Table II. Mean urine volume (ml) and urinary excretion of amphetamine and total ^{14}C expressed as percent of the dose administered ($N = 6$)

Dose	Total dose excreted (%) and urine volumes (in brackets)			Total dose as unchanged amphetamine (%) (0–6 h)	Total dose excreted in 24 h (%)
	0–6 h	6–12 h	12–24 h		
Amphetamine (10 mg/kg)	70.6 (1.10)	4.5 (1.02)	2.9 (1.61)	31.0	77.9
Amphetamine + 2 g/kg ethanol	62.2 (0.99)	4.8 (0.14) [a]	7.5 (0.59)	39.8	74.6
Amphetamine + 3 g/kg ethanol	55.9 (0.59)	18.7 (0.27)	9.1 (0.90)	38.5	83.7
Amphetamine + 4 g/kg ethanol	55.3 (0.57)	20.4 (0.44)	3.5 (0.67)	39.8	79.2

[a] 2 animals only.

peaking at 50 mg/kg, and a subsequent decline at 70 mg/kg. This phenomenon has been reported by several workers[12,13] but generally with grouped mice, where the mortality at low amphetamine doses is very much greater. In this study ethanol was effective in reducing or eliminating this small initial mortality response. Because of this non-linear response, the LD_{50} calculations were based on the final increase in mortality beginning at 70 mg/kg.

The effects of ethanol on the urinary excretion of amphetamine and its metabolites in the mouse are shown in Table I. Individual metabolites were determined in the 0–6 h urine only. The urine pH was 6.6–7.0. As the ethanol dose increased, the excretion of unchanged amphetamine, expressed as percent of total ^{14}C excreted, increased from 44% to 72%. All other metabolites, with the exception of hippuric acid, decreased as the ethanol dose increased. On the other hand, expressed as a percent of the dose administered (Table II), excretion of total radioactivity decreased from 71% to 55% resulting in only a slight net increase (31% to 39%) in the excretion of amphetamine in the presence of ethanol (Table II). Similarly, at 24 h the excretion of total radioactivity, expressed as percent of the dose administered, was only slightly greater (77.9% to 79.2%) in the presence of 4 g/kg ethanol.

Discussion. The lack of effect of ethanol on the acute toxicity of amphetamine would suggest that the alcohol is not significantly altering interaction of the amine with its receptor site. A decrease in the initial mortality phase at 50 mg/kg was noted, but it is difficult to explain this action since the phenomenon is probably seen as a residual effect of grouping stress.

The inhibitory effect of ethanol on metabolism is striking if one considers the percentage increase in unchanged amphetamine appearing in the 0–6 h urines (Table I). In terms of percent of amphetamine administered, the inhibitory effect of ethanol is not as marked, increasing unchanged amphetamine in the urine from 31.0% to 39.8% (Table II). In this context, 2 g/kg ethanol was just as effective as 4 g/kg in increasing 0–6 h urinary output of unchanged amphetamine.

The major effects of ethanol appear to be a decrease in urine volume, noted at higher levels of ethanol, coupled with an inhibition of metabolism or excretion of metabolites in the 0–6 h period. This is consistent with the large increase of urinary radioactivity (20%) in the 6–12 h urines at 4 g/kg ethanol compared to the 4% output with amphetamine alone. Some effect on metabolism would be expected since the metabolism of ethanol itself would change the NAD/NADH ratio of the liver. These effects however do not have a significant effect on toxicity since the LD_{50} value at 2.5 h is little different from that at 24 h.

Summary. Ethanol, 3 g/kg i.p., did not significantly alter the acute toxicity of amphetamine in the mouse. However, the urinary metabolite pattern was changed, suggesting that ethanol suppressed metabolism of the stimulant during the initial 6 h period. After 24 h, the mouse metabolized the same fraction of a given dose of amphetamine, whether it was given as amphetamine alone or amphetamine mixed with 2, 3 or 4 g/kg ethanol.

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In vivo and in vitro Studies on Irreversible Binding of Halothane Metabolites to Proteins

The halothane hepatitis is a very rare but extremly serious incident. The reason for its development has not been elucidated. Its course and symptoms point to an allergic process[1–3]. However, the halothane molecule does not posess the chemical properties essential for reacting as hapten with proteins in order to form an antigenic molecule[4–6]. This seems to be the reason why many attempts have failed to elicit with halothane a transformation of lymphocytes from patients having survived a halothane hepatitis[7,8]. Efforts to sensitize rabbits against trifluoracetic acid[9], the main metabolite of halothane, did not present convincing results. The chemical coupling

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of trifluoroacetic acid to proteins, however, produced an antigen able to sensitize guinea-pigs[5], but there is no evidence that such a conjugation, which requires particular reaction conditions, occurs in vivo.

UEHLEKE et al.[10] presented evidence that halothane, similarly to CCl_4, binds irreversibly to microsomal proteins, if it is incubated with microsomes and a NADPH-generating system. The experiments presented here suggest that a halothane metabolite is bound covalently to microsomal protein and also to proteins outside the microsomal membrane, e.g. BSA added to the test tubes or cytoplasmic proteins in vivo.

Liver microsomes of mice were incubated with ^{14}C-halothane (1 m*M*) and a NADPH-regenerating system as described elsewhere[11]. After 90 min 0.5 ml of TCA (15%) was added to 1 ml of the incubated suspension. The precipitate was extracted twice with methanol, twice with methanol/chloroform 1:1 and again with 80% methanol, until no further radioactivity could be removed. The remaining protein was solubilized in hyamin and, after dissolving in Bray's solution, counted. The procedure corresponds to the method used for the demonstration of covalent binding of estrogens to microsomal proteins[12]. 3.5 $\pm$ 0.8 nmoles of halothane per mg microsomal protein were bound irreversibly. If the mice were pretreated with phenobarbital, the value increased up to 7.2 $\pm$ 0.9. The amount of radioactivity which could not be removed was dependent on the duration of incubation up to 90 min and on the concentration of halothane up to 1 m*M*. Without a NADPH-regenerating system, the value was insignificant and did not exceed 8% of the amount measured in the presence of the reducing system.

The same experiments were repeated with bovine albumin added to microsomes of untreated mice. About 20% of the total radioactivity bound to proteins in and outside the membranes was found in albumin isolated from the microsomes by ultracentrifugation and purified by dissolving in ethanol and precipitation by addition of diethyl ether in the cold (−30 °C). This indicates that reactive metabolites can also leave the microsomal membranes. 2 h after i.p. injection of 5 µl ^{14}C-halothane (spec. act. 1 mCi/mmole) to mice, proteins of microsomes contained 0.74 and those of the cytosol of the mouse liver 0.39 nmoles per mg protein of halothane bound irreversibly. The two liver fractions were lyophilized and extracted as mentioned above. The high amount of activity in the proteins of cytosol indicates that the labelling occurs also outside the endoplasmic membranes under in-vivo-conditions.

30 mg of microsomal protein from in vitro and in vivo experiments loaded with the halothane radioactivity were digested with trypsine (38 °C, 72 h, *tris* buffer pH 8.0) and subdued to ion exchange chromatography. Under these conditions, the radioactivity which could be eluted was present in several peptide fragments, indicating that amino acids bound a halothane metabolite covalently. The elution profile of digested microsomal proteins from in vivo experiments resembled that of the protein loaded in vitro, demonstrating that the same type of covalent binding occurs in vivo and in vitro (Figure).

In analogy to the well established formation of radicals in the course of CCl_4 metabolism, a similar mechanism could be assumed to explain the origin of radicals[13,14] if halothane is metabolized by the microsomal hydroxylating system. Reducing equivalents, flowing from NADPH to the iron of cytochrome P-450, should be involved in this reaction. The real nature of the radical is not yet known.

If one assumes and average molecular weight of microsomal proteins of around 50,000, it can be estimated that under the conditions we used for the in vivo experiments, which should be similar to the situation of patients under general anaesthesia, only every 30th protein molecule is loaded with a hapten. This number increases if the microsomal drug metabolizing system is induced. This small amount of altered protein molecules which also originate outside the endoplasmic membranes by reacting with a halothane radical seems to be too low to produce liver injury, but might be sufficient for acting as antigens, if a particular immunological situation prevails.

Zusammenfassung. Die Bindung von ^{14}C-Halothanmetaboliten an mikrosomales Protein wurde in vitro in mikrosomalen Inkubationen und in vivo and der Maus untersucht. Tryptische Spaltung des Proteins zeigt, dass die Radioaktivität kovalent gebunden ist.

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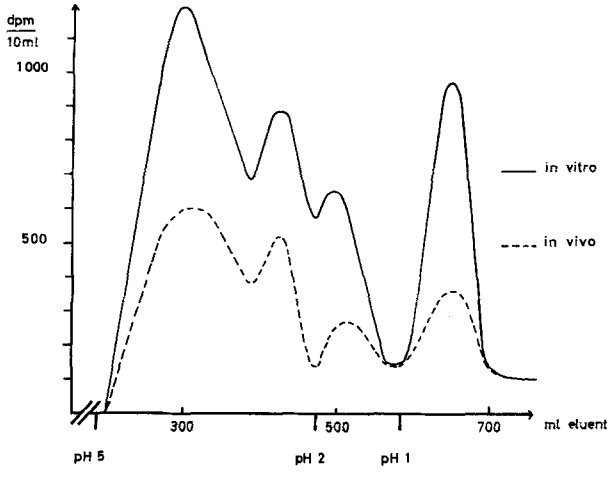

Elution profile of polypeptides loaded with radioactivity from ^{14}C-halothane. Column: Dowex 1×8, 50–100 mesh. Eluents: collidine/acetate pH 8 and pH 5, acetic acid pH 2 and pH 1.

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