EFFECT OF TRIARIMOL ON CHOLESTEROL BIOSYNTHESIS IN RAT-LIVER SUBCELLULAR FRACTIONS

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SUMMARY

Cholesterol biosynthesis was studied in rat-liver subcellular fractions incubated with DL-[2-^{14}C]mevalonic acid in the presence and absence of triarimol (α-(2,4-dichlorophenyl)-α-phenyl-5-pyrimidine methanol). Triarimol strongly inhibits incorporation of radioactivity into cholesterol and this results in a large accumulation of radioactive lanosterol and 24,25-dihydrolanosterol. The inhibition of lanosterol 14α-demethylase by triarimol was confirmed by assay of the enzyme in rat-liver microsomal fraction in the presence and absence of the inhibitor. Apart from a slight inhibition of Δ^7-sterol-Δ^5-dehydrogenase, triarimol did not affect the activity of any other enzyme involved in cholesterol biosynthesis from acetate.

The fungitoxicity of triarimol (α-(2,4-dichlorophenyl)-α-phenyl-5-pyrimidine methanol)(1-4) has been ascribed to its inhibition of ergosterol synthesis (5) and *in vivo* studies with *Ustilago maydis* have shown that triarimol inhibits the removal of the 14α-methyl group of lanosterol and its metabolites.

Studies in this laboratory have been concerned with the mechanism of 14α-demethylation during cholesterol biosynthesis by rat-liver enzymes *in vitro* (6-8) and in view of the close similarity between this process and that leading to the formation of ergosterol in fungi (9,10), the present study was undertaken for the purpose of investigating the effect of triarimol on various steps during cholesterol biosynthesis by rat-liver subcellular fractions.

Systematically, lanosterol is 5α-lanosta-8,24-dien-3β-ol; 24,25-dihydrolanosterol is 5α-lanost-8-en-3β-ol; 14-demethyllanosterol is 4,4-dimethyl-5α-cholesta-8,24-dien-3β-ol; cholesterol is cholest-5-en-3β-ol; cholest-7-enol is 5α-cholest-7-en-3β-ol; and ergosterol is ergosta-5,7,22-trien-3β-ol.

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MATERIALS AND METHODS

Cell-free fractions were prepared according to methods described previously (6,7) except that the livers were homogenized either in 4 volumes of 0.1 M potassium phosphate buffer, pH 7.4, containing nicotinamide (30 mM), EDTA (1 mM) and GSH (10 mM) for the preparation of the S_{10} fraction, or in 4 volumes of the same buffer containing nicotinamide (30 mM) and EDTA (1 mM) only for the preparation of microsomes. Incubations were started by the addition of either [2-^{14}C]mevalonic acid (2 μCi/incubation; 7.10 μCi/μmol) or [2-^{14}C]acetate (10 μCi/incubation; 58 μCi/μmol) as a solution in potassium phosphate buffer (pH 7.4) and were conducted for 1 hour, at 37° C, with air as the gas phase.

Incubations were terminated by the addition of 20 volumes of a mixture of chloroform and methanol (2:1, v/v). Isolation of the total lipid fraction, separation of the 4,4-dimethyl sterols (lanosterol and companion C_{30} and C_{29} sterols) from the 4-demethyl sterols (cholesterol and companion C_{27} sterols) and squalene by t.l.c. on silicagel H using chloroform as the moving phase (system 1), acetylation of sterols, analytical g.l.c. and measurement of radioactivity have been described previously (6,11). After the addition of non-radioactive cholest-7-enol (200 μg), labelled cholesterol and cholest-7-enol were isolated individually as the acetates from the 4-demethyl sterol fraction by argentation chromatography (12,13). Radioactive lanosterol and 24,25-dihydrolanosterol were also isolated as the acetate derivatives from the 4,4-dimethyl sterol fraction (after the addition of non-radioactive carrier materials) by thin-layer argentation chromatography (6,12). Sterols were crystallized with a mixture of chloroform and methanol as solvent.

Cholest-7-enol was prepared by catalytic hydrogenation of cholesta-5,7-dien-3β-ol (14). Lanosterol and dihydrolanosterol were prepared as previously described (6).

HMG-CoA reductase activity was assayed by a modification of the radioisotope incorporation method described elsewhere (15). [^{14}C]-HMG-CoA for this assay was prepared as published elsewhere (16). The activity of lanosterol 14α-demethylase was assayed as described previously (17).

RESULTS

Inhibition of cholesterol biosynthesis in the presence of triarimol

An S_{10} fraction of rat-liver homogenate was incubated with [2-^{14}C]-mevalonic acid in the presence of various concentrations of triarimol. The lipid extract from each incubation was chromatographed and the 4-demethyl and 4,4-dimethyl sterol and the squalene fractions were isolated and assayed for radioactivity. Fig. 1A shows the distribution of radioactivity in the three fractions at various concentrations of the inhibitor. The total amount of radioactivity incorporated into the three fractions was similar in all incubations. However, with increasing concentration of triarimol there was a decrease in the amount of radioactivity that appeared in the 4-demethyl fraction concomitant with a comparable increase of radioactivity in the 4,4-

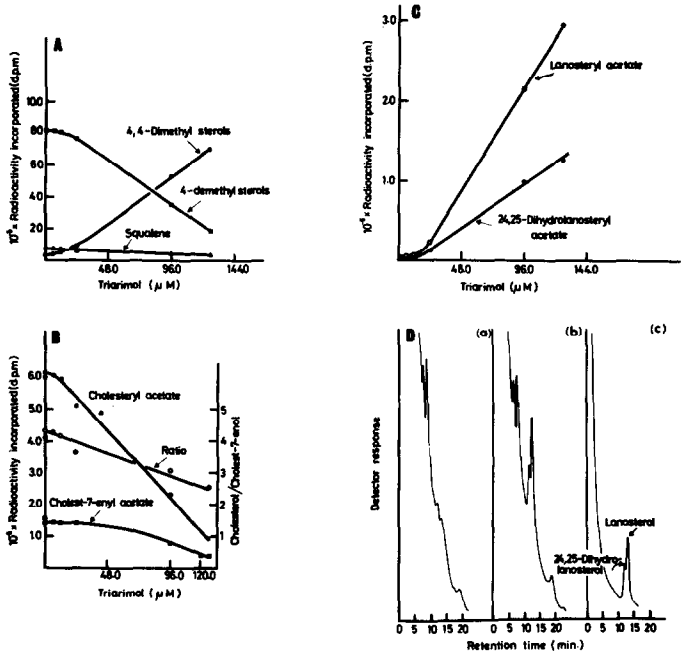

Fig. 1. The biosynthesis of cholesterol and its triterpenoid precursors during incubations of the S_{10} fraction of rat-liver homogenate with [2-^{14}C]-mevalonic acid in the absence and in the presence of various concentrations of triarimol. The incubation mixture contained, in a total volume of 5 ml, 4 ml of S_{10} fraction (15.5 mg of protein/ml), NAD^+ (1.6 mM), fructose 1,6-diphosphate (16 mM), $MgCl_2$ (4 mM), [2-^{14}C]mevalonic acid (2 μCi; 7.10 μCi/μmol) and the indicated concentration of triarimol added as a solution in 25 μl of methanol. Incubations in the absence of triarimol received 25 μl of methanol. Portions of the lipid extract from each incubation mixture were fractionated for the isolation of various sterols. A, incorporation of radioactivity into squalene, 4-demethyl and 4,4-dimethyl sterols. B, radioactivity into cholesterol and cholest-7-enol isolated as the acetate derivatives. C, radioactivity into lanosterol and 24,25-dihydrolanosterol isolated as the acetate derivatives. D, g.l.c. of the 4,4-dimethyl sterol fraction obtained (a) in the absence and (b) in the presence of triarimol (126 μM) and g.l.c. of a mixture of authentic lanosterol and 24,25-dihydrolanosterol (c). Results on the incorporation of radioactivity into various sterol fractions are not corrected for recoveries during chromatography and the yield achieved on acetylation. Other experimental details are given in the text.

dimethyl fraction. The radioactivity in the squalene fraction declined gradually with increasing triarimol concentration.

After acetylation of the 4-demethyl sterols and chromatography on silver nitrate-impregnated plates of alumina, more than 75 % of the radioactivity of this fraction in all kinds of incubations was in the bands of the cholesteryl and cholest-7-enyl acetate fractions. However, the ratio of Δ^5- to Δ^7-sterol

changed gradually from 4.3 in the absence of the inhibitor to 2.5 at 126 μM of triarimol. At this concentration of the inhibitor the amount of radioactivity isolated in the cholesteryl acetate and the cholest-7-enyl acetate bands was 15.3 and 26.7 % respectively of that incorporated in the two bands in the absence of triarimol.

To a portion of the eluate from the cholesteryl acetate band from the incubations in the absence of triarimol 50 mg of cholesteryl acetate was added and the mixture was crystallized to constant specific radioactivity. The specific radioactivity of the crystals obtained remained constant during four recrystallizations (18,037, 17,951, 15,712, 17,081 dpm/mg) and was similar to the initial calculated value (18,049 dpm/mg). The radiochemical purity of the isolated cholest-7-enyl acetate was also confirmed by dilution analysis. The specific radioactivity remained constant during four recrystallizations (3,896, 3,816, 3,957, 3,709 dpm/mg) and similar to the initial calculated value (4,113 dpm/mg).

Nature of the radioactive 4,4-dimethyl sterols accumulating in the presence of triarimol

Carrier lanosterol and 24,25-dihydrolanosterol (150 μg of each) were added to portions of each of the radioactive 4,4-dimethyl sterol fractions isolated in the absence of, and at various concentrations of, triarimol (Fig. 1A). The mixtures were acetylated and the radioactive components separated by argentation chromatography. The radioactivity associated with the acetates of lanosterol and 24,25-dihydrolanosterol in each incubation is shown in Fig. 1C. These values are not adjusted for losses incurred during the two chromatographic procedures involved. At a triarimol concentration of 126 μM, all the radioactivity contained in the original 4,4-dimethyl sterol fraction was associated with lanosterol and its 24,25-dihydro derivative. However, in the absence of triarimol, only a small fraction of the substantially reduced radioactivity contained in the 4,4-dimethyl sterol fraction (Fig. 1A) was associated with lanosterol and dihydrolanosterol and we have shown previ-

ously (6) that under these conditions the major radioactive sterols are 14-demethyl lanosterol and 14-demethyldihydrolanosterol. The radiochemical identities of lanosterol and the 24,25-dihydrolanosterol isolated as the acetates from incubations containing triarimol (126 μM) were confirmed by addition of the appropriate "carrier" material followed by several recrystallizations. In the case of lanosteryl acetate, the original value was 4951 dpm/mg and this showed no decline during recrystallization (4827, 4853, 5462 and 4630 dpm/mg). With dihydrolanosteryl acetate, the corresponding values were 2577 dpm/mg (initially) followed by 2168, 2257, 2176 and 2248 dpm/mg.

Further evidence for the accumulation of lanosterol and dihydrolanosterol in the presence of triarimol was afforded by analytical g.l.c. of a portion of the 4,4-dimethyl sterol fraction (Fig. 1D). In the presence of triarimol (126 μM) two peaks appeared, the least polar and most polar of which had retention times identical with those of 24,25-dihydrolanosterol and lanosterol respectively. No peaks were observed in this region of the chromatogram upon analysis of the 4,4-dimethyl sterol fractions obtained in the absence of triarimol.

Effect of triarimol on the activity of lanosterol 14α-demethylase and HMG-CoA reductase in the liver microsomal fraction

Incubation of 24,25-dihydro[32-^{14}C]lanosterol with rat-liver microsomes results in the release of [^{14}C]formic acid and a measure of the rate of formation of this material provides a direct assay method for lanosterol 14α-demethylase (17). The presence of triarimol (126 μM) inhibited the rate of [^{14}C]HCOOH formation by more than 90 % (Table 1). Furthermore, in the presence of triarimol there was more radioactivity associated with the organic material, due, presumably, to less substrate being metabolized in the presence of the inhibitor.

The activity of HMG-CoA reductase in the liver microsomal fraction was similar in incubations conducted in the presence of 126 μM triarimol (179 ±

TABLE 1

Effect of triarimol on the rate of release of [^{14}C]formic acid from 24,25-dihydro[32-^{14}C]lanosterol by the microsomal fraction from rat liver

	Radioactivity (dpm) in		Relative effect
	HCOOH	Organic phase	of triarimol
Control	6169 ± 503	40,070 ± 936	100
Triarimol (126 μM)	486 ± 21	47,190 ± 661	7.9
Not incubated	54	47,400	

24,25-Dihydro[32-^{14}C]lanosterol (47,500 dpm; 3030 dpm/nmol) was incubated with rat-liver microsomes (0.1 ml; 17.5 mg protein/ml) for 30 min at 37° C. The incubation mixture contained in a total volume of 1 ml:- potassium phosphate buffer (0.1 M), pH 7.4; ATP (6.9 mM); $NADP^+$ (2.6 mM); glucose-6-phosphate (10.3 mM), glucose-6-phosphate dehydrogenase (1 unit), and 5 μl of methanol with or without triarimol (126 nmol). All incubations were started by the addition of 24,25-dihydro[32-^{14}C]lanosterol as an emulsion with Tween-80. Incubations were terminated by the addition of chloroform-methanol and carrier formic acid (14.4 mg). Radioactivity due to formic acid in the aqueous phase was separated from the radioactivity with the organic phase and was assayed. Each type of incubation was carried out in triplicate and each value is the mean ± SD. Other experimental details are given in the Methods section.

7 pmol/min/mg protein) to that observed in its absence (184 ± 8 pmol/min/mg protein). In agreement with this is the observation that in incubations of the S_{10} fraction of liver with [2-^{14}C]acetate the presence of triarimol (126 μM) had no effect on the total amount of radioactivity incorporated into the sum of the 4,4-dimethyl and 4-demethyl sterol fractions. However, in the presence of triarimol the incorporation of radioactivity into the 4-demethyl sterol fraction was reduced by 90 % and there was a concomitant increase in the amount of radioactivity associated with the 4,4-dimethyl sterol fraction.

DISCUSSION

The inhibition of lanosterol 14α-demethylase by triarimol in rat liver is evidenced by the accumulation of radioactive lanosterol and 24,25-dihydrolanosterol during cholesterol biosynthesis from [2-^{14}C]acetate and [2-^{14}C]-

MVA. These C_{30} sterols have been identified both radiochemically and by analytical g.l.c. The 14α-methyl carbon (C-32) is released as formic acid (17,18) and, in the present study, the decreased rate of formation of $H^{14}COOH$ from 24,25-dihydro[32-^{14}C]lanosterol by liver microsomes in the presence of triarimol provides further evidence for the inhibitory effect of this compound on 14α-demethylation.

The effect of triarimol, therefore, in rat liver, is similar to its inhibitory behaviour in the biosynthesis of ergosterol in Ustilago maydis (5) where it is also known to block 14α-demethylation. This provides another example of the close similarity between the enzymes participating in 14α-demethylation in the two organisms (17). The enzymes of U. maydis, however, are able to remove the 4α-methyl group of a sterol containing a 14α-methyl group, a capacity which reflects the metabolic similarity of this fungus to green plants which normally synthesize their sterols by this sequence of events. In U. maydis, therefore, 4,4,14α-trimethyl sterols do not accumulate to any large extent in the presence of triarimol. Higher animals do not retain this capacity; 4-demethylation will not occur until the 14α-methyl group has been removed and this leads to the accumulation of 4,4,14α-trimethyl sterols in the presence of 14α-demethylase inhibitors (6,11).

Apart from a relatively minor inhibition of cholest-7-enol-Δ^5-dehydrogenase (as judged by the decrease in the cholesterol : cholest-7-enol ratio in the presence of triarimol, Fig. 1B), triarimol appears specifically to inhibit the 14α-demethylase during cholesterol biosynthesis. The similarities in the amounts of radioactivity associated with the total sterol fraction (4-demethyl plus 4,4-dimethyl sterols) in the presence and absence of inhibitor when either [2-^{14}C]acetate or [2-^{14}C]MVA was used as the cholesterol precursor supports the idea that the enzymes between acetate and squalene are unaffected. This is corroborated by the absence of any effect of triarimol on liver microsomal HMG-CoA reductase.

During cholesterol biosynthesis from [2-^{14}C]MVA in the absence of any inhibitors, the small amount of radioactivity associated with the 4,4-dimethyl

sterol fraction is due to 14-demethyllanosterol and 14-demethyldihydrolanosterol; lanosterol is present, proportionately, to only a small extent and dihydrolanosterol not at all (6). In the present study, this explains the small contribution (<10 %) of radioactive lanosterol and dihydrolanosterol to the total radioactivity of the 4,4-dimethyl sterol fraction biosynthesized in the absence of triarimol. Triarimol, by virtue of its inhibition of lanosterol 14α-demethylase and consequent accumulation of lanosterol and dihydrolanosterol, resembles the behaviour of carbon monoxide in this respect (6,11). In this case carbon monoxide inhibits cytochrome P-450, which participates in one or more reactions leading to the oxidative elimination of the 14α-methyl group (7). It appears, therefore, that triarimol may be a specific inhibitor of cytochrome P-450.

The formation of 24,25-dihydrolanosterol in the presence of triarimol does not mean that this compound is a normal intermediate in cholesterol biosynthesis. On the contrary, as shown previously under similar conditions (6), its presence is due merely to the decreased rate of oxidation of lanosterol, thus providing a static pool of the latter for Δ^{24} bond reduction by an enzyme which does not normally utilize lanosterol as a substrate.

The severe inhibition of cholesterol biosynthesis in the presence of triarimol and the concomitant accumulation of lanosterol and 24,25-dihydrolanosterol provides a simple method for the biosynthesis of stereospecifically labelled 4,4,14-trimethyl-Δ^{8}-sterols of high specific radioactivity.

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