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The differential migration of sterol acetates on silica gels and its application to the fractionation of sterol mixtures*

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SUMMARY

The retention volumes of 12 sterol acetates relative to cholesteryl acetate were determined on four well-characterized silica gels of different body structure. These values illustrate the contribution of silica gel structure to the chromatographic process and permit the selection of appropriate gels for a given separation. The application of these values to the fractionation of model and biological mixtures of sterol acetates is illustrated.

The fractionation of sterols by chromatographic means has been the subject of considerable interest. To date, the most prominent of such means has been gas-liquid chromatography (1–3). Several groups have reported the fractionation of free sterol mixtures by adsorption chromatography on silicic acid (4–6). While gas-liquid chromatography offers the considerable advantage of speed, adsorption chromatography offers the possibility of preparative scale isolations that facilitate metabolic studies by permitting the recovery of intermediates for chemical and radiochemical examination. Our experiences with the ability of silica gel columns to fractionate esters of cholesterol according to the constituent fatty acid (7) suggested that different sterols as the same ester (acetate) might be resolved under similar conditions. The fractionation of sterols as acetates offers, as a further advantage over the chromatography of free sterols, a means of establishing sterol composition quantitatively by the isotope derivative technique employing C^{14}-acetic anhydride as the acetylating agent (8). Such a technique could reduce the problems of obtaining appropriate colorimetry standards for the Liebermann–Burchardt reaction as well as those evolving from two types of spectra and two types of reaction rates in this reaction.

Early experiences with the fractionation of sterol acetates led to the realization that both the column efficiency and the separation factors in such a fractionation were strongly dependent on the structure of the silica gel employed. Several features contributing to column efficiency (pore diameter and surface energy) have been investigated (9, 10). We wish to report here the mapping of retention volumes for 13 sterols on four silica gels whose average pore diameters range from 20 to 170 Å. These data permit the selection of a silica gel appropriate for a given separation.

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EXPERIMENTAL METHODS

Materials. Dihydrocholesterol, cholesterol, 7-dehydrocholesterol, and ergosterol were purchased from Nutritional Biochemicals Corp., Cleveland, Ohio, and were recrystallized before use. The following sterols were obtained as gifts: a 1:1 mixture of 24,25-dihydrolanosterol-lanosterol from Dr. Paul O'Connell (Upjohn Company); zymosterol from Dr. O. N. Breivik (Fleischman Laboratories); 25-ketonorcholesterol from Dr. William Gebert (Schering Corporation); sitosterol and stigmasterol from Dr. E. R. Diller (Eli Lilly); agnosterol from Dr. C. H. Duncan (University of Louisville); methostenol from Dr. W. W. Wells (University of Pittsburgh), triparanol from Dr. T. R. Blohm (William S. Merrill Co.). Δ^7-Cholestenol was prepared by hydrogenation of 7-dehydrocholesterol over Raney nickel by the method of Fieser and Herz (11); 24-dehydrocholesterol was prepared from 25-ketonorcholesterol by the method of Ryer, Gebert, and Murrill (12).

Sterol acetates were prepared by the micro method of Johnston, Gautschi, and Bloch (13) and were purified by column chromatography (7) before use.

Silica gels were products of the Davison Company, Baltimore, Md.; they will be designated by their Davison code numbers as #12, #950, MS, and #62. The lots used in these studies were characterized by the independent measurements of surface area and pore volume listed in Table 1; these properties were found to agree closely with the specifications listed for them by Davison (see Ref. 9). All gels were screened to obtain the fraction between 200 and 325 mesh.[1] The final fraction was rescreened twice to remove all particles smaller than 325 mesh.

It is advisable to equilibrate the gels at the relative humidity that provides the lowest retention volume consistent with maximum efficiency (10). This humidity must be determined for each type of silica gel structure; for #12, #950, and MS, a relative humidity of 7% appears optimal, for #62, 11%.[2]

Operation of the Column. The column consisted of 110 cm of glass tubing (0.45 cm i.d.). At the lower end, a Luer tip was fused to the tubing; at the upper end, a 24/40 standard tapered female joint provided a reservoir above the column bed. A small pad of glass wool was tamped into the bottom of the column. The column could be shut off by slipping a sealed needle shank over the Luer tip. The column was filled with 16% benzene in hexane (pure grade solvents) and the appropriate gel was introduced into the reservoir through a funnel. The introduction of the gel was followed by stirring and tapping to permit the gel to settle evenly. The column was filled to about 110 cm and then allowed to settle while draining (assisted, if necessary, by a slight positive pressure from a well-controlled air pressure source). When the settling was complete, the column was shut off and the level of the bed was brought to exactly 100 cm by removal of excess gel. At no time was the bed permitted to become dry. After the bed was prepared, the supernatant solution was removed and the sample (2–10 mg total sterol acetates in 16% benzene in hexane) was pipetted onto the top of the column. The sample was passed into the bed and rinsed in with a few milliliters of 16% benzene in hexane. A reservoir (500, 1,000, or 2,000 ml capacity), made of a round-bottomed flask with an outlet of a 24/40 tapered male joint at the bottom, having an inner drip tip, was placed on top of the column and filled with 16% benzene in hexane. The column reservoir was filled from the upper reservoir and elution was begun.

TABLE 1. PROPERTIES OF SILICA GELS USED IN STEROL FRACTIONATIONS

Division Code No.	#12	#950	MS	#62
Surface area, m^2/g	805	672	547	274
Pore volume, ml/g	0.41	0.45	0.71	1.16
Avg. pore diameter, Å	20	27	67	170
Bulk density, g/ml	0.61	0.60	0.40	0.29
Equilibration, % humidity	7.05	7.05	7.05	11.02
Retention volume of cholesteryl acetate per ml column volume, ml 16% benzene in hexane	62	85	72	27

The eluate was led through a needle and a length of small-diameter polyethylene tubing to the fraction collector. It was found advisable to collect the fractions directly in the tubes without the intervention of the funnel commonly used. The flow rate was set between 15 and 20 ml/hr, or fractions of 5 to 7 ml in each 20-min period. Each fraction, or an aliquot of each, was evaporated to dryness and the sterol content was determined by the Liebermann-Burchardt reaction. It was found convenient to use two colorimeters, each set at the appropriate wavelength, to measure the blue-green reactions at 660 mμ and the yellow reaction at 440 mμ.

Determination of Relative Elution Volumes. The relative elution volume of a sterol acetate was defined to be the ratio of the volume of solvent required to elute 50% of that sterol to the volume of solvent required to elute 50% of the cholesteryl acetate. The graphic method of determining the 50% point, described earlier, was used in all instances (9). In all runs, 2 mg of cholesteryl acetate was used for reference purposes; samples of 0.2 to 2.0 mg of the other sterols (depending upon the color yield of the sterol) were added and the mixture was chromatographed. In those instances in which the relative elution volumes were within 10–15% of the standard volume (i.e., the elution volume of cholesterol), final calculations were made on separate elutions of each sterol from two columns run in parallel.

RESULTS

The relative elution volumes of 13 sterols on the four Davison gels are listed in Table 2. The column desig-

[1] Coarser particles can be reduced to the proper mesh size by repeatedly subjecting the dry gel to high-speed shearing forces in a Waring blendor for short periods of time (30 sec to 2 min). After each blendor treatment, the gel is screened to remove finer particles. It is advisable to use a dozen or so 1/2-in. porcelain balls on each screen to reduce clogging.

[2] These are provided by desiccators containing saturated solutions of sodium hydroxide and of lithium chloride respectively. The equilibration is hastened by daily stirring of the gel, followed by re-evacuation of the desiccator to the vapor pressure of the solution, and is essentially completed by 2 weeks' storage.

TABLE 2. RELATIVE ELUTION VOLUMES* OF STEROL ACETATES ON VARIOUS SILICA GELS

Gel	#12	Mallinckrodt	#950	MS	#62
Avg. Pore Diam. (Å)	20	Unknown†	27	67	170
Dihydrolanosterol	0.74	0.72	0.72	0.72	0.89
Dihydrocholesterol	0.86	0.90	0.98	0.93	0.97
Cholesterol	1.00	1.00	1.00	1.00	1.00
Sitosterol	1.04		0.93	0.94	1.07
Stigmasterol	1.09		0.96	0.99	1.08
Lanosterol	1.34	1.28	1.21	0.91	1.10
Methostenol	1.22	1.38	1.17	1.15	1.18
Δ^7-Cholestenol	1.40	1.40	1.39	1.41	1.45
Agnosterol	1.64	1.48	1.43	1.09	1.20
24-Dehydrocholesterol	1.98	1.58	1.67	1.32	1.32
7-Dehydrocholesterol	1.77	1.68	1.40	1.34	1.43
Ergosterol	2.04	1.91	1.66	1.62	1.52
Zymosterol	2.42	2.20			1.69

* All values are reproducible to within 3%.

† Pore diameter of this preparation was unavailable. The properties suggest a pore diameter of approx. 24 Å.

nated Mallinckrodt lists values obtained in the early stages of this work with a silica gel product of undetermined structure; on the basis of elution values, it appears to have been between 20 and 25 Å average pore diameter. Subsequent products from this manufacture, have had different properties.

The largest range of relative elution volumes appears to be achieved on a small-pore gel (#12); in part, this is due to the selective retardation of sterols with unsaturated bonds in the side chain. Fig. 1 illustrates the effect of pore diameter on the chromatography of sterols that are unsaturated in the 24, 25 position. The three sterols shown all exhibit an increased retention volume relative to cholesterol as the pore diameter of the gel is decreased. A further distinction can be noted in the retardation as a function of variations in the size and configuration of the side-chain (Fig. 2). The retardation of ergosterol begins at a lower pore diameter and does not decrease to the same extent as does that of the 24-dehydrosterols. Futhermore, substitution of an ethyl group in the side chain almost completely abolishes the selective retardation. We interpret this as being caused by a "shielding" of the adsorptive group by the ethyl addition.

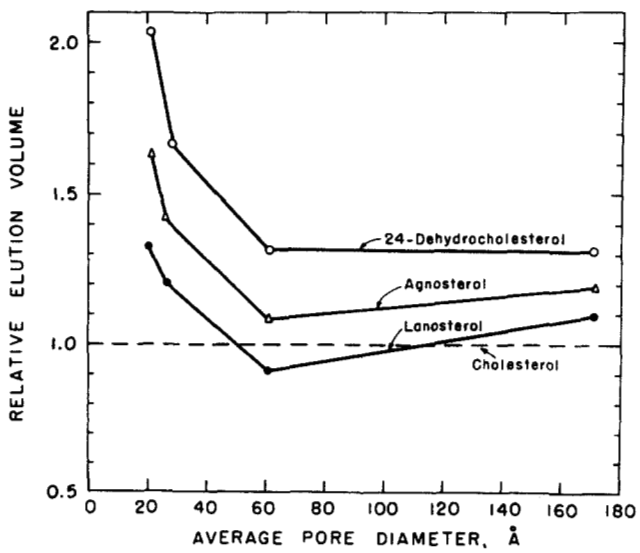

FIG. 1. Relative elution volumes of acetyl esters of 24-dehydrocholesterol, agnosterol, and lanosterol as a function of average pore diameter.

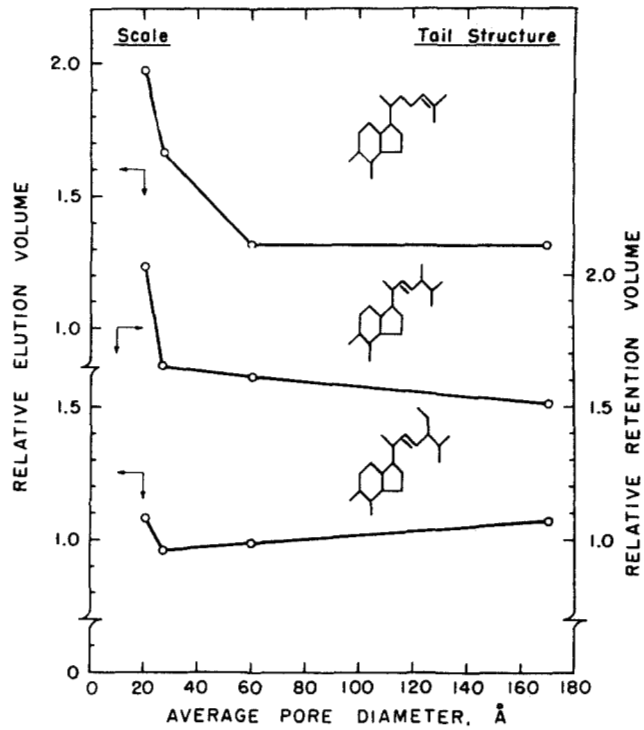

FIG. 2. Effect of side chain structure on the relative elution-volume changes produced by small-pore gels. Top curve, 24-dehydrocholesteryl acetate; middle curve, ergosteryl acetate; lower curve, stigmasterol acetate. Arrows indicate ordinate and abscissa applicable to each curve.

Several other effects of gel structure are discernible in Table 2. For example, the relative elution position of sterols may be changed by a change in pore diameter. Thus, in the region between 40 and 100 Å, lanosterol will migrate ahead of cholesterol; below and above this range, it will migrate behind cholesterol. Similar effects are noted in the behavior of sitosterol and stigmasterol, while agnosterol and Δ^7-cholestenol as well as 24-dehydrocholesterol and 7-dehydrocholesterol each exhibit "crossover" behavior with respect to the

other. Indeed, the only sterol that maintains the same elution volume relative to cholesterol throughout the whole pore diameter range is Δ^7-cholestenol.[3]

Recoveries of each sterol acetate were calculated during these studies; all but three of the 13 showed consistent recoveries of 80–100%. Zymosterol, 7-dehydrocholesterol, and ergosterol recoveries ranged downward from 75–15%. Of these three, it is likely that the zymosterol sample had already undergone decomposition during prolonged storage in solution, but ergosterol and, in particular, 7-dehydrocholesterol were found to be extremely prone to decomposition upon adsorption on the silica surface. This decomposition was accompanied by the formation of dark blue-green bands at the top of the column reminiscent of that seen in the Liebermann-Burchardt reaction. The reaction apparently involved the initial formation of a more polar molecule that could be eluted with ethyl ether but proceeded eventually to yield an insoluble brown resin-like material.

Application to Model and Biological Mixtures. Several model separations are illustrated in Fig. 3. These are the resolution of dihydrocholesterol and cholesterol (Fig. 3*a*); cholesterol and 24-dehydrocholesterol (Fig. 3*b*); 24,25-dihydrolanosterol, dihydrocholesterol, cholesterol, Δ^7-cholestenol, and 24-dehydrocholesterol (Fig. 3*c*). A preparative scale fractionation of 40 mg of commercial "lanosterol" into 24,25-dihydrolanosterol and lanosterol is shown in Fig. 4.

In Fig. 5 is shown a chromatogram of the digitonin-precipitable sterols from the liver of an animal that had received 20 mg/kg body weight of triparanol for 7 days and 20 μc of mevalonic acid-C^{14} 30 min before sacrifice. Only two definite peaks are detectable colorimetrically–cholesterol and 24-dehydrocholesterol–although traces of color were found in the interval between these two peaks (indicated by dots). The radioactivity in these fractions, measured by liquid scintillation counting(15), showed a number of other interesting features. In addition to Δ^5 and $\Delta^{5,24}$ sterols, peaks migrating with the elution volumes of lanosterol, 7-dehydrocholesterol, and zymosterol were observed. Two unknown sterols, one with a migration rate consistent with a $\Delta^{7,24}$ sterol (16) and the other with a rate consistent with a $\Delta^{5,7,24}$ structure, bracketed the zymosterol peak but were clearly distinguishable from it.

[3] The relative elution positions and the "cross-overs" described here are independent of benzene concentration in the range between 12 and 20% benzene in hexane. Thus, there appears to be a distinction between "crossovers" between members of the same class caused by differences in pore diameter, and those "cross-overs" between *classes* caused by solvent composition such as have been described by Hirsch and Ahrens (14).

DISCUSSION

The establishment of the relative elution volumes of these sterol acetates as a function of silica gel structure serves two purposes: it illustrates the contribution of gel structure to the chromatographic process and it permits the rational selection of a silica gel for a desired separation.

It is evident that the elution order of the sterol acetates in these systems is not the same as that reported for the free sterols by Kandutsch and Russell or by Wells and Lorah in their systems (5, 6). How many of these differences are attributable to the structure of the silica gels used by them and how many to the difference between the chromatographic properties of free and acetylated sterols cannot be established at this time. In all three systems (including ours), the addition of methyl groups to the sterol nucleus reduces its retention volume (e.g., methostenol *vs* Δ^7-cholestenol, lanosterol *vs* zymosterol). The pivotal difference appears to be in the effect of the $\Delta^{8(9)}$ bond, which, in the system of Kandutsch and Russell, *reduces* the retention volume compared to that of the Δ^5 bond, and in our system, *increases* the retention volume (zymosterol *vs* 24-dehydrocholesterol).

The utility of these retention volumes in planning separations will, of course, hinge on the column efficiencies attainable. Within the load ranges described in these studies, a 1-meter column can resolve two components whose retention volumes differ by a factor of 1.15–1.20, the nominal equivalent of 500 to 750 plates. A more detailed prediction of column efficiency cannot be given at this time because it appears to be intimately related to the orientation assumed by the sterol acetate molecules in the adsorbed state. These considerations, however, do not affect the retention volumes reported here.

The liver sterols from a triparanol-treated animal provide an excellent measure of the utility of this fractionation system. Four previously reported features of sterol metabolism in the treated animal are demonstrable in the chromatogram (Fig. 4): (1) there is negligible incorporation of mevalonic acid into cholesterol (17), (2) there is an accumulation of desmosterol (18), (3) there are appreciable quantities of radioactivity associated with zymosterol indicating a block in its hydrogenation to zymostenol (19), and (4) a sterol with the properties of a $\Delta^{7,24}$-stenol is detectable (16). The close correlation of colorimetric and radiochemical findings in this chromatogram with those results reported by others suggested that the fractionation system described here may be of considerable use in studies of sterol metabolism.

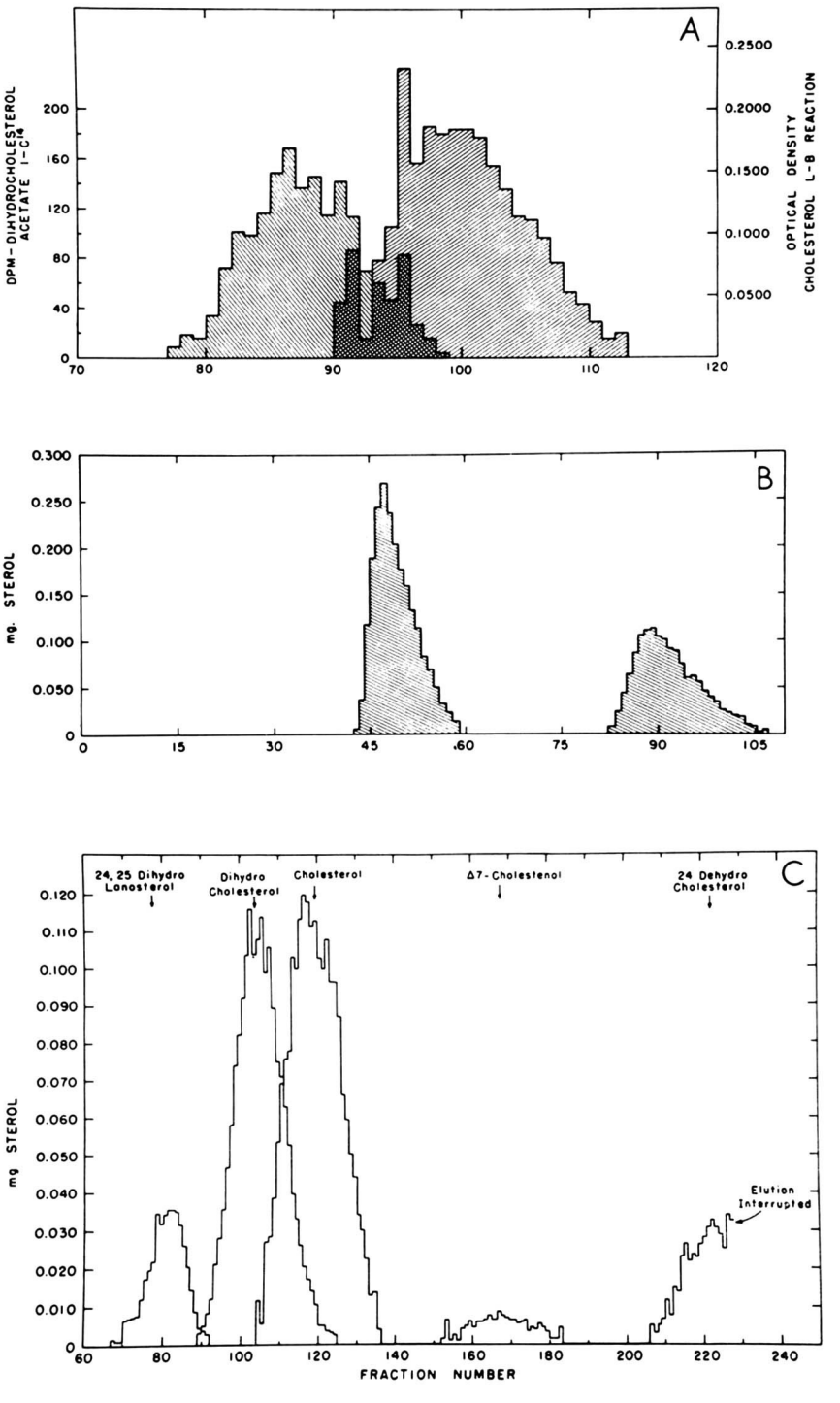

FIG. 3. Fractionation of model mixtures. All compounds are listed in order of emergence.

A, gel #12; column dimensions, 0.45 x 100 cm; load, 0.6 mg dihydrocholesteryl acetate-C^{14}, 1.2 mg cholesteryl acetate; fractions, 2.9 ml/10 min. Double cross-hatched areas indicate region and extent of cross-contamination.

B, gel #12; column dimensions, 0.25 x 40 cm; load, 2.0 mg cholesteryl acetate, 2.0 mg 24-dehydrocholesteryl acetate; fractions, 1.5 ml/15 min.

C, gel #12; column dimensions, 0.45 x 103 cm; load, 0.4 mg 24,25-dihydrolanosteryl acetate, 2.0 mg dihydrocholesteryl acetate, 2.0 mg cholesteryl acetate, 0.2 mg Δ^7-cholesteryl acetate, 2.0 mg 24-dehydrocholesteryl acetate; fractions, 6.0 ml/15 min.

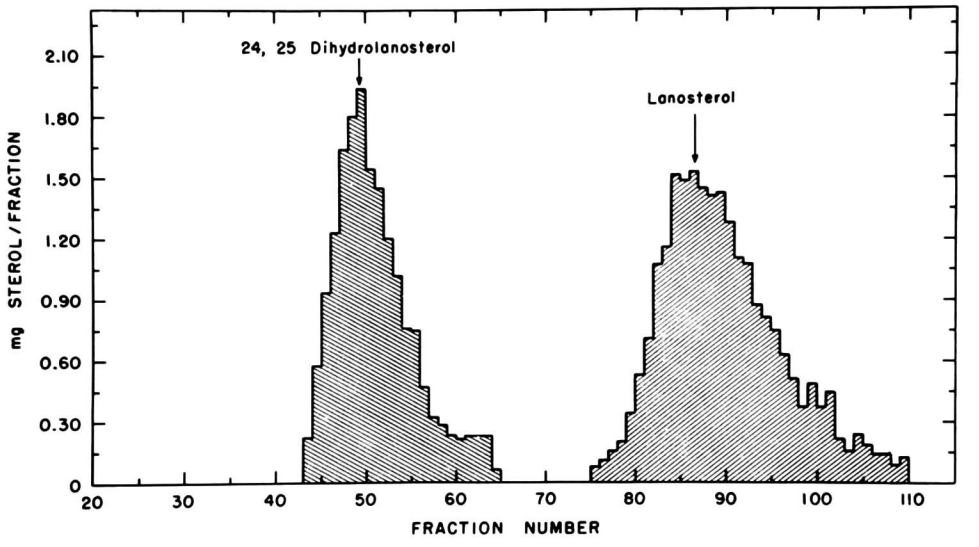

FIG. 4. Preparative-scale fractionation of crude lanosterol. Gel, #12; column dimensions, 0.90 x 50 cm; load, 40 mg crude lanosterol; fractions, 15 ml/20 min.

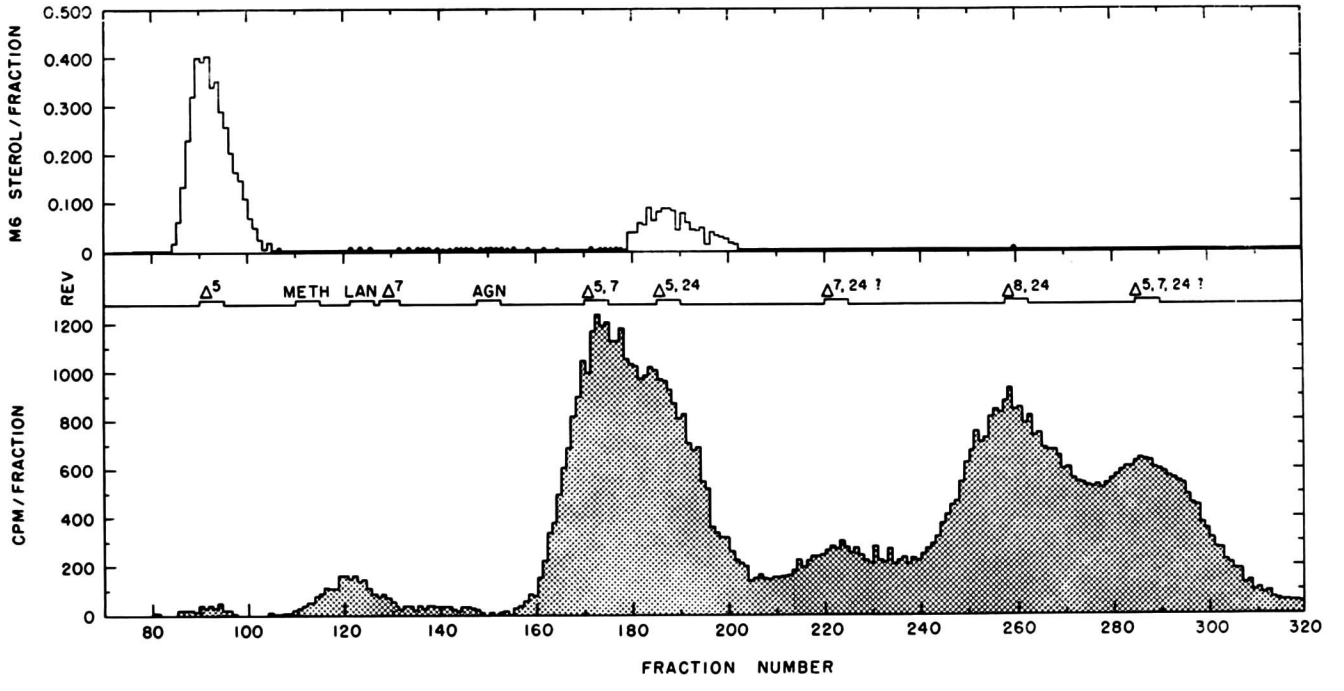

FIG. 5. Fractionation of liver digitonin-precipitable sterols from a triparanol-treated rat. Gel, #12; column dimensions, 0.45 x 75 cm; load, 6 mg sterol acetates, 130,000 cpm; fractions, 5.5 ml/22 min.

We wish to thank the individuals who so generously contributed samples of sterols to make positive identification of retention values possible. The technical assistance rendered by Mrs. Rita M. Dahl and Barbara A. Johnson is gratefully acknowledged.

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Erratum

In the paper by Peter D. Klein and Patricia A. Szczepanik (Vol. 3, No. 4, October 1962), the last part of the sentence on page 460, second column, last three lines, should have read:

24-dehydrocholesterol was prepared from 25-ketonorcholesterol via the 25-hydroxycholesterol intermediate (12) by the method of Bergmann and Dusza (*J. Org. Chem.* **23:** 459, 1958).