pression of Nahas, *et al*(2) were otherwise acceptable, it would nevertheless need to be corrected by replacing the urinary $RNH_3^+$ term with a term representing solely the filtered $RNH_3^+$ ($UV_{H^+} = UV_{TA} + UV_{NH_4^+} - UV_{HCO_3^-} + C_{IN}P_{RNH_3^+}$). c) Values corresponding to filtered $RNH_3^+$ may be derived from total urinary THAM values by calculating the extent of ionization at blood pH. Hence, "$UV_{H^+}$" values corresponding to the "corrected" formulation may be calculated from the present experimental data.

Cumulative excretion values for filtered $RNH_3^+$ (calculated in the manner suggested by the reviewer) and for "$H^+$" calculated from the "corrected" formulation ("$H^+$" = "net acid" + filtered $RNH_3^+$) are shown in Fig. 4. Since the corrected values (Fig. 4) are not appreciably different from the uncorrected values shown in Fig. 1, it is evident that this correction eliminates only a relatively minor source of error. This result is in accord with the views already stated. Since $RNH_3^+$ cannot be metabolized, its presence in the organism can have no appreciable effect on acid-base status beyond the initial change brought about by its formation at the expense of hydrogen ion removed from blood buffers by interaction of the latter with infused $RNH_2$. The experimental data confirm that no further removal of hydrogen ion is effected as a consequence of $RNH_3^+$ excretion.

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## Comparison of Intestinal Absorption and Esterification of 4-$C^{14}$ Vitamin $D_3$ and 4-$C^{14}$ Cholesterol in the Rat.* (31534)

NORMAN H. BELL†
*Department of Medicine, Northwestern University Medical School, Chicago, Ill.*

Cholesterol and other sterols are esterified during the process of intestinal absorption in rats(1-4). Further evidence is available that vitamin D also may undergo esterification (5). The purpose of the present study is to show that vitamin D is esterified during absorption. Further, the esters will be characterized on the basis of the degree of unsaturation of the fatty acid moiety.

*Methods.* Male Wistar rats weighing between 175 and 225 g were lightly anesthetized with ether. The thoracic duct was cannulated with a polyethylene catheter and exteriorized through a stab wound(1). The rats were placed in restraining cages and allowed free access to tap water and the Rockland "D free" diet (Teklad, Inc., Monmouth, Ill.). Twenty-four hours later they were given by intubation under light ether anesthesia $10^6$ dpm 4-$C^{14}$ cholesterol (145 $\mu$c/mg) (New England Nuclear Corp., Boston, Mass.) or 4-$C^{14}$ vitamin $D_3$ (15 $\mu$c/mg) (N. V. Philips-Duphar, Weesp, Netherlands) dissolved in a drop of ethanol and diluted with 0.5 ml propylene glycol(5). The labeled sterols were purified immediately before use by chromatography on 20 × 20 cm glass plates covered with Silica Gel H (Brinkman Instruments, Westbury, N. Y.) in the solvent system methylene chloride. Lymph from the rats was collected in the next 24 hours and frozen until analyzed.

Aliquots of lymph were extracted by the method of Bligh and Dyer(6). The chloro-

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$N_2$ and chromatographed as described above. Standard markers of the sterols and their esters were spotted on the plates adjacent to form extracts were dried under a stream of the samples and were visible under UV light when sprayed with a solution of Rhodamine G in water after chromatography. The solvent front was allowed to run 15 cm, and the silica gel was fractionated at intervals of one cm and scraped into counting vials. 15 ml of a solution of 40 g Cab-O-Cil, 4 g 2-3 diphenyloxazole (PPO) and 40 mg 1-4 bis 2- (4-methyl-5-phenyloxazolyl) benzene (dimethyl POPOP) (Packard Instrument Corp., Downers Grove, Ill.) per liter of toluene(7) was added and, with shaking, a gel formed. Radioactivity was determined with a liquid scintillation spectrometer. When necessary, quenching was corrected with an external gamma source(8).

To separate the esters, the chloroform extracts were chromatographed in the dark under $N_2$ over 24 hours on plates 20 cm wide and 40 cm long covered with a mixture made up of 20 g Silica Gel G (Brinkman Instruments) and 50 ml 1.4% silver nitrate(9). The solvent system hexane:benzene 1:2 was used for the esters of vit. $D_3$(9) and hexane: benzene 1:1 for those of cholesterol(10). In these systems, the sterols remain at the origin and the esters are separated into saturated, monounsaturated, diunsaturated, triunsaturated and tetraunsaturated groups(9,10). Stearate or palmitate, oleate, linolate, linolenate and arachidonate esters of vit. $D_3$ and cholesterol were synthesized from fatty acid chlorides or from fatty acid chlorides made from the fatty acids and oxalyl chloride by the method of Kuksis and Beveridge(11) as previously described(9). The markers were spotted on the plates next to the extracts and identified after chromatography as described above. The silica gel adjacent to the markers was scraped into counting vials after chromatography, and counted.

TABLE I. Percent Absorption and Composition of Lymph of Rats Given $C^{14}$ Vitamin $D_3$ and $C^{14}$ Cholesterol.

| Rat No. | Unknown % | Unknown dpm | Sterol % | Sterol cpm | Ester % | Ester cpm | Absorbed % |
|---|---|---|---|---|---|---|---|
| | | | $C^{14}$ vitamin $D_3$ | | | | |
| 1 | 25.6 | 1226 | 64.0 | 3066 | 8.1 | 389 | 36.8 |
| 2 | 24.4 | 888 | 56.5 | 2065 | 2.8 | 102 | 43.6 |
| 3 | 18.4 | 679 | 71.8 | 2654 | 7.2 | 268 | 40.1 |
| | | | $C^{14}$ cholesterol | | | | |
| 4 | 11.3 | 235 | 39.7 | 826 | 42.1 | 877 | 24.8 |
| 5 | 2.0 | 79 | 35.6 | 1392 | 62.0 | 2425 | 46.5 |
| 6 | 5.0 | 173 | 27.3 | 942 | 66.8 | 2306 | 66.6 |

*Results.* The fraction of 4-$C^{14}$ vit. $D_3$ recovered from lymph was similar to that of 4-$C^{14}$ cholesterol during the period of 24 hours after oral administration (Table I).

After fractionation by thin-layer chromatography, the extracts of lymph were found to contain the sterols, their esters and other unidentified material which remained at or near the origin (Fig. 1a and 1b). Whereas most of the administered 4-$C^{14}$ vit. $D_3$ was recovered as the vitamin and very little as esters, most of the administered 4-$C^{14}$ cholesterol was recovered as esters (Table I). More of the 4-$C^{14}$ vit. $D_3$ than of the 4-$C^{14}$ cholesterol was converted to unidentified more polar compounds. Two-thirds or more of the esters of the 2 sterols was composed of saturated or monounsaturated fatty acids (Table II).

TABLE II. Sterol Ester Composition of Lymph of Rats Given $C^{14}$ Vitamin $D_3$ and $C^{14}$ Cholesterol.

| Rat No. | Saturated % | Saturated dpm | Unsaturated mono- % | mono- dpm | di- % | di- dpm | tri- % | tri- dpm | tetra- % | tetra- dpm |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | $C^{14}$ vitamin $D_3$ | | | | | |
| 1 | 55.1 | 336 | 20.8 | 127 | 6.9 | 42 | 12.1 | 74 | 5.1 | 31 |
| 2 | 37.1 | 266 | 52.2 | 374 | 2.5 | 18 | 1.4 | 10 | 6.8 | 49 |
| 3 | 53.3 | 340 | 18.5 | 118 | 5.0 | 32 | 3.3 | 21 | 19.9 | 127 |
| | | | | | $C^{14}$ cholesterol | | | | | |
| 4 | 28.9 | 122 | 37.4 | 158 | 26.3 | 111 | 0 | 0 | 7.3 | 31 |
| 5 | 36.8 | 716 | 32.8 | 638 | 30.4 | 591 | 0 | 0 | 0 | 0 |
| 6 | 57.2 | 2236 | 12.3 | 483 | 29.1 | 1138 | 0 | 0 | 1.4 | 54 |

*Discussion.* The results show that, like cholesterol, vit. $D_3$ is esterified during absorption and that the esters of both sterols are composed principally of saturated and monounsaturated fatty acids (Table II). Although the percent absorption of the sterols was quite similar, the esterification of vit. D was considerably less (Table I). There appeared to be an inverse relationship between esterification and the formation of unidentified more polar compounds.

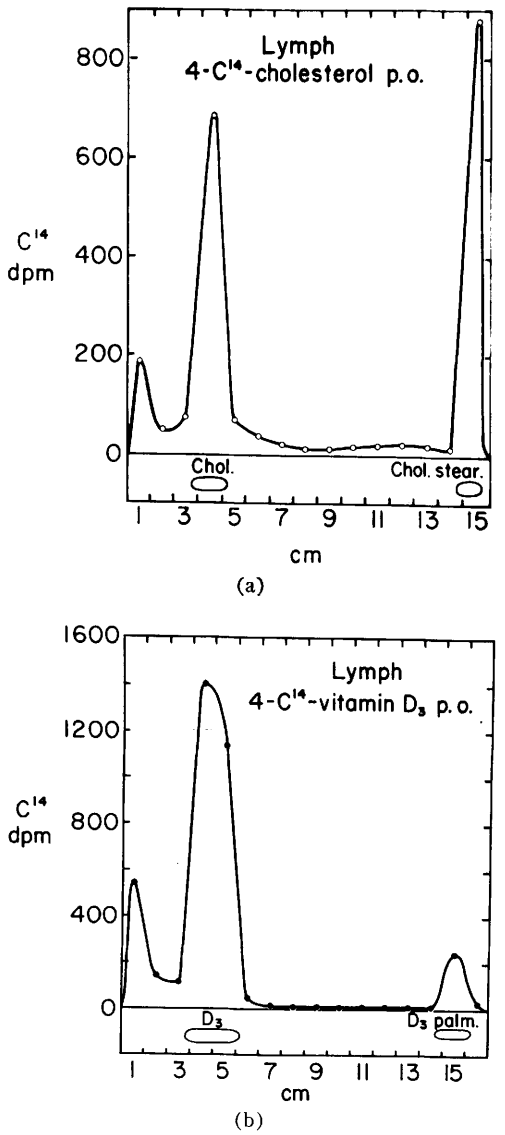


FIG. 1. Thin-layer chomatography of extracts of lymph after oral administration of 4-$C^{14}$ vitamin $D_3$ (a) and 4-$C^{14}$ cholesterol (b). Markers are vitamin $D_3$ ($D_3$), vitamin $D_3$ palmitate ($D_3$ palm.) cholesterol (chol.) and cholesterol stearate (chol. stear.).

In studies with extracts of hog pancreas (12,13), rat pancreas and rat small intestine (14), the relative order of esterification of sterols has been shown to be dihydrocholesterol > cholesterol > $\beta$-sitosterol > stigmasterol > ergosterol > epicholesterol and 7-dehydrocholesterol. This relative order has also been found to occur during intestinal absorption in rats with lymph fistulas(1-4). Thus, it appears that the extent of esterification of sterols is significantly determined by their structure. It is related inversely to the number of unsaturated double bonds in the B ring and, less importantly, to the occurrence of a double bond at carbon 22 in the side chain and is markedly inhibited when the hydroxyl group at carbon 3 is oriented in the $\alpha$ rather than $\beta$ position(13). In keeping with this view is the demonstration that vit. $D_3$, with unsaturated double bonds at carbons 5, 7 and 10-19, is poorly esterified. The fraction of esterified vit. $D_3$ in the present report (Table I) is somewhat lower than the fraction (range: 12.7 to 28%) reported by Schacter, Finkelstein and Kowarski(5).

For intestinal absorption to occur, sterol esters evidently must undergo hydrolysis, and absorption apparently is dependent upon the extent to which the ester is hydrolysed. Cholesterol is readily absorbed when administered as one of the naturally occurring esters(15), but is absorbed either poorly or not at all when given as cholesteryl trimethyl acetate, $\alpha,\alpha$-methyl ethyl caproate(15,16) or $\alpha$-ethyl caproate(17), esters which are poorly hydrolysed by pancreatic extracts or juice. Following oral administration to rachitic rats, the esters, acetate(18), ethyl carbonate(19) and phosphate(20) of irradiated provitamin $D_2$ have the same antirachitic activity as vit. $D_2$ but the esters benzoate(19) and palmitate (21,22) are less effective and the esters allophanate, cinnamate, diphenylacetate, oxalate, $\alpha$1 naphthylurethane and phenylurethane (21) are completely ineffective. After column chromatography of extracts of intestinal mucosa of rats given $H^3$-vitamin $D_3$, the antirachitic activity of the least polar peak ("peak 1") was 75% that of $H^3$ vitamin $D_3$ ("peak