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Conversion of Cholest-4-en-3-one* to 5α-Cholestan-3β-ol† by Centrifugal Fractions of Rat Liver.‡ (27507)

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The double bond between carbon atoms 4 and 5 conjugated to the 3 carbonyl group of ring A is a molecular configuration essential for physiological activity of many 19-carbon and 21-carbon steroid hormones(1). These hormones are inactivated in part by 2 enzymatic reactions: A) a 5α- or 5β-hydrogenase reduces the double bond between carbon atoms 4 and 5, thus producing a saturated 3-ketosteroid; and B) a 3α- or 3β-dehydrogenase reduces the latter to a secondary steroid alcohol. The localization of these reducing enzymes in cell fractions prepared by differential centrifugation of liver homogenates has been investigated extensively(2-6).

Although the reductions of the 4-5 double bond and the 3-keto group of the 27-carbon steroid, cholest-4-en-3-one, have been studied *in vivo*(7,8), no attempt has been made to localize the enzymes responsible for these reactions in cellular compartments. Since this unsaturated 3-ketosteroid may be an intermediate in conversion of cholesterol to 5α-cholestan-3β-ol, we studied the localization of the 5α-hydrogenase and the 3β-dehydrogenase that act on cholest-4-en-3-one in rat liver cells.

Experimental. Livers of female rats weighing 200-230 g were homogenized with 2.5 volumes of 0.25 M sucrose in a Potter-Elvehjem homogenizer provided with a loose fitting Teflon pestle. The homogenate was separated into cellular debris, mitochondria, microsomes and supernatant fractions by the centrifugation procedures of Hogeboom(9). The mitochondria and the microsomes were suspended separately in 0.25 M sucrose, by gentle hand homogenization, before being incubated with the substrate, labeled cholest-4-en-3-one.

The cholest-4-en-3-one-4-C^{14} was purchased from the Nuclear-Chicago Corporation and was purified by chromatography on silicic acid(10). About 0.4 to 0.8 μc was added to each flask along with 0.55 mg of unlabeled cholest-4-en-3-one. The steroid was solubilized with Tween 20 as described in(11). To each incubation flask were added 10 ml of a centrifugal fraction containing about 90 mg of protein, which was assayed by the method of Gornall(12). The final incubation mixture, prepared with 0.025 M phosphate buffer of pH 7.4, contained 2×10^{-4} M oxidized triphosphopyridine nucleotide, 0.7×10^{-3} M potassium isocitrate, 0.6×10^{-3} M magnesium chloride, and 50 units of isocitric dehydrogenase (Sigma) in a total volume of 20 ml. Incubations were carried out with shaking for 2 hours at 37° in a nitrogen atmosphere.

The enzymatic reaction was stopped by addition of 2 volumes of ethyl alcohol containing enough KOH to provide a 15% concentration of KOH in the mixture. After 300 mg of unlabeled 5α-cholestan-3β-ol were added to each flask, the contents were gently refluxed on a steam bath for 3 hours. The

* This sterol is also known as cholestenone.

† This sterol is also known as dihydrocholesterol and 3β-cholestanol.

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TABLE I. Conversion of Cholest-4-en-3-one to 5α-cholestan-3β-ol by Centrifugal Fractions of Female Rat Liver.

Centrifugal fraction	Cholest-4-en-3-one converted to 5α-cholestan-3β-ol	Calculated sum of activities of centrifugal fractions	Diff. between observed and calculated activities
	μmoles × 10^{-3}/mg protein		%
Boiled whole homogenate	.00074		
Whole homogenate	.85		
Mitochondria	.71		
Microsomes	.94		
Supernatant	.02		
Mitochondria + microsomes	.65	.83	− 21
Supernatant + mitochondria	.52	.37	+ 42
Supernatant + microsomes	.99	.48	+108

reaction mixture was transferred to a separatory funnel with a volume of water equal to that of the reaction mixture, and the lipids were extracted 3 times with equal volumes of petroleum ether. The combined petroleum ether extracts were washed with water, dried, and were then evaporated to dryness. The sterol residue was precipitated with digitonin (13). The digitonides were washed once with 95% alcohol, once with acetone-ether mixture (1:2), and twice with ether. The digitonides were dried and split with dry pyridine. The crude sterol fraction was isolated in the usual manner and was treated with excess peroxybenzoic acid dissolved in chloroform. This converted the unsaturated sterols to epoxide derivatives. The reaction mixture was allowed to stand at room temperature for 2 hours, and was treated as described elsewhere (14). Labeled 5α-cholestan-3β-ol was isolated by chromatographic methods(14). After its recrystallization from methanol, it was dissolved in 15 ml of toluene containing 45 mg of 2,5-diphenyloxazole, and its radioactivity was assayed on a Packard scintillation spectrometer (model #314). The labeled 5α-cholestan-3β-ol isolated from the incubation mixtures was radiochemically homogeneous(14). It is unlikely that this labeled sterol contained coprosterol, for coprosterol has never been found in animal tissues.

Mitochondria were sonically disrupted in a 9 kc, 100 W Raytheon magneto-striction oscillator operated at full voltage as described by Morton(15). The first precipitate is designated P_1, the second P_2, and the final supernatant fraction, S_f.

Results. Table I shows that 5α-hydrogenase and 3β-dehydrogenase, which sequentially reduce cholest-4-en-3-one to 5α-cholestan-3β-ol, are present in the microsomal and mitochondrial fractions prepared from rat liver. Each of these centrifugal fractions was about equally effective, as was the whole homogenate, in reducing cholest-4-en-3-one to 5α-cholestan-3β-ol. The minute reduction by the supernatant fraction can probably be ascribed to its contamination by mitochondria and microsomes.

When mitochondria were combined with microsomes, the conversion of cholest-4-en-3-one to 5α-cholestan-3β-ol was 21% less than that expected from the sum of their individual activities. Addition of the supernatant fraction to the microsomes caused a marked stimulation of the reduction of cholest-4-en-3-one to 5α-cholestan-3β-ol. A less pronounced stimulation was observed upon adding the supernatant fraction to the mitochondria.

Only one-third of the reducing activity of the mitochondria remained after their disruption by sonic oscillation (Table II). Most of the remaining activity per mg of protein was associated with P_2 (148,000 × g), which was 3 to 4 times more active than the disrupted mitochondria.

Discussion. The difference in stimulation of enzymatic reduction induced by addition of the supernatant fraction to the microsomes and mitochondria may have been due to an unequal concentration of 5α-hydrogenase and 3β-dehydrogenase in the microsomes as compared with a more evenly balanced distribution of these 2 enzymes in the mitochondria. The supernatant fraction, by providing one of these enzymes (the rate limiting one), might

TABLE II. Conversion of Cholest-4-en-3-one to 5α-Cholestan-3β-ol by Centrifugal Fractions of Disrupted Rat Liver Mitochondria.

Centrifugal fraction	C^{14} of cholest-4-en-3-one converted to 5α-cholestan-3β-ol per mg of protein: % of total	Calculated sum of activities of centrifugal fractions	Difference between observed and calculated activities %
Disrupted mitochondria before centrifugation	.12		
P_1	.017		
P_2	.41		
S_f	.012		
$P_1 + S_f$	.005	.014	−64
$P_2 + S_f$	.03	.21	−86

preferentially stimulate the microsomes. Alternatively, if the enzyme systems involved in conversion of cholest-4-en-3-one to 5α-cholestan-3β-ol differ in the microsomes and mitochondria, that difference might also explain the inhibition or activation observed upon mixing the various centrifugal fractions.

Our failure to find additive reduction when mitochondria and microsomes were mixed may have been due to the presence of a 27-carbon 3β-dehydrogenase inhibitor in one of these fractions. This idea is supported by the work of Tomkins(16), who found evidence for a 21-carbon 3α-dehydrogenase inhibitor (substrate dihydrocortisone) in subcellular fractions of rat liver homogenates. Similarly, Rubin and Strecker(4) observed 19-carbon 3β-dehydrogenase inhibition in whole homogenates of rat liver.

Tomkins found a 27-carbon reductase in the microsomal fraction of rat liver(17). Our data confirm this observation and demonstrate that this enzyme is a 5α-hydrogenase. The presence of the same or a similar 27-carbon 5α-hydrogenase in the mitochondria (Table I), apparently not hitherto reported, was not unexpected because Forchielli and Dorfman(3) found that a particulate fraction, prepared by centrifugation of rat liver homogenate at 6000 × *g*, reduced 17,21-dihydroxypregn-4-ene-3,20-dione to a 5α-steroid.

Summary. The whole homogenate of female rat liver, as well as the centrifugal fraction of mitochondria and microsomes prepared from it, actively converts cholest-4-en-3-one to 5α-cholestan-3β-ol, thereby demonstrating the presence in these fractions of 5α-hydrogenase and 3β-dehydrogenase activities. One or both of these enzymes was absent in the supernatant fraction. A particulate fraction (148,000 × *g*) prepared from sonically disrupted mitochondria was more active in reducing cholest-4-en-3-one to 5α-cholestan-3β-ol than were the disrupted mitochondria.

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Effect of a Liver Extract on Hepatic Injury Produced by Carbon Tetrachloride.* (27508)

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It has recently been stated(1) that a protein-free, water soluble fraction of the liver of cattle ("Ripason")‡ can significantly modify the hepatotoxic effects of carbon tetrachloride (CCl_4). If experimental animals are treated with this extract for some time before, and also during, the period of administration of CCl_4, it is stated that sections of their livers show considerably less damage on histological examination than those of their controls not given liver extract. The liver preparation in question apparently also accelerates the disappearance of the fatty deposits in the damaged liver during the period of convalescence following cessation of injections of CCl_4(2), prevents impairment of liver function by this agent(3), and is stated to exhibit lipotropic properties in other experimental situations as well(3,4). To some extent these claims are similar to older observations in which a favorable effect of liver preparations on hepatic damage caused by CCl_4 or by deficient diets has been reported (5,6,7). The possibility that the liver may contain one or several protective principles which prevent liver damage caused by various hepatotoxic factors in other species is of great theoretical, and possibly of practical, interest and has found its clinical expression in the notion of "organotherapy". There are available in the older, as well as in the more recent literature(8,9), reports describing favorable effects produced in patients with a variety of hepatic disorders by liver extracts, including the preparation to be studied here. It was, therefore, of interest to see whether some of the claims made for this particular preparation could be confirmed experimentally. Carbon tetrachloride was chosen as the most suitable hepatotoxic agent, not only because it is still one of the most widely used poisons for this purpose, but also because it was hoped that an understanding of the reported protective effects of liver extracts against this agent might lead to a better understanding of the mode of action of CCl_4 itself.

Methods. A total of 58 Sprague-Dawley rats was used. Because of the death of some animals only 50 rats, or 25 pairs, were included in the final analysis. Twelve of these animals, all belonging to the first experiment, were females; the remainder were males. The weight of the animals at the start of experiment ranged from 42 to 152 g. This large variation in age and weight of the animals was offset by the use of littermates of the same sex, so that each treated animal was compared only to its untreated brother or sister. It was assumed that the variations in sex, age and weight of the experimental rats, controlled by the littermate arrangement adhered to throughout, would help to define the dependence of any effects to be demonstrated

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