPERNATANTS OF CONTROL AND PHENOBARBITAL-TREATED RATS.

Enzyme source*	System	% Distribution of extracted ^{3}H after 1 hr incubation†			
		Diazepam	I	II	Oxazepam
Control rat	AB′	62	8·9	10	Nil
	AC	71	9·7	12	1·1
Phenobarb.-tr. rat	AB′	20	29	21	10
	AC	20	25	19	10

* The 9000 *g* supernatant of liver was a pooled sample obtained from 4 control rats or from 4 phenobarbital-treated rats.

† Virtually all (99%) of the ^{3}H was extracted after incubation with control rat liver supernatant, while 71 per cent was extracted after incubation of liver supernatant from phenobarbital-treated rats.

Table 4 shows that the metabolism of diazepam by control rat liver supernatant was qualitatively and quantitatively very similar to that seen in the experiments with control dog liver supernatant (Table 1). The increased diazepam metabolism resulting

from phenobarbital treatment again led to the formation of increased amounts of oxazepam. However, the stimulated rat enzymes differed in their activity from those of the dog in that there was an apparent increase in I rather than in II and there was a marked amount (equivalent to 0·105 μmole) of nonextractable metabolite(s) formed.

TABLE 4. METABOLISM *IN VITRO* OF ^{3}H-DIAZEPAM BY 9000 *g* SUPERNATANTS* OF RAT LIVER

Rat treatment	Substrate and extractable metabolites after 1 hr incubation†				
	Diazepam (μmole)	I (μmole)	II (μmole)	Oxazepam (μmole)	Unaccounted for (μmole)
Control	0·23	0·032	0·038	nil	0·045
Phenobarbital	0·049	0·066	0·049	0·025	0·057

* See table 3, first footnote.

† The amount (μmoles) of each compound was calculated from the percent extraction of ^{3}H (see Table 3, † after incubation of 0·351 μmole ^{3}H-diazepam and the average distribution of the ^{3}H found in Table 3.

DISCUSSION

The demonstration that drug-metabolizing enzymes of the dog liver were capable of forming I, II and oxazepam is consistent with the formation *in vivo* of these 3 metabolites from diazepam in dog and man.[1,2] The liver enzymes of the rat were also capable of forming these metabolites, but only one of them, I, was found in the intact rat.[3] Although the most prominent pathway of diazepam metabolism *in vivo* in the rat appeared to be hydroxylation of the 5-phenyl ring at the *para* position,[3] no significant amounts of these phenolic metabolites (which are ethyl acetate-extractable) were evident *in vitro*. Therefore, if in the intact rat the liver is a major site of synthesis of phenolic metabolites, then the system employed *in vitro* in these studies must have favored *N*-demethylation and hydroxylation at the C-3 position over hydroxylation of the phenyl ring.

The stimulatory effect of phenobarbital on drug-metabolizing enzymes, which has been widely demonstrated,[5] was also evident in these studies. On incubation of diazepam with liver supernatant from phenobarbital-treated dogs, the metabolite produced in greatest amounts was II. It is of interest that II was a major metabolite in the blood of a dog given a 10 mg/kg i.v. dose of ^{3}H-diazepam.[2] The blood levels of diazepam fell rapidly in this dog, while the levels of II were higher than those of diazepam at 1 hr and stayed at these relatively high levels for 12 hr. In the human, de Silva *et al.*[6] did not detect II in the blood after a 10 mg oral, i.v. or i.m. dose of diazepam. However, after chronic administration of 30-mg daily doses of diazepam, the blood levels of II increased with time and eventually equalled those of diazepam. These results suggest that when diazepam is given in high or repeated doses its conversion to II in the liver of dog and man is faster than the further metabolism of II.

Oxazepam glucuronide is a major urinary metabolite of diazepam in both man and dog.[2] The above studies *in vitro* indicated that oxazepam could be produced *in vivo* from both possible intermediates, I and II.

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