Biochemical Pharmacology, Vol. 19, pp. 2493–2499. Pergamon Press. 1970. Printed in Great Britain

METABOLISM OF ETHANOL BY RAT LIVER MICROSOMAL ENZYMES

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(*Received* 13 *December* 1969; *accepted* 5 *February* 1970)

Abstract—In confirmation of other reports, rat liver microsomes were found to oxidize ethanol to acetaldehyde *in vitro* in the presence of NADPH and oxygen, at an optimal pH of 7·0–7·4. This activity was inhibited markedly by addition of deoxycholate *in vitro*, cyanide and azide, but only weakly by digitonin and not at all by pyrazole or SKF 525-A (β-diethylaminoethyl-diphenylpropyl acetate). 3-Amino-1,2,4-triazole inhibited the activity when added *in vitro* or pre-injected *in vivo*. Hydrogen peroxide and barium peroxide were effective in place of NADPH and oxygen when initial reaction rates were considered: this activity was also inhibited by cyanide and by aminotriazole. It is concluded that oxidation of ethanol by hepatic microsomes *in vitro* is brought about by a combination of catalatic activity plus a system which generates H_2O_2 from the oxidation of NADPH, rather than by the intact mixed-function oxidase (drug-metabolizing) system.

LIVER alcohol dehydrogenase (ADH) is generally regarded as the principal enzyme responsible for oxidizing ethanol to acetaldehyde *in vivo*.[1–6] It was reported by Orme-Johnson and Ziegler[7] that hepatic microsomes can oxidize ethanol to acetaldehyde in the presence of NADPH and oxygen *in vitro*, possibly by the same enzyme system involved in the metabolism of barbiturates and many other drugs. This work was confirmed and extended recently by Lieber and DeCarli[8] and by Roach *et al.*[9] Indirect evidence for the existence of an ethanol oxidizing system in hepatic microsomes was provided by Lind and Parkes,[10] who found that an inhibitor of microsomal drug-metabolizing enzymes, SKF 525-A (β-diethylaminoethyl diphenylpropyl acetate), injected intraperitoneally in a dose of 25 mg/kg 45 min before the ethanol, resulted in four-fold prolongation of ethanol sleeping time in mice. They also observed a significant decrease in ethanol sleeping time by pretreatment with pentobarbital, chlorpromazine, amitriptyline or imipramine, given 21 hr before ethanol. On the basis of these works, an alternate major pathway for ethanol metabolism has been suggested[7, 8] and it has been invoked as a metabolic basis of cross-tolerance between ethanol and barbiturates.[11] A number of relevant studies by other workers have been reviewed,[12] and additional evaluation has been submitted elsewhere.*

These studies left a number of unanswered questions: (1) It was not clear whether the hepatic microsomal oxidation of ethanol was catalyzed by the same enzyme system as that which oxidizes barbiturates; (2) the microsomal ethanol-oxidizing system was not proven to be active *in vivo*; (3) its relation to cross-tolerance between ethanol and barbiturates *in vivo* was not demonstrated. It has been shown subsequently that microsomal oxidation of ethanol probably does not occur to any significant extent

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in vivo[12, 13] and that this system is probably not involved in ethanol-barbiturate cross-tolerance.* The present work deals with experiments *in vitro*, which indicate that hepatic microsomal oxidation of ethanol is probably effected by a catalase-like activity rather than by the mixed-function drug metabolizing system. A preliminary report of this work was presented elsewhere.[14]

MATERIALS AND METHODS

Male Wistar rats of 200–300 g body weight, maintained on a diet of normal rat chow, were killed by decapitation. The livers were removed and washed with ice-cold 1·15% KCl solution, homogenized in 4 vol. of the same solution and centrifuged at 8000 *g* for 15 min in the SM head of a Servall RC-2B. The supernatant was then centrifuged at 105,000 *g* in the Al47 head of a model B-60 International preparative ultracentrifuge for 1 hr at 0°. The resulting supernatant was then poured off and the pellet suspended in 1·15% KCl solution. This was again centrifuged at 105,000 *g* for 1 hr. The washed microsomes were resuspended in 1·15% KCl solution. Microsomal protein content was determined by a slightly modified biuret method,[15] and adjusted by appropriate dilution to 25 mg/ml; 1 ml of the microsome suspension was used for each flask. Each ml of incubation mixture contained 50 μmoles of ethanol; 0·5 μmoles of NADPH; 200 μmoles of phosphate buffer, pH 7·4; 5 μmoles of $MgCl_2$ and 5 mg of microsomal protein. The concentrations of H_2O_2, BaO_2 and inhibitors used in different experiments are indicated at appropriate places. The reaction mixtures were pre-incubated for 5 min at 37°. After pre-incubation, the reaction was started by the addition of the microsomal preparation. A portion of the same preparation heated in a boiling water bath for 10 min was used for the blank. The incubation technique and acetaldehyde determination were as described by Lieber and DeCarli.[8] A standard curve for acetaldehyde, using four different concentrations from 0·0125 to 0·05 μmoles/ml, was obtained in the same way. The stock solution for the preparation of these standards was first analysed by the titration method of Siggia and Maxcy.[16]

RESULTS

Oxidation of ethanol by liver microsomes. In confirmation of earlier reports,[7, 8] liver microsomes were found to catalyze the oxidation of ethanol to acetaldehyde in the presence of NADPH and oxygen. NADH was approximately one-third as effective as NADPH, whereas NAD and NADP were inactive. The average activity, expressed as rate of acetaldehyde production, was 3·8 μmoles/mg of microsomal protein per minute between the third and sixth minute of incubation. Depending on the concentration of the microsomal suspension, acetaldehyde production was roughly linear with time for 6–15 min, after which it levelled off. For a 5-min reaction period, the pH optimum for the reaction was found to lie in the range 7·0–7·4 (Fig. 1).

Effects of various inhibitors. The effects of various inhibitors are shown in Table 1. Addition of sodium deoxycholate resulted in a reduction of acetaldehyde formation, the magnitude of which depended on the concentration added; $7{\cdot}0 \times 10^{-2}$ M deoxycholate completely abolished the reaction. In contrast, digitonin in comparable concentrations had relatively little effect on acetaldehyde production. Pyrazole, a potent inhibitor of liver alcohol dehydrogenase,[17] had no effect on the microsomal production of acetaldehyde at concentrations of up to 20 mM.

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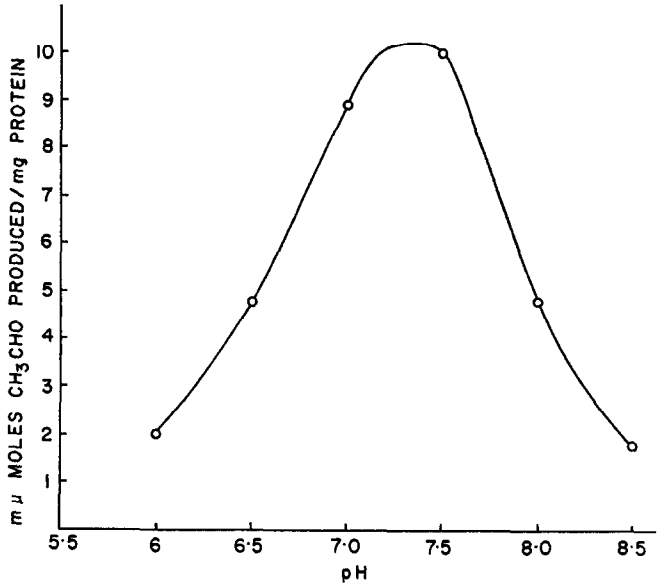

FIG. 1. Effect of pH on microsomal oxidation of ethanol *in vitro*. Acetaldehyde production was measured at the end of a 5-min incubation at 37°.

TABLE 1. EFFECT OF VARIOUS INHIBITORS ON ACETALDEHYDE PRODUCTION FROM ETHANOL AT VARIOUS TIMES OF INCUBATION

Inhibitor	Concentration (M)	Acetaldehyde produced (mμmoles/mg protein)			
		3 min	6 min	9 min	12 min
Deoxycholate	0	8·8*	20·0	23·4	24·6
	$1{\cdot}4 \times 10^{-3}$	4·0	8·4	14·7	17·7
	$1{\cdot}4 \times 10^{-2}$	0	0	1·4	2·4
	$7{\cdot}0 \times 10^{-2}$	0	0·2	0·2	0·2
Digitonin	0	7·6	16·2	22·6	24·1
	2×10^{-4}	8·4	19·6	20·6	22·7
	4×10^{-3}	5·2	12·4	19·2	19·8
	8×10^{-3}	4·2	10·1	15·8	17·0
Pyrazole	0	14·2	23·8	26·8	27·6
	5×10^{-3}	11·6	21·6	24·6	25·2
	1×10^{-2}	12·4	21·6	23·2	23·2
	2×10^{-2}	13·6	22·4	26·0	28·4
KCN	0	12·4	24·2	26·1	28·8
	1×10^{-4}	7·8	16·6	17·4	18·4
	5×10^{-4}	2·6	7·2	6·2	6·2
	1×10^{-3}	1·6	1·8	0·2	0·2
Na azide	0	11·2	22·4	25·8	26·0
	1×10^{-4}	5·6	10·0	13·0	13.0
	1×10^{-3}	2·4	6·8	7·4	7·8
	1×10^{-2}	0	0·2	0	0
SKF	0	8·4	18·6	20·4	22·0
	1×10^{-4}	8·2	16·2	17·6	18·0
	1×10^{-3}	11·2	17·0	17·6	18·8
	$2{\cdot}5 \times 10^{-3}$	10·0	16·2	20·6	19·4

* The various inhibitors were tested on different microsomal preparations, as indicated by differences in the respective control values.

The addition of KCN was found repeatedly to inhibit acetaldehyde production strongly, the extent depending on the concentration added. In all cases, 10^{-3} M KCN inhibited the process completely. This concentration of KCN does not affect microsomal drug metabolism.[18] Sodium azide had a similar but less marked effect, 10^{-2} M NaN_3 being required for complete inhibition.

SKF 525-A had no effect upon the production of acetaldehyde when added in concentrations ranging from 10^{-4} to 10^{-3} M; these concentrations are known to inhibit the metabolism of many drugs by microsomal preparations *in vitro*.[18]

3-Amino-1,2,4-triazole was found to decrease the microsomal production of acetaldehyde to about the same degree when added *in vitro* in a final concentration of 80 mM, and when injected *in vivo* in a dose of 2 g/kg intraperitoneally 4 hr before preparation of the microsomes (Fig. 2). The decrease after addition *in vitro* was greater

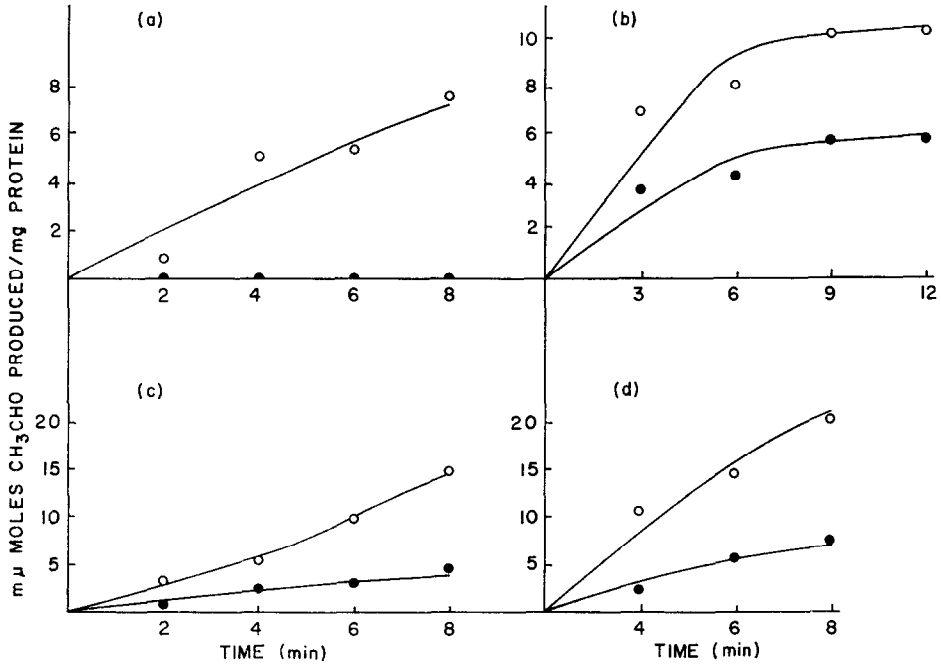

FIG. 2. Oxidation of ethanol by hepatic microsomal preparations in the absence (○) and presence (●) of 3-amino-1,2,4-triazole. In (a) and (b) amino-triazole was added *in vitro* to give a final concentration of 80 mM, and pre-incubated with the microsomes for 10 min before addition of the substrate at zero time. In (c) and (d) aminotriazole was injected intraperitoneally in a dose of 2 g/kg, and the animals were killed 4 hr later for preparation of hepatic microsomes. In (b) and (d) the oxidation of ethanol proceeded at the expense of 0·5 mM NADPH; in (a) and (c) NADPH was replaced by 59 mM BaO_2.

when aminotriazole was pre-incubated with the microsomes for 10 min at 37° before addition of ethanol than when the microsomes were added to the complete system. This is in keeping with the observations by Tephly *et al.*[19]

Replacement of NADPH and oxygen by peroxides. Microsomal production of acetaldehyde from ethanol was sustained even in the absence of NADPH when either H_2O_2 or BaO_2 was added. The initial rate of acetaldehyde formation was directly related to the concentration of H_2O_2 employed (Fig. 3). However, the production of

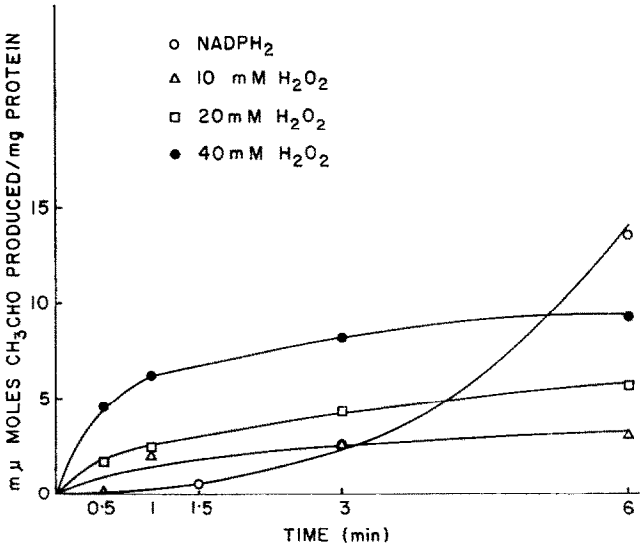

FIG. 3. Oxidation of ethanol by hepatic microsomes in the presence of NADPH or of various concentrations of H_2O_2.

TABLE 2. EFFECT OF KCN ON MICROSOMAL PRODUCTION OF ACETALDEHYDE FROM ETHANOL AND 59 mM BaO_2 AT VARIOUS TIMES OF INCUBATION

Time of incubation (min)	Acetaldehyde production (mμmoles/mg protein)			
	Control	KCN		
		10^{-4} M	10^{-3} M	10^{-2} M
1·5	2·5	0·3	0	0
3	4·4	0·5	0·1	0
6	5·2	1·2	0·4	0
9	6·3	1·2	1·1	0

acetaldehyde was initially rapid when H_2O_2 or BaO_2 was used and then levelled off, while an initial lag of 2–3 min was noted when NADPH was employed, followed by the rapid phase and later plateau.

Addition of 10^{-3} M KCN inhibited sharply the production of acetaldehyde in the presence of BaO_2 (Table 2). Similarly, injection of aminotriazole into the rats, 4 hr before removal of the liver, resulted in a reduction of about 50 per cent in the acetaldehyde production with 59 mM BaO_2 (Fig. 2).

DISCUSSION

The present results confirm the claims of earlier investigators[7, 8] that hepatic microsomal preparations convert ethanol to acetaldehyde *in vitro* by a system dependent upon NADPH and oxygen. As shown in Fig 3, this reaction reaches its maximal rate after a lag of 2–3 min. Our preparations showed an average activity during the 3- to 6-min period equivalent to 3·8 μmoles of acetaldehyde produced per milligram of

microsomal protein per minute. This is less than the value of 7·7 found by Lieber and DeCarli,[8] but is very close to that reported by Roach *et al.*[9] for twice-washed microsomal preparations which appear to be similar to the preparations used in the present study. The fact that deoxycholate which is known to disrupt the microsomal drug-metabolizing activity[20] abolished the oxidation of ethanol (Table 1), while pyrazole, a potent inhibitor of hepatic cytoplasmic ADH, did not, suggests that the activity of these preparations resides in microsomal components themselves rather than in adsorbed cytoplasmic ADH. This is further supported by the effectiveness of NADPH and the ineffectiveness of NAD in maintaining the ethanol-oxidizing activity of the microsomes.

However, the hepatic microsomal ethanol-oxidizing system does not appear to be identical with the microsomal system which metabolizes barbiturates. Gillette *et al.*[18] found that digitonin, which is a weaker inhibitor than deoxycholate, did not disrupt microsomal NADPH oxidase activity at a concentration which effectively inhibited drug metabolism; the same concentration produced only about 25 per cent reduction of ethanol-oxidizing activity (Table 1). Moreover, SKF 525-A, in a concentration known to inhibit barbiturate metabolism, had no effect on oxidation of ethanol. This is in accord with the observations of Tephly *et al.*[13] and of Khanna and Kalant.[12]

In contrast, the microsomal oxidation of ethanol was markedly inhibited by KCN and NaN_3 in concentrations which do not affect barbiturate metabolism. The report by Lieber and DeCarli[8] that KCN had little effect on acetaldehyde production is explainable by the low concentration of KCN which they used. Aminotriazole, a relatively selective inhibitor of catalatic activity, also reduced markedly the ethanol-oxidizing activity. These results are in excellent agreement with those of Roach *et al.*[9]

It seems probable, therefore, that ethanol is being oxidized by catalatic activity of either a constituent or a contaminant of the microsomal preparation. This is further supported by the ability of inorganic peroxides to replace NADPH in the system. Other investigators[7, 9] have found that acetaldehyde production in the presence of H_2O_2 was only 5–12 per cent of that observed in the presence of NADPH. This is probably explainable in terms of the experimental procedure. Roach *et al.*[9] measured acetaldehyde production at the end of a 30-min incubation. The initial rate of production in the presence of H_2O_2 (Fig. 3) or BaO_2 is greater than with NADPH but falls off rapidly, probably because of rapid decomposition of the peroxide.[21] If initial velocities are considered, it is clear that peroxides can replace NADPH and oxygen. Roach *et al.*[9] have shown that a H_2O_2-generating system, giving a continuing supply of peroxide, can replace NADPH completely.

Our results with aminotriazole are also in close agreement with those reported by Roach *et al.*,[9] in that the microsomes from animals pre-injected with this catalase inhibitor showed somewhat over 50 per cent inhibition of ethanol oxidation, regardless of whether NADPH or BaO_2 was used. However, an unexpected finding was that addition of aminotriazole *in vitro*, with a brief pre-incubation period before addition of the substrate, caused complete inhibition of ethanol oxidation by BaO_2 but only 50–75 per cent inhibition when NADPH was used. No explanation of this difference can be offered at present, but it suggests that Roach *et al.*[9] may not be justified in concluding that "60% of the activity of the system is due to catalase and about 40% to some other system".

Gillette *et al.*[18] have shown that hepatic microsomes can generate H_2O_2 from

NADPH *in vitro*. It seems probable that cytochrome P 450, which transfers "active oxygen" to drug substrates via drug-specific enzymes, is the immediate generator of H_2O_2 when the system is studied *in vitro*. Since it has been shown[12, 13] that inhibitors and inducers of barbiturate metabolism do not affect the rate of ethanol metabolism *in vivo*, it appears that microsomal oxidation of ethanol is an artifact which arises *in vitro* when the H_2O_2-generating function is divorced from drug metabolism and is linked to a catalatic activity. This suggestion would be compatible with the observation by Rubin and Lieber[22] that 50 mM of ethanol inhibits the hydroxylation of pentobarbital *in vitro*, if it could be shown that oxidation of ethanol and hydroxylation of pentobarbital compete for the available O-cytochrome P 450 intermediate under these conditions.

Although ethanol is probably not metabolized by hepatic microsomes *in vivo*,[12] and chronic treatment with ethanol does not affect the metabolism of pentobarbital *in vivo*,* an explanation is still required for the observation that chronic treatment with ethanol enhances the pentobarbital hydroxylase activity *in vitro* of microsomes isolated from the livers of the treated subjects.[22]

Acknowledgements—This work was supported by a grant from the Alcoholism and Drug Addiction Research Foundation of Ontario. We are indebted to Mr. N. I. Rangaraj, Mr. K. Niederhuber and Mr. J. Zarek for technical assistance.

* H. KALANT, J. M. KHANNA and J. MARSHMAN, *J. Pharmcc. exp. Ther.* in press.

REFERENCES

1. E. JACOBSEN, *Pharmac. Rev.* **4**, 107 (1952).
2. W. W. WESTERFIELD, *J. Clin. Nutr.* **9**, 426 (1961).
3. C. S. LIEBER and C. S. DAVIDSON, *Am. J. Med.* **33**, 319 (1962).
4. H. KALANT, *Q. J. Stud. Alcohol* **23**, 52 (1962).
5. K. J. ISSELBACHER and N. J. GREENBERGER, *New Engl. J. Med.* **270**, 351 (1964).
6. F. LUNDQUIST, in *International Encyclopaedia of Pharmacology and Therapeutics*, Section XX, *Alcohols and Derivatives*, Vol. 1, (Ed. J. TRÉMOLIÈRES), p. 95. Pergamon Press, Oxford (1970).
7. W. H. ORME-JOHNSON and D. M. ZIEGLER, *Biochem. biophys. Res. Commun.* **21**, 78 (1965).
8. C. S. LIEBER and L. M. DECARLI, *Science, N.Y.* **162**, 917 (1968).
9. M. K. ROACH, W. N. REESE and P. J. CREAVEN, *Biochem. biophys. Res. Commun.* **36**, 596 (1969).
10. N. LIND and M. W. PARKES, *J. Pharm. Pharmac.* **19**, 56 (1967).
11. E. RUBIN and C. S. LIEBER, *Gastroenterology* **54**, 642 (1968).
12. J. M. KHANNA and H. KALANT, *Biochem. Pharmac.* **19**, 2033 (1970).
13. T. R. TEPHLY, F. TINELLI and W. D. WATKINS, *Science, N.Y.* **166**, 627 (1969).
14. J. M. KHANNA and H. KALANT, *4th Int. Congr. Pharmac., Basel, Switzerland*, p. 239 (1969).
15. H. KALANT, R. K. MURRAY and W. MONS, *Cancer Res.* **24**, 570 (1964).
16. S. SIGGIA and W. MAXCY, *Analyt. Chem.* **19**, 1023 (1947).
17. W. D. WATKINS, J. I. GOODMAN and T. R. TEPHLY, *Fedn Proc.* **28**, 1594 (1969).
18. J. R. GILLETTE, B. B. BRODIE and B. N. LADU, *J. Pharmac. exp. Ther.* **119**, 532 (1957).
19. T. R. TEPHLY, G. J. MANNERING and R. E. PARKS, JR., *J. Pharmac. exp. Ther.* **134**, 77 (1961).
20. J. R. GILLETTE, *Adv. Pharmac.* **4**, 219 (1967).
21. D. KEILIN and E. F. HARTREE, *Biochem. J.* **39**, 293 (1945).
22. E. RUBIN and C. S. LIEBER, *Science, N.Y.* **162**, 690 (1968).