Short Communications

Inhibition of Cholesterol Biosynthesis by Carbon Monoxide: Accumulation of Lanosterol and 24,25-Dihydrolanosterol

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It has been shown (Wada *et al.*, 1969) that CO inhibits the conversion of acyclic precursors into digitonin-precipitable sterols in 10000g supernatant fractions of rat liver homogenates. Although no direct experimental attempt was made to characterize the biosynthetic site of inhibition, this was attributed to an effect on the microsomal enzyme squalene 2,3-oxidase. More recent work, however, has demonstrated that squalene 2,3-oxidase is not inhibited by CO (Yamamoto & Bloch, 1969). The present investigation was undertaken to clarify this apparent contradiction by establishing the nature of the intermediates that accumulate during CO inhibition of cholesterol biosynthesis from selected precursors.

Cholesterol was a product of BDH Chemicals (Poole, Dorset, U.K.) and was recrystallized three times before use (m.p. 148–148.5°C). Lanosterol was obtained from Aldrich Chemical Co. (Milwaukee, Wis., U.S.A.) and contained 40% of 24,25-dihydrolanosterol as judged by g.l.c. Pure samples of each were obtained by the method described by Gibbons *et al.* (1971). [2-¹⁴C]Mevalonic acid lactone (10.3 Ci/mol) was a product of The Radiochemical Centre (Amersham, Bucks., U.K.) and was hydrolysed to the free acid before use. [¹⁴C]Squalene was prepared by incubation of a 10000g supernatant fraction of rat liver homogenate with [2-¹⁴C]mevalonate by the method described by Bucher *et al.* (1959). [¹⁴C]Squalene was isolated from the lipid fraction by t.l.c. on plates of silica gel H developed with chloroform. It was purified by further t.l.c. with hexane as the developing solvent. [¹⁴C]Lanosterol was prepared by the same method except that the incubation was carried out for 1 h under an atmosphere of CO+O₂ (see Table 1). These conditions provide a novel method for the biosynthesis of [¹⁴C]lanosterol of high specific radioactivity. [¹⁴C]Lanosterol was isolated from the total lipid fraction and purified as described by Gibbons *et al.* (1971). The specific radioactivity was 2.18×10^5 d.p.m./μg. Confirmation of the identity of this material with lanosterol is dealt with below. All 10000g supernatant fractions (S₁₀) of rat liver homogenates were prepared in the

homogenizing medium of Bucher *et al.* (1959). Male rats weighing 125–150 g, killed between 10:00 and 12:00 hours, were used for all experiments. The incubation conditions are given in Table 1. The total lipid fraction was extracted with chloroform-methanol (2:1, v/v), and the fractions containing squalene, lanosterol, 4α-methyl sterol and cholesterol were isolated by t.l.c. with chloroform as solvent after the addition of a mixture of carrier lanosterol and dihydrolanosterol (400 μg). Acetylation of steroids was carried out with pyridine-acetic anhydride (1:2, v/v).

The results of a typical experiment with [2-¹⁴C]-mevalonate as the cholesterol precursor are presented in Table 1. The amount of radioactivity incorporated into the 4-demethyl sterol fraction under a gas phase of CO+O₂ (90:10) was only 34.6% of that incorporated under N₂+O₂ (90:10). In the presence of the CO+O₂ mixture there was a sevenfold increase in the accumulation of radioactive 4,4-dimethyl sterols. Similar results were also obtained when NAD⁺, NADH, NADPH and ATP were used in place of NAD⁺ and fructose 1,6-diphosphate as cofactors. In another, similar, experiment the accumulated 4,4-dimethyl sterols were identified as lanosterol and 24,25-dihydrolanosterol by the following criteria. Acetylation, followed by t.l.c. on AgNO₃-impregnated silica gel, revealed only two major radioactive bands, which had the same mobilities as lanosteryl acetate and dihydrolanosteryl acetate. The separated acetates showed no significant decrease in specific radioactivity during four recrystallizations after the addition of non-radioactive material. The specific radioactivities of the alcohols obtained by hydrolysis of the respective acetates also remained virtually constant during three recrystallizations. Preparative g.l.c. of each 4,4-dimethyl sterol acetate fraction showed, in each case, only one radioactive peak, which coincided exactly with the peak of mass for lanosteryl acetate or dihydrolanosteryl acetate respectively.

The radioactive 4-demethyl sterol formed in relatively small amounts during CO inhibition was

Table 1. *Effect of CO on the incorporation of radioactivity from [2-¹⁴C]mevalonate, [¹⁴C]squalene and [¹⁴C]lanosterol into cholesterol by a rat liver S₁₀ fraction*

In experiments with [2-¹⁴C]mevalonate the reaction mixture contained, in a final volume of 2.5 ml, 2 ml of S₁₀ fraction, 1.6 mM-NAD⁺, 16 mM-fructose 1,6-diphosphate and 1 μ Ci of [2-¹⁴C]mevalonate. Incubations were done in triplicate and each result is an average of the three. When the lipid substrates were used the incubation mixture contained 2.0 ml of S₁₀ fraction, 0.8 mM-NAD⁺, 1.3 mM-NADH, 0.45 mM-NADPH, 1.3 mM-ATP and [¹⁴C]squalene (6.08×10^5 d.p.m.) or [¹⁴C]lanosterol (2.61×10^5 d.p.m.) in a total volume of 2.5 ml. Incubations were performed in duplicate and each result represents the average value. All flasks were sealed with rubber septa and the gas mixture (150 ml) was bubbled through the incubation mixture. The reaction was started by injection of the radioactive precursor and the incubation proceeded for 1 h at 37°C. The reaction was stopped by the addition of chloroform-methanol. The precipitated protein was filtered off and the organic phase washed with water. After evaporation of the solvent, the lipids were separated by t.l.c.

Substrate	T.l.c. fraction	Radioactivity incorporated (d.p.m.)	
		Under N ₂ +O ₂ (90:10)	Under CO+O ₂ (90:10)
[2- ¹⁴ C]Mevalonate	Squalene	44700	39500
	4,4-Dimethyl sterols	39200	267700
	4-Demethyl sterols	205000	71000
[¹⁴ C]Squalene	Squalene	165700	117100
	4,4-Dimethyl sterols	44200	146800
	4 α -Methyl sterols	12500	4700
	4-Demethyl sterols	32600	15400
[¹⁴ C]Lanosterol	4,4-Dimethyl sterols	25000	128000
	4 α -Methyl sterols	13900	8600
	4-Demethyl sterols	82800	46000

shown to consist entirely of cholesterol by t.l.c. of the acetylated material on AgNO₃-impregnated silica gel. Another portion of the 4-demethyl sterol fraction when subjected to preparative g.l.c. gave one radioactive peak with the same retention time as cholesterol. The squalene fractions from both types of incubations were subjected to t.l.c. with hexane. In each case only one radioactive band appeared, which had the same mobility as authentic squalene.

S₁₀ fractions were incubated with [2-¹⁴C]mevalonate under CO+O₂ for various times and the rates of incorporation of radioactivity into squalene, 4,4-dimethyl sterols and cholesterol were measured (Fig. 1). Parallel incubations under atmospheres of N₂+O₂ were used as controls. In the CO-inhibited incubations the accumulation of radioactive 4,4-dimethyl sterols was maximal after 60 min and the appearance of radioactive cholesterol was slower than in the controls. Again, in the controls there was a decrease in the amount of radioactive 4,4-dimethyl sterols over the whole time-period studied and maximal accumulation was reached after 45 min. The rates of [¹⁴C]squalene turnover appeared to be the same in both cases.

The effect of CO on the incorporation of radioactivity from [¹⁴C]squalene and [¹⁴C]lanosterol into cholesterol was also studied. The incubation con-

ditions and results are given in Table 1. When [¹⁴C]squalene was incubated under the CO+O₂ atmosphere, the amount of radioactivity incorporated into cholesterol was less than 50% of that in the control, and a threefold increase in the accumulation of ¹⁴C-labelled 4,4-dimethyl sterol was observed. With [¹⁴C]lanosterol as the cholesterol precursor the amount of [¹⁴C]cholesterol was decreased by approximately 50% and the amount of unchanged [¹⁴C]lanosterol was much greater. With both substrates the amount of radioactive 4 α -methyl sterols was much less in the CO-inhibited incubations.

The present results clearly show that cholesterol biosynthesis from [2-¹⁴C]mevalonate, [¹⁴C]squalene and [¹⁴C]lanosterol is inhibited in the presence of CO. Squalene 2,3-oxidase was not affected. This was evidenced by the observation that the kinetics of [¹⁴C]squalene turnover during cholesterol biosynthesis were the same irrespective of whether CO+O₂ or N₂+O₂ was used as the gas phase. The fact that the conversion of [¹⁴C]lanosterol into cholesterol was diminished in the presence of CO substantiates the belief that CO exerts an effect at a point beyond that of squalene 2,3-oxidase in the biosynthetic sequence. The observation that lanosterol and its 24,25-dihydro derivative accumulate exclusively

Fig. 1. Effect of incubation time on the incorporation of radioactivity from [2-¹⁴C]mevalonate into squalene and sterols

Incubations were carried out, as indicated in Table 1 legend and the text, under (a) N₂+O₂ (90:10) and (b) CO+O₂ (90:10). The incorporation of radioactivity into squalene (□), lanosterol (○) and cholesterol (Δ) is shown.

during cholesterol biosynthesis from [2-¹⁴C]mevalonate indicates that CO influences an early step in the oxidative elimination of the 14 α -methyl group of lanosterol. The exact nature of the intermediates involved and the sequence in which they participate in this process, however, remain matters of controversy. Until these biosynthetic problems are resolved more completely, it is difficult to ascertain the exact biosynthetic site of inhibition by CO. From our present knowledge of the effect of CO on biological hydroxylations it is reasonable to assume that, in the present case, it influences one or more steroidal hydroxylations during which the 14 α -methyl group of lanosterol is ultimately removed. If

this is the case, then cytochrome *P*-450 is probably involved and the use of CO as a specific inhibitor could be another tool for the elucidation of the sequence of steps beyond lanosterol in cholesterol biosynthesis.

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