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Stereospecificity of the microsomal ethanol-oxidizing system*

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The microsomal ethanol-oxidizing system (MEOS) has been interpreted by Lieber and DeCarli [1] to be a unique system contributing significantly to ethanol metabolism *in vivo*. A number of investigators have considered the possibility that MEOS activity is due to catalase or catalase combined with alcohol dehydrogenase activity [2–4], but a number of recent studies support an MEOS function independent of catalase [5–8]. Teschke *et al.* [5, 9] and Mezey *et al.* [10] reported the isolation of a microsomal preparation free of or containing only small amounts of catalase and capable of converting ethanol to acetaldehyde. Thurman and Scholz [3] isolated in a similar manner a catalase-free microsomal fraction, but found it devoid of MEOS activity. Barakat *et al.* [11] examined ethanol oxidation by components of rat liver microsomes and found that highly purified cytochrome P-450 did not oxidize ethanol appreciably.

Alcohol dehydrogenase shows stereospecificity, oxidizing the *R* hydrogen of ethanol in the formation of acetaldehyde [12]. Corrall *et al.* [13] reported that catalase shows the same stereospecificity. This is a report of a study to determine if, in the oxidation of ethanol by MEOS, the *R* or *S* hydrogen is retained in the acetaldehyde formed.

(*R*)-[1-$^3H_1$]ethanol and (*S*)-[1-$^3H_1$]ethanol were prepared and [2-^{14}C]ethanol was purchased from New England Nuclear Corp., Boston, Mass. Initially, media as described by Isselbacher and Carter [14] and Lieber and DeCarli [15] were employed. Isselbacher and Carter incubated ethanol with microsomal material in the presence of NADPH, magnesium chloride, and a sodium phosphate buffer at pH 7·4, while Lieber and DeCarli employed a potassium phosphate buffer at pH 7·4 containing magnesium chloride, an NADPH generating system consisting of NADP, sodium isocitrate and isocitrate dehydrogenase, and in the presence of nicotinamide. All later incubations were with a potassium phosphate buffer (80 mM) at pH 7·4 with 0·3 mM NADPH and 5 mM magnesium chloride.

Microsomes incubated in the medium of Isselbacher and Carter [14] were prepared from a 20% liver homogenate in 0·25 M sucrose made from livers of female Sprague–Dawley rats killed by decapitation. The microsomal fraction collected after centrifugation at 105,000 *g* was resuspended in 0·15 M KCl [14, 16]. Using the medium of Lieber and DeCarli [15] microsomes were prepared by homogenization in 0·15 M KCl and, after centrifugation at 9000 *g* for 30 min, the crude microsomes were collected by centrifugation at 105,000 *g* and resuspended in KCl and incubated, or further purification was attempted by washing. For the latter, the KCl suspension was centrifuged again at 105,000 *g*. The resulting pellet was resuspended in KCl, followed by a third centrifugation at 105,000 *g* and then resuspension of the resulting pellet in KCl for incubation. Protein in the final microsomal suspension was determined using biuret [17].

Livers were obtained from three monkeys. The first, a male, had been fasted 72 hr and his liver was removed under phencyclidine anesthesia. The other two were females which had been fasted overnight and were under barbiturate anesthesia. The first monkey, 1 yr before sacrifice, was under hyperthermia for 48 hr for another study. The second monkey was being sacrificed after accidental hemorrhage in preparation for another study; the third monkey had been oophorectomized and the liver was removed at the time its brain was being isolated and placed under perfusion for another study. It is assumed that these circumstances do not alter the stereospecificity of ethanol oxidation, but could alter the quantity of ethanol utilized. The use of these monkeys was preferred to killing of monkeys for the present study. The livers were collected in cold isotonic saline and homogenized within 40 min of removal.

To each of two flasks were added the potassium phosphate buffer, magnesium chloride and NADPH to give the final concentrations noted, and the microsomal suspension to give a final protein concentration of 3 mg/ml. The flasks were stoppered with inlets in the stoppers to permit subsequent injections into the media. The gas phase was air, except that oxygen was used when the conditions of Isselbacher and Carter [14, 18] were employed. Flasks with contents were incubated for 10 min at 37°, and then labeled ethanol was injected. To each flask, 0·4 to 1·2 μCi of [2-^{14}C]ethanol was added and 1–3 μCi of (*R*)-[1-$^3H_1$]ethanol was added to one flask and 1–3 μCi of (*S*)-[1-$^3H_1$]ethanol to the other flask. The ethanol was added to give a final concentration in the 40-ml volume of 2·3 mg/ml. Incubation was for 20 min. Two additional flasks serving as controls were incubated identically, except that that the microsomal suspension was boiled before addition or KCl was substituted for the suspension.

Incubation was terminated by the addition of equal volumes 0·3 N $Ba(OH)_2$ and 5% $ZnSO_4$ to the flasks and then 4·8 mg of unlabeled acetaldehyde in water was injected. The content of each flask was cooled and centrifuged. The supernatant was distilled and the initial distillate was collected in dimetol reagent. The dimetoacetaldehyde that precipitated was collected, washed, dried and weighed [12].

Catalase-free MEOS was prepared essentially as described by Mezey *et al.* [10], except that female Sprague Dawley rats [9] weighing 200–300 g were used. After isolation of the microsomal pellet and its solubilization, an ammonium sulphate precipitate was dialyzed. The dialyzed protein was passed through a Sephadex-G-25 column and was then placed on a DEAE cellulose column and eluted with KCl [10]. Catalase activity appeared at the beginning

* A preliminary account of these studies has appeared (*Biochem. Soc. Transactions* **2**, 994 1974).

of elution and a cytochrome P-450 peak followed which was devoid of catalase activity [3, 9, 10]. The tube fractions containing the P-450 peak were combined and ammonium sulfate was added to 50% saturation. The resulting precipitate was collected and dissolved in potassium phosphate buffer and incubated with NADPH, magnesium chloride and the labeled ethanols as just described, except that the total volume of the reaction mixture was 5 ml and protein was at 13 mg/ml. Catalase activity was determined by the method of Bergmeyer [19]; cytochrome P-450 was measured by the method of Omura and Sato [20].

Radioactivity in the acetaldehyde was assayed after dissolving dimetoacetaldehyde in a scintillant (Aquasol from New England Nuclear Corp., Boston, Mass.). All cpm were brought to dis./min by internal standardization. The dis./min actually incorporated into acetaldehyde were calculated from the 16–19 mg dimetoacetaldehyde collected and the 33·4 mg dimetoacetaldehyde to be expected from 4·8 mg of acetaldehyde, if there were no losses in isolation. Samples of the media incubated were assayed to obtain the total dis./min of ^{14}C and ^{3}H incubated.

^{14}C and ^{3}H present in the dimetoacetaldehyde isolated in the control incubations were due to contamination of the labeled ethanols with labeled acetaldehyde as evidenced by similar recoveries whether the microsomal suspension was boiled or 0·15 M KCl was substituted for the microsomal suspension and whether incubation was terminated at the beginning of incubation or after 20 min. By repeated distillations of the ethanols from semicarbazide before use [13], acetaldehyde contamination was reduced to less than 0·01 per cent of the dis./min added in the ethanols; the radioactivities in acetaldehydes in the control incubations were 1/10th or less of the activities in the acetaldehydes formed in the flasks incubated with the unboiled microsomal preparations. The activities in the acetaldehyde in the control flasks were subtracted from the corresponding activities in the acetaldehydes formed by MEOS. By incubating catalase-free MEOS at a protein concentration of 13 mg/ml, contaminating labeled acetaldehyde was also a minor quantity compared to the quantity of labeled acetaldehyde formed, despite the lower MEOS specific activities in these preparations.

While it is unlikely that a difference in stereospecificity would be observed consequent to the very small differences in conditions selected by Isselbacher and Carter [14, 18] compared to those of Lieber and DeCarli [15], stereospecificity was examined under both sets of conditions. The results are recorded in Table 1. The ratio is given of ^{3}H to ^{14}C in the acetaldehyde formed by the MEOS to the ratio of ^{3}H to ^{14}C in the ethanol incubated. There was 14 per cent or less retention of the *R* hydrogen, while the retention of the *S* hydrogen ranged from 64 to 100 per cent under these conditions. Using the modified incubation medium as described in Methods in all subsequent experiments, results were similar, with little or no tritium in acetaldehyde formed from the (*R*)-[1-$^{3}H_1$]ethanol, while 80 per cent or more of the tritium in the (*S*)-[1-$^{3}H_1$]ethanol oxidixed was retained.

The small retention of the (*R*)-hydrogen and incomplete retention of the (*S*)-hydrogen may indicate oxidation by more than one reaction. With the monkey preparations, the ratio with the (*R*)-hydrogen appears somewhat closer to 0; with the (*S*)-hydrogen it is closer to 1·0. Secondary isotopic effects could be responsible for the failure to obtain theoretically complete retention of the (*S*)-hydrogen. The approach to the theoretical ratio of 1·0 using crude monkey preparations may be due to the higher percentage of conversion of ethanol in these incubations, so that an isotopic effect would be obscured. Rognstad [8] has considered the contribution of catalase to ethanol oxidation, assuming it is the (*R*) and not the (*S*) atom which is responsible for isotopic discrimination. Gang *et al.* [21] reported a single experiment showing that racemically labeled [1-$^{3}H_1$]ethanol, on incubation with rat MEOS, yields acetaldehyde with much less than half of the specific activity of the [1-$^{3}H_1$]ethanol. This would suggest considerable isotopic discrimination for the (*S*)-hydrogen. The quantity of ethanol oxidized was not reported. The (*R*)-hydrogen from (*R*)-[1-$^{3}H_1$]ethanol was removed in accordance with the present report.

The stereospecificity of MEOS, the removal of the *R*-hydrogen in acetaldehyde formation, is the same stereospecificity that has been observed for alcohol dehydrogenase [12] and catalase [13]. However, we observed acetaldehyde formation with the same stereospecificity in an MEOS preparation [10] in which catalase and alcohol dehydrogenase have not been detected, although admit-

Table 1. Oxidation of (*R*)-[1-$^{3}H_1$]ethanol and (*S*)-[1-$^{3}H_1$]ethanol in the presence of [2-^{14}C]ethanol by microsomal preparation from rat and monkey

Animal	Microsomal preparation	Expt. No.	$^{3}H/^{14}C$ in CH_3CHO / $^{3}H/^{14}C$ in C_2H_5OH (*R*)-1-^{3}H	$^{3}H/^{14}C$ in CH_3CHO / $^{3}H/^{14}C$ in C_2H_5OH (*S*)-1-^{3}H	C_2H_5OH oxidized (nmoles/min/mg protein)
Rat	Crude	1*	0·00	0·79	2·1
		2*	0·10	0·64	1·4
		3†	0·14	0·82	3·5
		4†	0·13	1·00	2·8
		5	0·11	0·86	1·4
	Washed	1	0·03	0·87	0·7
		2	0·16	0·81	1·4
	Catalase-free	1	‡	0·81	0·6
		2	0·07	‡	0·4
Monkey	Crude	1	0·07	0·98	453
		2	0·05	1·03	238
		3	0·03	1·13	151
	Washed	1	0·02	0·95	73·8
		2	0·02	0·88	8·2
		3	0·03	0·91	12·5
	Catalase-free	1	0·02	0·80	1·5

* Incubation medium as described [14].
† Incubation medium as described [15].
‡ Incubations not done.

tedly with less formation of acetaldehyde than in the less purified microsomal preparations. A decrease in activity would be expected with solubilization of the microsomes.

The amount of ethanol oxidized to acetaldehyde, in nmoles per min of incubation per mg of microsomal protein, has been calculated from the specific activity of the [2-^{14}C]ethanol and the quantity of ^{14}C incorporated into the acetaldehyde. The quantity oxidized in the flask containing (*R*)-[1-$^{3}H_1$]ethanol was, as expected, essentially the same (within 10 per cent) as that in the flask with (*S*)-[1-$^{3}H_1$]ethanol in each experiment, and the average of these two values is recorded in the last column of Table 1. The quantity was greater with the monkey preparations. Little change in acetaldehyde formation as a result of the repeat washings of the rat microsomal preparation is evident, but the specific activity in nmoles of ethanol oxidized to acetaldehyde per mg of protein was least with the catalase-free preparation.

The acetaldehyde formed is less than the 14·7 nmoles/min/mg of protein reported by Lieber and DeCarli [22] for a 10-min incubation period. We have assumed linear formation of acetaldehyde over the 20-min period of incubation [15]. The greater rate of formation of acetaldehyde in the monkey could suggest that in the primate MEOS is more active. However, Mannering *et al.* [23] reported monkey liver has a larger quantity of catalase than rat liver, but is less active peroxidatively. MEOS prepared from human liver obtained by surgical biopsy is reported to have somewhat less ethanol-oxidizing activity than MEOS prepared from rat liver [15]. The amount of ethanol oxidized in the incubation with MEOS from monkey liver exceeds by many fold the amount of NADPH added. Vatsis and Schulman [24], making a similar observation, postulated the existence of a constituent in microsomes that regenerates NADPH during ethanol oxidation.

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Department of Medicine,
Case Western Reserve University
School of Medicine,
Cleveland, Ohio 44106, *U.S.A.*

ROGER J. M. CORRALL
LING C. YU
BYRON A. ROSNER
JOSEPH M. MARGOLIS
HARVEY M. RODMAN
WING KAM
BERNARD R. LANDAU

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