Effect of 22,25-Diazacholestanol on Synthesis of Cholesterol by Rat Liver Homogenates. (29299)

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On the basis of experiments *in vitro*, Ranney and Counsell(1) suggested that 20α-(2-dimethylaminoethyl)amino-5α-pregnan-3β-ol dihydrochloride (22,25-diazacholestanol, or SC-11952) affected hepatic cholesterol synthesis by a "cholesteromimetic" type of inhibition, *i.e.*, by suppressing primarily the formation of mevalonate. *In vivo*, such interference should produce sterol contents which differ from controls only in their reduced concentrations. In contrast, Sachs and Wolfman(2) detected 24-dehydrocholesterol (desmosterol) in the serum of hypercholesterolemic patients treated with 22,25-diazacholestanol. Prompted by this observation we have found(3) high concentrations of desmosterol in the serum, liver and adrenals of normal rats fed with this diazasterol. From this we concluded that *in vivo*, 22,25-diazacholestanol interfered with sterol synthesis by a triparanol-like(4) mechanism of inhibition. Ranney, Cook, Hambourger and Counsell(5) have recently reached a similar conclusion.

Since rats are usually used in *in vitro* studies of hepatic cholesterogenesis, we considered it important to clarify the apparent discrepancy as to the site of inhibition of 22,25-diazacholestanol between *in vitro* and *in vivo* experiments. We have therefore investigated the effect of this agent on the incorporation of acetate, and of mevalonate and desmosterol(6) into cholesterol by rat liver homogenates.

Methods. Livers taken from hooded rats were homogenized and incubated according to Bucher and McGarrahan(7) in the presence of cofactors as described by Popjak, Cornforth and Clifford(8). Each flask contained 4 ml of homogenate, 1.9 μmoles of 2-C^{14}-acetate (5.0 μC) (Merck, Sharp & Dohme of Canada Ltd.) or, 2.0 μmoles of 2-C^{14}-mevalonate (0.5 μC) (Nuclear Chicago Corp.) or, 0.042 μmoles of 26,27-C^{14}-desmosterol (1.0 μC) (Volk Radiochemical Co.) respectively; 2 μmoles of $NADH_2$, 2 μmoles of NADP, 5 μmoles of ATP and 4 μmoles of glucose-6-phosphate. 22,25-Diazacholestanol* was added at a final concentration of 1×10^{-5} and 1×10^{-6}M in water. With triparanol,† added in a 2% gelatin suspension, the final concentrations were 1×10^{-4} and 1×10^{-5}M respectively. After one hour at 37°C, with air as the gas phase (with 2-C^{14}-acetate, 95% oxygen and 5% carbon dioxide), enzyme action was arrested by addition of 20 N KOH (1.0 ml). Carrier cholesterol (150 mg) was added, the suspension heated for one hour at 75-80°C and the nonsaponifiable lipids extracted repeatedly with petroleum ether (b.p. 30-40°C). After bromination(9), 5,6-dibromocholestan-3β-ol was isolated and crystallized from methanol-ethyl acetate to constant radioactivity. With acetate and mevalonate 3-4 crystallizations were necessary and with desmosterol up to 9. Specific radioactivity was verified by debromination to cholesterol(10). Samples were counted in a D-47 gas flow counter mounted with a Mylar Micromil window (Nuclear Chicago Corp.).

Results. The effects of 22,25-diazacholestanol and of triparanol on the conversion to cho-

TABLE I. Effect of 22,25-Diazacholestanol (SC-11952) and of Triparanol (MER-29) on Incorporation of 2-C^{14}-Acetate into Cholesterol by Rat Liver Homogenates.

	Total radioactivity, as cpm*	
	Nonsaponif.	Dibromo-cholestanol
Control	29,882	19,539
SC-11952, 1×10^{-5}M†	30,110	1,133
1×10^{-6}M	29,595	1,833 (–91%)
MER-29, 1×10^{-4}M	31,390	1,939 (–91%)

* Avg values of duplicate experiments.
† Final concentration.

* Kindly provided by Dr. V. A. Drill, G. D. Searle and Co.

† Kindly provided by Dr. C. A. Bunde, Wm. S. Merrell Co.

TABLE II. Effect of 22,25-Diazacholestanol (SC-11952) and of Triparanol (MER-29) on Conversion of 2-C^{14}-Mevalonate and of 26,27-C^{14}-Desmosterol to Cholesterol by Rat Liver Homogenates.

	Total radioactivity, as cpm*				
	Mevalonate as precursor			Desmosterol as precursor	
	Non-saponif.	Dibromo-cholestanol	Cholesterol	Dibromo-cholestanol	Cholesterol
Control	60,972	37,492	38,585	88,797	90,677
SC-11952, 1×10^{-5} M†	60,405	60	24	3,625	3,149
1×10^{-6} M	61,957	967	861 (–98%)	4,242	2,050 (–98%)
MER-29, 1×10^{-4} M	62,247	151	109	8,941	7,081
1×10^{-5} M	60,967	11,703	11,893 (–69%)	26,408	25,313 (–72%)

* Avg values of duplicate experiments. † Final concentration.

lesterol in rat liver homogenates of (a) 2-C^{14}-acetate are presented in Table I and, (b) of 2-C^{14}-mevalonate and 26,27-C^{14}-desmosterol respectively in Table II. 22,25-Diazacholestanol, at a final concentration of 1×10^{-6}M, inhibited to the same extent the conversion to cholesterol of both acetate and mevalonate as well as of desmosterol. As expected, similar results were obtained with triparanol.

Discussion. It has been reported that, *in vitro*(1), 22,25-diazacholestanol (a) inhibited acetate incorporation into cholesterol by rat liver homogenates to a greater extent than that of mevalonate, (b) depressed acetate transformation to mevalonate but not to β-hydroxy-β-methylglutarate and (c) did not affect acetate or mevalonate incorporation into desmosterol(6). From this, it was concluded that 22,25-diazacolestanol acted by inhibiting β-hydroxy-β-methylglutaryl coenzyme A reductase. Since according to Siperstein and Guest(11,12) the same enzyme is affected by exogenous cholesterol, 22,25-diazacholestanol was named "cholesteromimetic"(1).

In contrast, according to our results, 22,25-diazacholestanol prevents the conversion of desmosterol to cholesterol in rat liver homogenates. Furthermore, at the same final concentration the agent blocked to the same extent incorporation into cholesterol of acetate, of mevalonate and of desmosterol. The results with 22,25-diazacholestanol parallel those obtained simultaneously with triparanol in the same homogenate. Like triparanol(4), 22,25-diazacholestanol did not affect count incorporation into the total nonsaponifiable lipid fraction. Inhibition of incorporation into cholesterol was revealed only after desmosterol and other "high counting companions" (10,4) were removed by purification *via* the 5,6-dibromo derivative. Hence, *in vitro*, 22,25-diazacholestanol acts by a triparanol-like mechanism, *i.e.* its primary site of inhibition is at the level of "24-dehydrosterol Δ^{24}-reductase." These findings *in vitro* are thus in complete agreement with our earlier observation *in vivo*(3).

Thompson, Dupont and Robbins(13) have recently detected high levels of demosterol in livers and carcasses of rats fed with 20,25-diazacholesterol, another diazasterol for which a "cholesteromimetic" inhibition of sterol biosynthesis was surmised(14). Hence, it appears that diazasterols act by inhibiting "24-dehydrosterol Δ^{24}-reductase."

Summary. In rat liver homogenates 22,25-diazacholestanol blocked to the same extent the incorporation into cholesterol of 2-C^{14}-acetate, 2-C^{14}-mevalonate and of 26,27-C^{14}-desmosterol. Thus, direct evidence is provided for a triparanol-like interference with hepatic cholesterogenesis *in vitro*. This is in agreement with the finding of appreciable desmosterol levels in humans and rats treated with 22,25-diazacholestanol *per os*.

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Effect of Triparanol on Synthesis of Fatty Acids by *Tetrahymena pyriformis*.* (29300)

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Triparanol, an inhibitor of cholesterol synthesis in animals, also is effective as a growth inhibitor of protozoa(1). The triparanol-induced delay of synchronized first division(2) and growth inhibition in *Tetrahymena*(3) and *Ochromonas danica*(4,5) could be reversed by oleic or palmitic acids, among other compounds, suggesting interference with metabolism of long-chain fatty acids.

It was of interest to investigate the effect of triparanol on the synthesis of specific fatty acids, since Shorb(6,7) had demonstrated the synthesis of odd and even-numbered, as well as branched-chain fatty acids by *Tetrahymena pyriformis* in response to acetate, propionate, isobutyrate and α-methyl-*n*-butyrate as medium supplements.

Experimental. Strains H, S and W of *T. pyriformis* were grown in the dark at 24-26°C in 125 or 250 ml volumes in 500 or 1000 ml flasks (except as noted in footnote, Table I) on the synthetic medium D of Kidder and Dewey(8). The medium was modified by dilution to one-quarter strength; acetate, Tween 80 and glucose were omitted and vitamin B_{12}, biotin and thioctic acid were added (10, 3 and 10 μg/l, respectively). Growth supplements and inoculum treatment were as indicated in each experiment. Triparanol was used as an alcoholic emulsion, keeping alcohol to a minimum. Triparanol citrate was used as a water solution. The inhibitors were added only to the larger volumes of medium (250 ml) so that approximately equal numbers of organisms could be harvested around the mid-point of logarithmic growth. Because growth varied with the supplement added, the exact time of incubation is given in the footnotes of each table. Cells of *T. pyriformis* incubated with triparanol were more fragile than the control cells, making harvesting of the organisms more difficult.

Total lipids were extracted from centrifuged, frozen and thawed cells with chloroform-methanol (2:1, v/v) and methylated with BF_3 methylating reagent.‡ Fatty acid methyl esters were separated from other lipid components on a silicic acid column(9) with a benzene-hexane solvent (1:1, v/v). The methyl esters were further separated into saturated and unsaturated fatty acids by silicic acid chromatography of mercuric acetate adducts(10). Gas-liquid chromatograms of methyl esters were made on a 6 foot glass "U" column of 16% ethylene glycol succinate

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