THE ESTERIFICATION OF CHOLESTEROL BY RAT LIVER PARTICLES

Daniel Deykin and DeWitt S. Goodman

Section on Metabolism
National Heart Institute
National Institutes of Health
U. S. Public Health Service
Bethesda 14, Maryland

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Recent studies on the hydrolysis of long-chain fatty acid esters of cholesterol by rat liver (Deykin and Goodman, 1962) have indicated that the bulk of hydrolytic enzyme activity is present in the soluble fraction of the liver homogenate. The different fatty acid esters were hydrolyzed at different rates, with the relative rates of hydrolysis being: cholesteryl oleate $\geqslant$ linoleate $>$ palmitate $\geqslant$ stearate. No cofactors were required, and the system was incapable of esterifying free cholesterol.

We have now performed experiments designed to study the esterification of cholesterol with the same long-chain fatty acids. Incubations were conducted with rat liver homogenates, using 7 α-H^3-cholesterol as substrate. Reactions were terminated, and the lipids extracted, with $CHCl_3$-MeOH (2:1), and the free and esterified cholesterol separated on small columns containing 2 gm alumina (Woelm, neutral, grade II) (Deykin and Goodman, 1962). In experiments in which C^{14}-fatty acids were also employed, the cholesterol ester fraction was isolated by chromatography on silicic acid columns.

Maximum esterification of H^3-cholesterol was observed when the substrate was added to the 3 ml incubation dissolved in 100 µl acetone. In contrast to the hydrolytic activity, the esterifying enzymes were exclusively particulate. Mitochondria and microsomes were equally active, and there was no further increase in esterifying activity on addition of soluble fraction to washed cell particles.

In experiments with either washed microsomes or mitochondria, there was an absolute requirement for the addition of both ATP and coenzyme A (CoA) (see Table I). The system was strongly inhibited by the addition of potassium oleate to a final concentration of 10^{-4} M or greater. A similar inhibition of cholesterol esterification, on the addition of free fatty acid to whole liver homogenates, was observed by Swell et al. (1962), using 100-times the above concentration of oleic acid. We have found that this inhibition could be completely overcome by the addition of small quantities of fatty acid-poor human serum albumin (Goodman, 1957) to bind the free fatty acid anions. The amount of albumin employed was usually 2 to 3 mg per 0.25 µmole of fatty acid.

Similar yields of H^3-cholesterol ester were obtained whether or not exogenous fatty acid was added (see Table I). In the absence of added fatty acid, the addition of serum albumin was without effect. Direct participation of added fatty acid in the esterification could, nevertheless,

Table I

The complete system contained, in a final vol. of 3 ml: 0.4 mg microsomal protein; 300 µmole K-PO_4 buffer, pH 7.4; 3 mg serum albumin; 5 µmole ATP; 50 mµmoles CoA; 50 mµmoles K-oleate; and 20 mµmoles H^3-cholesterol added in 100 µl acetone. Incubations were for one hour at 37°.

Omission or addition	H^3-Cholesterol esterified (per cent)
None (Complete system)	9.5
No microsomes	0.0
No CoA	0.0
No ATP	0.6
No K-oleate*	9.2
No CoA, no ATP, no K-oleate; + 50 mµmoles oleyl-CoA	8.9

*From several other experiments, prorated for per cent esterification achieved by complete system.

be demonstrated by the addition of both C^{14}-oleate and H^3-cholesterol to the same incubations. These experiments employed 0.5 mg microsomal protein as enzyme. In one experiment in which there were added 20 μmoles of H^3-cholesterol and 5 μmoles of C^{14}-oleate, the purified cholesterol ester fraction, obtained after a one hour incubation, contained 14.9% of the total H^3 and 18.5% of the total C^{14} in the system. In another experiment employing 412 μmoles of H^3-cholesterol and 5 μmoles of C^{14}-oleate, the corresponding recovery of H^3 and C^{14} in the cholesterol ester fraction was 1.7% and 31.8% respectively.

From these and similar experiments using different amounts of the two substrates, estimates could be made of the size of the endogenous microsomal pools of cholesterol and fatty acids available for this reaction. These estimates involved the assumption of rapid, complete mixing of the endogenous and exogenous pools of each substrate. The estimates for the pool size of both cholesterol and fatty acid were approximately 25 to 35 μmoles of each per mg of washed microsomal protein. These values were in close agreement with analytic measurements of the amounts of cholesterol and free fatty acids in the same microsome preparations. Subsequent experiments have indicated that equilibration of exogenous and endogenous cholesterol is complete within two minutes; the degree and rate of fatty acid mixing is not accurately known. It is probable that the endogenous fatty acid pool is sufficiently large to permit maximum rate of cholesterol esterification to occur, and that exogenous increments of fatty acid provide no further stimulation.

In view of the absolute requirement for ATP, a requirement for Mg^{++} was also expected. However, repeated experiments in which Mg^{++} was omitted showed full activity, and microsomes washed in a buffer solution containing 10^{-3} M-EDTA were equally active. The addition of glutathione was inhibitory, perhaps by providing a competing substrate for thiotransacetylase (Stern and Ochoa, 1956).

The dependence of the reaction on ATP and CoA suggested that the reaction proceeded through the formation of a fatty acyl-CoA intermediate, which in turn reacted with free cholesterol, as suggested by Mukherjee et al. (1958). Formal proof for this mechanism was obtained by demonstrating that long-chain fatty acyl-CoA esters, synthesized by the method of Goldman and Vagelos (1961), could completely replace ATP and CoA in the incubations. The data in Table I illustrate one such experiment.

The dependence of this reaction on the concentration of added oleyl-CoA is illustrated by the experiment shown in Table II.

Using oleyl-CoA in incubations similar to those of Table II, the reaction was found to have a rather broad pH optimum range between 7 and 8. The reaction rate was constant for the first 20 minutes, with less than 30% deviation from linearity after one hour.

Table II

Each one hour incubation contained microsomes, buffer, and serum albumin, in a total volume of 3 ml, as described in Table I. Fifty mμmoles of H^3-cholesterol were added in 100 μl acetone, the flasks incubated at 37° for two minutes, and the reactions begun by the addition of oleyl-CoA in amounts listed below.

Oleyl-CoA added (mμmoles)	H^3-Cholesterol esterified (per cent)
5	4.1
15	13.0
30	15.3
60	21.1

Preliminary experiments have been conducted comparing reaction rates, using palmitoyl-, stearoyl-, oleyl-, and linoleyl-CoA. Concentration curves for each fatty acyl-CoA ester have been constructed for each of two levels

of added cholesterol. The relative rates of reaction observed with the derivatives were oleyl > palmitoyl > stearoyl-CoA. The results with linoleyl-CoA were uncertain because of the possibility that the compound was oxidized during its chemical synthesis.

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