Inhibition of Cholesterol Biosynthesis in the Rat by 3β-(2-diethylaminoethoxy)androst-5-en-17-one, hydrochloride. (28003)

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Current interest in hypercholesterolemia has stimulated a search for agents capable of reducing plasma cholesterol levels by reducing endogenous synthesis. Several synthetic agents have been shown to inhibit cholesterol biosynthesis and cause accumulation of desmosterol(1,2,3). The latter phenomenon has been correlated with inhibitory effect of the compound on reduction of desmosterol to cholesterol(4) and lanosterol to dihydrolanosterol (5). This report deals with the effect of a potent hypocholesterolemic agent 3β-(2-diethylaminoethoxy) androst-5-en-17-one, hydrochloride (U-18666A),* and with effects of several compounds on sterol metabolism. The hypocholesterolemic activity of the dimethyl derivative of this compound has been reported previously(3).

Methods. Ten male Sprague-Dawley rats weighing approximately 220 g were used in each group. Compounds were given orally in 0.5% solution of carboxymethylcellulose; control animals were given vehicle only. Rats were permitted food and water *ad libitum* except for 17-hour fasting period prior to drawing blood at end of experiment. Rats used in radioactive acetate test were not fasted. Blood samples were taken, without anesthesia, from external jugular vein near the junctions of the cephalic and anterior jugular veins.

Ferric chloride-sulfuric acid reagent was used for colorimetric determination of total sterols according to Zak *et al.*(6) and samples were analyzed by means of an AutoAnalyzer.† This method gives equal color yields for cholesterol and desmosterol(7). Desmosterol was determined colorimetrically according to Avigan *et al.*(2) and was confirmed for several samples by gas-liquid chromatography(8). Moreover, desmosterol was isolated from a larger sample by column chromatography of p-phenyl-azo-benzoate derivatives and identified by its IR spectrum(9,2).

In the Triton test, rats were given 200 mg/kg of Triton WR-1339‡ intravenously as an 8% solution in isotonic saline after a 24-hour fast. At the same time, U-18666A was given orally at 100 mg/kg. Sterols were determined on blood obtained 18 hours later.

Rats treated for 15 days with U-18666A at 15 mg/kg/day were injected with 25 μc of acetate-1-C^{14} per 100 g of body weight and sacrificed 4 hours later. Liver sterols were isolated by method of Duncan(10) and analyzed by method of Kabara(11) (Liebermann-Burchard reaction). Fieser's procedure(12) was used for purifying the isolated cholesterol by way of the dibromide. Liver fatty acids were isolated gravimetrically according to the method of Srere(13). Radioactivity was measured in a Tricarb scintillation spectrometer.§

Results. U-18666A caused marked decreases in serum total sterols at all doses tested, i.e., 1-40 mg/kg given for 14 days and at 0.1-1 mg/kg given for 26 days (Table I). There was no reduction in weight gain at doses of 20 mg/kg or less. At 40 mg/kg, the compound suppressed weight gain and food consumption. Preliminary experiments had revealed that cholesterol-lowering effect was equal with oral or subcutaneous administration of U-18666A, but the compound appeared to be more toxic as reflected by weight gain when given subcutaneously. Analyses of livers from rats in the above 14-day test indicated that maximum effects of U-18666A on concentrations of total sterols and of desmosterol were reached at doses of 5-10 mg/kg daily (Table II).

* Compound generously supplied by Drs. B. J. Magerlein and F. Kagan and Mr. R. D. Birkenmeyer, Upjohn Co. This compound has been reported as a coronary vasodilator(19).

† Technicon Controls, Inc., Chauncey, N. Y.

‡ Tyloxapol or oxyethylated tertiary octylphenol-formaldehyde polymer obtained from Winthrop Laboratories, New York.

§ Packard Instrument Co., LaGrange, Ill.

TABLE I. Changes in Total Serum Sterol Levels, Weight Gain and Food Intake at Several Doses of U-18666A.

Duration of treatment (days)	Dose (mg/kg/day)	Serum sterols (mg/100 ml)	Wt increase (g)	Food intake (g)
14	0	71 ± 2.3	70 ± 3.1	241 ± 6.5
"	1	46 ± 2.1	71 ± 3.2	236 ± 6.5
"	5	41 ± 2.9	70 ± 3.1	233 ± 6.5
"	10	41 ± 2.9	72 ± 3.4	238 ± 9.0
"	20	37 ± 2.9	66 ± 3.1	226 ± 5.9
"	40	32 ± 3.0	35 ± 5.9	184 ± 10.1
26	0	80 ± 3.1	132 ± 3.2	526 ± 49.4
"	0.1	66 ± 3.0	132 ± 3.4	546 ± 13.1
"	0.5	61 ± 3.8	130 ± 4.5	542 ± 14.4
"	1	55 ± 2.5	131 ± 5.3	523 ± 16.5

Values expressed with standard errors of means.
Ten rats per group.

Simultaneous administration for 2 weeks of a mixture of the 2 moieties of U-18666A, dehydroepiandrosterone and diethylaminoethanol, did not affect serum sterol levels, even when given at a dose greater than that used for the U-18666A (Table III). Negative results not shown here were also obtained when the 2 moieties were administered separately.

U-18666A caused a moderate but significant (22%) diminution of Triton-induced hypercholesterolemia (Table IV).

U-18666A caused increased incorporation of radioactive acetate into total sterols precipitable by digitonin (Table V). Concentration of total digitonin-precipitable sterol in liver was reduced about 35% with U-18666A. When the sterols were purified by way of the dibromide for removal of chloesterol precursors, no cholesterol dibromide was precipitated from samples from U-18666A-treated animals. Absence of precipitate after bromination of sterol mixture appears to be characteristic for preparations containing desmosterol(2). Bromination of pooled samples from each group was repeated after adding carrier cholesterol until constant specific radioactivity was obtained. After several fractionation steps through brominations and debrominations, cholesterol from U-18666A-treated rats showed about 85% reduction in specific activity (Table VI). No reduction was observed for labeled sterols from control animals. This compound altered slightly the specific radioactivity of the fatty acids. However, since total amount of fatty acids was

TABLE II. Effect of U-18666A on Liver Total Sterol and Desmosterol Concentrations.*

Dose (mg/kg/day)	Total sterols (mg/g)	Desmosterol (%)
0	3.64	0
1	2.86	23
5	2.02	44
10	1.88	44
20	2.06	48
40	2.00	46

* Determined by differential colorimetric method (2).

TABLE III. Ineffectiveness of the Diethylaminoethanol and Dehydroepiandrosterone, Moieties of U-18666A.

Test material and dose	Serum sterols (mg/100 ml ± S.E.)
Control	70 ± 3.2
Dehydroepiandrosterone 50 mg/kg/day + Diethylaminoethanol 15 mg/kg/day	69 ± 2.4
U-18666A 10 mg/kg/day	44 ± 1.9

TABLE IV. Effect of U-18666A on Triton-Induced Hypercholesterolemia.

Test material	Serum sterols (mg% ± S.E.)
Saline (I.V.)	78 ± 2.8
Triton (I.V.) 200 mg/kg	300 ± 8.1
Idem + U-18666A (Oral) 100 mg/kg*	233 ± 8.0

Rats fasted 24 hr prior to Triton treatment. Serum samples collected 18 hr after Triton injection (fasting continued throughout).
* One rat died.

TABLE V. Effect of 15 mg/kg of U-18666A on Composition and Incorporation of Acetate-1-C^{14} into Liver Lipids.

	Control	U-18666A
Liver wt, g	11.81	13.25
Digitonin precipitable sterols*		
Concentration, mg/g	1.67	1.09
Total amt per liver, mg	19.7	14.4
Specific radioactivities, (cpm/mg)	607	3249
Total radioactivity per liver, cpm	1.20×10^4	4.68×10^4
Fatty acids		
Concentration, mg/g	22.3	29.3
Total amt per liver, mg	263	388
Specific radioactivities (cpm/mg)	1251	1580
Total radioactivity, cpm	3.29×10^5	6.13×10^5

Each rat injected with 25 μc of acetate-1-C^{14} per 100 g body wt.
* Samples were combined for determination of digitonin precipitable sterols.

greater in livers of treated rats than in controls, total radioactivity of the fatty acid fraction was increased almost 2-fold (Table V).

Preliminary studies were also carried out with several other compounds containing the diethylaminoethoxy moiety. Results (Table VII) reveal that hexesterol bis-β-diethylaminoethyl ether caused a significant reduction in serum sterols after 3 weeks. Desmosterol was identified in the liver, but its concentration was less than that found with similar doses of U-18666A (Table II). Diethylaminoethanol and hexesterol were ineffective in this test. The latter, given at a relatively small dose, caused increased serum sterols and arrested weight gain of animals.

Discussion. Results presented clearly demonstrate pronounced reduction in serum and liver cholesterol and establish appearance of desmosterol after administration of U-18666A. The finding that a single large dose of U-18666A reduces significantly Triton WR-1339 induced hypercholesterolemia is consonant with the hypothesis that this compound inhibits endogenous synthesis of cholesterol. It is believed that Triton WR-1339 induces hypercholesterolemia by altering properties of plasma lipoproteins and by increasing endogenous synthesis of cholesterol by the liver(14, 15). Marked stimulation of incorporation of acetate-1-C^{14} into nonsaponifiable lipids could be secondary to reduction of liver sterol concentration since it has been shown that the rate of sterol synthesis in liver is inversely related to liver sterol concentration(16). Reason for small increase in the synthesis of fatty acids is not clear. Several compounds containing the diethylaminoethoxy *moiety* have been shown to reduce sterol content of serum and tissues and to cause partial or almost complete replacement of cholesterol by desmosterol(2,3,17). Because of the small number of active compounds studied to date, correlation between chemical structure and effec-

TABLE VI. Purification of Radioactive Liver Sterols.*

	Specific radioactivity (cpm/mg)	
	Control	U-18666A
Digitonin-precipitable sterols	607	3249
Cholesterol fraction reprecipitated after adding carrier cholesterol	81	917
Cholesterol fraction after purification through several brominations	76	127

* Via bromination according to Fieser(12).

TABLE VII. Effects of Other Diethylaminoethoxy Compounds on Serum and Liver Sterols After 3 Weeks.

Compound	Dose (mg/kg/day)	Serum total sterols (mg/100 ml ± S.E.)	Weight gain (g ± S.E.)	Liver sterols (mg/g)	Liver sterols % Desmosterol
Control	—	79 ± 2.4	115 ± 5.9	1.45	0
β-Diethylaminoethanol	100	81 ± 2.8	105 ± 4.9	3.29	0
p-(β-Diethylaminoethoxy) toluene·HCl	50	77 ± 4.1	106 ± 4.7	4.31	0
Hexestrol bis-β-diethylaminoethyl ether·2HCl*	50	63 ± 2.6	53 ± 6.2	3.52	16.5
Hexestrol	0.7	116 ± 12.5	−2 ± 7.4	2.58	0

* Sample obtained from Chemo Puro Manufacturing Corp.

tiveness in blocking conversion of desmosterol to cholesterol is not clear. However, studies by Palopoli(18), as well as evidence presented here, indicate that activity is limited to certain compounds and is neither shared by all those containing the diethylaminoethoxy group *per se,* nor limited to any other single moiety in the molecule. Testing of additional substances for inhibitory effects on cholesterol synthesis might bring about a better understanding of enzymatic processes involved and help to establish the most favorable chemical structure for maximum inhibitory activity and minimum toxic side effects.

Summary. 1. U-18666A, a new inhibitor of cholesterol biosynthesis, caused marked reduction in serum and liver sterols and appearance of desmosterol in livers of rats. 2. Reduction of serum sterols by U-18666A was obtained in animals in which hypercholesterolemia was induced with Triton WR-1339. 3. U-18666A increased incorporation of acetate-1-C^{14} into liver digitonin precipitable sterols fourfold and into liver fatty acids twofold. 4. Of several other compounds tested for cholesterol inhibitory activity, only hexestrol bis-β-diethylaminoethyl ether was found to cause appearance of desmosterol.

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1. Blohm, T. R., Kariya, T., Laughlin, M. W., *Arch. Biochem. Biophys.,* 1959, v85, 250.

2. Avigan, J., Steinberg, D., Vroman, H. E., Thompson, M. J., Mosettig, E., *J. Biol. Chem.,* 1960, v235, 3123.

3. Gordon, S., Cantrall, E. W., Cekleniak, W. P., Albers, H. J., Littell, R., Bernstein, S., *Biochem. Biophys. Res. Comm.,* 1961, v6, 359.

4. Steinberg, D., Avigan, J., *J. Biol. Chem.,* 1960, v235, 3127.

5. Avigan, J., Goodman, D. S., Steinberg, D., *Fed. Proc.,* 1962, v21, 300.

6. Zak, B., Moss, N., Boyle, A. J., Zlatkis, A., *Anal. Chem.,* 1954, v26, 776.

7. Frantz, I. D., Jr., Mobberley, M. L., Schroepfer, G. J., Jr., *Progress Card. Dis.,* 1960, v2, 511.

8. Steinberg, D., Avigan, J., Feigelson, E. B., *J. Clin. Invest.,* 1961, v40, 884.

9. Stokes, W. M., Hickey, F. C., Fish, W. A., *J. Biol. Chem.,* 1958, v232, 347.

10. Duncan, C. H., Best, M. M., *Am. J. Physiol.,* 1958, v194, 351.

11. Kabara, J. J., *J. Lab. & Clin. Med.,* 1957, v50, 146.

12. Fieser, L. F., *J. Am. Chem. Soc.,* 1953, v75, 5421.

13. Srere, P. A., Chaikoff, I. L., Dauben, W. G., *J. Biol. Chem.,* 1948, v176, 829.

14. Friedman, M., Byers, S. O., *J. Exp. Med.,* 1953, v97, 117.

15. Frantz, I. D., Jr., Hinkelman, B. T., *ibid.,* 1955, v101, 225.

16. Frantz, I. D., Jr., Schneider, H. S., Hinkelman, B. T., *J. Biol. Chem.,* 1954, v206, 465.

17. Wong, H. Y. C., Avigan, J., *Fed. Proc.,* 1962, v21, 98.

18. Palopoli, F. P., *Progress Card. Dis.,* 1960, v2, 489.

19. Cavallini, G., Massarani, E., *Farm. sci. e tec.* (Pavia), 1951, v6, 291.

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Cell Cultures for Detection of Trachoma Virus from Experimental Simian Infections.* (28004)

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Two methods have been used to detect the causative agent of trachoma in infected eyes, *i.e.,* microscopic examination of direct smears, and culture of conjunctival specimens in the yolk sac of embryonated eggs(1). Although the latter method is valuable as a research tool, it is not satisfactory as a routine diagnostic procedure. Similar methods have been

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