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Sterols of skin in the normal and triparanol-treated rat

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SUMMARY

Skin of normal rats and of rats treated with triparanol were incubated with acetate-2-H^3 for 2 hr, and the sterols were extracted and separated chromatographically. Treatment with triparanol resulted in grossly visible changes (scaliness, roughness, and atrophy of the skin) and in a marked alteration in the metabolism of skin sterols. There were considerable reductions in the concentrations of compounds with saturated side chains: cholesterol, methostenol, and dihydrolanosterol; and increases in the concentrations of sterols with unsaturated side chains: desmosterol, lanosterol, and sterols tentatively identified as dehydromethostenol and Δ^7,24-cholestadienol. Isotope data showed that after incubation for 2 hr, a large proportion of the counts were in squalene in skin from both normal and triparanol-treated rats. There was a reduction in the incorporation of radioactivity into the Δ^5-stenol fraction and an increase in the incorporation into lanosterol and Δ^7-sterols, in skin from triparanol-treated rats. Triparanol inhibits the enzyme responsible for reduction of the 24,25-double bond in the sterols of rat skin.

The mechanism of sterol biosynthesis in mammalian skin is generally believed to be similar to that in the rest of the organism. The composition of the skin sterols, however, differs markedly from their composition in other organs. Skin contains significantly higher concentrations of various sterols that serve as normal intermediates in the biosynthesis of cholesterol. Skin, then, would appear to be an ideal site for studying the biosynthesis of cholesterol in normal animals and for observing the effects of inhibitors of biosynthesis.

Previous studies on the composition of rat skin include those of Moore and Baumann (1), who identified 7-dehydrocholesterol among the "fast-acting" sterols from rat skin, and Idler and Baumann (2), who found that Δ^7-cholestenol represents up to 50% of the total sterol fraction. It is interesting to note that Δ^7-cholestenol is less abundant in other mammals (3). Neiderhiser and Wells (4) reported the presence of 4α-methyl-Δ^7-cholestenol (methostenol) in rat skin and other tissues, and Stokes et al. (5) identified 24-dehydrocholesterol (desmosterol) as a normal constituent of rat skin. Gaylor (6) found the most abundant sterol in rat skin to be cholesterol (52%); Δ^7-cholestenol constituted 31.2%, cholestanol 7.5%, desmosterol 2.4%, and 7-dehydrocholesterol 1.9%.

An earlier study of sterol biosynthesis in rat skin was that of Srere et al. (7), who demonstrated the incorporation *in vitro* of radioactive acetate into digitonin-precipitable sterols of rat skin. Later, several workers (8, 9, 10) found that most of the label so incorporated was in sterols other than cholesterol. Gaylor (6) showed that, following incubation *in vitro* of rat skin with C^{14}-labeled acetate, the specific radioactivity of Δ^7-cholestenol was 13 times that of cholesterol, suggesting that it was an important precursor of cholesterol. Similar results were obtained in studies *in vivo* by Wells and Lorah (10), who found that 5 min after injection of labeled acetate the specific activity of skin methostenol was 17 times, and that of the Δ^7-cholestenol fraction 4 times, the specific activity of cholesterol.

Triparanol was first reported, by Blohm and MacKenzie (11), to block biosynthesis of cholesterol at a late stage. Desmosterol was later identified as a major sterol in the tissues of rats treated with triparanol, indicating an inhibition of the reduction of the 24,25-

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double bond (12). Steinberg and Avigan (13) subsequently suggested that the drug might also inhibit reduction of this double bond in compounds with different nuclear structures, thus interfering with cholesterol biosynthesis at several stages and directing the available pathway toward desmosterol as the major end product. The peculiar characteristics of sterol metabolism in skin, namely the tendency for accumulation of intermediates, suggested to us that it would be advantageous to study the effects of triparanol in this organ. This consideration, and the availability of new chromatographic techniques for the separation and identification of closely related sterols, prompted the present study.

PROCEDURES

Male rats of the Sprague-Dawley strain weighing 200–250 g were used. Some of the rats received 0.1% triparanol in their diet for periods of 2–6 weeks. The animals were killed by a blow on the back of the neck, their backs were shaved, and the dorsal skin was removed and freed from fat and loose connective tissue by scraping. Duplicate samples of 1 g were taken from each animal and cut with scissors into small pieces. All these procedures were carried out in a cold room. The incubated sample consisted of Krebs-Ringer phosphate medium (pH 7.4), 6 ml; acetate-2-H^3, 9 μc, 16 μmoles; and 1 g of finely minced skin. The flasks were flushed with 95% O_2 + 5% CO_2 and were stoppered and shaken in a water bath for 2 hr at 37°. At the end of the incubation period, ethanol (6 ml) and enough pellets of KOH to make a 20% solution were added. The flasks were flushed with nitrogen, and were stoppered and heated on a steam bath for 4 hr. The contents of the flasks were then transferred to separatory funnels, and the flasks were washed with 10 ml distilled water and 25 ml hexane, and the nonsaponifiable lipids were extracted three times with hexane. The combined hexane extracts were washed once with 10% KOH and several times with distilled water. They were then evaporated almost to dryness under nitrogen and, finally, were made up to appropriate volumes in toluene or benzene for chromatographic analysis. Gas-liquid chromatography (GLC) was carried out at 235° on a 1% SE 30 column using an argon ionization detector. In most instances, the sterol mixture was applied directly to the column. Some samples, however, were first chromatographed by thin-layer technique (TLC) (14) and separated into major component groups. Chromatography was carried out on 40-cm plates coated with silica gel, into which Rhodamine-6G had been incorporated. The solvent was benzene–ethyl acetate 20:1 (v/v) and the plates were run in the dark for 24 hr. Several zones could be clearly distinguished; these were scraped from the plates and the sterols were eluted with chloroform or methylene chloride. After evaporation of the solvent under nitrogen, the sterols were taken up in benzene or toluene, for counting or GLC analysis. Radioactivity was measured with a Packard Tricarb Scintillation Spectrometer. Samples of cholesterol, Δ^7-cholestenol, 7-dehydrocholesterol, desmosterol, dihydrolanosterol, lanosterol, and methostenol were used for identification of the various peaks obtained by GLC and as standards for quantitative analysis. The standard solutions were chromatographed at various concentrations, both separately and in combination, and the mass-area relationships were determined for each component.

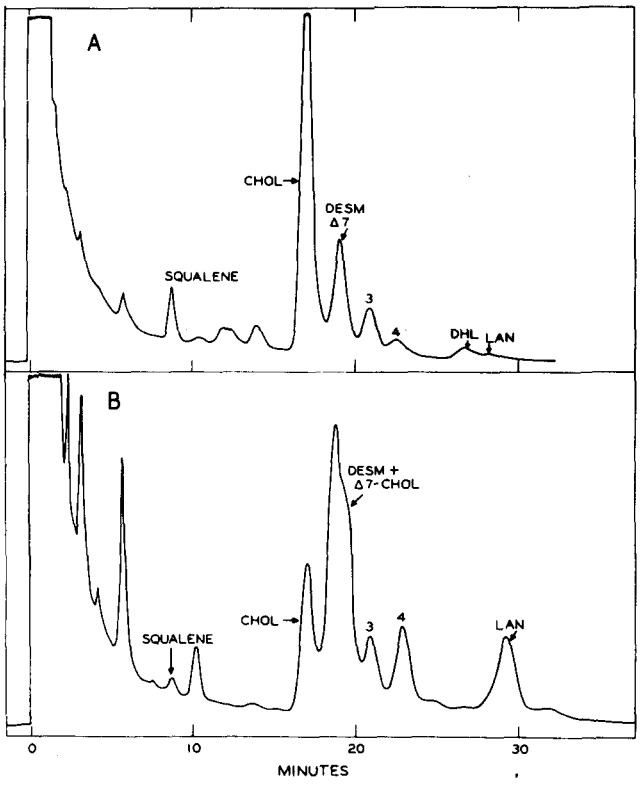

FIG. 1. GLC patterns of nonsaponifiable lipids in skin of (A) normal rat, (B) triparanol-treated rat.

RESULTS

Normal Rat Skin. Figure 1A shows the pattern obtained by GLC analysis of the total sterol extract of normal rat skin. Six peaks may be seen; four of them were identified as cholesterol, Δ^7-cholestenol (coinciding with desmosterol), dihydrolanosterol, and lanosterol.

The two peaks (labeled 3 and 4) between Δ^7-cholestenol and 24,25-dihydrolanosterol are probably methostenol and 24,25-dehydromethostenol. The standard methostenol sample gave two peaks that coincided with peaks 3 and 4. Peaks 3 and 4 did not separate on TLC and ran together with the standard methostenol. Table 1 shows the distribution of the various components, as calculated from the GLC pattern of (1) total nonsaponifiable material, and (2) fractions obtained by preliminary separation of the sterols by TLC. This second procedure showed that some of the peaks obtained by GLC of the total nonsaponifiable lipids consisted of several components, and, therefore, permitted a more precise quantitative determination of the various sterols. It was performed as follows.

The entire TLC plate was separated into zones. Figure 2A shows the distribution of the zones and the corresponding GLC analysis of each of them. Zone 1 gave only one peak, which was identified as squalene. Zone 2 contained 24,25-dihydrolanosterol and lanosterol. Zone 3 contained a number of more volatile components (fatty alcohols), not shown on the figure and not further studied, and the two peaks 3 and 4, tentatively identified as methostenol and dehydromethostenol. Zone 4 contained cholesterol and desmosterol. Zone 5, on the boundary between zones 4 and 6, was dark in ultraviolet light; it contained small amounts of cholesterol, desmosterol, Δ^7-cholestenol, and 7-dehydrocholesterol. The last named was detected on TLC but, because of its low concentration, did not appear on GLC. Because of the incomplete separation of 7-dehydrocholesterol from Δ^7-cholestenol on GLC, we have grouped it with the Δ^7-cholestenol. Zone 6 contained most of the Δ^7-cholestenol and only traces of less saturated sterols, and zone 7 did not contain any sterol material. The distribution of radioactivity among the sterols after incubation of skin with acetate-2-H^3 is shown in Table 2.

The compounds eluted from zone 6 were acetylated (14) in the presence of 1 mg each of carrier zymosterol ($\Delta^{8,24}$-cholestadienol) and of Δ^7-cholestenol and were rechromatographed on long plates coated with Silica Gel G with a solvent mixture of hexane–benzene 6:1. This was done because the nuclear structure of the sterol component(s) of zone 6, which have an unsaturated side chain, was uncertain ("$\Delta^{7,24}$" or "$\Delta^{8,24}$", or a mixture of both). The two can be expected to have very similar chromatographic mobilities. The distribution of radioactivity in the fractions obtained is shown in Table 3.

Skin of Triparanol-Treated Rats. The skins of the treated rats showed gross morphological changes from

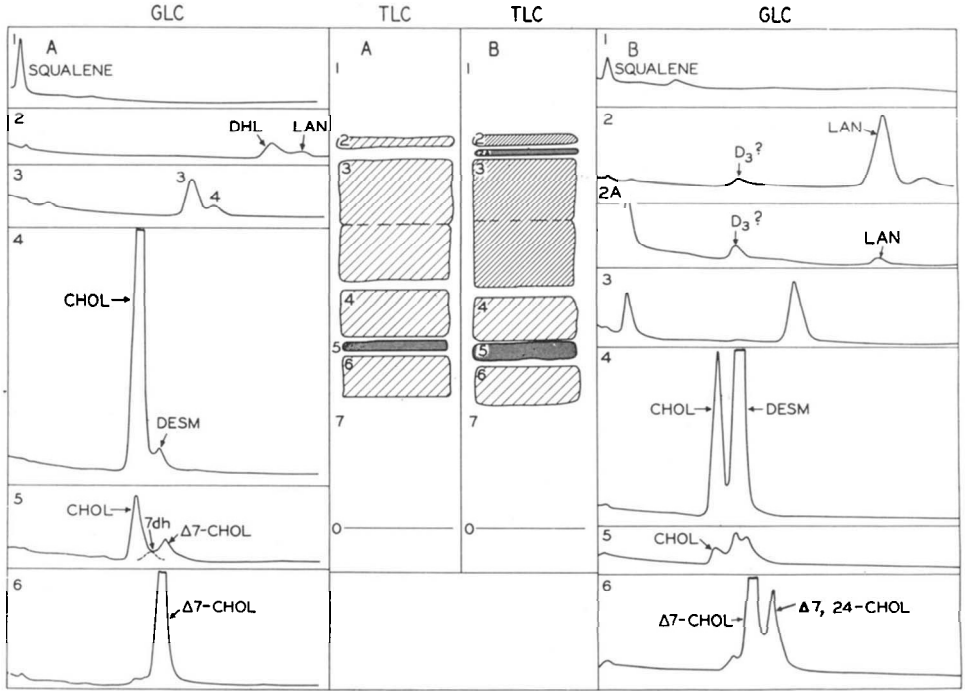

FIG. 2. **TLC patterns and the corresponding GLC peaks** of nonsaponifiable lipids of skin of: (A) normal rat. **(The position in which 7-dehydrocholesterol** should have appeared is indicated on the chromatogram of zone 5. **Small amounts would not** have been visible because of some trailing of the cholesterol peak.) (B) triparanol-treated rat.

TABLE 1. COMPOSITION OF RAT SKIN STEROLS ANALYZED BY GLC WITH AND WITHOUT PRELIMINARY FRACTIONATION BY TLC

	Normal		Triparanol-Treated	
	Without TLC*	With TLC	Without TLC†	With TLC
	%	%	%	%
Cholesterol	55.0 ± 4.5	62.6	7.4 ± 2.2	12.4
Desmosterol		5.2		28.3
Δ^7-cholestenol (+ $\Delta^{7,24}$-cholestadienol)	24.9 ± 3.3	19.3	51.1 ± 7.7	25.5
Methostenol‡	8.4 ± 1.9	3.8	7.5 ± 1.2	0
Dehydromethostenol‡	3.0 ± 0.8	1.4	8.7 ± 1.2	6.5
Dihydrolanosterol	6.0 ± 1.3	5.8	0	0
Lanosterol	2.7 ± 0.5	2.0	25.3 ± 6.0	27.3

* Mean of results in 4 animals ± standard deviation.
† Mean of results in 5 animals ± standard deviation.
‡ Tentative identification.

TABLE 2. DISTRIBUTION OF RADIOACTIVITY IN THE STEROLS OF SKIN OF NORMAL AND TRIPARANOL-TREATED RATS AFTER INCUBATION WITH ACETATE-2-H^3

Chromatographic Zones Analyzed	Normal	Triparanol-Treated
	%	%
1 (squalene)	56.7	29.1
2 (dihydrolanosterol, lanosterol)	7.3	26.5
3 (GLC peaks 3 and 4)	5.0	12.9
4 (cholesterol, desmosterol)	12.5	2.1
5 (cholesterol, 7-dehydrocholesterol, Δ^7-cholestenol)	6.0	5.4
6 (Δ^7-cholestenol)	11.9	21.9
7 (no sterol)	0.6	2.1

normal. They were almost hairless, thin, roughened, scaly, and distinctly atrophic. These changes are analogous to those described in some patients treated with triparanol (15). Figures 1B and 2B and Table 1 show that treatment with triparanol produced striking changes in the sterol pattern of rat skin. Most marked were the reduction in cholesterol, and the great increases in desmosterol and in lanosterol. Moreover, 24,25-dihydrolanosterol and methostenol virtually disappeared. A heavy UV-absorbing band, appearing at the border of zones 2 and 3, behaved chromatographically on TLC and had the same retention time on GLC as vitamin D_3. There was a definite increase in the concentration of the fatty alcohols as determined by GLC. Upon GLC of the compounds in zone 6 (Δ^7-stenols), a new peak appeared, which had not been seen in the normal rat, and which we believe to be $\Delta^{7,24}$ (or $\Delta^{8,24}$-)cholestadienol for reasons given below. This new compound has been grouped with Δ^7-cholestenol in Tables 1 and 2.

The distribution of radioactivity among the sterols following incubation of the skin with acetate-2-H^3 is shown in Tables 2 and 3. After acetylation of the compounds in zone 6 and rechromatography as described before, we noted three well-resolved fluorescent bands. The most rapidly migrating fraction was Δ^7-cholestenol. The middle band included zymosterol and possibly $\Delta^{7,24}$-cholestadienol, and contained almost all the radioactivity of zone 6 (Table 3). The identity of the most polar component was not known.

Treatment with triparanol resulted in a moderate change in total sterol content of skin, and in striking changes in composition of the sterol constituents. There was an increased amount of sterol per gram of skin in the triparanol-treated animals (from 2.80 to 5.22 mg/g), but there was less tissue because the skin of treated animals was considerably thinner than normal skin. The total amount of radioactivity incorporated *in vitro* into the sterols of skin was not changed significantly by triparanol (Table 4). The incorporation of labeled acetate into the fatty acid fraction of skin (not shown here) was also unaffected by treatment with triparanol.

DISCUSSION

A combination of TLC and GLC has permitted adequate separation of most of the sterols in rat skin with the exception of 7-dehydrocholesterol, which was not

TABLE 3. DISTRIBUTION OF RADIOACTIVITY AMONG THE Δ^7-STEROLS FROM SKIN OF NORMAL AND TRIPARANOL-TREATED RATS AFTER INCUBATION WITH ACETATE-2-H^3

Compound*	Normal	Triparanol-Treated
	%	%
Δ^7-cholestenol	69.5	7.0
$\Delta^{7,24}$-(or $\Delta^{8,24}$-)cholestadienol	30.5	34.0
Unknown		59.0

* The compounds were isolated as a group (zone 6) by TLC and acetylated in the presence of nonradioactive carrier compounds, and the acetylated derivatives were separated by TLC as described in the text.

TABLE 4. TOTAL STEROL CONCENTRATION AND INCORPORATION OF ACETATE-2-H^3 INTO TOTAL SKIN STEROLS OF NORMAL AND TRIPARANOL-TREATED RATS

	Total Sterols*	Acetate-2-H^3 Incorporated*
	mg/g skin	*cpm/g skin*
Normal	(4) 2.80 ± 0.56	(3) 10,741 ± 3,812
Triparanol	(5) 5.22 ± 0.90	(4) 7,418 ± 3,980

* Mean ± standard deviation. Figures in parentheses indicate number of animals.

properly separated from Δ^7-cholestenol. The concentrations of cholesterol, desmosterol, and Δ^7-cholestenol are in good agreement with those previously reported by Gaylor (6). GLC peaks 3 and 4 probably represent methostenol and 24,25-dehydromethostenol. This identification is based on similarities in chromatographic behavior, and on the fact that peak 3 disappeared in the triparanol-treated animal while the area of peak 4 increased. The combined concentration of the compounds represented by peaks 3 and 4 approached the previously reported values for methostenol (4). Of considerable interest is the finding of dihydrolanosterol and lanosterol in normal rat skin in a ratio of 3:1. This is in agreement with the findings in preputial gland tumor tissue (16), but is in contrast with those in liver, where lanosterol is found in concentration similar to that of 24,25-dihydrolanosterol (17).

Treatment with triparanol caused a marked reversal of the cholesterol-to-desmosterol ratio from 12:1 in normal skin to 1:2 in the skin of triparanol-treated rats. The relative amount of desmosterol was thus higher than that previously reported for other tissues of the triparanol-treated rat (12). There was no significant change in the Δ^7-cholestenol content, but two more polar components appeared, containing nearly all the radioactivity of zone 6. One of these components appeared identical to a compound isolated from triparanol-treated rat liver by Goodman, Avigan, and Steinberg[2], and very probably consisted of either $\Delta^{7,24}$- or $\Delta^{8,24}$-cholestadienol, or a mixture of both. $\Delta^{7,24}$-Cholestadienol has been also tentatively identified by Frantz and Mobberley in the liver of the triparanol-treated rat (18). Methostenol and dehydromethostenol also underwent significant changes in concentration following triparanol, as did 24,25-dihydrolanosterol and lanosterol. In both instances, the saturated component virtually disappeared, and there was an accumulation of the unsaturated component. The absolute amount of lanosterol increased to several times the sum of lanosterol and dihydrolanosterol found in normal skin.

Results of incubation *in vitro* of triparanol-treated skin indicate that triparanol treatment blocks reduction of the side-chain double bond in skin in at least four sterols of different configuration: desmosterol→cholesterol; $\Delta^{7,24}$-($\Delta^{8,24}$-)cholestadienol→Δ^7-(Δ^8-)cholestenol; dehydromethostenol→methostenol; lanosterol→dihydrolanosterol.

Reduction of desmosterol and of lanosterol has been shown to be inhibited in liver *in vitro*. The inhibition of the reduction of $\Delta^{7,24}$-(or $\Delta^{8,24}$)-cholestadienol is consistent with the results obtained previously (14) in liver homogenates of triparanol-treated rats after injection of C^{14}-mevalonic acid. Schroepfer (19) recently reported that labeled zymosterol was converted to desmosterol in the triparanol-treated rat, whereas labeled zymostenol (Δ^8-cholestenol) was efficiently incorporated into cholesterol, suggesting an analogous block in the reduction of the side chain of zymosterol.

Thus, there is now evidence that reduction of the side chain in five different sterol intermediates can be inhibited by triparanol. This, combined with the finding that other inhibitors of desmosterol reductase also inhibit lanosterol reduction, makes it likely that there is a single "side-chain reductase" for all the sterols, as proposed by Avigan, Goodman, and Steinberg (14). The marked increase in the concentration of lanosterol after treatment with triparanol may reflect either some direct effect of the drug on the reactions modifying the sterol nucleus as well, or, possibly, that reduction of the nucleus occurs more slowly in sterol substrates that retain the unsaturated side chain than in sterols containing a saturated side chain. Fish et al. (20) have suggested that in some tissues the reduction of the side-chain double bond may occur immediately after formation of lanosterol, whereas in others (e.g., brain of the chick embryo) the nuclear alterations proceed to completion first, producing desmosterol as the immediate precursor of cholesterol. Kandutsch and Russell (16) have suggested that in preputial gland tumor, and probably in skin and intestine, the pathway from lanosterol to cholesterol is via the 24,25-saturated intermediates. Our data clearly show that in the normal rat skin 24,25-unsaturated intermediates are present, and that their concentration is greatly increased in the triparanol-treated rat. The results suggest the existence of an alternate pathway in which saturation of the double bond in the side chain is the last step.

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