INVESTIGATION OF THE COMPONENT REACTIONS OF OXIDATIVE STEROL DEMETHYLATION. ALCOHOL DEHYDROGENASE[1]

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The alcohol dehydrogenase of microsomal oxidative sterol demethylation is removed from rat liver microsomes by extraction with Tris-acetate buffer. Demethylase activity is reconstituted by addition of either the crude extract or crystalline alcohol dehydrogenase to washed microsomes. The overall reaction catalyzed by the reconstituted system is the same stoichiometrically as that catalyzed by unwashed microsomes. The dehydrogenase of sterol demethylation that catalyzes the oxidation of the primary alcohol may be identical to liver alcohol dehydrogenase.

Microsomal enzymes catalyze the oxidative demethylation of lanosterol and related 4,4-dimethyl sterols to yield CO_2 from the 4α- and 4β-methyl groups (Lindberg et al., 1963). This work is part of our continuing attempt to isolate, purify, and characterize these microsomal enzymes.

Each demethylation apparently follows the sequence (Olson et al., 1957; Miller et al., 1967): $R\text{-}CH_3 \xrightarrow{A} RCH_2OH \xrightarrow{B} RCHO \xrightarrow{C} RCOOH \xrightarrow{D} RH + CO_2$. Gaylor and coworkers (Miller et al., 1967), using Triton-treated microsomes, experimentally divided demethylation into two processes: aerobic hydroxylation of the methyl group (enzyme A); and anaerobic conversion of the oxygenated intermediates to CO_2 (enzymes B, C, and D). The hydroxylase has been isolated from rat liver microsomes and purified (Gaylor and Mason, 1968). The anaerobic process has now been further resolved experimentally.

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The alcohol dehydrogenase activity (enzyme B) may be removed from rat liver microsomes by extraction with Tris-acetate buffer. This report describes briefly the extraction procedure and restoration of demethylase activity.

EXPERIMENTAL PROCEDURES

Washing procedure. Livers (33 g) from adult, male rats were homogenized in 100 ml of cold 0.05 M Tris-acetate buffer (pH 7.4 at 37°, containing 2 mM glutathione and 30 mM nicotinamide). The suspension was centrifuged for 5 minutes at 1,000 x g and 20 minutes at 10,000 x g. The resulting supernatant fraction then was diluted to 140 ml and centrifuged for 60 minutes at 105,000 x g (37,000 RPM, No. 50.1 rotor, Beckman Model L2-65B centrifuge). The supernatant fraction from the first high-speed centrifugation was discarded. For washing, the pellets from high-speed centrifugation were vigorously homogenized in 140 ml of fresh buffer, and the suspensions were centrifuged at 105,000 x g for 60 minutes. For incubation, the microsomal pellets were suspended in 15 ml of fresh buffer.

Incubation. Each incubation flask contained, in 2.2 ml, approximately 20 to 30 mg of microsomal protein, 2.2 μmoles of NAD+, and 100 mμmoles of [3α,3β-di-^{14}C]4,4-dimethyl-5α-cholest-7-en-3β-ol suspended in the same buffer by the aid of 2 mg of Triton WR-1339 (Ruger Chemical Co.). Samples were incubated 10 minutes at 37°. Assay of $^{14}CO_2$ release was carried out as described by Swindell and Gaylor (1968).

Materials. The ^{14}C-labeled substrate (41,300 dpm/mg) was prepared by Dr. Swindell. 4α-Methyl-5α-cholest-7-en-3-one was prepared by t-butyl chromate oxidation of 4α-methyl-5α-cholest-7-en-3β-ol (Swindell and Gaylor, 1968). Purities have been established (Swindell and Gaylor, 1968).

Crystalline horse liver alcohol dehydrogenase (HLADHL, Lot 8BA, 2.6 U/mg) and yeast alcohol dehydrogenase (ADHL, Lot 8EB, 350 U/mg) were purchased from the Worthington Biochemical Corp. Protein concentrations were determined by the method of Lowry et al. (1951).

RESULTS AND DISCUSSION

Repeated extraction of rat liver microsomes with Tris-acetate buffer resulted in almost complete loss of demethylase activity (without supernatant fraction, Fig. 1). Full activity was restored when the supernatant fraction was added to the washed microsomes. Considerable protein was washed from the microsomes (Fig. 1). The specific activity of the restored microsomal system was actually increased by washing.

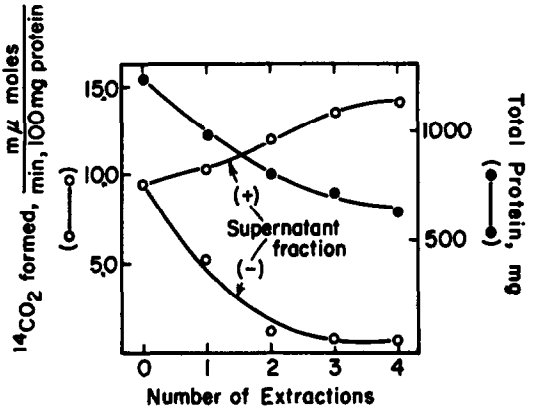

Fig. 1. Each sample was incubated with microsomes (18 to 35 mg protein) following the washing procedure. The supernatant fraction (1.0 ml) from the second high-speed centrifugation was substituted for buffer in some samples. The total protein is the amount recovered from 33 g of liver. Each value is the average of three determinations.

Demethylase activity was restored similarly when crystalline alcohol dehydrogenase was added (Table I). Addition of alcohol dehydrogenase from either horse liver or yeast fully restored demethylase activity; however, based on units of activity, horse liver alcohol dehydrogenase was far superior to the yeast enzyme. Although most of the liver alcohol dehydrogenase activity was destroyed by heat, the activity of the crude supernatant fraction somewhat resisted inactivation by heat treatment (Table I).

TABLE I

Restoration of Demethylase Activity

The samples were incubated with thrice-washed microsomes as described previously. An average of 20 mg of microsomal protein was added to each flask. Each value is the average of results from three separate incubations.

Addition	Amount of alcohol dehydrogenase		$^{14}CO_2$ formed
	Weight	Units	
	μg	EU	mμmoles/100 mg microsomal protein
None	--	--	5.8
Supernatant fraction (0.5 ml)*	--	--	56.3
Liver alcohol dehydrogenase	50	0.13	57.0
Yeast alcohol dehydrogenase	50	35.0	57.0
Yeast alcohol dehydrogenase	0.2	0.13	6.5
Liver alcohol dehydrogenase (boiled)	50	0.13	10.4
Supernatant fraction (0.5 ml boiled)*	--	--	25.2

*Approximately 1 mg protein.

Swindell and Gaylor (1968) have shown that CO_2 and 4α-methyl-5α-cholest-7-en-3-one are formed stoichiometrically during incubation of rat liver microsomes with the 4,4-dimethyl sterol substrate. Accordingly, the products of incubation of liver alcohol dehydrogenase and washed microsomes were collected and identified. Essentially equal amounts of $^{14}CO_2$ and ^{14}C-methyl-3-ketosteroid were recovered from an incubation of thrice-washed microsomes, NAD+, crystalline alcohol dehydrogenase, and 4,4-dimethyl sterol substrate (Table II).

Other buffers (e.g., phosphate, Tris-HCl) of various strengths did not yield similar extractions of the alcohol dehydrogenase activity.

The formation and metabolism of oxygenated intermediates during demethylation of 32-C (Fried et al., 1968) and of 30- and 31-C (Miller et al., 1967) has been clearly established. The oxidation of long-chain and complex cyclic alcohols by liver alcohol dehydrogenase (Waller et al., 1965; Merritt and Tompkins, 1959) is consistent with the hypothesis that liver alcohol dehydrogenase may oxidize the alcohols formed during demethylation. The physiological significance of the microsomal alcohol dehydrogenase is not clear at present, but there is an adequate amount of this enzyme activity present in microsomes to account for the observed rates of demethylation.

TABLE II

Stoichiometry of formation of $^{14}CO_2$ and ^{14}C-4α-methyl-5α-cholest-7-en-3-one*

Results are averages of four incubations in each experiment.

Experiment	$^{14}CO_2$ collected	^{14}C-4-methyl-3-ketosteroid collected	Recovery of steroid
	mμmoles	mμmoles	%
I	70.9	64.5	91
II	70.4	62.6	89

*Thrice-washed microsomes (40 mg protein) were incubated with 50 μg of liver alcohol dehydrogenase, 4.4 μmoles of NAD+, and 200 mμmoles of labeled substrate in a final volume of 3.6 ml. $^{14}CO_2$ was collected as described above. The labeled 3-ketosteroid was collected and purified with unlabeled carrier by gas-liquid chromatography as described by Swindell and Gaylor (1968).

The alcohol dehydrogenase isolated from rat liver microsomes is being purified. The substrate specificity of the purified enzyme will be examined shortly with hydroxymethyl sterol substrates and various primary and secondary alcohols.

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