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The skin sterols of normal and triparanol-treated rats*

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SUMMARY

The skin sterols of normal rats and of rats treated with the drug triparanol have been analyzed by means of chromatography on silicic acid followed by gas-liquid chromatography of the components of the peaks obtained from the silicic acid chromatogram. These two procedures complement each other in leading to complete separations of pairs of sterols that are poorly separated in either system alone. The results are shown to indicate the presence in rat skin of several previously undetected compounds for which molecular structures are proposed on the basis of the retention factor method. Evidence is presented for at least two effects of the drug triparanol, (1) in causing accumulation of the Δ^{24}-analogues of all the intermediates in cholesterol biosynthesis that normally occur in the 24,25-dihydro form, and (2) in causing marked alterations in the proportions of Δ^{7}- to Δ^{8}-isomers of these intermediates. The significance of these findings is discussed in the light of current views of the pathway of cholesterol biosynthesis.

The drug triparanol (1-*p*-(β-diethylaminoethoxy)-phenyl-1-(*p*-tolyl)-2-(*p*-chlorophenyl)ethanol) has been shown to inhibit cholesterol synthesis while causing concomitant accumulation of its Δ^{24}-analogue, desmosterol (1–3). This sterol is otherwise present in mammalian tissues in only trace amounts (4, 5), but has been found in larger quantities in embryonic vertebrate tissues (6) and in the adult tissues of invertebrates (7). Evidence that desmosterol may be a precursor in cholesterol biosynthesis has been presented (5, 6, 8) and it has been suggested that this compound is probably the immediate precursor of cholesterol in the liver (9).

Reports of the metabolic conversion of various 24,25-dihydrosterols (9–16) to cholesterol, however, have prompted several authors to discuss the question of whether the natural pathway of cholesterol synthesis involves reduction of the Δ^{24}-bond as a terminal step or at one or more earlier points in the metabolic sequence (8, 10, 15–18), but without reaching a satisfactory conclusion. Thus, when this work was begun, it was uncertain whether the accumulation of desmosterol under the influence of triparanol was due solely to a specific inhibition of the reduction of the Δ^{24}-bond of desmosterol, or whether the drug could inhibit the reduction of this double bond in any of several Δ^{24}-intermediates and so block a number of possible pathways from the Δ^{24}-series to the series of sterols with saturated side chains. The second possibility had been discussed by Steinberg and Avigan (8), though, at that time, in the absence of experimental data.

A detailed comparison of the sterols found in normal mammalian tissues with those found after administration of triparanol was undertaken in the hope that further light might be shed on the effects of this drug and, possibly, upon the sequence of events in cholesterol biosynthesis.

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We have previously presented evidence for the accumulation of $\Delta^{7,24}$-cholestadienol in the tissues of triparanol-treated rats (19) and of $\Delta^{5,7,24}$-cholestatrienol in the intestinal tissues of triparanol-treated guinea pigs (20). Evidence for the impaired conversion of zymosterol, but not of Δ^{8}-cholestenol, to cholesterol in the liver of triparanol-treated rats has been obtained by Schroepfer (21), and recently Klein and Szczepanik (22) have given chromatographic data consistent with all of these earlier findings. Further, Avigan et al. (23, 24) have reported results that indicate triparanol inhibition of the reduction of lanosterol to 24,25-dihydrolanosterol. These observations all point to the inhibition of the reduction of the Δ^{24}-bond at one or more stages prior to the conversion of desmosterol to cholesterol.

The sterols of skin were chosen for the present study since this tissue and its embryonic derivatives had already been shown (15–17, 25, 26) to contain relatively large amounts of several compounds present only in traces in other tissues but suspected to be intermediates in the biosynthesis of cholesterol. When the rat was treated with triparanol, a still more complex mixture of sterols appeared in the skin.

These sterol mixtures could be resolved to a considerable extent by chromatography on silicic acid-Super-Cel. Furthermore, on the basis of the known effects of certain variations of molecular structure upon chromatographic behavior and by the use of the Liebermann-Burchard reaction to distinguish between "fast-" and "slow-reacting" sterols, reasonable guesses could be made as to the structures of the materials present in the various peaks.

The resolution on silicic acid columns, however, was not complete; in some instances, single peaks were obtained, corresponding to unresolved pairs of components, while in other regions of the chromatogram, there was only partial separation of peaks.

In the present study, a further examination of the materials eluted from the silicic acid column was carried out by means of gas-liquid chromatography (GLC). For this purpose, the eluted sterols were converted to their methyl ethers and chromatographed on the highly polar liquid phase, polydiethyleneglycol succinate. It has been shown (27) that under these conditions subtle differences of polarity, which apparently are not sufficient to give satisfactory resolution of sterol mixtures on silicic acid columns, lead to clear-cut separations on the gas-liquid chromatogram. Separations in this system, unlike those obtained with silicic acid columns, are also influenced markedly by differences in molecular weight. Moreover, it has been demonstrated that the retention times of sterol methyl ethers undergoing GLC may be calculated accurately from the retention factors characteristic of the various individual structural features of the molecule. This technique therefore provided independent evidence as to the structures of the compounds recovered from the silicic acid chromatogram.

Although chromatographic evidence is insufficient to establish structure, it can provide a valuable basis for further work. In this investigation, two different chromatographic methods have been used in complementary fashion to yield information, on the basis of which some tentative structural assignments can be proposed for the components of this complex sterol mixture. The results of this study are offered as an interim report.

MATERIALS AND METHODS

Female Sprague-Dawley rats weighing about 225 g were maintained on a diet of Purina laboratory chow. Triparanol was fed in an amount of approximately 10 mg/day by dissolving the drug in ether, spraying the solution on the pellets of food, and allowing the ether to evaporate. Triparanol feeding was continued for three months before the animals were killed. For the experiments described below, the skins from three normal and three triparanol-fed rats were used. The skins, which weighed 92 g and 110 g, respectively, were cut into small pieces and refluxed for 3 hr in 15% potassium hydroxide in 95% ethanol (5 ml/g wet tissue). An equal volume of water was added, and the nonsaponifiable fraction was removed by extracting three times with petroleum ether (boiling range 30–60°; volume used for each extraction equal to twice that of the aqueous layer). The petroleum ether extracts were filtered and evaporated to dryness, and the residues redissolved in benzene. For chromatography on silicic acid-Super-Cel, one-fourth of each extract, containing about 50 mg of sterols, was applied to a column 2 cm in diam and 1 m in length. This technique is described in detail in the addendum to this paper. Color development of aliquots of chromatographic fractions in the Liebermann-Burchard reaction after 1.5 and 30 min was measured with reagents prepared according to Abell et al. (28) with readings at 620 mμ.

For the purpose of further characterization of the sterols eluted from the silicic acid chromatograms, selected fractions, usually corresponding to the central, more polar and less polar regions of each peak, were subjected to GLC by the method previously described, the quantitative aspects of which have been fully discussed (27).

To obtain the values for the approximate percentage abundance of each sterol in the total material recovered from the silicic acid columns (Table 4), a further aliquot of the total nonsaponifiable material was chromatographed on silicic acid and the Liebermann-Burchard reaction applied to each fraction as before. The total weight of each fraction was also recorded and the fractions were then recombined into chromatographic zones, corresponding to suitable classes of compounds on the basis of the Liebermann-Burchard analyses and the earlier GLC findings. Aliquots of these reconstituted zones from the silicic acid chromatograms were then analyzed by GLC. The percentage of each individual sterol in the sample was determined by calculation of the area of each peak on the gas-liquid chromatogram as a percentage of the combined areas of all peaks given by the sample. Since the percentage by weight of the total material recovered from the silicic acid columns present in each zone could be calculated, it was possible to derive an approximate value for the amount of each sterol as a percentage of the total recovered material.

7-Dehydrocholesterol, which was not separated from desmosterol in either chromatographic system, was estimated by measuring the ultraviolet absorption of aliquots taken from the appropriate region of the silicic acid chromatogram (peaks corresponding to cholesterol and Δ^7-cholestenol and the intervening region). Measurements were made in spectroscopic grade hexane at 271, 276, 281.5, 289, and 293 mμ; in calculating the total amount of material on the basis of the absorption at 281.5 mμ, due allowance was made for nonspecific absorption by contaminating materials.

Sterols Used as Reference Standards for GLC. Δ^7-Cholestenol (lathosterol), Δ^8-cholestenol, $\Delta^{5,24}$-cholestadienol (desmosterol), 4α-methyl-Δ^7-cholestenol (methostenol), 4α-methylcholestanol, 4,4-dimethyl-Δ^7-cholestenol, and 4,4-dimethyl-Δ^8-cholestenol were made available to us by Professor Konrad Bloch. 4,4,14α-Trimethyl-Δ^7-cholestenol was kindly supplied by Professor R. B. Woodward. 4,4,14α-Trimethyl-$\Delta^{8,24}$-cholestadienol (lanosterol) was prepared from "*iso*-cholesterol" via the acetate dibromide as described by Johnston et al. (29). 4,4,14α-Trimethyl-Δ^8-cholestenol (24,52-dihydrolanosterol) was prepared by hydrogenation of lanosterol in the presence of Raney nickel. $\Delta^{5,7}$-Cholestadienol (7-dehydrocholesterol) was obtained from the Aldrich Chemical Co., Milwaukee, Wis. Cholesterol was obtained from Nutritional Biochemicals Co., Cleveland, Ohio, and was purified via the dibromide before use. Triparanol was obtained from the W. S. Merrill Co., Cincinnati, Ohio.

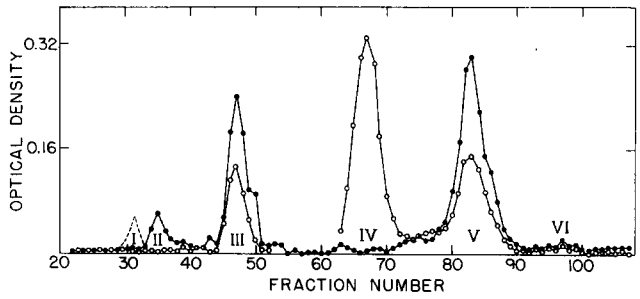

FIG. 1 Chromatography of sterols of normal rat skin on silicic acid. Conditions as in the addendum. "Fast-reacting" sterols, —●—●—●—; "slow-reacting" sterols, —○—○—○—

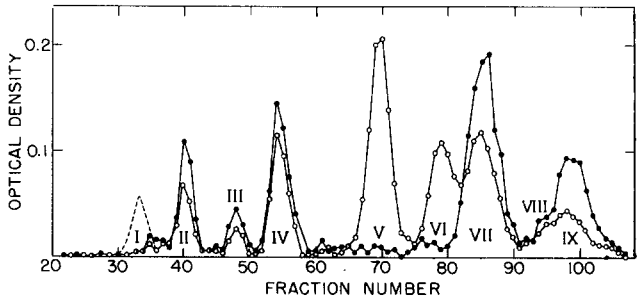

FIG. 2 Chromatography of sterols of triparanol-treated rats on silicic acid. Conditions and symbols as for Fig. 1.

For purposes of shipping and storage, fractions from the silicic acid chromatograms were evaporated to dryness under nitrogen in tubes that were sealed with rubber stoppers after being flushed with nitrogen.

RESULTS AND DISCUSSION

Silicic Acid Chromatography. Figures 1 and 2 represent the results obtained by silicic acid chromatography of the sterols of normal and triparanol-treated rats, respectively. In each figure, the full points represent the Liebermann-Burchard reading after 1.5 min ("fast-reacting" sterols) and the open points represent the Liebermann-Burchard reading after 30 min ("slow-reacting" sterols).

It is immediately clear that there is a more complex array of materials present in the sterol mixture of the triparanol-treated animals than in the controls. Experience gained in our laboratory over a number of years and corroborated by the work of others (15, 16) shows that these substances are eluted from the column in three primary structural groups differentiated from each other according to the degree of methyl substitution in ring A and, hence, according to the degree of hindrance of the 3β-hydroxyl group. Thus, a 4α-methyl sterol (e.g., methostenol) emerges consistently before cholesterol, while 4,4-dimethyl sterols

(e.g., lanosterol) precede methostenol. Hence, the retention volume on the silicic acid column gives some preliminary evidence as to the structure of ring A.

Superimposed on this basic pattern is a secondary one, resulting from the presence or absence of various double bonds in the nucleus and side chain of the molecules, and influenced in a consistent manner by the positions of the double bonds in question. It is found that Δ^7- and Δ^8-compounds are eluted after the corresponding Δ^5-compound. Thus, lathosterol follows cholesterol (10, 15) and $\Delta^{7,24}$-cholestadienol follows desmosterol (19). Moreover, Δ^7- and Δ^8-compounds (e.g., methostenol and its Δ^8-analogue) are difficult to a separate on this system (16, 26). Finally, the presence of a Δ^{24}-bond causes a compound to separate by an appreciable factor[1] from its 24,25-dihydro analogue; e.g., lanosterol from dihydrolanosterol (15), desmosterol from cholesterol (21), and $\Delta^{7,24}$-cholestadienol from Δ^7-cholestenol (19).

With these considerations in mind, and making the reasonable assumption that triparanol is likely to cause accumulation of the Δ^{24}-analogues of compounds that are normally present in the 24,25-dihydro form, it is possible to speculate as to the most probable structures of the materials corresponding to the various peaks in Figs. 1 and 2.

The large peak of slow-reacting material occurring near the center of each chromatogram (Fig. 1, peak IV; Fig. 2, peak V), a major component in each case, corresponds to cholesterol. These two different elution patterns are most conveniently compared by considering in turn the regions to the right and left of the cholesterol peak. These regions correspond to materials that are more and less polar, respectively, then cholesterol.

A large peak of fast-reacting material, more polar than cholesterol and separated from it to the same degree, is found in each chromatogram (Fig. 1, peak V; Fig. 2, peak VII). This peak corresponds to lathosterol (Δ^7-cholestenol), long recognized as an important component of the sterol mixture found in normal rat skin (25).

Interposed between the Δ^7-cholestenol and cholesterol peaks from the triparanol-treated animals is a relatively large peak of slow-reacting material (Fig. 2, peak VI), which is not seen in the controls, although evidence will be given for its presence as a minor constituent.

[1] A separation factor incurred by the Δ^{24}-bond can be calculated as the ratio of the elution volume of a Δ^{24}-compound and that of its 24,25-dihydro analogue. If this calculation is carried out for each pair of known or suspected Δ^{24}- and 24,25-dihydro analogues represented in Figs. 1 and 2, an almost constant factor (1.10–1.13) is obtained (20).

TABLE 1. COMPARISON OF OPTICAL ROTATION OF "PEAK III" WITH ITS PROBABLE COMPONENTS

	Alcohol		Acetate			Benzoate		
	$[\alpha]_D$	$[M]_D$	$[\alpha]_D$	$[M]_D$	Δ	$[\alpha]_D$	$[M]_D$	Δ
4α-Methyl-Δ^7-cholestenol	+5	+20	+28	+124	+104	+43	+217	+197
4α-Methyl-Δ^8-cholestenol	+55.2	+221	+64	+283	+62	+82	+414	+193
"Peak III"	+27.0	+108	+44.2	+196	+88	+61.5	+310	+202
Calc. for 56.2% Δ^7, 43.8% Δ^8	+27.0	+108	+43.8	+194	+86	+60.1	+303	+195

This material, the only slow-reacting component apart from cholesterol, and more polar than cholesterol, is desmosterol.

The double peak of fast-reacting materials (Fig. 2, peaks VIII and IX), still more polar than Δ^7-cholestenol, which is conspicuous in the chromatogram from the triparanol-treated animals but barely apparent in the controls, is suspected to contain partially resolved zymosterol (VIII) and $\Delta^{7,24}$-cholestadienol (IX). The latter compound has been isolated in a fairly pure state and considerable chemical evidence for its structure has been accumulated and reported previously (19). A full account of the study of this material is in preparation.

Turning to the materials less polar than cholesterol, the first peak of fast-reacting material to the left of cholesterol in the normal chromatogram (Fig. 1, peak III) may contain methostenol (lophenol, 4α-methyl-Δ^7-cholestenol) and possibly its Δ^8-analogue. 4α-Methyl-Δ^7-cholestenol was isolated from rat feces by Wells and Neiderhiser (30), and from the cactus *Lophocereus schottii* by Djerassi et al. (31). 4α-Methyl-Δ^8-cholestenol was isolated from a preputial gland tumor of the mouse by Kandutsch and Russell (26). We reported a new sterol in rat skin (32, 33) for which we suggested the structure later assigned to methostenol, but were unable to obtain the material in sufficiently pure form to permit positive identification. Neiderhiser and Wells (34) obtained this same mixture from rat skin. They considered the predominant component to be methostenol, but their melting point, 140–141°, agreed with that reported by us, as compared with the value 147–148° for the purest methostenol isolated from feces, and 149–151° for the compound isolated from the cactus by Djerassi et al. (31). Kandutsch and Russell (26) suggested that the mixture from rat skin contained both the Δ^7- and Δ^8-isomers. Table 1 shows the optical rotation of one of our early preparations and of its acetate and benzoate, with values for the Δ^7-isomer, measured by

Djerassi et al. (35), and for the Δ^8-isomer, measured by Kandutsch and Russell (26), for comparison. Calculated values for a mixture containing 56.2% of the Δ^7-isomer and 43.8% of the Δ^8-isomer are also shown. It is apparent that these rotation differences are compatible with the assumption that the material of peak III is, in fact, such a mixture. As noted below (Table 4), the results of gas-liquid chromatography are also in qualitative agreement, although the percentage composition apparently differed slightly from that of the mixture reported in Table 1.

It can be seen from Figs. 1 and 2 that in the triparanol-treated animal the proportion of material present in peak III is much less than in the controls, but that a relatively large peak (peak IV) of fast-reacting material occurs on the more polar side of peak III in a position that contains only very minor components in the control chromatogram. Tentative structural assignments of $\Delta^{7,24}$- and $\Delta^{8,24}$-4α-methyl cholestadienols for components of peak IV, Fig. 2, seem reasonable.

Peak II in both chromatograms is likely to correspond to compounds with the gem-dimethyl structure at C_4. Peak II contains fast-reacting compounds in both cases, but the two peaks do not have identical positions. After triparanol treatment (Fig. 2), peak II emerges later than in the control (Fig. 1); hence, it is reasonable to expect that peak II in Fig. 2 might correspond to a Δ^{24}-4,4-dimethyl sterol while peak II in Fig. 1 might correspond to its 24,25-dihydro analogue. As will be shown below, the results of gas-liquid chromatography lend support to this expectation.

It was not at first known to what extent silicic acid chromatography would resolve the various possible 4,4-dimethyl sterols that might be encountered, and, in particular whether the 4,4,14α-trimethyl sterols would be separated from their 14-desmethyl derivatives. A detailed examination by GLC of materials eluted from the silicic acid column prior to the first peak of Liebermann-Burchard positive sterols indicated the presence of a peak of 4,4,14α-trimethyl sterols in this early portion of the chromatogram. These compounds, although not apparent in the Liebermann-Burchard test, are designated peak I in both Fig. 1 and Fig. 2.

Gas-Liquid Chromatography. Tables 2, 3, and 4 summarize the main results obtained from both silicic acid and gas-liquid chromatography of the skin sterols from normal and triparanol-treated rats, respectively.

Tables 2 and 3 show the following: Col. 1, the regions of the silicic acid chromatogram in terms of peak numbers shown in Figs. 1 and 2; Col. 2, their elution volumes relative to that of cholesterol on the same chromatogram; Col. 3, the fraction numbers of samples from the regions shown in Col. 1 analyzed by GLC; Col. 4, the retention times of peaks corresponding to this fraction that were observed on GLC; Col. 5, the suggested structure corresponding to each peak appearing in the gas-liquid chromatogram; Col. 6, the approximate percentage of the total sample accounted for as each component; Col. 7, the retention time of the assigned structure either as measured by means of a known standard or calculated by the retention factor method on the basis of some known subsidiary standard; Col. 8, the percentage errors between the observed values in Col. 4 and the corresponding standard or calculated values in Col. 7.

In general the reproducibility of relative retention times and the agreement between observed values and values calculated on the basis of retention factors for double bonds fall within the range of $\pm 2\%$ (27). Inspection of the last column in each table, therefore, indicates good agreement between the observed values in column 4 and those listed in column 7 for the retention times of the proposed structures. The retention times of the hitherto unknown Δ^{24}-compounds were derived in each case as the product of a measured retention time for the appropriate known 24,25-dihydro compound and the value (1.44) of the retention factor characteristic of the Δ^{24}-bond. The findings recorded in Tables 2 and 3 will be discussed in turn. Discussion of the Δ^{24}-compounds will, in general, be given in relation to Table 3, in which these compounds are more prominent.

Proposed Structural Assignments (Table 2).

Peak I: 4,4,14α-Trimethyl-$\Delta^{8,24}$-cholestadienol (lanosterol). The least polar identifiable material to be recovered from the silicic acid chromatography of the sterols from the normal skin appears to be lanosterol. This material, which was originally identified as a major component of the wool wax of sheep (36) and was subsequently implicated as a probable intermediate in the biosynthesis of cholesterol in the rat (37), has been isolated more recently from a preputial gland tumor of the mouse (17). On the basis of its retention time, this compound is clearly distinguishable from its Δ^7-analogue (7.5) and from its 24,25-dihydro derivative (4.0). 24,25-Dihydrolanosterol could not be detected in more than trace amounts in this or any other fraction, though every fraction subsequent to fraction 7 was examined by GLC.

Lanosterol fails to give a blue color (λ max = 620 mμ) in the Liebermann-Burchard test, but yields instead a yellow color (λ max = 460 mμ) and hence is not detected in the standard assay procedure used here (17). It is thus clearly differentiated from the group of 14-desmethyl compounds that follow it on the silicic acid chromatogram and give the usual 620 mμ absorp-

TABLE 2. SKIN STEROLS OF NORMAL RATS: GAS-LIQUID CHROMATOGRAPHY OF FRACTIONS FROM SILICIC ACID CHROMATOGRAM

Peak No.[1]	Rel. Elution Vol.[2]	Frac. No.	Rel. Ret. Times of GLC Peaks[3]	Proposed Structures Corresponding to GLC Peaks	% of GLC Sample[4]	Rel. Ret. Time of Standard[5]	% Error in Observed Rel. Ret. Time[6]
I	0.48	32	5.70	4,4,14α-Trimethyl-$\Delta^{8,24}$ cholestadienol(lanosterol)	50	5.70	0.0
II	0.52	35	5.15	4,4-Dimethyl-Δ^{7}-cholestenol	20	5.25	−2.0
			4.24	4,4-Dimethyl-Δ^{8}-cholestenol	80	4.32	−1.8
More polar edge of II	0.58	39	5.15	4,4-Dimethyl-Δ^{7}-cholestenol	39	4.25	−2.0
			4.24	4,4-Dimethyl-Δ^{8}-cholestenol	28	4.32	−1.8
			7.40	4,4-Dimethyl-$\Delta^{7,24}$-cholestadienol	Trace	(7.55)	−2.0
			6.11	4,4-Dimethyl-$\Delta^{8,24}$-cholestadienol	33	(6.23)	−1.9
III	0.70	47	4.75	4α-Methyl-Δ^{7}-cholestenol (Methostenol)	40	4.75	0.0
			3.90	4α-Methyl-Δ^{8}-cholestenol	60	(3.95)	−1.3
More polar edge of III	0.81	54	4.75	4α-Methyl-Δ^{7}-cholestenol	60	4.75	0.0
			3.90	4α-Methyl-Δ^{8}-cholestenol	10	(3.95)	−1.3
			6.75	4α-Methyl-$\Delta^{7,24}$-cholestadienol	4	(6.85)	−1.5
			5.60	4α-Methyl-$\Delta^{8,24}$-cholestadienol	26	(5.69)	−1.6
IV	1.00	67	3.85	Δ^{5}-Cholestenol (cholesterol)	100	3.90	−1.3
Less polar edge of V	1.19	80	5.75	$\Delta^{5,24}$-Cholestadienol[7] (desmosterol)	30	5.65	+1.8
			4.70	Δ^{7}-Cholestenol (lathosterol)	70	4.70	0.0
			3.90	Δ^{8}-Cholestenol	Trace	3.88	0.0
V	1.24	83	4.68	Δ^{7}-Cholestenol	95	4.70	−0.4
			3.88	Δ^{8}-Cholestenol	5	3.88	0.0
More polar edge of V	1.30	87	4.70	Δ^{7}-Cholestenol	~100	4.70	0.0
			3.92	Δ^{8}-Cholestenol	Trace	3.88	+1.0
VI	1.45	97	6.80	$\Delta^{7,24}$-Cholestadienol	100	(6.77)	−1.4

[1] Peak numbers refer to Fig. 1.

[2] Unless otherwise stated, these values are for the center of each peak eluted from silicic acid and are given relative to that of cholesterol = 1.

[3] Retention times relative to cholestane = 1.

[4] Estimates by triangulation of emergent peaks.

[5] Where no standard was available for direct comparison, the relative retention time for the proposed structure was calculated as described in the text. The calculated values are entered in parentheses.

[6] Calculated as: $\frac{[\text{Observed retention time}] - [\text{Standard (or calculated) retention time}]}{[\text{Standard (or calculated) retention time}]} \times 100.$

[7] Contains some unresolved 7-dehydrocholesterol (see Table 4).

tion in the Liebermann-Burchard reaction (38).

A companion substance, equal in amount to lanosterol, having a retention time of 2.8, was present in this fraction. No structural assignment is proposed for this material, which may not be a sterol.

Peak II: 4,4-Dimethyl-Δ^{7}-cholestenol and 4,4-dimethyl-Δ^{8}-cholestenol. Neither of these compounds has hitherto been detected as a biological material, though there is good evidence (39) that the $\Delta^{8,24}$-compound (14-desmethyl-lanosterol) is a precursor of cholesterol in the rat (see below).

Evidence for the separation of lanosterol from the 14-desmethyl group of compounds has been cited. That this separation is complete is evident from the fact that no materials of retention time 5.25 or 5.70 could be detected in the fractions separating peak I from peak II.

4,4-Dimethyl-Δ^{8}-cholestenol has a retention time close to that of 24,25-dihydrolanosterol (4.0) and 4,4-dimethyl-$\Delta^{8(14)}$-cholestenol (4.07). Confusion with the former is excluded on the basis of a discrepancy of 6% in retention time and of the evidence for the separation of this material from lanosterol. The $\Delta^{8(14)}$-compound is an unlikely structure on biogenetic grounds as well as on the grounds of a discrepancy (3.7%) in the observed retention times that exceeds the normal error.

The more polar edge of peak II contained, besides

the 4,4-dimethyl-Δ^7- and Δ^8-cholestenols, an approximately equal amount of 4,4-dimethyl-$\Delta^{8,24}$-cholestadienol and a trace of the corresponding $\Delta^{7,24}$-isomer. The $\Delta^{8,24}$-compound, a very minor component in the sterol spectrum of normal skin, is more prominent in the triparanol-treated animal.

Peak III: 4α-Methyl-Δ^7-cholestenol and 4α-methyl-Δ^8-cholestenol. Evidence obtained by ourselves and others in support of the structures assigned to the components of peak III has been discussed above.

The retention time of the Δ^8-compound, which was not available as a standard, was calculated as the product of the retention time of the fully saturated 4α-methylcholestanol (=3.66) and the retention factor characteristic of the Δ^8-bond (=1.08).

Both these compounds are clearly distinguished in retention time from their Δ^{24}-analogues (see Table 3). Δ^7-Cholestenol, which has a similar retention time to that of 4α-methyl-Δ^7-cholestenol, appears in a region of the silicic acid chromatogram that is widely separated from the 4α-methyl derivatives. Similarly, cholesterol, with a relative retention time identical with that of 4α-methyl-Δ^8-cholestenol, is clearly separated from peak III by silicic acid chromatography. The remarks made concerning the possibility of confusion of the Δ^8-compound in peak I with its $\Delta^{8(14)}$-isomer apply equally in this case.

As in the case of the 4,4-dimethyl-compounds (peak II), the more polar side of peak III was found to contain minor amounts of the Δ^{24}-analogues of both the principal components.

Peak IV: Cholesterol. This material is well established as the major sterol of normal rat skin.

Peak V: Δ^7-Cholestenol, $\Delta^{5,24}$-cholestadienol, and Δ^8-cholestenol. The major component of this peak (cf. middle fraction 83) is Δ^7-cholestenol, as was to be expected from much earlier work. Desmosterol is present as a minor component of the leading edge of this peak. Also detected, as a minor component in the central portion and as traces in the leading and following parts of this peak, was Δ^8-cholestenol. There is little possibility of confusion of the Δ^7- and $\Delta^{5,24}$-compounds with other materials. The retention time of the Δ^8-stenol, however, is identical with that of cholesterol and the only evidence for the presence of Δ^8-cholestenol rather than cholesterol as a contaminant in this peak is the rise and fall of the proportions of the Δ^8-compound within peak V corresponding to the presence of a very small but, nevertheless, distinct peak of Δ^8-cholestenol in that region. A more convincing example of the same kind of distribution is found in the chromatogram from the drug-treated animals in which the proportion of the Δ^8-material is greater.

Peak VI: $\Delta^{7,24}$-Cholestadienol is only a trace component in the total silicic acid chromatographic spectrum in the normal animal but occurs in much larger amounts in the triparanol-treated rat (19) (cf. Fig. 2 and Tables 3 and 4). Its retention time is calculated from the observed retention time of Δ^7-cholestenol (4.70). The retention time of this material is highly characteristic and distinguishes it clearly from other related materials in the C_{27} series.

Proposed Structural Assignments (Table 3).

Peak I: 4,4,14α-Trimethyl-$\Delta^{7,24}$-cholestadienol and 4,4,14α-trimethyl-$\Delta^{8,24}$-cholestadienol. The appearance of a peak of 4,4,14α-trimethyl sterols as the first identifiable materials to be recovered from the silicic acid column is consistent with the findings in the case of the normal animal. This fraction was not detected by the Liebermann-Burchard reaction.

The retention time of the major component, 4,4,14α-trimethyl-$\Delta^{7,24}$-cholestadienol, was calculated on the basis of that of its synthetic 24,25-dihydro analogue (=5.2). Its retention time was also found to be identical with that of a sterol isolated from rat skin by Dr. J. L. Gaylor, which had been assigned the same structure (40) and was made available to us for comparison. The highly characteristic value for the retention time of this material makes its confusion with other compounds quite unlikely.

Lanosterol was identified by direct comparison with the pure substance.

An unidentified material of retention time 2.2 was also present in this sample. This material is probably not a sterol, though this is not certain.

Peak II: 4,4-Dimethyl-$\Delta^{8,24}$-cholestadienol has been shown to be involved in the biosynthesis of cholesterol in liver (39), but its direct isolation has not previously been reported. Its retention time was calculated from that of the authentic 24,25-dihydro compound (=4.32). It is clearly distinguished from its dihydro analogue and from other 4,4-dimethyl sterols considered in the discussion of Table 2 above.

Peak III: 4α-Methyl-Δ^7-cholestenol and 4α-methyl-Δ^8-cholestenol. These materials appear as peaks with the same retention times as are observed in the controls and their characteristics have been discussed above. It is interesting that in the triparanol-treated rat the Δ^8-compound comprises a higher proportion of the total material than in the controls.

Peak IV: 4α-Methyl-$\Delta^{7,24}$-cholestadienol and 4α-methyl-$\Delta^{8,24}$-cholestadienol are two previously unknown sterols. Their calculated retention times are derived from those of their respective 24,25-dihydro analogues. As in the case of peak III of the same chromatogram, the Δ^8-compound is by far the major component and

TABLE 3. SKIN STEROLS OF TRIPARANOL-TREATED RATS: GAS-LIQUID CHROMATOGRAPHY OF FRACTIONS FROM SILICIC ACID CHROMATOGRAM

Peak No.[1]	Rel. Elution Vol.[2]	Frac. No.	Rel. Ret. Times of GLC Peaks[3]	Proposed Structures Corresponding to GLC Peaks	% of GLC Sample[4]	Rel. Ret. Time of Standard[5]	% Error in Observed Rel. Ret. Time[6]
I	0.47	33	7.45	4,4,14α-Trimethyl-$\Delta^{7,24}$-cholestadienol	64	(7.5)	+2.0
			5.70	4,4,14α-Trimethyl-$\Delta^{8,24}$-cholestadienol (lanosterol)	11	5.70	0.0
II	0.57	40	6.11	4,4-Dimethyl-$\Delta^{8,24}$-cholestadienol	100	(6.23)	−1.9
III	0.69	48	4.65	4α-Methyl-Δ^{7}-cholestenol (methostenol)	10	4.75	−2.0
			3.90	4α-Methyl-Δ^{8}-cholestenol	90	(3.95)	−1.3
IV	0.77	54	6.71	4α-Methyl-$\Delta^{7,24}$-cholestadienol	10	(6.85)	−2.0
			5.65	4α-Methyl-$\Delta^{8,24}$-cholestadienol	90	(5.69)	−0.7
V	1.00	70	3.85	Δ^{5}-Cholestenol (cholesterol)	100	3.90	−1.3
VI	1.13	79	5.75	$\Delta^{5,24}$-Cholestadienol[7] (desmosterol)	100	5.65	+1.8
Overlap VI & VII	1.2	82	5.75	$\Delta^{5,24}$-Cholestadienol[7] (desmosterol)	70	5.75	0.0
			4.78	Δ^{7}-Cholestenol (lathosterol)	15	4.70	+1.7
			3.92	Δ^{8}-Cholestenol	15	3.90	+0.5
VII	1.21	85	4.70	Δ^{7}-Cholestenol	90	4.70	0.0
			3.88	Δ^{8}-Cholestenol	10	3.88	0.0
VIII	1.34	94	5.70	$\Delta^{8,24}$-Cholestadienol (zymosterol)	80	(5.60)	+1.8
			6.90	$\Delta^{7,24}$-Cholestadienol	20	(6.77)	+1.9
Overlap VIII & IX	1.30	96	6.90	$\Delta^{7,24}$-Cholestadienol	60	(6.77)	+1.7
			5.68	$\Delta^{8,24}$-Cholestadienol (zymosterol)	40	(5.60)	+1.4
More polar edge of IX	1.46	102	6.85	$\Delta^{7,24}$-Cholestadienol	~100	(6.77)	+1.2
			5.68	$\Delta^{8,24}$-Cholestadienol	Trace	5.60	+0.3

[1] Peak numbers refer to Fig. 2.
[2–7] See Table 2.

constitutes approximately 90% of the total material of this peak. Compounds of closely similar retention time to the $\Delta^{8,24}$-compound are desmosterol and zymosterol, but these substances appear in widely separated portions of the silicic acid chromatogram (peaks VI and VIII, respectively).

Peak V: Cholesterol. As in the control animals, this sterol is still quantitatively the major single component of the total mixture.

Peak VI: Desmosterol accumulates in considerable amounts under the influence of triparanol, as has been reported by others. No contamination by other sterols was detected when the central portion of the peak was analyzed.[2] The compound is distinguished from all others in the chromatogram except cholesterol by its slow Liebermann-Burchard reaction. It is clearly separated on silicic acid from any other materials that have similar retention times on the gas-liquid chromatogram.

Peak VII: Δ^{7}-Cholestenol and Δ^{8}-cholestenol. As in the control, these two compounds occur together in the silicic acid chromatogram. In the triparanol-treated animals, however, the proportion of Δ^{8}-material is markedly increased. The Δ^{8}-stenol apparently moves somewhat more rapidly than the Δ^{7}-compound on silicic acid; in the overlapping area of peaks VI and VII (fraction 82), the ratio of the Δ^{7}- to the Δ^{8}-compound is approximately 1:1 whereas, in the center of peak VII (fraction 85), the Δ^{7}-compound predominates in the ratio of 9:1. It was pointed out above that cholesterol and Δ^{8}-cholestenol are indistinguishable on the basis of retention times, but the fact that the central fraction of peak VI (desmosterol) was obtained without contamination with any material of relative retention time 3.9 is good evidence for the identity of the material of peak VII with Δ^{8}-cholestenol and its clean separation from cholesterol.

Peak VIII: $\Delta^{7,24}$-Cholestadienol and $\Delta^{8,24}$-cholestadi-

[2] A minor proportion of 7-dehydrocholesterol is present but this material is unresolved in either chromatographic system. It may be detected by its ultraviolet absorption (cf Table 4).

enol (*zymosterol*) occur in this partly resolved peak in the ratio of 4:1; in the later region of overlap of peaks VIII and IX, the ratio has fallen to 3:2. This difference in relative amounts of the two substances is consistent with the observation noted above and by others (26) that a Δ^8-compound migrates on silicic acid slightly more rapidly than its Δ^7-isomer. Zymosterol has been shown by labeling techniques to be present in rat liver preparations, where it behaves as a precursor of cholesterol (9). In the present study, a thorough search was made for this compound in the normal skin, but no convincing chromatographic evidence for its presence was found.

The measured retention times of the $\Delta^{7,24}$- and $\Delta^{8,24}$-compounds are in good agreement with the calculated values based on the retention times of Δ^7-cholestenol (4.70) and Δ^8-cholestenol (3.88), respectively.

Peak IX: $\Delta^{7,24}$-Cholestadienol. The presence of a peak with retention time 6.85, which agrees well with the calculated value of 6.77 for cholesta-7,24-dienol based on the value 4.70 measured for Δ^7-cholestenol, furnishes further confirmation of the proposed structure of this substance (19), which is present only in traces in the control.

Total Composition of the Mixtures of Skin Sterols. The primary object of this study was to assemble evidence for the identity of the various compounds present in the skins of normal and triparanol-treated rats and to record the quantitative relationships in a way that would contribute to the structural identification (e.g., the change in proportions of Δ^7- and Δ^8-components in passing from the less polar to the more polar edge of a particular peak from the silicic acid chromatogram). However, from the GLC analyses of "reconstituted" zones, approximate percentages of most of the components present in the material eluted from the silicic acid chromatograms (shown in Figs. 1 and 2) were calculated and are recorded in Table 4. It is emphasized that these values must be regarded with reserve. Possible sources of error are: losses of more sensitive (especially Δ^{24}) components during manipulation and storage; and the presence (especially in the less polar chromatographic fractions) of variable amounts of unidentified materials (presumably not sterols), a portion of which often emerged close to the origin but in some cases failed to emerge from the chromatogram. It is on account of the presence of these materials that the sum of the percentages of the various sterols given in Table 4 falls appreciably short of 100%.

This survey of the various components of the mixture of sterols found in rat skin is more comprehensive than any hitherto published. Evidence has been presented for the detection of several compounds of hitherto unknown structure or known only from other sources. Substances not previously detected in any natural material, but which are most probably present in rat skin as indicated by the evidence assembled here, are: 4,4-dimethyl-Δ^8-cholestenol; 4,4-dimethyl-Δ^7-cholestenol; 4,4-dimethyl-$\Delta^{7,24}$-cholestandienol; Δ^8-cholestenol; 4α-methyl-$\Delta^{7,24}$-cholestandienol; and 4α-methyl-$\Delta^{8,24}$-cholestadienol. The percentage of 4α-methyl sterols in the normal skin as indicated by these analyses is of the same order as that (10.2%) reported by Neiderhiser and Wells (34) for the concentration of the Δ^7-compound in this tissue. The detection of a peak corresponding in retention time to 4,4-dimethyl-$\Delta^{8,24}$-cholestadienol is probably the first direct isolation of this intermediate in cholesterol biosynthesis (39). This compound appears to be present in the skin of the triparanol-treated rat in quantities that should render its purification and complete chemical characterization relatively straightforward. The separation of a substance of the calculated retention time of $\Delta^{7,24}$-cholestadienol from its companion $\Delta^{8,24}$-dienol (zymosterol) gives strong support to the $\Delta^{7,24}$-structure assigned to the major component of peak IX in the chromatogram from the triparanol-treated ani-

TABLE 4. APPROXIMATE PERCENTAGE COMPOSITION OF TOTAL STEROL MIXTURES ISOLATED FROM SKINS OF NORMAL AND TRIPARANOL-TREATED RATS

Sterol	Concentration[1] (%) Control Animals	Concentration[1] (%) Triparanol-Treated Animals
4,4,14α-Trimethyl-$\Delta^{7,24}$-cholestadienol	...	1
4,4,14α-Trimethyl-$\Delta^{8,24}$-cholestadienol-(Lanosterol)	1	<1
4,4-Dimethyl-Δ^7-cholestenol	3	...
4,4-Dimethyl-$\Delta^{7,24}$-cholestadienol	<0.1	...
4,4-Dimethyl-Δ^8-cholestenol	10	...
4,4-Dimethyl-$\Delta^{8,24}$-cholestadienol	3	8
4α-Methyl-Δ^7-cholestenol(methostenol)	7	<1
4α-Methyl-$\Delta^{7,24}$-cholestadienol	<0.1	1
4α-Methyl-Δ^8-cholestenol	7	3
4α-Methyl-$\Delta^{8,24}$-cholestadienol	<0.1	8
Δ^5-Cholestenol(cholesterol)	32	25
$\Delta^{5,24}$-Cholestadienol(desmosterol)	4	20
Δ^7-Cholestenol (lathosterol)	21	12
$\Delta^{7,24}$Cholestadienol	<0.1	9
Δ^8-Cholestenol	<0.1	1
$\Delta^{8,24}$-Cholestadienol(zymosterol)	...	3
$\Delta^{5,7}$-Cholestadienol (7-dehydrocholesterol)	0.5–1.0	0.5–1.0

[1] Estimated as a percentage of the materials recovered from the silicic acid chromatograms over the elution range indicated in Figs. 1 and 2.

mals (19). The recent report (40) of the presence of the Δ^7-analogue of lanosterol in rat skin is supported by the present findings in the case of the drug-treated animals. The percentages of Δ^7-cholestenol and 7-dehydrocholesterol found in the normal skin in this study are in reasonably good agreement with the values found for these sterols by Idler and Baumann (25).

The various new structures proposed in the foregoing discussion require verification by the established techniques of organic chemistry, and this work is presently in progress. The probability that these proposed structures are correct, however, is sufficiently high to warrant some consideration of their significance.

It is now well established that the biosynthesis of cholesterol (18, 41) involves the union of a series of precursors of lower molecular weight to give the C_{30} triterpenoid hydrocarbon, squalene, and that this compound cyclizes to a 4,4,14α-trimethyl-(C_{30}) sterol that has been characterized as lanosterol (42). There is strong evidence for the conversion of this compound to cholesterol via 4,4-dimethyl-(C_{29}) (39), and 4α-methyl-(C_{28}) (13, 16, 43) derivatives by a series of predominantly oxidative reactions. It is clear from the control chromatogram (Table 2) that the pathway of cholesterol biosynthesis beyond the C_{30} (lanosterol) stage is represented in the normal skin by the accumulation of a series of neutral sterols having saturated side chains, with only minor amounts of some of the Δ^{24}-analogues of these compounds. Lanosterol itself is a striking exception to this general rule, since it is by far the major C_{30} sterol and appears to be accompanied by only traces of its 24,25-dihydro derivative. This last observation is surprising in view of the identification of comparable amounts of both lanosterol and dihydrolanosterol in preputial gland tumor of the mouse (17).

The most obvious effect of triparanol is to cause accumulation of the Δ^{24}-analogues of all those compounds that are found in the normal skin almost entirely in the 24,25-dihydro form. It is clear, therefore, that triparanol cannot be a specific inhibitor of the reduction of the Δ^{24}-bond of desmosterol, but may inhibit the reduction of this double bond in at least one, and possibly several other compounds also. This conclusion is in accord with the findings of Avigan et al. (23, 24).

A second effect of triparanol, suggested by the results of this study and not detected hitherto, is to cause a marked distortion of the normal ratios of Δ^8- to Δ^7-isomeric compounds that accumulate in the skin. In the various groups of compounds following lanosterol in the biosynthetic (and chromatographic) sequence, the effect is in the direction of increasing the proportion of Δ^8- vs Δ^7-isomers. It should be noted, however, that in the normal animal the ratios of Δ^8- to Δ^7-isomers in the Δ^{24}-series are generally different from those of their 24,25-dihydro analogues. Moreover, the direction of these differences is not always the same. For example, in the 4α- and 4,4-dimethyl sterols of the Δ^{24}- series, the ratio of Δ^8 to Δ^7- is higher than in the corresponding 24,25-dihydro series; but this does not hold for the C_{27} sterols, where $\Delta^{7,24}$-cholestadenol is detectable while $\Delta^{8,24}$-cholestadienol (zymosterol) is not.

The effect of the drug on the position of the double bond in the case of the C_{30} sterols is the reverse of its effect on later steps in the biosynthetic sequence, since lanosterol ($\Delta^{8,24}$) appears to be largely replaced by its $\Delta^{7,24}$-isomer in the triparanol-treated animal. This observation is of great interest in view of Gaylor's recent report (40) of the isolation of $\Delta^{7,24}$-lanostadienol from rat skin, though it would be hazardous to speculate on its significance on the basis of the limited data presented here. The possibility must be considered, however, that some of the differences observed between the sterol composition of the skin of the normal and drug-treated animals were not due to specific effects of triparanol. Animals treated for such a long period of time in the dosage used in this study fail to gain weight at a normal rate, and their skin shows rather severe pathologic changes.

A study of this type cannot supply a definite answer to the much discussed question of whether the biosynthesis of cholesterol takes place via a pathway involving reduction of the Δ^{24}-bond at an early stage or as the final step. There is extensive evidence for the biological conversion of both 24,25-dihydro derivatives (9–16, 21) and Δ^{24}-derivatives (8, 9, 21, 44) to cholesterol; and triparanol has been shown to inhibit the conversion of desmosterol to cholesterol both *in vivo* and *in vitro* (8), of lanosterol to dihydrolanosterol *in vitro* (23, 24), and of zymosterol to cholesterol *in vivo* (21). Moreover, good evidence has been obtained for the role of both Δ^8- (9, 21, 39) and Δ^7- (10–13, 15, 16) derivatives as probable intermediates in cholesterol biosynthesis. In view of these observations, the highly complex pattern of sterols found in the skin of the normal animal and the accumulation of several Δ^{24}-sterols under triparanol treatment strongly suggest that the enzymes responsible for transformation of the nucleus are capable of acting independently of the enzyme (or enzymes) responsible for side-chain reduction, and vice versa. Under these circumstances, as has been indicated by others (8, 15, 18, 24), it becomes unnecessary to view the process of cholesterol biosynthesis in the skin as taking place by one or the other of two mutually exclusive pathways. It seems reasonable to assume

that the array of accumulated intermediates of cholesterol biosynthesis that appears in the skin does so mainly because of the absence of enzymes for the further metabolism of cholesterol, and because, as has been suggested by Bloch (18), there is a low level of activity in this tissue of the enzymes responsible for some of the oxidative steps involved in the later stages of cholesterol biosynthesis.

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Addendum

Chromatography of unesterified sterols on silicic acid-super-cel

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Methods have been reported from several laboratories for the separation of free sterols (1) and of their esters (2, 3, 4) by chromatography on silicic acid. The purpose of this addendum is to present such a method for

the separation of free sterols that has evolved in this laboratory over a period of several years (5–9) and that was used in the studies reported above. Its advantages are simplicity, ready availability of materials, satisfactory reproducibility, and good resolution.

For preparation of a column 1 m long and 1 cm in diam,[1] 40 g of silicic acid[2] and 20 g of Super-Cel[3] are mixed in a beaker. Two hundred and seventy-five milliliters of benzene[4] are added, and a uniform slurry is prepared by vigorous stirring with a glass rod. A few milliliters of benzene are poured over a cotton plug in the lower end of the column. The columns are simple, straight glass tubes, with a short glass delivery tube of smaller diameter at the bottom. Reservoirs are made by sealing a narrow glass tube to the bottom of a long-necked, round-bottomed flask. The reservoir is attached with a rubber stopper. If a column is to be stopped and saved for re-use, the clamp holding it is loosened and the delivery tube is allowed to rest on a solid rubber stopper. The weight of the column and of the solvent in the reservoir is sufficient to stop the flow. Columns may be re-used many times.

Materials dissolved from the rubber stoppers are easily removed by allowing a small volume of solvent to run through the column before the sample is added. The only other disadvantage of this technique is the tendency of the stoppers to swell after prolonged contact with benzene vapor. These disadvantages in the apparatus are greatly outweighed by its cheapness, simplicity, and freedom from leaks and potential obstructions to flow, as compared with the use of ground glass joints, fritted glass discs, and stopcocks.

The column is filled to the top with the freshly stirred slurry. A little pressure from a tank of compressed gas is applied at the top of the column, but not enough to convert the dropwise flow of benzene to a steady stream. Soon the pressure can be increased to 10 lb/in.[2] It is maintained at this level until settling of the stationary phase has stopped. A convenient source of pressure is a tank of compressed nitrogen fitted with a diaphragm-type regulator. The handle of the regulator may be turned in until the pressure reaches the desired level, and then turned back out. If the system is tight, the pressure will be maintained with little change. If the rubber stopper should blow out while the column is unattended, little gas will be wasted, and the effect on the packing will be less disastrous.

The excess of benzene is drawn off, more freshly stirred slurry is added, and pressure is re-applied. This routine is repeated until the desired length has been attained. The required number of additions of slurry may be reduced by using a glass column longer than the required length of packing, or by fitting the column with a temporary extension. The sample may be added immediately after completion of the packing. We have the impression, however, that the resolution is sometimes improved if solvent is allowed to run through the column overnight under gravity before the sample is added.

When the sample is to be added, the solvent is siphoned from the reservoir, most of the benzene is pipetted from the top of the column, and the surface of the column is allowed to run just dry. Before the top of the column has run dry, the surface may be straightened and flattened with a stirring rod, if it is grossly irregular. The sample is added as a solution in 2 ml or less of benzene. It is run in slowly from a pipette held near the surface of the packing, in order to avoid disturbing the surface. The sample should be free of suspended or emulsified material, which can obstruct the flow. The surface is allowed to run just dry, and the part of the wall of the glass tube that has come in contact with the sample is rinsed with about 1 ml of benzene.[5] After the surface is again

[1] A column of these dimensions will separate a mixture of about 12 mg of sterols without signs of overloading, regardless of the percentage composition of the mixture; i.e., the column will not be overloaded even if most of the 12 mg is a single compound. If larger quantities are to be separated, the amount of stationary phase specified and the cross-sectional area of the column should be increased in proportion to the weight of sample. The resolution is equally good with columns 1 and 2 cm in diam. The resolution decreases with larger diameters, but I have obtained useful separations with columns up to 15 cm in diam. For certain mixtures, shorter columns give adequate separation and allow a saving of time. Increasing the length of the column appears to improve the resolution more than would be predicted on theoretical grounds.

[2] Mallinckrodt Chemical Works, 100 mesh, analytical reagent, suitable for chromatographic analysis by the method of Ramsey and Patterson. Other brands have been found unsatisfactory.

[3] Johns-Manville Corp. Various grades of "Celite" can also be used, but none has been found that gives as good resolution as do the best lots of Super-Cel. It was found expedient to obtain samples of a number of lots and then order 50–100 lb of the lot that performs best. Most lots are perfectly satisfactory.

[4] The volume of benzene is not critical; the volume specified has been found convenient. All brands of benzene tried have been equally good. Even Merck reagent-grade benzene should be redistilled if compounds are to be isolated from the effluent. Redistillation also facilitates the application of the Liebermann-Burchard color reaction if colors are to be read at early time intervals, such as $1^1/_2$ min after the addition of the reagent. Benzene as supplied by the manufacturer leaves a gummy residue that interferes with the rapid mixing of the sterols with the color reagent. Redistillation does not affect the resolution obtainable with the columns.

[5] The operation of adding the sample and washing it into the column may be hastened by the use of pressure, but care should be exercised to remove the pressure before the column is quite dry. Otherwise, channels may be introduced that will spoil the resolution.

dry, the washing is repeated, a few milliliters of benzene are added, and the reservoir is installed.

Fractions 2–5 ml in volume are collected. The column should flow at a rate of about 5–7 ml/hr. Cholesterol should emerge when about 200–300 ml has been collected, depending on the temperature and the tightness of the packing.

Typical chromatograms are shown in references previously cited (5–9). The foregoing article gives data on the relative retention volumes of a number of naturally occurring sterols. Although more elaborate processing of the stationary phase might be desirable for some purposes, this method using commercially available materials has proved very useful.

The following workers in this laboratory have made substantial contributions in the development and application of this method: Mrs. Veronica M. Anderson, Mrs. Ann G. Davidson, Mrs. Elinor Dulit, Mrs. Mary L. Mobberley, Mr. Albert N. Nelson, and Mr. Ajitkumar T. Sanghvi.

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