Effect of a new inhibitor of cholesterol biosynthesis (AY 9944) on serum and tissue sterols in the rat

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ABSTRACT AY 9944 is a novel inhibitor of cholesterol biosynthesis which appears to act on the Δ^7-reductase enzyme, interfering with the conversion of 7-dehydrocholesterol to cholesterol. In all tissues studied, with the exception of the lens, there was a reduction in cholesterol and a marked accumulation of 7-dehydrocholesterol. Isotope studies showed a reduced uptake of acetate into cholesterol, and an increased uptake into 7-dehydrocholesterol, in various tissues in vivo and by skin samples in vitro. There was no apparent accumulation of lanosterol or of the methostenols in the rat skin.

KEY WORDS AY 9944 · inhibitor · biosynthesis · cholesterol · 7-dehydrocholesterol · accumulation · skin · aorta · liver · adrenal · kidney · lens · serum · rat · lanosterol · methostenol · Δ^7-reductase

In the biosynthesis of cholesterol, the steps leading from acetate to lanosterol are generally agreed upon (1). Two possible pathways leading from lanosterol to cholesterol have been identified, but their relative importance is not yet known. Fish, Boyd, and Stokes (2) have suggested that in some tissues the reduction of the side-chain double bond (C_{24}-C_{25}) may occur immediately after lanosterol synthesis, whereas in other tissues (brain of chick embryo) the nuclear alterations proceed to completion first, producing desmosterol as the immediate precursor of cholesterol. Kandutsch and Russell (3) have suggested that in preputial gland tumor, skin, and intestine the pathway from lanosterol to cholesterol is via the C_{24}-C_{25} saturated intermediates. Following the administration of triparanol (MER-29) to rats we observed (4) the accumulation of several sterol intermediates with a C_{24}-C_{25} double bond in the skin of the treated rats. Similar observations were also made by Clayton, Nelson, and Frantz (5) and confirmed the existence of a pathway from lanosterol to cholesterol by way of the C_{24}-C_{25} unsaturated intermediates.

Goodman, Avigan, and Steinberg (6) presented evidence, however, that the normal pathway from lanosterol to cholesterol in rat liver involves the saturation of the Δ^{24} double bond at a stage between C-30 and C-27. Thus, neither dihydrolanosterol nor desmosterol appears to lie on the major normal pathway of cholesterol biosynthesis in the liver.

Recent studies with a new inhibitor of cholesterol biosynthesis, AY 9944 [(*trans*-1,4-bis(2-chlorobenzylaminomethyl) cyclohexane dihydrochloride)] have shed additional light on the final steps in the "saturated side-chain" pathway from lanosterol to cholesterol. This inhibitor has been shown to interfere with the conversion of 7-dehydrocholesterol to cholesterol in the rat liver, so that serum cholesterol levels fall and serum 7-dehydrocholesterol levels increase (7). The data reported below indicate that this compound

Abbreviations: GLC, gas–liquid chromatography; TLC, thin-layer chromatography; 7-DH, 7-dehydrocholesterol; AY, AY 9944.

Systematic names of the sterols referred to in the text by their trivial names only are as follows:

Cholesterol: cholest-5-en-3β-ol
Δ^7-Cholestenol: 5α-cholest-7-en-3β-ol
7-Dehydrocholesterol: 5α-cholesta-5,7 dien-3β-ol
Methostenol: 4-methyl-5α-cholest-7-en-3β-ol
Dehydromethostenol: 4α-methyl-5α-cholesta-7,24-dien-3β-ol
Lanosterol: 4,4,14α-trimethyl-5α-cholesta-8,24 dien-3β-ol
Dihydrolanosterol: 4,4,14α-trimethyl-5α-cholest-8-en-3β-ol.

TABLE 1 BODY AND ORGAN WEIGHTS AT TIME OF SACRIFICE

Group	Body	Liver	Adrenal*	Aorta	Kidney†
	g	*g/kg body wt*	*mg/kg body wt*	*mg/kg body wt*	*g/kg body wt*
Control (6)	559 ± 64	27.6 ± 3.4	105.1 ± 18.2	296.6 ± 57.3	3.01 ± 0.23 (3)
AY 9944 (6)	451 ± 18	32.7 ± 2.5	197.5 ± 33.2	286.9 ± 86.9	3.49 ± 0.24 (3)
P	>0.01	>0.002	>0.001		

Numbers in parentheses indicate number of animals in each group.
* Pooled wt. of both adrenals.
† One kidney.

inhibits cholesterol biosynthesis in many tissues and invariably results in accumulation of 7-dehydrocholesterol in substantial amounts.

PROCEDURE

Male Wistar rats weighing approximately 250 g were fed standard rat cubes, and the experimental group received the drug (AY 9944) dissolved in 5% dextrose in water at a concentration of 84 mg/liter. The drug was consumed ad libitum for 3 months. Postulating an average intake of 20 ml/day, the average daily dose would be 10 μmole/kg per day. At the end of the experiment both the experimental and control rats received acetate-^{3}H, 244 uc/250 g of body weight intraperitoneally. Thereafter, they were sacrificed in pairs at 24 hr intervals. Samples of blood, liver, kidney, and skin, as well as both adrenals and the whole aorta were removed for analysis. In a few animals the lenses of the eyes were removed and pooled for analysis.

Duplicate 1 g samples of skin were minced with scissors into fine fragments. Liver slices weighing between 400 and 500 mg were prepared with a Stadie-Riggs microtome. The skin and liver samples were incubated in Krebs-Ringer phosphate buffer (pH 7.4) with 12.2 uc of acetate-2-^{14}C for 2 hr at 37°. Carrier sodium acetate was present at a concentration of 60 μM. All the tissue samples were digested in alcoholic KOH under nitrogen and the nonsaponifiable fraction was extracted with hexane. An aliquot was examined by GLC (SE-52, 1.16% on silanized Gas Chrom S) at 220° and the remainder was chromatographed on thin layers of silica gel pre-stained with Rhodamine 6G (Allied Chemicals, New York).

The TLC methods used have been described in detail by Avigan, Goodman, and Steinberg (8). Plates were developed under nitrogen. A preliminary separation of the skin sterols was carried out using benzene–ethyl acetate 20:1 as the solvent. Five UV-absorbing or fluorescent zones could be identified and have been characterized in a previous study by Horlick and Avigan (4). 7-DH, which absorbs strongly in the UV, lay between cholesterol and Δ^7-cholestenol and there was some overlap between these zones. Therefore, the three zones were scraped from the plate together, eluted with chloroform, and rechromatographed on silver nitrate-impregnated silica gel. A clear separation of all three components was obtained. No corrections were applied for possibly incomplete recoveries. The nonsaponifiable material from liver, adrenal, kidney, aorta, and lens was chromatographed on silver nitrate-impregnated plates in order to isolate the 7-DH for quantification by GLC against standards of known concentration.[1] Δ^7- and Δ^8-stenols cannot be clearly separated by the methods used here. Therefore, values given under Δ^7 may contain an unknown amount of Δ^8. Aliquots were assayed for radioactivity in a Packard liquid scintillation spectrometer.

Serum cholesterol and 7-dehydrocholesterol were determined by digitonin precipitation in duplicate samples. One sample was subjected to the Liebermann-Burchard color reaction and the "fast" and "slow" reacting sterols were determined at 1.5 and 30 min as described by Moore and Baumann (9). The second sample of digitonide was cleaved with pyridine and the free sterols were separated by TLC on silver nitrate-impregnated silica gel, eluted with chloroform, and quantified by GLC against standards of known concentration.

RESULTS

Body weights and organ weights (Table 1). The AY 9944-treated rats were almost 100 g lighter than the controls at the time of sacrifice. Their adrenal glands, however, were markedly hypertrophied. Their livers were slightly heavier than those of the controls expressed as grams per kilogram of body weight, but the kidneys and aortas were unchanged in weight.

Serum sterols (Table 2). Serum cholesterol was substantially reduced in the AY 9944-treated animals. There was, however, considerable accumulation of 7-

[1] Cholesterol was obtained from British Drug Houses, Δ^7-cholestenol and 7-DH from Nutritional Biochemicals, lanosterol (contaminated with dihydrolanosterol) and methostenol from Dr. J. Avigan.

TABLE 2 EFFECT OF AY 9944 ON SERUM STEROLS

Group	Cholesterol	7-DH*	7-DH†	Total sterols
	mg/100 ml	*mg/100 ml*	*mg/100 ml*	*mg/100 ml*
Control (7)	73.8 ± 9.37	0	0	73.8
AY 9944 (6)	23.9 ± 8.19	29.03 ± 11.5	30.49 ± 18.76	52.98
P	>0.001			

Numbers in parentheses indicate number of animals in each group.
* By differential spectrometry.
† By GLC.

TABLE 3 TISSUE STEROLS OF CONTROL AND AY-TREATED RATS

	Control	AY 9944	
	Cholesterol	Cholesterol	7-DH
	mg/100 mg tissue		
Liver (4)*	0.18 ± 0.07	0.06 ± 0.02	0.24 ± 0.07
Adrenals (4)	2.38 ± 0.38	0.18 ± 0.08	0.47 ± 0.25
Kidney (3)	0.45 ± 0.10	0.25 ± 0.04	0.37 ± 0.04
Aorta (5)	0.10 ± 0.02	0.05 ± 0.01	0.10 ± 0.03
Lens (6)†	0.30	0.16	

* Number of samples studied.
† Single pool of 6 lenses.

TABLE 4 RADIOACTIVITY OF TISSUE STEROLS IN CONTROL AND AY-TREATED RATS

	Control	AY 9944	
	Cholesterol	Cholesterol	7-DH
	cpm/100 mg tissue		
Liver	170.1	37.4	219.4
Adrenals	1051	161	250
Kidney	191.3	27.8	170.6
Aorta	36	22	40

Values represent incorporation of acetate-^{3}H injected intraperitoneally 24 hr before sacrifice.

dehydrocholesterol in the serum. The values for 7-DH obtained by two separate methods showed close agreement. Total sterol content of the serum was lower in the treated animals than in the controls.

Tissue sterols (Table 3). Cholesterol was markedly reduced in all the tissues examined, and 7-DH accumulated to a considerable degree in all tissues except the lens of the eye. In some tissues, such as the liver, kidney, and aorta the accumulation of 7-DH was great, so that the total sterol content was greater than in the control sample. In the adrenals, cholesterol was reduced to one-tenth of its original content and despite the substantial increase in 7-DH, total sterol content was only about one-quarter of the control values. No 7-DH was found in the pooled samples of lens tissue from the treated rats. Radioactivity incorporated from injected acetate-^{3}H into the tissue sterols is shown in Table 4.

Skin sterols (Tables 5, 6). Figure 1A is a GLC trace of the crude nonsaponifiable fraction from a control skin sample. At least six components can be separated and have been identified as shown. Cholesterol appears to be the major component, but Δ^7-cholestenol constitutes as much or more when corrections for mass area response are applied. The standard methostenol gave one major peak on GLC, which corresponded to the major peak of the pair marked M and DHM in Fig. 1. The "methostenols" obtained from skin could be separated into two components by TLC on silver nitrate-impregnated silica gel. The major component, with the higher R_F value, had the same mobility as methostenol, whereas the minor component presumably corresponded to dehydromethostenol. The identification of the dehydromethostenol must be considered tentative. The dihydrolanosterol and lanosterol peaks corresponded with those of authentic samples on GLC, and could be clearly separated into two spots by silver nitrate TLC. Figure 1B is from an AY-treated rat. The cholesterol peak is greatly reduced, and there is a very large peak consisting of Δ^7-cholestenol and 7-dehydrocholesterol. The other components do not appear to be appreciably changed. The combined cholesterol, Δ^7-cholestenol and 7-DH bands were rechromatographed on silver nitrate plates. 7-DH and Δ^7-cholestenol were separated and the results from three rats in each group are shown in Table 5. In the skin of AY-treated rats, the cholesterol was reduced, the Δ^7-cholestenol was unchanged, and there was a great increase in 7-DH.

Radioactivity incorporated by each fraction (in vitro) is shown in Table 6. Because of the small number of samples and the large scatter of the results, only the mean values are shown. Only the 7-dehydrocholesterol fraction differed significantly from the control.

Aorta sterols (Table 3, Fig. 2). The nonsaponifiable material extracted from the aortas of control and AY-treated rats was subjected to TLC on silica gel. GLC patterns for the three bands removed are shown. Of interest was the increase in total sterol in the AY-treated rat, accounted for mainly by the large amount of 7-DH present.

DISCUSSION

The work of Mercer and Glover (10) suggested that cholesterol, 7-dehydrocholesterol, and Δ^7-cholestenol

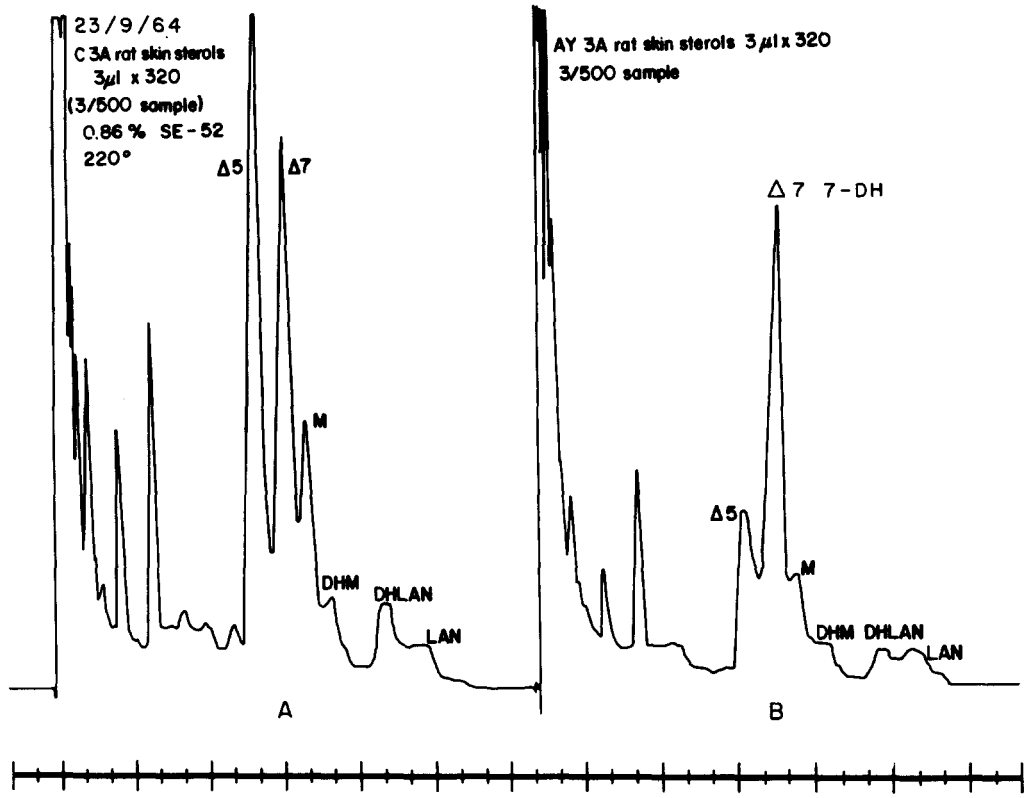

FIG. 1. GLC of free sterols of rat skin. C3A is a control rat skin extract, AY 3A is from an AY-treated rat. Each trace represents 3/500 of the nonsaponifiable fraction extracted from 1 g of skin. Δ^5 = cholesterol, Δ^7 = Δ^7-cholestenol, 7-DH = 7-dehydrocholesterol, M = methostenol, DHM = 24-25 dehydromethostenol, DHLAN = dihydrolanosterol, LAN = lanosterol.

TABLE 5 SKIN STEROLS IN CONTROL AND AY-TREATED RATS

	Control (3)		AY (3)		
	mg/g	%	*mg/g*	%	*P*
Cholesterol	0.65 ± 0.16	32.0	0.20 ± 0.06	15.2	>0.02
7-Dehydrocholesterol	0.08 ± 0.01	3.8	0.35 ± 0.12	26.9	>0.02
Δ^7-Cholestenol	0.66 ± 0.34	32.3	0.37 ± 0.11	28.1	
Methostenol	0.28 ± 0.14	13.9	0.15 ± 0.09	11.2	
Dehydromethostenol	0.10 ± 0.04	5.1	0.07 ± 0.04	5.2	
Dihydrolanosterol	0.16 ± 0.04	8.0	0.09 ± 0.04	7.0	
Lanosterol	0.10 ± 0.03	4.9	0.08 ± 0.04	6.2	
Total	2.03 ± 0.62		1.31 ± 0.45		

Numbers in parentheses indicate number of animals in each group.

TABLE 6 IN VITRO INCORPORATION OF ACETATE-^{14}C BY 1-g SAMPLES OF SKIN FROM CONTROL AND AY-TREATED RATS

	Control (4)		AY-Treated (4)		
	cpm/fraction	*% of total*	*cpm/fraction*	*% of total*	*P*
Cholesterol	188.2	5.8	63.3	3.5	
7-Dehydrocholesterol	47.5	1.5	195	10.6	<0.001
Δ^7 Cholestenol	189.9	5.8	171	9.3	
Methostenol	234.2	7.2	156.4	8.5	
Lanosterol	209.1	6.4	133.8	7.3	
Squalene, hydrocarbon	2369.9	72.7	1093.6	59.6	
(Origin)	21.3	0.6	22.2	1.2	
Total	3260.1	100.0	1835.3	100.0	

Numbers in parentheses indicate number of animals in each group.

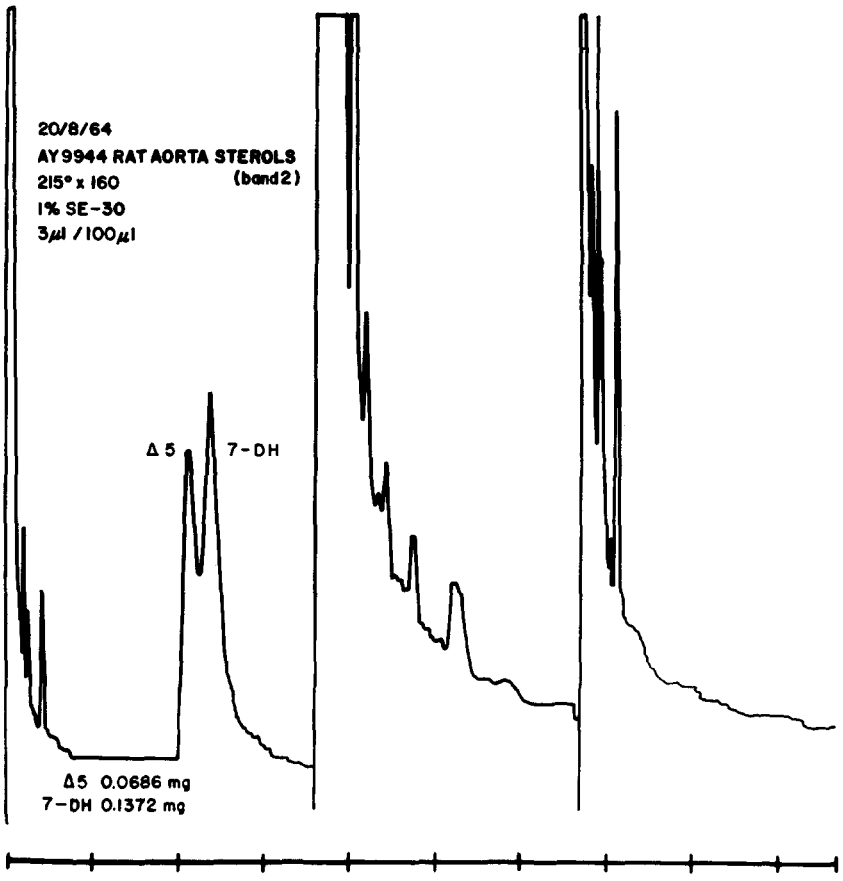

FIG. 2. GLC of free sterols from aorta of rat treated with AY 9944. The nonsaponifiable material from one aorta was subjected to TLC on silica gel. GLC patterns for the three bands removed are shown. On the left, Δ^5 (cholesterol) and 7-DH (7-dehydrocholesterol). In the middle panel a homologous series of peaks (probably alcohols) may be seen. The panel on the right showed only one early peak (unidentified). In the normal (control) aorta (not shown), only Δ^5 (cholesterol) and the alcohols are seen.

are in equilibrium in animal tissues. They reported the conversion of cholesterol to 7-dehydrocholesterol and also the conversion of 7-dehydrocholesterol to Δ^7-cholestenol, with the latter reaction predominating. Frantz, Sanghvi, and Schroepfer (11), who repeated Mercer and Glover's experiments, found that the biogenetic reaction sequence from Δ^7-cholestenol through 7-dehydrocholesterol to cholesterol was essentially irreversible. AY 9944 appears to be a specific inhibitor of the Δ^7-reductase enzyme and blocks the transition from 7-DH to cholesterol.

In AY-treated rats, all of the tissues studied (with the exception of the lens) showed a very significant accumulation of 7-DH. In some tissues (liver, kidney, aorta) this was so great that the total sterol content was increased despite a marked reduction in cholesterol. In other organs, skin, adrenals, and in serum the total sterol content was reduced, despite the accumulation of 7-DH. In the skin, where other precursors of cholesterol could be studied, we found no significant changes other than a reduction in cholesterol and increase in 7-DH. In other organs (liver, adrenal) we were unable to detect sterols other than cholesterol and 7-DH. The inhibiting effect of AY 9944 on cholesterol biosynthesis was also demonstrated in vivo and in vitro using radioactive acetate. In both instances radioactivity of the cholesterol fraction declined and radioactivity accumulated in the 7-DH fraction.

Of interest was the accumulation of 7-DH and the increase in total sterol in the rat aorta after 3 months of treatment (see Fig. 2). It has been shown that triparanol, which inhibits the sterol Δ^{24}-reductase on the alternative pathway to cholesterol, also lowers cholesterol levels but results in accumulation of desmosterol in many tissues and in the aorta (12, 13). Thus, it would appear that interference with cholesterol metabolism in either of the postulated pathways at a stage beyond cyclization of squalene may result in the accumulation of substantial amounts of precursors whose atherogenic potential is as yet unknown.

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