The metabolism of the fluorescent probe cholesta-5,7,9(11)-trien-3 β -ol by rat liver

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The chemical synthesis and metabolism of [3 α -³H]cholesta-5,7,9(11)-trien-3 β -ol is described. This radioactive fluorescent sterol probe cannot be rearranged or converted to cholesterol by rat liver but is readily esterified.

Cholesta-5,7,9(11)-trien-3 β -ol is a non-perturbing fluorescent analogue of cholesterol which we have used to investigate the organization of sterols in lipid bilayers (Rogers *et al.*, 1979). Although this fluorescent sterol is readily incorporated into the cell membranes of the protozoan, *Tetrahymena pyriformis* (Rogers *et al.*, 1979), attempts to incorporate the probe into MRC-5 fibroblasts in culture resulted in eventual cell death (Shakespeare & Wilton, 1980). This toxicity has been ascribed to the fact that the fluorescent triene or its possible metabolites are potent inhibitors of cholesterol biosynthesis (Shakespeare & Wilton, 1980). In separate studies we have demonstrated the capacity of liver systems to convert sterols containing a $\Delta^{7,9(11)}$ -diene system into cholesterol (Akhtar *et al.*, 1972; Tavares *et al.*, 1977) and therefore it became important to investigate the possible conversion of the $\Delta^{5,7,9(11)}$ -triene system into cholesterol or other sterol metabolites. By analogy to the pathway for cholesta-7,9(11)-dien-3 β -ol metabolism (Tavares *et al.*, 1977) a possible metabolic route for the fluorescent triene would be:

An investigation of the possible metabolism of the fluorescent probe is also relevant in view of its recent use for labelling of membranes and lipoproteins *in vivo* (Bergeron & Scott, 1982).

In this paper I describe the synthesis of [3 α -³H]cholesta-5,7,9(11)-trien-3 β -ol and its metabolism by rat liver systems.

Materials and methods

The preparation of the 10000 g_{av} supernatant (S_{10}) from rat liver, incubation and isolation procedures have been described previously (Tavares *et al.*, 1977).

Preparation of [3 α -³H]cholesta-5,7,9(11)-trien-3 β -ol

Cholesta-4,7-dien-3-one (1g) (Shepherd *et al.*, 1955) was dissolved in pyridine (2.5 ml) and acetic

anhydride (2.5 ml) and refluxed for 3 h. After addition of diethyl ether (25 ml) the solution was washed four times with cold HCl (0.1 M) and once with saturated NaHCO₃. After evaporation to dryness the residue was dissolved in petroleum spirit (b.p. 40–60°C), applied to a silica gel column (20 cm $\times$ 2 cm) and eluted with petroleum spirit (b.p. 40–60°C)/diethyl ether (19:1, v/v). Cholesta-3,5,7-trienol acetate was eluted in the first 100 ml and was recrystallized to yield material (300 mg) having the characteristic λ_{max} at 331, 316 and 303 nm of this enol acetate. To the enol acetate (30 mg) in propan-2-ol (30 ml) and water (0.3 ml) was added NaB³H₄ (~2 mg; 2 mCi/mg) (Holick *et al.*, 1977). After refluxing (1 h) under N₂ excess NaBH₄ was added (10 mg) and refluxing was continued for 30 min. The products were extracted into diethyl ether, purified by preparative t.l.c. developed in petroleum spirit (b.p. 60–80°C)/acetone (3:1, v/v) and the more polar 3 β isomer [3 α -³H]cholesta-5,7-dien-3 β -ol was eluted. This material was acetylated overnight in pyridine (1 ml) and acetic anhydride (0.1 ml) at room temperature and after work-up was purified by t.l.c. on silica gel containing 10% (w/w) AgNO₃ developed in petroleum spirit (b.p. 60–80°C)/benzene (1:1, v/v). The [3 α -³H]cholesta-5,7-dien-3 β -ol acetate band was eluted (1 $\times$ 10⁹ d.p.m.). To one-half of this material was added non-radioactive cholesta-5,7-dien-3 β -ol acetate (50 mg) and the mixture was dissolved in chloroform (1 ml). Acetic acid (2 ml) containing mercuric acetate (100 mg) was added and the mixture was stirred in the dark at room temperature for 24 h. After work-up the [3 α -³H]cholesta-5,7,9(11)-trien-3 β -ol acetate was purified as above by argentation t.l.c. and after elution was hydrolysed by excess LiAlH₄ in dry diethyl ether to give [3 α -³H]cholesta-5,7,9(11)-trien-3 β -ol (10 mg; 1 $\times$ 10⁷ d.p.m./mg). This material had the characteristic λ_{max} at 325 nm and no absorption at 282 nm due to cholesta-5,7-dien-3 β -ol. The triene was stored in

Table 1. *Metabolism of cholesta-5,7,9(11)-trien-3 β -ol by a post-mitochondrial supernatant from rat liver*

For radioactive incubations [3α - ^3H]cholesta-5,7,9(11)-trien-3 β -ol (50 μg ; 5×10^5 d.p.m.) or [3α - ^3H]cholesta-7,9-dien-3 β -ol (50 μg ; 1×10^5 d.p.m.) were incubated aerobically with a 10000 g_{av} supernatant of rat liver homogenate (4 ml) for 90 min. Cholesterol was analysed by the dibromide method. Sterol esters and polar metabolites were separated by t.l.c. and after detection using a radiochromatogram scanner were eluted and the radioactivity was measured. The radioactivity in the aqueous phase of an incubation was measured after exhaustive solvent extraction to remove sterols.

Substrate incubated	Product analysed for radioactivity	Radioactivity in product (%)
[3α - ^3H]Cholesta-7,9-dien-3 β -ol	Cholesterol dibromide	61
[3α - ^3H]Cholesta-5,7,9-trien-3 β -ol	Cholesterol dibromide	0
	Sterol esters from t.l.c.	24
	Polar material from t.l.c.	19
	C ₂₇ sterols from t.l.c.	57*
	Aqueous phase after extraction	<5

* Shown by subsequent argentation t.l.c. and u.v. analysis to be cholesta-5,7,9(11)-trien-3 β -ol.

liquid N₂ until required. The preparation of [3α - ^3H]cholesta-7,9(11)-dien-3 β -ol has been described previously (Rahimtula *et al.*, 1969).

Results

In order to maximize the possibility of metabolism of the fluorescent triene, incubations were performed with the post-mitochondrial supernatant of rat liver (S₁₀) under aerobic conditions. A number of parallel incubations were performed and the results are summarized in Table 1. The S₁₀ was unable to convert the fluorescent triene into cholesterol under conditions in which the related $\Delta^{7,9(11)}$ -diene was transformed in over 60% yield. However, there was considerable conversion of the fluorescent triene into less polar material of similar R_F to sterol esters on t.l.c., and this is consistent with the ability of the S₁₀ system from rat liver to esterify a wide variety of added sterols (Brady & Gaylor, 1971). Hydrolysis of this radioactive non-polar material with LiAlH₄ gave on t.l.c. a single radioactive peak corresponding to the fluorescent triene alcohol.

When incubations were saponified with methanolic KOH before extraction a single radioactive peak corresponding to the fluorescent triene starting material was obtained on t.l.c. When this radioactive material was acetylated and separated by argentation t.l.c. a single major peak of radioactivity was obtained corresponding to the fluorescent triene (R_F 0.3) which was well separated from cholesterol (R_F 0.4) and cholesta-5,7-dien-3 β -ol (R_F 0.2) and had the correct u.v. absorption spectrum. A minor broad band (R_F 0–0.1) was also noted but this must represent chemical decomposition products of the triene because cholesta-5,7-dien-3 β -ol is the most

polar of the possible monohydroxy C₂₇ sterol acetates in this chromatographic system. Radioactive polar material (R_F 0–0.2) was also noted after t.l.c. of initial incubation extracts and, under the conditions of the incubation described in Table 1, this material represented up to about 20% of the extracted radioactivity. Again this material probably represents chemical decomposition products due to the relative instability of the fluorescent triene (Rogers *et al.*, 1979) and was also obtained with inactivated S₁₀ preparations.

A potential problem when studying the metabolism of 3 β -hydroxysterols which are labelled with hydrogen at the 3 α position is the possible lability of this hydrogen as the result of 3 β -hydroxysterol dehydrogenase activity. Since labilization of this radioactive hydrogen might obscure potential metabolism of the sterol, in one experiment the percentage of radioactivity that remained in the aqueous phase at the end of an incubation after solvent extraction was determined. This radioactivity was less than 5% of that incubated and hence there was no significant labilization of the 3 α -hydrogen of the fluorescent triene.

Discussion

The results described above demonstrate that the fluorescent sterol cholesta-5,7,9(11)-trien-3 β -ol is not susceptible to any nuclear transformation catalysed by the S₁₀ system from rat liver. However, a large proportion of the triene is esterified under the conditions of the incubation. Some polar material was produced during the incubations but this material was also present after incubations with an inactivated S₁₀ and reflects the relative lability of

this particular sterol triene (Rogers *et al.*, 1979). It would therefore appear that formation of this polar material is a possible explanation for the effectiveness of the triene as an inhibitor of 3-hydroxy-3-methylglutaryl-CoA reductase and the subsequent cell death in MRC-5 fibroblasts (Shakespeare & Wilton, 1980). It is noteworthy that the fluorescent triene is readily incorporated into the membranes of the protozoan, *Tetrahymena pyriformis*, *in vivo* without any effects on growth (Rogers *et al.*, 1979). However, we have observed that this organism is unaffected by oxygenated sterols that are inhibitory in mammalian systems (J. Rogers, M. Wakeel & D. C. Wilton, unpublished observations).

The results described above clearly demonstrate that the enzyme system in rat liver that isomerizes the $\Delta^{7,9(11)}$ -diene system is unable to do so in the presence of a further double bond at the Δ^5 -position. We have previously shown that this isomerization also cannot occur in the presence of two methyl groups at C-4 (Tavares *et al.*, 1977) and indicates that this unusual enzymic reaction that is present in rat liver shows a high degree of specificity. Why this enzymic activity is required in the liver is not fully understood at this stage.

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