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EFFECT OF ETHANOL ON THE FATE OF PENTOBARBITAL IN THE RAT*

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Abstract—The effect of ethanol on the tissue distribution of radioactivity after administration of ^{14}C-pentobarbital was studied in the rat. The effect of ethanol on the renal excretion of pentobarbital and its metabolites was also investigated. The ethanol-treated rats had higher tissue levels of radioactivity than control animals at 3 hr after administration, but not at 1 hr. No particular redistribution of radioactivity between the blood and tissues was observed. The brain was found to contain only unchanged pentobarbital at 3 hr, which confirmed the pharmacological importance of the elevated brain level in the ethanol-treated rats at this time. Ethanol inhibited the excretion of radioactivity during the first 6 hr after dosing. This was found to be due to reduced excretion of a metabolite of low R_f; the structure of this metabolite has not been positively determined. It is postulated that the lower excretion rate of radioactivity, and consequent higher tissue levels, are due to inhibition of pentobarbital metabolism by ethanol.

MANY studies have been made of the interaction between ethanol and barbiturates,[1–16] but there is disagreement as to whether the pharmacological and toxicological interaction is additive,[1–5] potentiating[1,6–11] or even antagonistic.[12,13]

The quantitative measurement of the interaction has usually been in terms of sleeping time or acute toxicity. In some laboratories, it was considered that the interaction would be better understood if the effect of ethanol on the tissue distribution and elimination of barbiturates was investigated. Seidel[15] studied the effect of ethanol on the tissue distribution of pentobarbital, barbital and thiopental in mice, and observed increased concentrations of pentobarbital in blood and several tissues, including brain, up to 70 min after administration. More recently Coldwell *et al.*[16] studied the effect of ethanol on the distribution in rat blood and brain of five commonly used barbiturates. They found that ethanol increased the concentration of phenobarbital in both blood and brain 249 min after administration, but decreased the brain concentration of pentobarbital and had no effect on the blood level 225 min after injection. Both laboratories used a conventional assay method involving ultraviolet absorption spectrometry. In the experiments reported in this paper, ^{14}C-labeled pentobarbital was used and estimated by liquid scintillation counting.

MATERIALS AND METHODS

Pentobarbital-2-^{14}C [5-ethyl-5-(1-methylbutyl) barbituric-2-^{14}C acid], specific activity 12·5 mc/g, was obtained from New England Nuclear Canada Limited. Unlabeled pentobarbital-Na was purchased from BDH Canada Limited.

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Experiment 1—*Tissue distribution study.* Male Wistar rats weighing approximately 200 g were used in this study.

Pentobarbital (30 mg/kg) at a specific activity of 0·8 mc/g was injected intraperitoneally. Ethanol (3 g/kg as a 15%, w/v, solution in saline) or an equivalent volume of saline was administered simultaneously by intraperitoneal injection. The rats were killed at either 1 or 3 hr and the tissues immediately dissected and frozen.

Blood and brain from the rats killed by decapitation at 3 hr were estimated for metabolites and unchanged pentobarbital by paper chromatography. The brain homogenate (prepared as described under the "Estimation of radioactivity") and blood were extracted prior to chromatography as follows: 35% HCl (0·1 ml) was added to 2 ml of either brain homogenate or blood. After centrifugation, the supernatant was extracted three times with 2 ml aliquots of chloroform. The chloroform layers were pooled and dried in a current of dry nitrogen. The residue was dissolved in 0·1 ml of ethyl acetate. A sample of approximately 20 μl was applied to the chromatography paper which was run according to the method of Algeri and McBay.[17]

Experiment 2—*Blood concentration decay profile.* Pentobarbital, ethanol and saline were administered in the same doses as in experiment 1. Samples of tail blood (10 μl) were collected at intervals up to 6 hr after dosing.

Experiment 3—*Urine excretion.* Male rats (weighing about 200 g), starved of food overnight, were given water by gavage at a dose of 40 ml/kg; 30 min later the treatment was repeated. At this point, the rats were divided into three treatment groups and dosed by intraperitoneal injection with unlabeled drugs as follows: group 1 received pentobarbital (30 mg/kg); group 2, pentobarbital (30 mg/kg) and ethanol (3 g/kg); and group 3, saline as a control. Eight rats were used per group and they were housed two per metabolism cage. Urine was collected at 1, 2, 4 and 8 hr after dosing and its volume measured.

Experiment 4—*Excretion of pentobarbital and its metabolites in urine.* As in the previous experiments, male Wistar rats were starved of food overnight. On the day of treatment the rats were divided randomly into two groups of six animals each and dosed orally with dilute solutions of pentobarbital in order to promote urine excretion. The control group received ^{14}C-pentobarbital (30 mg/kg) alone and the test group a solution of ^{14}C-pentobarbital (30 mg/kg) and ethanol (3 g/kg). Both groups received their doses in a volume of 40 ml/kg. Once dosed, each rat was placed in an individual metabolism cage (Acme Research Products, Cincinnati, Ohio) and urine, free from feces, was collected at 3, 4, 6, 12 and 24 hr after dosing. The volume of urine was measured and the pH determined. The urines were frozen until analyzed for radioactivity. The proportions of unchanged drug and metabolites were determined in urines obtained from three randomly chosen rats in each group by the paper chromatography system of Algeri and McBay,[17] using 50-μl aliquots of urine applied directly to the paper.

Estimation of radioactivity

Tissues and blood. Triplicate samples of tissue weighing 25–100 mg were randomly cut from each organ (except the brain), weighed, and transferred to scintillation counter vials containing 1 ml Soluene (Packard Instrument Company). In the case of blood and plasma, 10-μl samples were added to the Soluene. The vials were incubated for 18 hr at 37° to ensure complete dissolution of the tissue. The whole brain was homo-

genized with an equal volume of water and triplicate 0·1-ml aliquots were added to Soluene in vials as above. After tissue digestion, 10 ml of toluene-based scintillation fluid, containing 0·6% 2,5-diphenyloxazole (PPO) and 0·02% *p*-bis-[2-(4-methyl-5-phenyloxazolyl)]-benzene (dimethyl POPOP), was added to each vial. The radioactivity was determined using a liquid scintillation counter.

Urine. Samples of up to 0·2 ml were added to Aquasol scintillation fluid obtained from New England Nuclear Canada Ltd. This fluid gives high counting efficiencies with aqueous samples.

Paper chromatograms. After complete drying, developed chromatograms were cut into numbered 1-cm wide strips between the origin and solvent front. Each paper strip was placed in a scintillation counter vial with 10 ml of the standard toluene-based scintillation fluid.

Counting statistics. All vials were counted three times in the scintillation counter. In each experiment standards and controls were included in order to obtain machine efficiency, background count, and specific activity of the drug being used. Quench corrections were made by either the channel ratio or external standard method. All calculations were performed on an IBM-360 computer using a purpose-designed program.

RESULTS

Tissue distribution

Pentobarbital. Figure 1 shows the results of the tissue distribution at 1 hr after dosing from three ethanol-treated and three control rats. No statistically significant changes ($P > 0·05$) were observed in any of the tissues at this time.

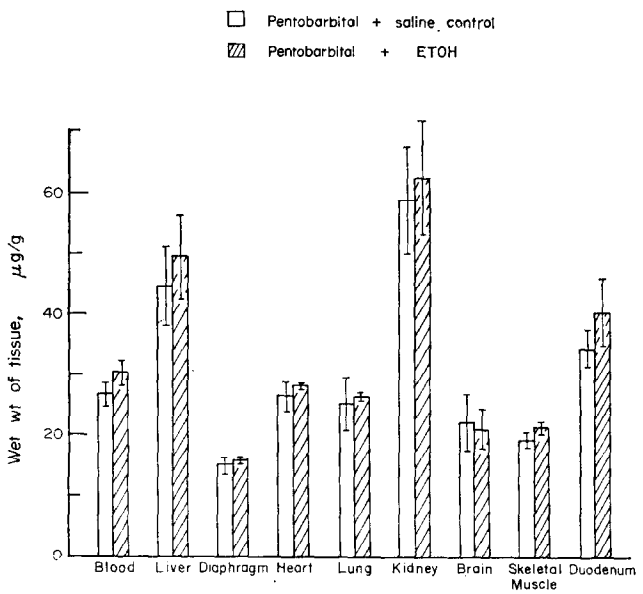

FIG. 1. Tissue distribution of radioactivity expressed as micrograms of pentobarbital per gram wet wt. in groups of three rats 1 hr after an intraperitoneal injection of ^{14}C-pentobarbital (30 mg/kg) and either 15% ethanol (3 g/kg) or saline control. The figures are means with standard errors.

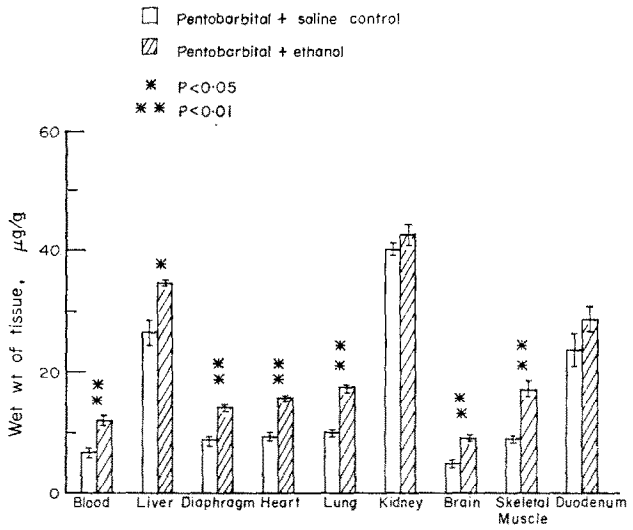

FIG. 2. Tissue distribution of radioactivity expressed as micrograms of pentobarbital per gram wet wt. in groups of three rats 3 hr after an intraperitoneal injection of ^{14}C-pentobarbital (30 mg/kg) and either 15% ethanol (3 g/kg) or saline control. The figures are means with standard errors.

At 3 hr (Fig. 2), all tissues showed a marked reduction in pentobarbital concentration from those observed at 1 hr. The results revealed that ethanol had a marked effect on the tissue concentration of pentobarbital. Most tissues had a higher concentration in the ethanol-treated group, significant differences ($P < 0{\cdot}05$) being observed in all tissues except the kidney and duodenum.

Paper chromatography results obtained from the bloods and brains of the 3-hr treatment groups are shown in Table 1. The brains from both groups of rats contained

TABLE 1. EFFECT OF ETHANOL ON THE PROPORTION OF PENTOBARBITAL AND ITS METABOLITES IN BLOOD AND BRAIN*

	Blood			Brain		
Treatment group	R_f 0·35 metabolite (%)	R_f 0·46 metabolite (%)	Pento-barbital (%)	R_f 0·35 metabolite (%)	R_f 0·46 metabolite (%)	Pento-barbital (%)
Pentobarbital + saline (3)	5·6 ± 1·3	22·4 ± 7·5	71·9 ± 8·5	0	0	100·0
Pentobarbital + ethanol (3)	10·0 ± 1·0	17·8 ± 3·7	72·2 ± 4·7	0	0	100·0
Difference $P < 0{\cdot}05$	NS	NS	NS			

* Rats were killed 3 hr after an intraperitoneal dose of ^{14}C-pentobarbital (30 mg/kg) with either ethanol (3 g/kg) or saline control. Pentobarbital and its metabolites were separated by paper chromatography.[17] The figures are means with standard errors. Numbers of rats are shown in parentheses. NS = not significant.

no measurable quantities of metabolites; all the radioactivity was located at the R_f of unchanged pentobarbital. No significant changes were observed in the proportions of unchanged drug and metabolites in the blood from the ethanol-treated and control rats. The metabolite with an R_f of 0·46 was identified by Algeri and McBay[17] as 5-ethyl-5-(3-hydroxy-1-methylbutyl) barbituric acid. The R_f 0·35 metabolite was not identified, but was shown to have an intact barbituric acid ring.[17]

Blood concentration decay profile

Pentobarbital. The blood concentration profiles obtained with ^{14}C-pentobarbital from four control and four ethanol-treated rats are shown in Fig. 3. The variability during the uptake phase prevents any firm conclusions regarding the effect of ethanol. During the elimination phase, significantly higher blood levels were obtained in the ethanol-treated group at 2 and 4 hr, which coincides with the tissue changes observed at 3 hr.

Analysis of the blood profiles, using a computer program designed to measure the area under the curve, revealed a considerable difference between the two treatment groups. The area for the control group was 4756·7 ± 406·0 and 7641·3 ± 751·4 μg/ml min for the ethanol-treated group. The difference between the two groups was highly significant ($P < 0·02$). These results are interpreted as indicating that ethanol inhibits the elimination of pentobarbital.

Urine excretion. The effect of pentobarbital, alone and combined with ethanol, on the volume of urine excreted by rats given large doses of water, is shown in Table 2. Pentobarbital when given alone causes a depression in the volume of urine excreted which lasts about 1 hr. When ethanol is given, there is still a considerable reduction

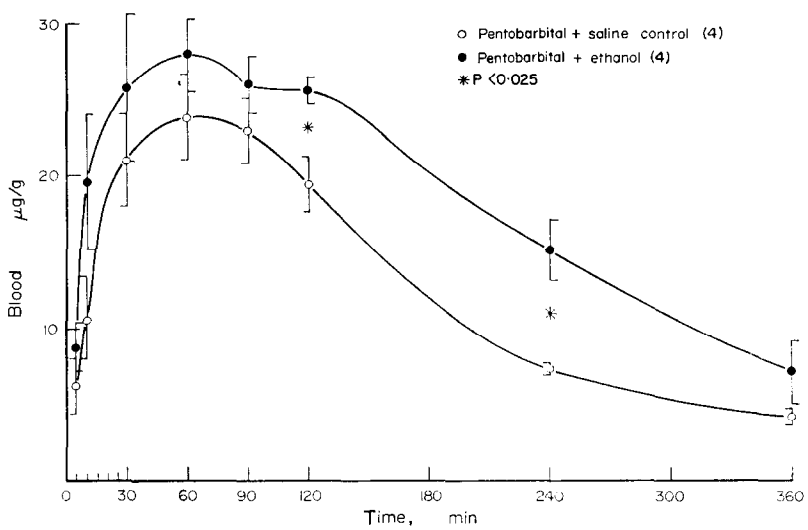

FIG. 3. Blood concentration of radioactivity expressed as micrograms of pentobarbital per milliliter in rats given intraperitoneal injections of ^{14}C-pentobarbital (30 mg/kg) with either 15% ethanol (3 g/kg) or saline control. Number of rats is in parentheses. The figures are means with standard errors.

TABLE 2. EFFECT OF PENTOBARBITAL AND ETHANOL ON URINE EXCRETION*

Time (hr)	Urine excreted (ml/hr)		
	Saline control	Pentobarbital + saline	Pentobarbital + ethanol
0–1	13·85 ± 1·67	7·80 ± 1·08	4·63 ± 2·19
Different from control		P < 0·025	P < 0·02
1–2	11·75 ± 1·23	14·50 ± 1·19	15·20 ± 0·95
Difference from control		NS	NS
2–4	2·53 ± 1·13	3·38 ± 0·78	8·30 ± 0·62
Difference from control		NS	P < 0·005
4–8	0·31 ± 0·10	0·43 ± 0·03	0·91 ± 0·17
Difference from control		NS	P < 0·025

* Rats (four pairs per group) were dosed orally twice with water (40 ml/kg) at 30-min intervals. Pentobarbital (30 mg/kg) and ethanol (3 g/kg) were given by intraperitoneal injection. Figures are means with standard errors. NS = not significant.

in urine volume during the first hour. After 2 hr ethanol causes the expected diuresis. A pilot study with a small number of rats showed that ethanol alone produced an effect similar to that seen in the pentobarbital plus ethanol group, but the initial antidiuresis was less pronounced.

Excretion of pentobarbital and its metabolites in urine. The smaller dose of water given to the rats in this experiment resulted in little or no urine being excreted during the first 2 hr after dosing. After this period, both groups of rats excreted adequate volumes of urine (> 1 ml) which allowed a collection to be made at 3 hr. The total radioactivity excreted by the control and ethanol-treated rats during 24 hr after dosing is shown in Fig. 4. It is seen that the radioactivity excreted by the ethanol-treated rats is significantly less ($P < 0·05$) than that of the controls during the first 6 hr. By 12 hr the two groups are not significantly different.

The most important results obtained by paper chromatography are shown in Fig. 5. Very little unchanged pentobarbital was detected. The most marked change induced by ethanol was a reduction in the excretion of M–I. This metabolite of R_f 0·04 was not positively identified by Algeri and McBay,[17] but has an intact barbiturate ring and is acidic. We ran some urines on the paper chromatography system of Titus and Weiss[18] and formed a tentative conclusion, on the basis of comparable R_f, that it is 5-ethyl-5-(1-methyl-3-carboxypropyl) barbituric acid. Incubation of the urine specimens with Glusulase (Endo Products Inc.), which splits drug conjugates, failed to affect the quantity and R_f of this component, making it unlikely that it is a conjugate. The reduced elimination of radioactivity seen in Fig. 4 is entirely accounted for by the reduced excretion of M–I. Subsequently the ethanol-treated rats eliminate more of metabolite IV. M–IV (R_f 0·40) was identified by Algeri and McBay[17] as 5-ethyl-5-(3-hydroxy-1-methylbutyl) barbituric acid. Table 3 shows the complete results obtained at each time. M–II and M–III are of unknown structure and both constituted only 4 per cent of the dose in 24 hr. In the individual time periods, it is observed that

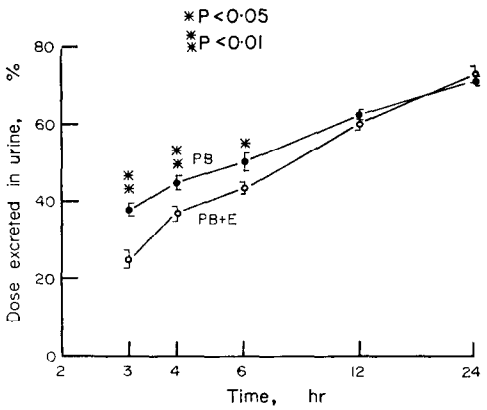

FIG. 4. Excretion of radioactivity in rat urine expressed as a percentage of the dose of ^{14}C-pentobarbital (30 mg/kg). Control animals received pentobarbital and saline (●) and the treated group received pentobarbital and ethanol (3 g/kg) (○). The figures are means with standard errors.

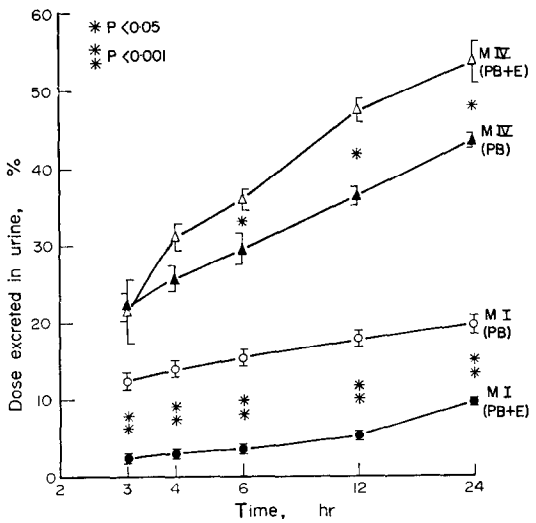

FIG. 5. Excretion of pentobarbital and its metabolites in rat urine after either pentobarbital and ethanol (PB + E) or pentobarbital and saline control (PB). The figures are means with standard errors.

ethanol inhibits the excretion of M–I only up to 6 hr; in the 12–24 hr period, the situation is even reversed. Less than 1 per cent of the dose was excreted as unchanged pentobarbital in 24 hr.

DISCUSSION

Absorption. The large standard errors observed in the blood levels of radioactivity during the absorption phase do not allow any precise conclusions with regard to absorption rate. The percentage of the dose of radioactivity recovered in the urine

Table 3. Excretion of pentobarbital and its metabolites in urine*

Time (hr)	Treatment	Metabolites (% dose) M–I R_f 0·04	M–II R_f 0·15	M–III R_f 0·24	M–IV R_f 0·40	Pentobarbital (% dose) R_f 0·70
0–3	PB + E	2·15 ± 0·55	1·00 ± 0·28	0·33 ± 0·21	21·47 ± 4·28	0·42 ± 0·15
	PB	12·44 ± 0·83 P < 0·001	1·47 ± 0·19	1·16 ± 0·13 P < 0·05	21·86 ± 1·95	0·84 ± 0·33
3–4	PB + E	0·90 ± 0·14	0·62 ± 0·10	0·63 ± 0·13	9·71 ± 3·00	0·17 ± 0·04
	PB	1·50 ± 0·20	0·68 ± 0·09	0·65 ± 0·03	4·01 ± 0·37	0·023 ± 0·003 P < 0·02
4–6	PB + E	0·50 ± 0·18	0·55 ± 0·18	0·54 ± 0·21	4·78 ± 1·45	0·11 ± 0·03
	PB	1·37 ± 0·21 P < 0·05	0·62 ± 0·11	0·62 ± 0·09	3·72 ± 0·67	0
6–12	PE + E	1·83 ± 0·33	1·59 ± 0·28	1·57 ± 0·33	11·36 ± 2·25	0·27 ± 0·21
	PB	2·51 ± 0·05	1·07 ± 0·18	1·25 ± 0·25	6·83 ± 0·89	0
12–24	PB + E	4·13 ± 0·59	0·64 ± 0·09	0·67 ± 0·13	6·25 ± 0·07	0
	PB	1·90 ± 0·14 P < 0·025	0·64 ± 0·04	0·65 ± 0·01	7·04 ± 0·65	0

* Groups of three rats were dosed orally with ^{14}C-pentobarbital (30 mg/kg) and either ethanol (3 g/kg; PB + E) or saline control (PB). The figures are means with standard errors. M–I, M–II, M–III, M–IV = metabolites I, II, III and IV.

during 24 hr after administration was the same in both treatment groups (Fig. 4), indicating that the total amount of pentobarbital absorbed is not influenced by ethanol.

Tissue distribution. Ethanol did not cause any apparent redistribution of radioactivity. The changes effected in barbiturate tissue levels by ethanol occurred in all tissues to about the same extent. One hr after dosing no statistically valid changes were produced. At 3 hr there were markedly higher tissue levels in the ethanol-treated rats compared with the controls. The brain levels were greater in the ethanol-treated rats, which means that these changes will tend to prolong the pharmacological action of the barbiturate. The results are in agreement with those of Seidel[15] and of Leslie *et al.*,[19] but contradict those of Coldwell *et al.*[16] obtained at 3 hr 45 min after dosing in the blood and brain of the rat. The latter results may be due to incomplete extraction or lack of specificity in the ultraviolet method used by these authors. Pentobarbital, at the dose used in this study, has been reported[16] to have no significant effect on ethanol levels in the blood and brain of the rat. The blood level at 1 hr after a 3 g/kg dose of ethanol was 3·3 mg/ml and at 4 hr it had declined to 2·0 mg/ml.[16] Hence at 3 hr, significant levels of ethanol would still be present. The paper chromatography results obtained from the blood and brain of rats killed at 3 hr showed that ethanol did not alter the proportion of metabolites to unchanged drug at this time. Since no metabolites of pentobarbital were detected in the brain, the observed changes in brain level were of unchanged pentobarbital. This confirms the pharmacological importance of the ethanol-induced increase in brain barbiturate levels. Since the relative increase in brain level produced by ethanol runs parallel with the blood pentobarbital level, there is no evidence that ethanol effects the passage of pentobarbital from blood to brain.

Ethanol caused a marked increase in the blood concentration of radioactivity at 2 and 4 hr (Fig. 3), and a concomitantly large increase in the area under the blood curve. It seems unlikely that these changes are due to any change in the distribution of pentobarbital and its metabolites, since the changes observed in blood occurred to approximately the same extent in the tissues at the two times they were estimated. Consequently, it is concluded that ethanol must be causing changes in metabolism and excretion.

Excretion and metabolism. When rats were loaded with large amounts of water by gavage, it was found that pentobarbital, at the dose used in this study, inhibited urine excretion during the first hour. Co-administration of ethanol did not reverse this effect until 2–4 hr after dosing. Since ethanol did not significantly reduce urine excretion, this cannot be a factor in the apparent inhibition of barbiturate elimination.

The inhibition by ethanol of the renal excretion of radioactivity appears to account completely for elevated blood and tissue levels of radioactivity observed in the ethanol-treated rats. Leslie *et al.*[19] also observed that ethanol decreased the renal excretion of radioactivity after administration of ^{14}C-pentobarbital, but their observations were restricted to the first 30 min after dosing.

The pentobarbital metabolism results obtained by paper chromatography of urine showed that excretion of M–I was inhibited by ethanol during the first 6 hr (Table 3) after dosing and accounted for the reduced excretion of radioactivity (Fig. 4). In contrast, after the first 3 hr, the amount of metabolite (M–IV) excreted in the urine was greater in the ethanol-treated rats. The net result was that the total radioactivity excreted was the same in both groups by 12 hr. The reduced excretion of M–I could

be due to inhibition of its renal excretion, but in this case one would expect to see significant levels of this metabolite in the plasma of the ethanol-treated rats. In fact this metabolite was not detectable in plasmas taken 3 hr after dosing. It appears therefore that M–I is rapidly eliminated and the most plausible explanation for the reduced quantity excreted in the urine of the ethanol-treated rats is inhibition of this route of pentobarbital metabolism. It is not possible at present to explain why ethanol had these effects on pentobarbital metabolism, since neither the identity nor the metabolic pathway producing M–I has been determined. Recently Rubin *et al.*[20] published results obtained with rat liver microsomes which showed that ethanol inhibits pentobarbital metabolism *in vitro*. Our results *in vivo* are therefore in agreement with their data. Schuppel[21] has also produced data *in vivo* showing that acute doses of ethanol similar to the one used in this study inhibited microsomal *N*-demethylation and *C*-hydroxylation.

It is concluded that ethanol at the dose used in this study causes higher blood and tissue levels of radioactivity during the immediate post-absorbative phase. From a pharmacological point of view, the increased brain level of pentobarbital observed at 3 hr after dosing is of the most significance and supports the contention that the interaction between pentobarbital and ethanol is more than purely additive. The results obtained in urine show that the higher tissue and blood levels of radioactivity observed when ethanol is administered are due to reduced renal elimination of a pentobarbital metabolite during the first 6 hr after dosing, probably due to an inhibition of pentobarbital metabolism by ethanol.

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