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An Azasterol that Inhibits Cholesterol Synthesis *in vitro*. (27346)

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The site of physiological regulation of cholesterol synthesis has been suggested by Siperstein and Guest(1,2) to be the reductive transformation of hydroxy-methylglutarate (HMG) to mevalonate. These investigators demonstrated that liver slices from rats previously fed cholesterol incorporated C^{14}-labeled acetate into cholesterol at significantly reduced rates. When, however, mevalonate-2-C^{14} was the labeled substrate, utilization of this material for cholesterol synthesis proceeded at near normal rates. Furthermore, they observed that conversion of labeled acetate to fatty acids and to keto-acids was essentially unimpaired by cholesterol feeding. They reasoned, therefore, that the homeostatic mechanism regulating cholesterol synthesis in the liver was a negative feedback system in which the sensitive element, HMG-CoA reductase, was inhibited by greater than normal amounts of cholesterol in the liver-plasma cholesterol pool.

The present report deals with one of a series of azasterols structurally related to cholesterol that seemingly inhibits cholesterol synthesis in a manner similar to that reported for dietary cholesterol. This compound, synthesized by one of us (R.E.C.), is 20α-(2-dimethylaminoethyl) amino-5α-pregnan-3β-ol dihydrochloride, or SC-11952.*

Methods. The homogenate system of Bucher(3) was used in all studies. The livers of female rats (100-120 g) were briefly homogenized in a "loose" glass homogenizer with 2.5 volumes of ice cold phosphate buffer at pH 7.2 and centrifuged at 500 $\times$ *g* for 3 minutes. The supernatant was incubated in the presence of co-factors with or without SC-11952. Animals were either pre-treated with SC-11952 or the compound was added as a suspension to homogenates prepared from untreated animals. After a 5-minute equilibration period, labeled substrate was added, and the incubation was continued in an oxygen atmosphere. When the same homogenate was used for all studies the per cent incorporation of substrate C^{14} was determined. In other studies where different homogenates were compared the results were calculated in terms of the dry weight of the preparations. Further details are included in the data presentation of each experiment. After incubation an aliquot of the incubated mixture was saponified in 10% KOH in 50% ethanol. The non-saponifiable fraction was extracted with n-hexane and an aliquot of this solution was placed on a silicic acid column (10 mm $\times$ 120 mm) that had been prepared according to Hirsh and Ahrens(4). Gradient elution of the non-saponifiable lipids was accomplished using n-hexane in a mixing flask and 15% ethyl ether in n-hexane in a reservoir. A pressure of 60 mm Hg of N_2 was impressed on the reservoir contents. Fractions (10 ml) were collected in a GME Model T-15 fractionator† from 7 columns simultaneously

* SC-11952 has also been termed 22,25-diazacholestanol.

† This fraction collector was purchased from Gilson Medical Electronics Co., Middleton, Wisc.

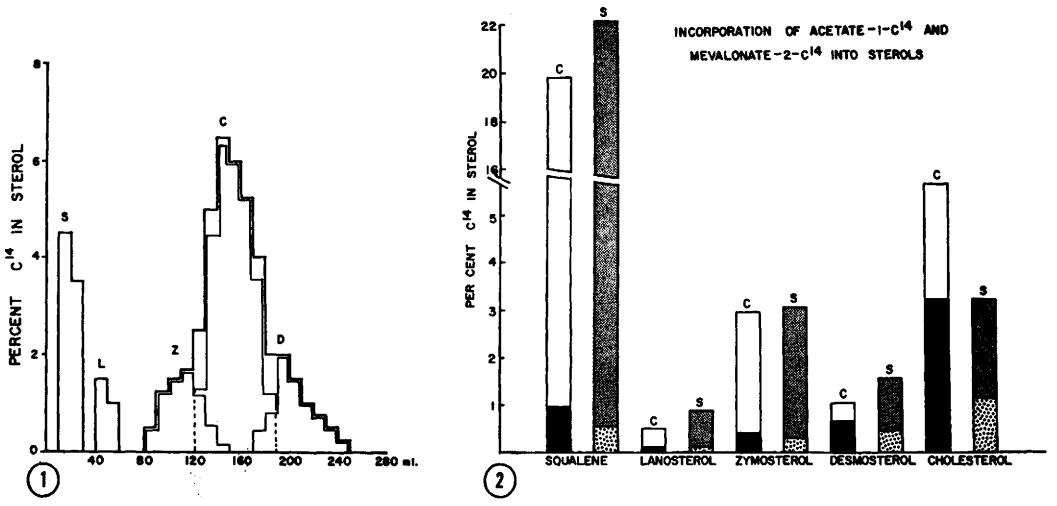

FIG. 1. Chromatogram of non-saponifiable lipids of untreated homogenate incubated 1 hr in O_2. Symbols: S = squalene, L = lanosterol, Z = zymosterol, C = cholesterol, and D = desmosterol. Dotted and light lines indicate extrapolated curves described in text.

FIG. 2. Effect of SC-11952 on acetate and mevalonate incorporation into cholesterol and its precursors. The lower portion of each bar indicates incorporation of acetate into sterols; upper portion shows mevalonate transformation to sterols. C = untreated controls; S = SC-11952 treated homogenate. The homogenizing medium was: potassium phosphate buffer pH 7.2, 0.044 M; nicotinamide, 0.028 M; magnesium chloride, 0.007 M; and sucrose, 0.125 M. The incubation mixture contained 2.0 ml of homogenate supernatant, 0.0008 M ATP, and 0.0015 M DPN. Substrate concentrations were: acetate-1-C^{14}, 1.0 μmole/ml; or mevalonate-2-C^{14}, 0.2 μmole/ml. SC-11952 was added as a suspension in 0.5% sodium alginate at a final concentration of 0.5 μmole/ml. Control beakers received an equal volume (0.1 ml) of 0.5% sodium alginate alone. The final volume was 2.3 ml. Incubation was carried out for one hour at 37° in O_2. Incubations and analyses were done in duplicate.

by regulating the outflow of each column to be identical with the others. A volume of 280 ml per column eluted the following lipids in sequence: squalene, lanosterol, zymosterol, cholesterol, and desmosterol. The location of each substance was initially determined gravimetrically by chromatographing the pure sterols alone and in combination. The sterols were obtained from commercial sources with the exception of zymosterol, which was kindly supplied by Dr. O. N. Breivik, Fleishmann Laboratories. The peaks for zymosterol, cholesterol, and desmosterol overlap one another and radioactivity of each component was determined by extrapolating the 3 curves to the base line and integrating the area under each curve. A typical chromatogram of an untreated homogenate showing the extrapolated portions of the curves is shown in Fig. 1.

The analysis of keto-acids was by the method of Kronfeld and Kleiber(5) scaled down for small samples (2 ml). Determination of fatty acids was carried out on saponified samples after non-saponifiable lipids had been removed by extraction with several portions of n-hexane. The aqueous phase was acidified to pH 4; the fatty acids were extracted with n-hexane; and the amounts of fatty acids in the extracts were measured gravimetrically.

Oxygen utilization by homogenates was measured with a Warburg apparatus. After equilibration of a liver homogenate prepared from normal animals with co-factors and SC-11952 for 10 minutes, acetate-1-C^{14} was added and oxygen consumption recorded for 15 minutes. The reaction was stopped with the addition of 0.2 ml 6N H_2SO_4. $C^{14}O_2$ released from the incubation mixture was absorbed on 10% KOH-saturated filter paper in the center well during a subsequent 30-minute period of shaking. The contents of the center well were removed quantitatively, diluted with water, and an aliquot of this extract taken for C^{14} analysis.

Radioactivity measurements were made by liquid scintillation spectrometry. Aqueous samples were dissolved in 0.4% PPO (2,5-di-

TABLE I. Acetoacetate, β-Hydroxybutyrate, Fatty Acid and Non-saponifiable Lipid Analyses of Liver Homogenates Prepared from Control Rats and Rats Treated with SC-11952.*

Constituent	Concentration (mg/g dry wt)		Specific activity (% C^{14}/mg)	
	Control	SC-11952	Control	SC-11952
Acetone & acetoacetate	1.15	.187	.13	1.9
β-hydroxybutyrate	7.75	6.93	.070	.25
Non-saponifiable lipid	5.80	5.70	230	14
Total fatty acids	10.7	13.1	4.0	8.4

* Rats were treated daily for 10 days with SC-11952 (5 mg/kg orally). At the end of treatment pooled livers from 3 control and 3 treated rats were homogenized and incubated for 40 min. Each value is avg of 3 samples of incubated homogenate. The buffer and co-factors were the same as for Fig. 2. Substrate: acetate-1-C^{14}, 1.5 μmole/ml.

phenyloxazole), 0.005% POPOP (2,2-p-phenylenebis(5-phenyloxazole)), 35% absolute ethanol, and 65% toluene. Lipids were counted in 0.4% PPO-toluene and solid samples (keto-acids as the acetone-mercury complex) on horizontally oriented filter paper in 0.4% PPO-toluene. Appropriate corrections were made for differences in the efficiency of scintillator solutions.

Results. Effect of SC-11952 on acetate and mevalonate incorporation into sterols. Incorporation of acetate-1-C^{14} and mevalonate-2-C^{14} into the sterols of an untreated homogenate prepared from normal rat livers and that treated with SC-11952 is shown in Fig. 2. When acetate was the substrate, C^{14} incorporated into cholesterol and its precursors in the SC-11952 treated mixtures was consistently less than or equal to the control values.

Utilization of mevalonate for sterol synthesis by SC-11952 treated homogenates showed a lesser degree of inhibition than when acetate-1-C^{14} was the substrate. Squalene, lanosterol, and desmosterol C^{14} all exceeded control values. Cholesterol C^{14} was less than the control.

Because of the variability of the incorporation of C^{14} of the 2 substrates into the precursor materials, we feel only consistent changes seen with both mevalonate and acetate are meaningful. With such a criterion, it must be concluded that SC-11952 had little effect on the formation of the precursor lipids but did reduce cholesterol synthesis, and that the effect upon acetate incorporation into cholesterol (67% reduction) was greater than the effect upon mevalonate incorporation (46%).

Effect on keto-acid and fatty acid synthesis. Since SC-11952 apparently inhibited synthesis of cholesterol to a greater extent when acetate was the labeled substrate than when mevalonate was used, it seemed desirable to examine the effect of the compound on reactions preceding mevalonate formation. In this study, a liver homogenate from rats pre-treated with SC-11952 was compared with that prepared from control animals. The details of treatment and results of the study are given in Table I. The treatment with SC-11952 affected only the amount of acetoacetate fraction in the homogenate, bringing about a 6-fold reduction of this component. SC-11952 profoundly affected the transformation of labeled acetate into all fractions measured. In the homogenate from treated rats the marked inhibition of utilization of acetate for sterol synthesis resulted in an apparent increase in the specific activity of the acetate and acetoacetate pools of the system which was reflected by an increased specific activity of β-hydroxybutyrate and fatty acids. One must conclude from this study that the site of action of SC-11952 was at one of the enzymatic reactions that succeeds the formation of acetoacetyl-CoA. Certainly it is evident from the specific activity of both keto- and fatty acids that the compound did not inhibit the CoA-catalyzed reactions that lead to formation of these metabolites.

Effect on oxygen utilization. An alternative hypothesis to that just proposed above might be that SC-11952 inhibited the oxidation of substrate acetate and in this way increased the specific activity of the acetate pool. This then would be reflected as the observed increases in keto- and fatty acid specific activities. To rule out this alternate in-

TABLE II. Oxygen Utilization and C^{14} Incorporation into CO_2 and Keto-Acids in a Rat Liver Homogenate Treated with SC-11952.*

Addition	Q_{O_2}†	% substrate C^{14} in: CO_2	Acetone + acetoacetate	β-hydroxy-butyrate	Residual acids‡
None	3.35	14.9	.292	.0717	4.16
SC-11952	3.12	12.7	.439	.121	3.60

* Rat liver homogenate prepared as for Fig. 2. Incubation for 15 min. at 37° in air. Substrate: acetate-1-C^{14}, 1.5 μmole/ml. SC-11952 present at concentration of 0.5 μmole/ml.

† Q_{O_2} = μl O_2 utilized per mg dry homogenate/hr.

‡ "Residual acids" includes all steam-labile materials remaining in acidified incubation mixture at end of experiment. This was assumed to be primarily acetic acid.

terpretation, oxygen consumption and $C^{14}O_2$ production of an SC-11952 treated homogenate was compared with a control incubation mixture. The details of the experiment and the results are given in Table II.

It is evident that SC-11952 induced no significant reduction in Q_{O_2}, nor in oxidation of substrate acetate-1-C^{14} to CO_2. The increased keto-acid radioactivities confirm the results obtained in the previous study. The values for residual steam labile substances left in the incubation mixture indicate that there was no significant change in utilization of the substrate by the treated homogenate system. These data reaffirm the hypothesis stated above, namely, the primary action of SC-11952 in reducing cholesterol synthesis must have its site at some enzyme system that follows acetoacetyl-CoA formation, and precedes mevalonate synthesis.

Discussion. The similarity of the apparent primary site of action of SC-11952 and that reported for cholesterol(1,2,6) has led us to term the azasterol as "cholesteromimetic" with respect to its action on the synthesis of cholesterol *in vitro*. The compound is not an antimetabolite as defined by Woolley(7), since, although it is structurally related to cholesterol, it does not block the action of cholesterol in its feedback control of sterol synthesis but rather mimics it. An alternative term might be "parametabolite" because the compound seemingly parallels the action of exogenous cholesterol in reducing cholesterol synthesis. The first alternative, cholesteromimetic, is preferred since it is an adjective that is more descriptive of the type of action the inhibitor exhibits.

SC-11952 does not inhibit acetate oxidation, fatty acid synthesis or keto-acid formation. One might argue that the effect of SC-11952 may not be inhibition of HMG-CoA-reductase but rather the potentiation of HMG-CoA deacylase, thereby reducing HMG-CoA available for mevalonate synthesis. We do not feel these data support this alternate hypothesis since stimulation of deacylase activity should not result in the enhanced fatty acid synthesis observed here. Furthermore, preliminary experiments have indicated that SC-11952 had little effect on HMG synthesis from acetate-1-C^{14} but that the inclusion of C^{14} into mevalonate was definitely depressed. SC-11952 inhibits acetate-1-C^{14} incorporation into cholesterol to a greater extent that mevalonate-2-C^{14} inclusion into the sterol product. By inference, then, we must conclude that its apparent primary site of action is at the level of HMG-CoA reductase. Experiments to test this hypothesis directly are in progress.

The measurable inhibition of cholesterol formation from mevalonate may indicate a second site of action of SC-11952. Such a response to cholesterol feeding was also found in the data of Bucher *et al.*(6) and Siperstein and Guest(1,2). Preliminary experiments have shown SC-11952 to reduce the rate of squalene cyclization to lanosterol when mevalonate-2-C^{14} was the substrate. Whether this is a direct effect of the azasterol on squalene synthetase or a reflection of a reduced rate of endogenous squalene precursor formation has not been determined.

SC-11952 is effective in reducing plasma cholesterol levels of normal and hypercholesterolemic rats, normal dogs and man. These results will be reported elsewhere.

Summary. A new inhibitor of cholesterol synthesis, SC-11952, closely related struc-

turally to cholesterol, has been characterized in terms of its effects on acetate and mevalonate metabolism *in vitro*. The compound did not reduce acetate conversion to CO_2, fatty acids, or keto-acids but did inhibit incorporation of this substrate into cholesterol. Inhibition of mevalonate conversion to cholesterol was less than when acetate was the substrate. The similarity of the effects of SC-11952 to those produced by cholesterol feeding is discussed.

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Water and Electrolyte Pharmacodynamics of Aspirin.* (27347)

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Acetylsalicylic acid (aspirin) was introduced into therapy by Dreser in 1899(1) and has since occupied a prominent position as an analgesic and antipyretic. Although aspirin analgesia is difficult to evaluate and explain, the antipyretic action has been resolved, tentatively, to increased heat loss *via* hydremia, vasodilation, and sweating(2,3,4). On this basis an assessment of aspirin-induced alterations of relatively simple parameters of fluid biodynamics would not only contribute to the pharmacology of the drug but also might provide a test for aspirin-like substitutes. Consequently, this study concerns the effects of aspirin on urinary sodium, potassium and volume and extra-cellular fluid space in influenza-infected and non-infected animals.

Methods. Male albino Webster Swiss mice (20-30 g) were hydrated with 5 ml of distilled water per 100 g of body weight *per os*. Aspirin dissolved in the hydration fluid was given in a dose of 10 mg/kg of body weight (0.02% solution for proper volume). Dilute acetic acid (placebo) having a pH (3.2) comparable to the aspirin solution was given to some animals instead of distilled water. Prior observations indicated that starvation for 16-24 hours before testing was unnecessary.

Immediately following hydration, urine was collected in 15 ml centrifuge tubes for 3 hours as follows: Silicone lubricant was applied to the inner surface of 10 glass funnels (diam. 150 mm, stem length 150 mm) held in a suitable rack. Watch glasses (diam. 100 mm) were treated similarly with silicone and each inverted and fixed to the inside of a funnel at 3 points with paraffin on surgical adhesive tape so that it rested at a slight angle. The fit of the watch glass and funnel sides allowed passage of urine but not feces. Three mice were placed in each funnel which was then covered with a watch glass (diam. 150 mm) and secured with tape. Urine volume was measured and specimens refrigerated in stoppered plastic tubes for Na^+ and K^+ determinations (Coleman Model 21 flame photometer).

Thiocyanate spaces also were calculated as a relative estimation of extracellular fluid volume(5,6). Each mouse received 0.5 or 0.75 mg of Na thiocyanate i.v.. Serum thiocyanate concentrations were determined at the end of the first, second and third hours on

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