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Involvement of Hepatic Mixed Function Oxidase Enzyme System in the Oxidative Metabolism of Methanol

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Methanol has been known to be oxidized by liver alcohol dehydrogenase (ADH) and catalase enzymes. The data presented here indicate that this primary alcohol is also metabolized by rat liver mixed function oxidase (MFO) induced by phenobarbital. Comparative studies on the oxidation of methanol and N-demethylation of benzphetamine by MFO reveal that SKF 525-A inhibits both reactions whereas aminotriazole, a known inhibitor of catalase, abolishes the activity of microsomal methanol oxidase with no effect on N-demethylase. While disulfiram depresses N-demethylase activity, pyrazole and cyanide have little or no effect on the microsomal enzyme activity. Although ethanol appears to be a competitive inhibitor of microsomal methanol oxidase it is a poor inhibitor of this enzyme system.

Introduction

Methanol poisoning in humans and animals has been a problem of considerable toxicological interest not because methanol is a potential toxicant, but because of its highly toxic metabolite formaldehyde. This metabolism of methanol to formaldehyde has been reported to be catalyzed by catalase, alcohol dehydrogenase and hepatic microsomes.[2-5] While Chance[2] reported that peroxidative removal of methanol by catalase is probably the principal pathway of its enzymatic oxidation, Kini and Cooper[3] claimed alcohol dehydrogenase (ADH) catalyzed metabolism of methanol as the major pathway. On the other hand, Theorell and Bonnichsen[6] found no measurable activity of crystalline horse liver ADH with methanol for the formation of formaldehyde. Thus it is not very clear which of these pathways is really important in the activation of methanol in body. Consequently, further information on the metabolism of methanol is needed. This communication reports on the kinetics of methanol oxidation catalyzed by rat liver mixed function oxidase and the effects of various compounds on this oxidative metabolism of methanol.

Experimental

Male Sprague-Dawley rats weighing 150—180 g were used in these experiments. The *ad lib* fed animals received *i.p.* dose of 50 mg/kg phenobarbital dissolved in saline daily for 4 consecutive days. Control animals received an equivalent amount of saline. The animals were sacrificed 24 hours following the last injection of phenobarbital sodium or saline. Their livers were rapidly removed, weighed, and perfused with ice-cold 1.15% KCl solution. The perfused livers of the five treated and five control animals were pooled separately and homogenized with 2 volumes of ice-cold 0.25M sucrose solution containing 0.05 mM ethylenediamine tetraacetic acid (EDTA) first in a waring blender and then in a Potter Elvehjem type homogenizer. The homogenate was centrifuged at 9000 $\times g$ for 20 minutes in a refrigerated Sorvall centrifuge. The microsomes from the supernatant fraction were then isolated by the procedure described by Cinti, *et al.*[7] The microsomal pellets were

1) Location: *Tuskegee, AL 36088, U.S.A.*
2) B. Chance, *Acta Chem. Scand.*, **1**, 236 (1947).
3) M.M. Kini and J.R. Cooper, *Biochem. Pharmacol.*, **8**, 207 (1961).
4) W.H. Orme-Johnson and D.M. Ziegler, *Biochem. Biophys. Res. Commun.*, **21**, 78 (1965).
5) R. Teschke, Y. Hasumura and C.S. Lieber, *J. Biol. Chem.*, **250**, 7397 (1975).
6) H. Theorell and R. Bonnichsen, *Acta Chem. Scand.*, **5**, 1105 (1951).
7) D.L. Cinti, R. Grundin, and S. Orrenious, *Biochem. J.*, **134**, 367 (1973).

repeatedly washed with 1.15% KCl solution containing 0.05 mM EDTA and were stored at −20° in nitrogen atmosphere until further use.

K_m and V_{max} values for the formation of formaldehyde from methanol were determined by incubating various substrate concentrations with microsomes from control and phenobarbital-treated rats. Incubations were carried out in 0.05 M Hepes buffer, pH 7.8, containing an NADPH generating system ($NADP^+$ 1 mM; glucose-6-phosphate 12 mM; glucose-6-phosphate dehydrogenase 1 unit) and 1.0—1.5 mg of microsomal protein per ml of incubation media in a final volume of 2 ml. Prior to the addition of substrate, incubations were temperature-equilibrated for 5 minutes at 37° in a gyrotory shaker waterbath. At the end of 15 minutes incubation period the reaction was stopped by adding $ZnSO_4$ followed by $Ba(OH)_2$. Formaldehyde formed was determined by Nash reagent as previously described.[8] Inhibition constant (K_i) of ethanol for the oxidative metabolism of methanol was determined according to the method of Dixon.[9] Comparative studies on the metabolism of methanol andb enzphetamine in the incubated hepatic microsomes were carried out with and without the addition to the incubation mixture of ampicillin, SKF 525-A, pyrazole, aminotriazole, or disulfiram.

Protein concentrations were determined by the biuret method modified to include 0.1 ml of 1% deoxycholate in each sample.

Results and Discussion

Apparent K_m and V_{max} values for methanol oxidation by liver microsomes isolated from control and phenobarbital-pretreated rats were determined from the Lineweaver-Burk plots (Fig. 1). As seen from these plots, the V_{max} value is increased about 2-fold probably as a result of the induction of mixed function oxidase (MFO) by phenobarbital pretreatment whereas the apparent K_m values remained almost unchanged, (control microsomes, $K_m=4.0\times10^{-2}$M; phenobarbital induced microsomes, $K_m=4.2\times10^{-2}$M). These data suggest that in addition to ADH and catalase enzymes, hepatic MFO is also capable of oxidizing methanol to formaldehyde in the presence of NADPH and molecular oxygen. Although the phenobarbital treatment of rats did not cause any significant change in the K_m value for the oxidation of methanol by induced microsomes, the increase in V_{max} value indicates that the concentration of microsomal methanol oxidase is almost doubled. The apparent K_m value (4.0×10^{-2}M) found for methanol oxidation by hepatic microsomes is quite comparable to the K_m values of 3.0×10^{-2}M, 1.7×10^{-2}M and 1.5×10^{-3}M reported for methanol oxidation by ADH in man and rhesus monkey, and by catalase in rat, respectively.[10] Therefore, it seems to be reasonable to support the view that methanol is metabolized at a considerable rate by hepatic MFO.

Next, experiments were carried out to determine whether oxidation of methanol and N-demethylation of benzphetamine were catalyzed by the same microsomal enzyme or two different enzymes. For this purpose, methanol and benzphetamine as substrates were separately incubated in incubation mixtures with and without the presence of SKF 525-A, pyrazole, aminotriazole, disulfiram, cyanide or ampicillin. The results of these experiments are presented in Table I. The fact that SKF 525-A abolished markedly the activity of both microsomal methanol oxidase and benzphetamine N-demethylase suggests that perhaps methanol and benzphetamine are metabolized by two different microsomal enzymes. On the contrary, Orme-Johnson and Ziegler[4] studied the effect of SKF 525-A and carbon monoxide on the microsomal methanol oxidase, and found no inhibition of the activity. Further, they reported 68% inhibition of microsomal methanol oxidase activity by cyanide and, based on their data, these workers concluded that methanol oxidase is a separate system from the microsomal N-dealkylase. In our experiments, when we incubated 1 mM cyanide in the incubations containing methanol or benzphetamine the MFO activity remained almost unchanged. This is in agreement with the view that microsomal oxidation of methanol is mainly catalyzed by MFO and not by contaminating catalase which should have been strongly inhibited by cyanide. Pyrazole, a potent inhibitor of ADH, was almost ineffective in the *in vitro* activity of MFO, thus ruling

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9) R.L. Dixon, *Biochem. J.*, **55**, 170 (1953).
10) W.A. Pieper and M.J. Skeen, *Biochem. Pharmacol.*, **22**, 163 (1970).

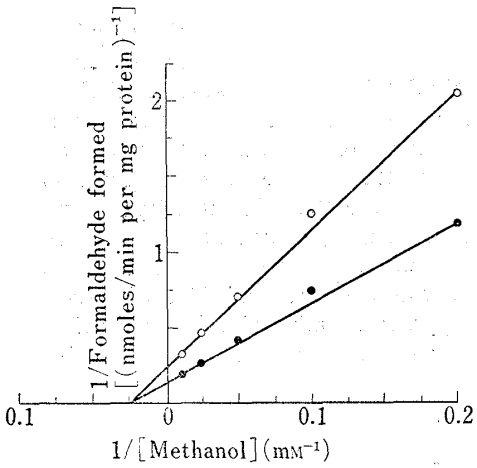

Fig. 1. Lineweaver-Burk Plots of the Oxidation of Methanol by Hepatic Microsomes isolated from Control (○) and Phenobarbital-pretreated Rats (●)

The microsomes and NADPH generating system were preincubated for 5 minutes at 37° in Hepes buffer (0.05M, pH, 7.8). Then methanol was added to a final concentration ranging 5 to 80 mM. After 15 minutes incubation, the formaldehyde formed was measured colori metricallyusing Nash reagent.[8]

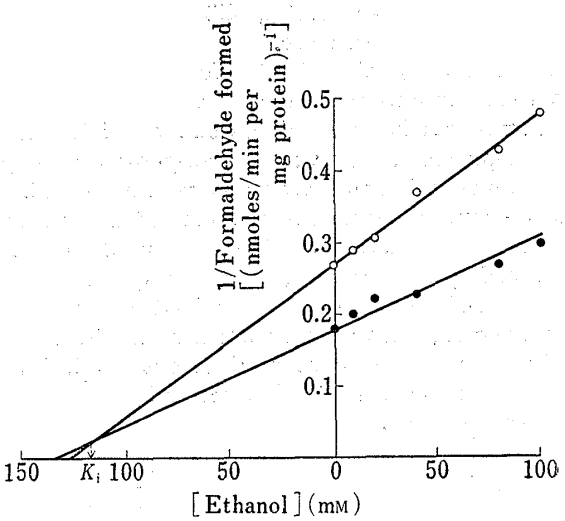

Fig. 2. Inhibition of Methanol Oxidation by Ethanol in Phenobarbital-pretreated Rat Liver Microsomes

The microsomes, NADPH generating system and various concentrations (10 to 100 mM) of ethanol were preincubated for 5 minutes at 37° in Hepes buffer (0.05M, pH, 7.8). Then methanol was added to a final concentration of 50 mM (○) or 200 mM (●). After 15 minutes incubation, the formaldehyde formed was measured colorimetrically using Nash reagent.[8] The inhibition constant is computed according to Dixon's method.[9]

TABLE I. Effect of Certain Compounds on the Activity of Hepatic Microsomal Methanol Oxidase and Benzphetamine N-demethylase

Addition	Concentration (mM)	% activity of control	
		Methanol	Benzphetamine
None	—	100	100
SKF 525-A	0.5	68	45
Potassium cyanide	1.0	98	96
Pyrazole	1.0	94	100
Aminotriazole	20.0	69	98
Ampicillin	1.0	81	101
Disulfiram	1.0	102	73

The microsomes isolated from phenobarbital-pretreated rats, NADPH generating system and the various compounds were preincubated for 5 minutes at 37° in Hepes buffer (0.05M, pH, 7.8). Then methanol (200 mM) or benzphetamine (5 mM) was added in a final volume of 2 ml. At the end of 15 minutes, formaldehyde formed was measured colorimetrically using Nash reagent.[8]

out any significant presence of ADH in the microsomes. It is interesting to note that aminotriazole at a concentration of 20 mM depressed the activity of microsomal methanol oxidase by about 30% but not that of N-demethylase suggesting the involvement of two different types of enzymes. Although this may appear to be because of catalase as a contaminant, the specific inhibitory effect of aminotriazole on MFO can not be excluded since this compound has been shown to inhibit *in vivo* activity of hepatic MFO.[11] Similarly, ampicillin and disulfiram, two different drugs, showed opposite effects on the activity of microsomal methanol oxidase and N-demethylase. These results suggest that methanol is metabolized by hepatic microsomal methanol oxidase which appears to be different from microsomal N-demethylase.

11) G.J. Traiger and G.L. Plaa, *Can. J. Physiol. Pharmacol.*, 15, 291 (1973).

Since ethanol and methanol are reported to be substrates for MFO, it was of interest to examine whether ethanol could competitively inhibit the mixed function oxidase catalyzed metabolism of methanol. The data plotted in Fig. 2 indeed demonstrate that ethanol competes with methanol for MFO but appears to be a poor inhibitor, ($K_i = 11.67 \times 10^{-2}$M) suggesting ethanol may not prove to be an effective antagonist of the microsomal metabolism of methanol.

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