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ACUTE INTERACTION OF DRUGS—I

THE EFFECT OF VOLATILE ANESTHETICS ON THE KINETICS OF ANILINE HYDROXYLASE AND AMINOPYRINE DEMETHYLASE IN RAT HEPATIC MICROSOMES

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Abstract—The *in vitro* effect of halothane, methoxyflurane, diethyl ether, and chloroform on the Michaelis constant (K_m) and maximal velocity (V_{max}) of microsomal aniline hydroxylase and aminopyrine demethylase was determined. The microsomes were obtained from rats pretreated with phenobarbital or 3-methylcholanthrene as well as from untreated rats. The halogenated anesthetics increased both the K_m and V_{max} of aniline hydroxylase in microsomes from untreated rats and this effect was magnified in microsomes from phenobarbital-induced animals. The K_m and V_{max} of aniline hydroxylase was not stimulated above control levels by halogenated anesthetics in microsomes from methylcholanthrene-induced rats. These anesthetics tended to inhibit the aminopyrine demethylase by lowering the V_{max}. Enzyme induction did not alter this inhibition. Diethyl ether inhibited aniline hydroxylase and aminopyrine demethylase by lowering the V_{max}.

STIMULATION of the drug-metabolizing activity by pretreatment *in vivo* with drugs or chemicals (enzyme induction) is well known and has been reviewed repeatedly.[1–3] In addition to the changes seen after chronic exposure to foreign compounds, Imai and Sato,[4] Anders,[5,6] and Van Dyke and Rikans[7] observed a stimulation of the rate of microsomal oxidations *in vitro* after the addition of ethyl isocyanide, acetone, or volatile anesthetics to microsomal suspensions. This finding was unexpected in view of the number of inhibited reactions, particularly those inhibited by the volatile anesthetics. Furthermore, it is important because many *in vitro* studies are performed on tissues removed from anesthetized animals or humans.

The reactions stimulated by the volatile anesthetics (type I binders) are limited to the oxidation of certain type II binding materials, aniline and zoxazolamine.[8] In rat microsomal preparations, the oxidation of type I binding drugs is inhibited by the volatile anesthetics.

In our attempt at further characterization of this effect, we present the results of studies on the effect of volatile anesthetics on the K_m and V_{max} of aniline hydroxylase and aminopyrine demethylase in microsomes from untreated rats and from rats pretreated with either 3-methylcholanthrene or phenobarbital.

MATERIALS AND METHODS

Tissue preparation. Adult male Sprague–Dawley rats were used. The animals were decapitated and exsanguinated. The livers were removed and placed on ice immediately. They were weighed and homogenized (Dounce homogenizer with a loose-fitting plunger) in 1·15% potassium chloride. The KCl volume equalled three times the wet wt of the livers. The homogenate was centrifuged in a refrigerated Sorvall centrifuge at 9000 g for 15 min. The supernatant fraction was then centrifuged in a Beckmann refrigerated ultracentrifuge at 105,000 g for 1 hr. The supernatant was decanted and the pellet resuspended in 0·05 m Tris buffer (pH 7·4 for the aminopyrine *N*-demethylase and pH 8·0 for the aniline hydroxylase) to yield a final concentration of microsomal protein of about 2·0 mg/ml and 3·0 mg/ml respectively.

Aniline hydroxylase assay. One ml aliquots of microsomal suspension were placed in 10 ml stoppered Erlenmeyer flasks. The flasks were flushed with oxygen or an anesthetic-oxygen mixture, and after equilibration to 37° and the substrate with an excess of a NADPH generating system was added rapidly. The final substrate concn ranged between 0·05 and 1·0 mM. The method of Imai *et al.*[9] was used to determine enzyme activity.

Aminopyrine assay. Two ml aliquots of the microsomal suspension were used per flask. The final substrate was determined by measuring the amount of formaldehyde formed.[10] Protein concentrations were determined by the method of Lowry *et al.*[11]

Time of incubation. Incubations were carried out for 8 min at 37°. The product formation was found to be linear with time for at least 8 min (but not longer than 10 min) for all substrate concentrations and for microsomes from induced and non-induced rats.

Enzyme induction. Rats were induced with 40 mg/kg phenobarbital (in pH 8·2 buffer) intraperitoneally (i.p.) daily for 3 days or with a single dose of 20 mg/kg 3-methylcholanthrene in corn oil i.p.; animals were killed 24 hr after the last injection.

Halothane concentrations. The Erlenmeyer flasks, containing aliquots of the microsomal suspension at 4°, were flushed for 10 sec with an anesthetic–oxygen mixture delivered by a standard anesthesia vaporizer. The total gas flow into the flasks was 5 l. min and was directed immediately above the surface of the suspension. The concentrations of anesthetics in the gas mixture were calculated from the flow rates of the oxygen through the anesthetic-containing copper kettle. (The concentrations of anesthetic agents mentioned later refer to these calculated concentrations in the gas mixture.) In order to determine the amount of anesthetic delivered to the microsomal suspension by this procedure, the anesthetic concentrations in the microsomal suspension were measured directly. After flushing the microsomal suspension, the anesthetic was extracted with an equal volume of heptane and measured by gas chromatography using a column packed with Carbowax 400/Porasil C, 100–280 mesh. A column temperature of 95° was used; the temperature of the injection port was maintained at 110° and that of the electron capture detector at 115°. In parallel experiments, rats were anesthetized by inhalation of anesthetic vapor in oxygen and maintained anesthetized for 30 min. They were sacrificed by decapitation and the livers were removed immediately. Microsomes were prepared, and aliquots were extracted with heptane and measured using the same gas chromatographic technique.

Data analysis. Values for V_{max} and K_m were determined for every experiment, by construction of Lineweaver–Burke plots and mean values as well as their standard

errors were calculated and the Student's t-test was used to determine the significance of the differences.[12] $P < 0.025$ was considered to be statistically significant.

RESULTS

Table 1 summarizes the results of the aniline hydroxylation reaction. In the presence of halothane (1,1,1-trifluoro-2, 2-chlorobromoethane), methoxyflurane (1,1-difluoro-2, 2-dichloroethyl methyl ether), or chloroform in low and in high concentrations, K_m and

TABLE 1. ACUTE EFFECTS OF VOLATILE ANESTHETIC AGENTS ON ANILINE HYDROXYLATION*

Concn of anesthetic† (%)	No.	V_{max} (mole/hr/mg protein × 10^8)	K_m (M × 10^4)
None	9	1·42 ± 0·04	0·36 ± 0·02
0·5 Halothane	7	2·81‡ ± 0·21	1·71‡ ± 0·08
4·0 Halothane	6	7·07‡ ± 0·67	2·50‡ ± 0·28
0·3 Methoxyflurane	8	6·70‡ ± 0·25	1·68‡ ± 0·21
1·5 Methoxyflurane	7	12·13‡ ± 1·10	3·48‡ ± 0·34
0·25 Chloroform	5	2·65‡ ± 0·16	4·26‡ ± 0·36
2·3 Chloroform	7	4·75‡ ± 0·20	4·49‡ ± 0·60
1·0 Diethyl ether	7	1·72 ± 0·17	6·20‡ ± 0·36

* Values are mean ± S.E. of the mean.
† Concentration of anesthetic volume as percent of total gas volume in gas used to flush the incubation flasks. Remainder of gas was oxygen.
‡ Significantly different from value obtained in absence of anesthetic.

V_{max} values were higher than under control conditions. This finding indicated a stimulation of enzyme activity. For diethyl ether, the V_{max} value was not significantly different from the control while K_m was greatly increased which explains the inhibition noted. Table 2 presents the results of the aminopyrine N-demethylation. It is apparent

TABLE 2. ACUTE EFFECTS OF VOLATILE ANESTHETIC AGENTS ON AMINOPYRINE N-DEMETHYLATION*

Concn of anesthetic† (%)	No.	V_{max} (mole/hr/mg protein × 10^8)	K_m (M × 10^4)
None	21	14·15 ± 0·89	3·50 ± 0·30
0·5 Halothane	10	17·93‡ ± 1·72	3·82 ± 0·32
2·5 Halothane	12	14·26 ± 0·73	5·18‡ ± 0·57
0·3 Methoxyflurane	6	16·25 ± 0·69	4·08 ± 0·94
1·5 Methoxyflurane	6	11·58 ± 1·07	5·36‡ ± 0·78
1·0 Diethyl ether	10	13·14 ± 1·46	3·24 ± 0·32
15 Diethyl ether	7	7·53‡ ± 1·24	2·70 ± 0·74

* Values are mean ± S.E. of the mean.
† See Table 1.
‡ Significantly different from value obtained in absence of anesthetic.

that high concentrations of halothane and methoxyflurane cause an increase in K_m, while the V_{max} is not significantly different from the control which is the reason the reaction is inhibited under these conditions. Ether at high concentrations also causes an inhibition of the reaction and this is the result of a definite decrease in V_{max} with no change in K_m. In low concentrations of halothane an increase in V_{max} was observed, but no significant change was noted in K_m, indicating an enhancement of aminopyrine oxidation.

Values for K_m and V_{max} of aniline hydroxylase were higher following pretreatment with phenobarbital or methylcholanthrene than in the control situation (Tables 3 and 4).

TABLE 3. HALOTHANE AND ENZYME INDUCTION EFFECTS ON ANILINE HYDROXYLATION

Microsomes from rats	Concn of halothane		
	None	0·5%	4·0%
No pretreatment			
No.	9	7	6
V_{max}* (mean ± S.E.)	1·42 ± 0·04	2·81† ± 0·21	7·07† ± 0·67
K_m* (mean ± S.E.)	0·36 ± 0·02	1·71† ± 0·08	2·50† ± 0·28
Phenobarbital pretreatment			
No.	6	6	9
V_{max}* (mean ± S.E.)	2·71‡ ± 0·38	4·56† ± 0·70	9·04† ± 1·09
K_m* (mean ± S.E.)	1·93‡ ± 0·34	3·41† ± 0·27	4·85† ± 0·61
3-Methylcholanthrene pretreatment			
No.	5	4	6
V_{max}* (mean ± S.E.)	4·79‡ ± 0·98	4·16 ± 1·07	3·50 ± 0·56
K_m* (mean ± S.E.)	2·36‡ ± 0·38	2·34 ± 0·47	1·92 ± 0·13

* V_{max} values, mole/hr/mg protein × 10^8. K_m values, M × 10^4.
† Significantly different from corresponding value in absence of halothane.
‡ Significantly different from corresponding value in noninduced group.

TABLE 4. DIETHYL ETHER AND ENZYME INDUCTION EFFECTS ON ANILINE HYDROXYLATION

Microsomes from rats	Concn of diethyl ether		
	None	1·0%	5·0%
No pretreatment			
No.	9	6	—
V_{max}* (mean ± S.E.)	1·42 ± 0·04	1·72 ± 0·17	—
K_m* (mean ± S.E.)	0·36 ± 0·02	6·20† ± 0·36	—
Phenobarbital pretreatment			
No.	6	5	3
V_{max}* (mean ± S.E.)	2·71‡ ± 0·34	3·45 ± 0·51	3·18 ± 0·52
K_m* (mean ± S.E.)	1·93‡ ± 0·34	7·06† ± 1·13	14·36† ± 3·59
3-Methylcholanthrene pretreatment			
No.	5	5	5
V_{max}* (mean ± S.E.)	4·79‡ ± 0·98	5·64 ± 0·81	4·04 ± 0·40
K_m* (mean ± S.E.)	2·36‡ ± 0·38	11·12† ± 1·42	22·14† ± 2·72

* V_{max} values, mole/hr/mg protein × 10^8. K_m values, M × 10^4.
† Significantly different from corresponding value in absence of diethyl ether.
‡ Significantly different from corresponding value in noninduced group.

In the presence of halothane a further increase in V_{max} and K_m for the aniline biotransformation was found when microsomes from phenobarbital-induced animals were used; however, halothane did not cause any further significant change in the rate of aniline breakdown when 3-methycholanthrene-induced animals served as the source of microsomes (Table 3). The effects of ether were the same in the induced as those in the non-induced livers. K_m was increased while V_{max} was not significantly changed, reflecting the inhibition of this reaction. The results of the aminopyrine *N*-demethylation reaction in induced animals are presented in Tables 5 and 6. Enzyme induction

TABLE 5. HALOTHANE AND ENZYME INDUCTION EFFECTS ON AMINOPYRINE *N*-DEMETHYLATION

Microsomes from rats	Concn of halothane		
	None	0·5%	2·5%
No pretreatment			
No.	21	10	12
V_{max}* (mean ± S.E.)	14·15 ± 0·87	17·93† ± 1·72	14·26 ± 1·73
K_m* (mean ± S.E.)	3·50 ± 0·30	3·82 ± 0·32	5·18† ± 0·57
Phenobarbital pretreatment			
No.	12	10	10
V_{max}* (mean ± S.E.)	19·46‡ ± 2·37	15·46 ± 1·47	9·15† ± 0·78
K_m* (mean ± S.E.)	3·86 ± 0·34	5·26 ± 0·61	4·81 ± 0·51
3-Methylcholanthrene pretreatment			
No.	7	7	7
V_{max}* (mean ± S.E.)	11·85 ± 1·46	10·79 ± 1·15	9·70 ± 1·35
K_m (mean ± S.E.)	4·17 ± 1·01	3·70 ± 0·36	6·77 ± 1·43

* V_{max} values, mole/hr/mg protein × 10^8. K_m values, M × 10^4.
† Significantly different from corresponding value in absence of halothane.
‡ Significantly different from corresponding value in noninduced group.

TABLE 6. DIETHYL ETHER AND ENZYME INDUCTION EFFECTS ON AMINOPYRINE *N*-DEMETHYLATION

Microsomes from rats	Concn of diethyl ether		
	None	1·0%	15·0%
No pretreatment			
No.	21	10	7
V_{max} (mean ± S.E.)	14·15 ± 0·87	13·14 ± 1·45	7·53† ± 1·24
K_m* (mean ± S.E.)	3·50 ± 0·30	3·24 ± 0·32	2·70 ± 0·74
Phenobarbital pretreatment			
No.	12	10	7
V_{max}* (mean ± S.E.)	19·46‡ ± 2·37	22·55 ± 0·84	8·43† ± 1·00
K_m* (mean ± S.E.)	3·86 ± 0·34	4·57 ± 0·52	8·80† ± 1·55
3-Methylcholanthrene pretreatment			
No.	7	7	7
V_{max}* (mean ± S.E.)	11·85 ± 1·46	9·01 ± 1·13	4·67† ± 0·38
K_m* (mean ± S.E.)	4·17 ± 1·01	4·34 ± 0·65	3·77 ± 0·62

* V_{max} values, mole/hr/mg protein × 10^8. K_m values, M × 10^4.
† Significantly different from corresponding value in absence of diethyl ether.
‡ Significantly different from corresponding value in noninduced group.

with phenobarbital caused an increase in the rate of aminopyrine breakdown with a rise in V_{max} and no significant change in K_m. 3-Methylcholanthrene induction did not cause a significant change in the rate of aminopyrine breakdown. In the presence of high concentrations of halothane, phenobarbital induction results in an inhibition with a lowered V_{max} and no significant change in K_m. After 3-methylcholanthrene induction, high concentration of halothane did not alter the rate of aminopyrine biotransformation significantly (Table 5). Diethyl ether decreased the rate of aminopyrine metabolism. V_{max} was lower with the three types of microsomes used (Table 6); K_m was significantly higher only when microsomes from phenobarbital-induced rats were used. Low concentrations of diethyl ether did not have a significant effect on the rate of aminopyrine breakdown by the three types of microsomes.

Halothane concentrations were measured in microsomes prepared from livers of anesthetized rats and were found to be 394 ppm. This value was within the limits of concentration of the anesthetic in microsomes exposed to halothane *in vitro* which were 96 ppm at 0·5% halothane in the gas mixture and 1703 ppm at 2·5% halothane in the gas mixture. Some halothane was lost (as expected) during the preparation of microsomes from livers of anesthetized rats, but it is demonstrated that the short exposure time represents an *in vitro* model, which is close to the *in vivo* situation.

DISCUSSION

The interpretation of the kinetic data of microsomal enzyme systems is difficult because of the complex nature of these reactions and, therefore, when changes in the kinetic parameters occur, several possible interpretations can be offered, based on other studies of this type. Thus the changes noted may reflect either a change in a rate-limiting step or a multiplicity of enzymes which carry out these oxidations or a combination of these two effects or a shift of electrons from the type I to the type II site.

The possibility that a rate-limiting step in the microsomal oxidase is altered by the anesthetics is derived from studies by Gigon *et al.*[13] in which they found evidence suggesting that drugs which produce a type I binding spectrum in microsomes accelerate the cytochrome P-450 reductase. In addition, evidence presented by Imai and Sato,[14] Davies *et al.*[15] and Schenkman and Cinti[16] suggests that the rate-limiting step in the microsomal mixed function oxidase system is the cytochrome P-450 reductase reaction. The volatile anesthetics produce a type I binding spectrum in rat liver microsomes, thus, if they react as other type I binding compounds, this would suggest that at least part of the effect noted is the result of an acceleration of the cytochrome P-450 reductase and thereby a release of this rate-limiting step. This explanation is weakened, however, since presumably the aminopyrine demethylase reaction utilized cytochrome P-450 reductase and yet is not stimulated in the studies reported in this paper.

The fact that not all reactions were stimulated by the anesthetic and that in many instances the K_m was altered but not the V_{max}, suggests another possible explanation. Recently, several reports have appeared concerning the possible multiplicity of aniline hydroxylases and aminopyrine demethylases in rat liver microsomes.[17–19]

The effects of the anesthetics on these two drug assays are consistent with the possibilities that they are inhibiting one or more of the component enzyme systems and thereby making the properties of the remaining enzyme(s) more apparent. The

fact that different levels of the same anesthetic produces different effects on the kinetics may reflect the different sensitivities of the various component enzymes to the anesthetic; a situation very similar to that found by Aust and Stevens[20] in the case of the effect of DDT and Dieldrin on the K_m of aminopyrine demethylase.

The fact that the K_m of the aniline hydroxylase is increased by phenobarbital and methylcholanthrene pretreatment of the animals has been previously found by Guarino *et al.*[21] However, the anesthetic does not have the same effect on the K_m of the aniline hydroxylase in both cases. For example, the K_m of aniline hydroxylase in the microsomes from the methylcholanthrene pretreated animals is unaffected by halothane, while in contrast halothane increased the K_m of aniline hydroxylase in microsomes from phenobarbital pretreated rats. This may indicate, as proposed by Aust and Stevens[20] for aminopyrine demethylase, that phenobarbital induces more than one microsomal enzyme capable of demethylating aminopyrine while methylcholanthrene does not induce the same systems. Rickert and Fouts[17] as well as Wada *et al.*[18] and Anders[5,6] have proposed that aniline hydroxylase exists in more than one form in hepatic microsomes and, thus, the effect of halothane may be to inhibit only one form.

Hildebrandt *et al.*[22] described the effect of metyrapone, a type I binding substance. The authors observed a stimulation of the metabolism of acetanilid and an inhibition of aminopyrine and hexobarbital metabolism. These results are comparable to those reported in this communication for aniline hydroxylation and aminopyrine demethylation and may indicate that it is possible to shift electrons from the type I to the type II binding site and thereby increase the activity of the type II binding site. The observation that the halogenated anesthetics stimulated aniline breakdown only when the type I site was available in microsomes from non-treated and phenobarbital treated rats supports this interpretation. Human liver microsomes do not produce the type I binding spectrum in the presence of halothane. The fact that halothane does not enhance aniline hydroxylation in human hepatic microsomes, is additional support for this interpretation (unpublished observation).

It is obvious that no complete interpretation of the data presented in this communication can be made now, however, two important points do emerge. First, since many tissues are removed from anesthetized animals and humans for *in vitro* studies, it should be recognized that the presence of the anesthetic may alter the reactions under study and, secondly, the volatile anesthetics may be useful in the study of the multiplicity of microsomal-mixed function oxidases.

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