Short Communications

Studies on the Control of Cholesterol Biosynthesis: The Adenosine 3':5'-Cyclic Monophosphate-Dependent Accumulation of a Steroid Carboxylic Acid

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Present evidence suggests that the effects of glucagon and adrenaline on hepatic gluconeogenesis are mediated by the intracellular participation of cyclic AMP (adenosine 3':5'-cyclic monophosphate) (Robison, Butcher & Sutherland, 1968). Studies on the effect of cyclic AMP on the metabolism of rat liver slices showed that cyclic AMP inhibited the synthesis of proteins, sterols and fatty acids in a co-ordinated fashion (Akhtar & Bloxham, 1970). It was implied that this effect might contribute to the control of hepatic gluconeogenesis. The present work shows that, in a cell-free system, cyclic AMP inhibits cholesterol biosynthesis from several precursors. This inhibition is accompanied by the accumulation of a new polar intermediate, compound A, which is tentatively formulated as structure (IV).

(3*R*)-[2-³H₂]Mevalonic acid (1.25 μ Ci, 12.5 nmol) was incubated for 15 min at 38°C with 1 ml of a 10000*g* supernatant of rat liver homogenate (Wilton, Munday, Skinner & Akhtar, 1968) supplemented with glucose 6-phosphate (5 μ mol), ATP (2.5 μ mol) and NADP (1.25 μ mol) in the presence of various concentrations of cyclic AMP. After addition of 2 ml of methanolic 20% (w/v) KOH followed by heating at 80°C for 2 min, the sterols were extracted with 5 $\times$ 3 ml of light petroleum (b.p. 60–80°C)–ether (1:1, v/v).

A portion of the organic extract was analysed for the incorporation of radioactivity into cholesterol (VI) (Akhtar, Rahimtula, Watkinson, Wilton & Munday, 1969*a*). Fig. 1 (curve *a*) shows a dose-dependent inhibition of cholesterol biosynthesis by cyclic AMP. The remaining organic extract was analysed by t.l.c. This revealed (Fig. 1, curve *b*) the progressive accumulation of a polar compound, A (*R_F* 0, solvent 1).

When radioactive compound A obtained by this technique was incubated with a 10000*g* supernatant of rat liver in the absence of cyclic AMP, then there was a 55.2% radiochemical conversion of compound A into cholesterol. This suggests that compound A is a genuine intermediate in cholesterol biosynthesis.

The inhibition of cholesterol biosynthesis in the presence of cyclic AMP was also observed when labelled acetate, squalene (I) or lanosterol (II) was used as precursor. However, cyclic AMP did not inhibit the conversion of cholest-7-en-3 β -ol into cholesterol. This suggests that cyclic AMP acts by inhibiting a step in the conversion of lanosterol into cholest-7-en-3 β -ol.

The following evidence suggested that compound A isolated as described above was the potassium salt of a hydroxy carboxylic acid. On acidification with formic acid the *R_F* of compound A was altered from 0 to 0.2 in solvent 1. The acid form of compound A on treatment with diazomethane gave the corresponding methyl ester (*R_F* 0.5, solvent 1; *R_F* 0.3,

Fig. 1. Inhibition of cholesterol biosynthesis (*a*, ○) and the accumulation of compound A (*b*, △) in the presence of cyclic AMP. A portion (1 ml) of 10000*g* supernatant from rat liver was incubated with (3*R*)-[(2*S*)-2-³H₁, 2-¹⁴C]-mevalonate (1.25 μ Ci, 12.5 nmol) in the presence of various concentrations of cyclic AMP. The incorporation into cholesterol was measured by the dibromide method (Akhtar *et al.* 1969*a*) and the incorporation into compound A was analysed by t.l.c. as described in the text.

Scheme 1. Location of labelled atoms in steroids derived from (3*R*)-[(2*S*)-2- $^3\text{H}_1$, 2- ^{14}C]mevalonate. The $^3\text{H}/^{14}\text{C}$ ratios shown are the theoretical values. ^{14}C atoms are represented by $\bullet$ and ^3H atoms by T.

solvent 2), which yielded a new compound on acetylation with acetic anhydride in pyridine (R_F 0.6, solvent 2). The cumulative biosynthetic and chemical evidence suggests that compound A corresponded to one of three possible structures, i.e. (III), (IV) or (V).

Further investigations involved the conversion of (3*R*)-[(2*S*)-2- $^3\text{H}_1$, 2- ^{14}C]mevalonate ($^3\text{H}/^{14}\text{C}$ ratio, 34; 23.4 μCi of $^3\text{H}/\mu\text{mol}$) into squalene, cholesterol and compound A. These compounds were found to have $^3\text{H}/^{14}\text{C}$ ratios relative to mevalonate of 0.91, 0.57 and 0.66 respectively.

The theoretical $^3\text{H}/^{14}\text{C}$ ratios of various key intermediates in Scheme 1 may be deduced from the labelling pattern of lanosterol and squalene biosynthesized from (3*R*)-[(2*S*)-2- $^3\text{H}_1$, 2- ^{14}C]mevalonate (Cornforth, Cornforth, Popják & Yengoyan, 1966). Prediction of the $^3\text{H}/^{14}\text{C}$ ratios of compounds (III), (IV) and (V) requires the following additional information. (a) The demethylation at C-14 is accompanied by the loss of 15 α -hydrogen atom (Akhtar *et al.* 1969*a*; Canonica *et al.* 1968*a*; Gibbons, Goad & Goodwin, 1968*b*). (b) The 2-methylene group of mevalonate is exclusively incorporated into the 4 α -position of lanosterol (Moss & Nicolaidis, 1969; Stone, Roeske, Clayton & van Tamelen, 1969). Thus the formation of a 4 α -carboxylic acid will result in the loss of the tritium atom at this position.

By using the $^3\text{H}/^{14}\text{C}$ ratios shown in Scheme 1, the experimentally determined $^3\text{H}/^{14}\text{C}$ ratio of 0.66 for compound A is in close agreement with the theoretical $^3\text{H}/^{14}\text{C}$ ratio of 0.67 for structure (IV). This experiment does not elucidate the saturation status of the side chain; however, structures containing 7,8-double bonds are eliminated, since the formation of the 7,8-double bond in cholesterol biosynthesis involves the additional loss of the 7 β -hydrogen atom (Akhtar & Rahimtula, 1968; Akhtar *et al.* 1969*a*; Gibbons, Goad & Goodwin, 1968*a*; Canonica *et al.* 1968*b*; Caspi, Greig, Ramm & Varma, 1968). Further, it should be noted that on the basis of the above evidence it is not possible to distinguish between structures (IV) and (V), since their $^3\text{H}/^{14}\text{C}$ ratios are fairly close.

The choice between structures (IV) and (V) was approached by an alternative technique involving the assumption that decarboxylation at C-4 involves the oxidation of the 3 β -hydroxyl group and in this process the 3 α -hydrogen atom is eliminated (Lindberg, Gautschi & Bloch, 1963; Swindell & Gaylor, 1968). Thus in the conversion of compound (IV) into compound (V) the 3 α -hydrogen atom will be labilized and this feature makes it possible to distinguish between these two structures.

[3 α - ^3H , 26, 27- ^{14}C]Lanosterol ($^3\text{H}/^{14}\text{C}$ ratio, 11.9; 6.8×10^5 c.p.m. of $^3\text{H}/\mu\text{mol}$) was incubated with a 10000*g* supernatant from rat liver in the absence

of cyclic AMP. The biosynthetic cholesterol had a $^3\text{H}/^{14}\text{C}$ ratio of 0.04, indicating the complete loss of the 3 α -tritium atom. The same sample of lanosterol, when incubated in the presence of cyclic AMP, directed the accumulation of a carboxylic acid chromatographically identical with compound A derived from mevalonate. The compound had a $^3\text{H}/^{14}\text{C}$ ratio of 12.70, which remained unchanged when the compound was treated with diazomethane and the resultant methyl ester was purified by t.l.c. The methyl ester (R_F 0.3, solvent 2) on oxidation with Jones's reagent (Bowden, Heilbron, Jones & Weedon, 1946) gave a less polar band (R_F 0.6, solvent 2), which had lost 82% of the tritium. This shows that, in the formation of the carboxylic acid from lanosterol, the 3 α -hydrogen atom remains intact. Thus, if the same compound accumulates from either mevalonate or lanosterol, then the carbocyclic skeleton of compound A may be tentatively formulated as structure (IV).

We have shown that in a cell-free system cyclic AMP inhibits the synthesis of cholesterol at the stage of a carboxylic acid of the type (IV). At present, the significance of this observation to the situation *in vivo* remains unestablished.

The formation of 4 α -carboxylic acid derivatives during cholesterol biosynthesis has been demonstrated by two other groups using a different approach from the present work (Miller & Gaylor, 1970*a,b*; Hornby & Boyd, 1970).

Materials and methods. Racemic mixtures containing (3*R*)-[2- $^3\text{H}_2$]mevalonate, (3*R*)-[(2*S*)-2- $^3\text{H}_1$]mevalonate and (3*R*)-[2- ^{14}C]mevalonate were obtained from The Radiochemical Centre (Amersham, Bucks., U.K.). [3 α - ^3H]Lanosterol was prepared by the reduction of lanost-8-en-3-one with NaB^3H_4 (3 mCi/mg). [26, 27- ^{14}C]Lanosterol was prepared by the method of Akhtar, Hunt & Parvez (1967). Cyclic AMP was obtained from Sigma (London) Chemical Co. Ltd. (London S.W.6, U.K.). T.l.c. was performed on Kieselgel GF₂₅₄ with light petroleum (b.p. 60–80°C)–acetone (14:3, v/v; solvent 1) or chloroform–toluene (3:1, v/v; solvent 2) as solvent systems. Squalene (R_F 0.95, solvent 1) was purified via formation of the thiourea clathrate. All radioactivity measurements were made on an Intertechnique ABAC SL40 liquid-scintillation spectrophotometer with automatic quench correction (Akhtar, Rahimtula & Wilton, 1969*b*).

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