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Investigations on Vitamin D Esters Synthesized in Rats

DETECTION AND IDENTIFICATION

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1. Vitamin D-deficient rachitic rats were given [1-^{3}H]cholecalciferol by gastric intubation. After 24hr., diethyl ether extracts of liver and kidney contained 5–11% and 4·5–20% respectively of total vitamin D apparently esterified with long-chain fatty acids. 2. A two-dimensional thin-layer chromatographic technique was devised that completely separated seven synthetic vitamin D esters according to the chain length and number of double bonds in the fatty acid component. When the 'vitamin D ester' fraction from liver or kidney was co-chromatographed with the standard esters, radioactivity appeared mainly in vitamin D palmitate, stearate, oleate and linoleate regions. The proportion of radioactivity in the saturated fatty acid esters was higher in kidney than in liver. 3. The same percentage of tissue vitamin D in the esterified form was found at each of two dosages of vitamin D. 4. The possible specificity of a vitamin D esterification mechanism is discussed.

Vitamin D after administration to rats is converted into a variety of metabolic products. Kodicek (1963) described experiments where [U-^{14}C]ergocalciferol was given orally or parenterally to rachitic or normal rats. Only 30% of the radioactivity could be recovered in the form of vitamin D alcohol 24hr. later, and the remainder appeared in faeces and intestinal contents (60%), liver (6%) and bones (3%) as biologically inactive metabolites. A small amount of the latter radioactive substances was diethyl ether-soluble, but the major portion was more hydrophilic and was extracted with ethanol–water (1:1, v/v).

Further characterization of the metabolites was attempted by Norman, Lund & DeLuca (1964), who administered cholecalciferol randomly labelled with tritium to vitamin D-deficient rats. Again, after 24hr., 49% of the radioactivity in intestinal mucosa and 17% in kidney was in hydrophilic material. The remainder was chloroform-soluble and separated on silicic acid column chromatography into four components. One of these contained 82–85% of the radioactivity and had the chromatographic properties and biological potency of vitamin D. The other three components were claimed to have some antirachitic activity but were not chemically defined.

None of these investigations has produced sufficient evidence for the presence of an esterified form of vitamin D in rat tissues. Indeed, in early attempts to find vitamin D esters in rats, with non-radioactive ergocalciferol (Kodicek, 1958) and [U-^{14}C]ergocalciferol of low specific radioactivity (0·46mc/m-mole) (Kodicek, 1957), all the vitamin was recovered in the non-esterified form. However, in a preliminary communication we reported the identification of long-chain fatty acid esters of vitamin D in rats given [1-^{3}H]cholecalciferol (6·46mc/m-mole) (Fraser & Kodicek, 1965), and a detailed account of that work is presented here. Lund & DeLuca (1966) found vitamin D ester in liver, serum, bone and faeces of rats given [1,2-$^3H_2$]-cholecalciferol, and Bell (1966) identified a series of these esters in the thoracic-duct lymph of rats absorbing [4-^{14}C]cholecalciferol from the intestinal tract.

MATERIALS AND METHODS

Animals. Male and female hooded rats were fed from weaning on the rachitogenic diet 2965 of Steenbock & Black (1925) as modified by Numerof, Sassaman, Rodgers & Schaefer (1955). Another group of weanling rats was raised on the vitamin D-free diet of DeLuca, Guroff, Steenbock, Reiser & Mannatt (1961). After 3–4 weeks on these diets, when the animals weighed 80–120g., 0·5mg. of [1-^{3}H]-cholecalciferol (6·46mc/m-mole) in 0·1ml. of arachis oil was administered by gastric intubation to each rat. In some experiments, rats under light ether anaesthesia were fitted, 8hr. before dosing, with tail cups as described by Barnes, Fiala & Kwong (1963). This stopped the 'recirculation' of excreted vitamin D by preventing coprophagy and also enabled total faecal collections to be made. The rats were killed by cervical dislocation 24hr. after administration of

the vitamin, and liver and kidneys were removed and stored at −15°.

Chemicals. The radioactive vitamin was prepared from [1-^{3}H]cholecalciferol 3,5-dinitrobenzoate kindly supplied by Dr R. K. Callow and Dr G. A. Thompson. The method of saponification was that of Havinga & Bots (1954), and the free cholecalciferol was purified by thin-layer chromatography by the method of Wilson, Lawson & Kodicek (1967).

Crystalline ergocalciferol was bought from Glaxo Laboratories Ltd. (Greenford, Middx.) and crystalline cholecalciferol from Koch–Light Laboratories Ltd. (Colnbrook, Bucks.). The methyl esters of stearic acid, palmitic acid and linoleic acid (Applied Science Laboratories Inc., State College, Pa., U.S.A.) and of oleic acid, linolenic acid and myristic acid (Sigma Chemical Co., St Louis, Mo., U.S.A.) and ethyl laurate (British Drug Houses Ltd., Poole, Dorset) were used in the chemical synthesis of vitamin D esters. Floridin earth was obtained from British Drug Houses Ltd. Anhydrous ethanol was prepared by refluxing and then distilling 95% (v/v) ethanol over magnesium with iodine as catalyst. Light petroleum (b.p. 40–60°) from May and Baker Ltd. (Dagenham, Essex) was shaken twice with conc. H_2SO_4, washed with water and dried with solid KOH before final distillation. All other chemicals and solvents were of analytical reagent grade and were used as received.

Measurement of radioactivity. Radioactivity was measured in a Packard Tri-Carb liquid-scintillation spectrometer on samples dissolved in 5ml. of scintillator solution in polythene counting vials. The scintillator solution contained, in toluene, 0·4% 2,5-diphenyloxazole and 0·01% 1,4-bis-(5-phenyloxazol-2-yl)benzene (Packard Instrument Co. Inc., La Grange, Ill., U.S.A.). Corrections for scintillation quenching were made with the use of *n*-[1,2-$^3H_2$]-hexadecane as an internal standard. The counting efficiency for tritium was 20%.

Synthesis of standard vitamin D esters. Seven long-chain fatty acid esters of vitamin D were prepared by transesterification of vitamin D acetate with the methyl esters of the various fatty acids, by a modification of a method for synthesizing retinyl esters (Futterman & Andrews, 1964*a*).

Ergocalciferol acetate was synthesized by dissolving 200mg. of ergocalciferol in 4ml. of pyridine with 2ml. of acetic anhydride and keeping the solution at room temperature overnight. The solvents were blown off at 40° under a stream of nitrogen and the residue, dissolved in light petroleum, was streaked on a silica gel G thin-layer plate and chromatographed with $CHCl_3$. Ergocalciferol acetate (R_F 0·71) was eluted with diethyl ether. Esterification was confirmed by comparing the transmittance-spectrum change of the product with that of ergocalciferol on a Unicam SP. 200 infrared spectrophotometer. The hydroxyl peak at 3500 cm.$^{-1}$ had virtually disappeared, but carbonyl (1740 cm.$^{-1}$) and acetate (1260 cm.$^{-}$) peaks were prominent. The ultraviolet absorption spectrum in ethyl alcohol gave a peak at 265mμ, identical with the unchanged vitamin. Transesterification to form the various esters was done in the presence of the catalyst, sodium ethoxide, prepared by treating small pieces of bright sodium with ethanol to give an ethoxide concentration of 40mg./ml.

Ergocalciferol acetate (25μmoles) and the required fatty acid methyl ester (30μmoles) were transferred in light petroleum to a 10ml. round-bottomed ground-glass-necked flask. The solvent was gently evaporated so that the residue remained in a small area at the bottom of the flask, and 0·025ml. of sodium ethoxide solution was quickly added. The flask was held at 50–60° for 10min. under reduced pressure to vaporize and remove the methyl acetate by-product.

The mixture was suspended in 1ml. of light petroleum and subjected to thin-layer chromatography with chloroform as before. Three major components were observed, two being comparable to vitamin D (R_F 0·35) and its acetate (R_F 0·71). The third and least polar substance was the required fatty acid ester of vitamin D (R_F 0·83). This was eluted and stored in light petroleum at −15°. Although high yields are obtained by this method when applied to the esterification of cholesterol (Mahadevan & Lundberg, 1962) or retinol (Futterman & Andrews, 1964*a*), in the case of ergocalciferol other reaction products occur such as the reduced forms, dihydrovitamins D_2 I and D_2 II (Windaus, Linsert, Lüttringhaus, & Weidlich, 1932). Also, some of the ester is hydrolysed to the free vitamin. Hence the yield of converted ester was never higher than 40–45% of the possible maximum.

Thin-layer chromatography. A two-dimensional chromatographic system was devised to separate the synthetic esters on a 20cm. × 20cm. plate coated with a 250μ layer of silica gel G. The plate was prepared by dipping one edge in aq. 5% (w/v) $AgNO_3$ and allowing it to ascend for 2·5cm. Re-activation was carried out at 110° for 30min., after which the plate was cooled and used immediately. Each ester and free vitamin D (10μg. of each) were applied in light petroleum to the $AgNO_3$-impregnated strip at a point 1·5cm. from the lower edge. The first dimension was developed for 35min. with diethyl ether–hexane (1:4, v/v) to a height of 16cm. along the strip, separating the esters into groups according to their saturated, monoenoic, dienoic and trienoic acyl functions (Morris, 1963). The plate was dried

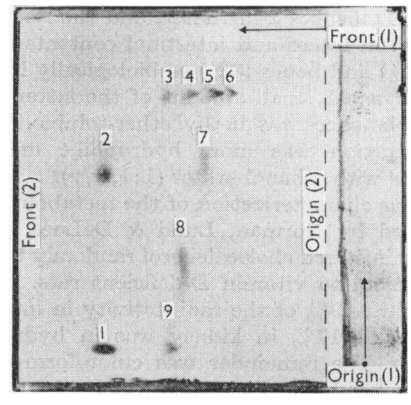

Fig. 1. Two-dimensional thin-layer chromatogram of synthetic vitamin D esters. The systems used were: system I: 15–16cm. chromatogram on silica gel G impregnated with aq. 5% (w/v) $AgNO_3$ and reactivated at 110°; developing solvent, diethyl ether–hexane (1:4, v/v); system II: 15–16cm. chromatogram on silica gel G impregnated with 1% liquid paraffin (B.P.) in light petroleum; developing solvent, acetic acid. 1, Vitamin D; 2, vitamin D acetate; 3, vitamin D laurate; 4, vitamin D myristate; 5, vitamin D palmitate; 6, vitamin D stearate; 7, vitamin D oleate; 8, vitamin D linoleate; 9, vitamin D linolenate.

at room temperature for 1 min., turned through 90° and developed with diethyl ether to a height of 4 cm., thus eluting the esters out of the $AgNO_3$ region, which was then removed. The second dimension used reversed-phase partition chromatography, which separated the esters mainly according to the acyl chain length. The plate was carefully lowered into 1% (w/v) liquid paraffin (B.P.) in light petroleum until the solution had reached the level of the esters. Drying was allowed for 2 min. at room temp. and development was completed (90 min.) with acetic acid ascending to the original left edge of the thin layer (Michalec, Sulc & Mestan, 1962). Acetic acid was removed by heating in a stream of air and the plate was cooled and sprayed with 10% (w/v) $SbCl_3$ to demonstrate the ester positions (Fig. 1).

RESULTS

Preliminary identification of ester. Liver and kidneys from rats given [1-^{3}H]cholecalciferol were ground separately with 30–40 g. of anhydrous granular sodium sulphate. The dry powder was extracted four times with 50 ml. volumes of diethyl ether, and the final volume was reduced by evaporation under vacuum. Extracted radioactivity was measured (Table 1) and a sample containing approx. 2000 counts/min. was chromatographed on thin layers with vitamin D side-markers, the plates being developed with chloroform to 16 cm. The silica gel was scraped off in 1 cm. bands and counted by liquid-scintillation spectrometry. Most of the radioactivity (80–90%) corresponded to the vitamin D standard (fractions 4–5, R_F 0·25–0·3). Some (1–2%) appeared at the origin and 5–11% was found in a fraction of low polarity at 12–13 cm. (R_F 0·75–0·8) (Fig. 2). This last fraction was eluted and re-chromatographed on a thin-layer plate, when the radioactivity again appeared at R_F 0·75–0·8.

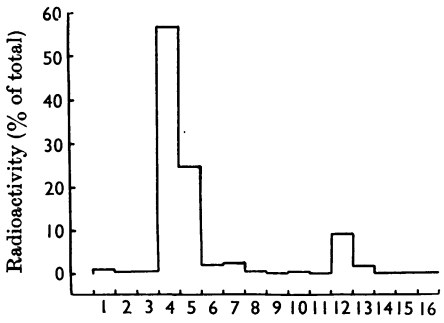

Fig. 2. Thin-layer radiochromatogram of diethyl ether extract of liver from a rat given 0·5 mg. of [1-^{3}H]cholecalciferol (6·46 mc/m-mole) 24 hr. previously. Silica gel G thin layers were developed with chloroform to a 16 cm. solvent front. Radioactivity was measured in 1 cm. bands by liquid-scintillation spectrometry.

A sample of this fast-running material was treated by refluxing it for 30 min. with M-potassium hydroxide in aq. 50% (v/v) methanol. The unsaponified material was chromatographed as before and radioactivity was found mainly in the vitamin D area (R_F 0·25–0·3). From these results it was concluded that a vitamin D metabolite had been observed, and, with properties of low polarity and conversion in alkali into free vitamin D, it seemed likely that it was a fatty acid ester of the vitamin.

As this material was heavily contaminated it was possible that free radioactive vitamin D had been carried ahead by cholesteryl esters. To check this, pure [1-^{3}H]cholecalciferol (2000 counts/min.) was chromatographed with 1 mg. of cholesteryl esters from rat liver. No radioactivity was found outside the vitamin D region of R_F 0·25–0·3, and it was considered that the ester-region radioactivity had been due to a true metabolite. The percentage of ether-extracted radioactivity appearing as 'ester' in liver ranged from 5 to 11%, and in kidney was more variable (4·5–20%) (Table 1).

Table 1. *Distribution of radioactivity in organs 24 hr. after administration of* [1-^{3}H]*cholecalciferol*

Diet for Expts. 1–3 was that of DeLuca *et al.* (1961) supplemented with vitamin D, and for Expts. 4–7 that of Steenbock & Black (1925) without vitamin D.

		Liver vitamin D		Kidney vitamin D	
Expt. no.	Dose of [1-^{3}H]cholecalciferol (μg.)	% of dose recovered	% of recovered vitamin D found as ester	% of dose recovered	% of recovered vitamin D found as ester
1	500	4·8	7·7	0·4	10
2	500	5·9	5·3	0·3	20
3	500	—	—	0·7	16
4	500	4·8	11·0	0·6	4·5
5	500	5·6	13·6	0·9	4·4
6	10	5·3	11·1	4·8	5·3
7	10	5·7	9·6	3·6	5·4

Further characterization of the 'ester' fraction. Because this isolated fraction was very small and contaminated, mainly by cholesteryl and retinyl esters, it was not possible to confirm its identity directly by saponification and isolation of the component vitamin and fatty acids. A comparison was therefore made of the chromatographic properties of the 'ester' fraction and of the synthetic standard esters by means of two-dimensional thin-layer chromatography described above. No chromatographic difference between the synthetic esters of ergocalciferol and cholecalciferol could be shown, and the ergocalciferol series was used in comparison with the supposed 'cholecalciferol ester' from rats.

Preliminary chromatography on floridin earth removed retinyl esters (Green, 1951) and separated the vitamin D 'ester' from free vitamin D. A column, 1 cm. int. diam., was filled to a height of 13 cm. with dry floridin earth, and 60 ml. of benzene–light petroleum (40:60, v/v) was run through under 5 lb./in.^{2} pressure of nitrogen. The diethyl ether extract of tissue was then applied in 2–3 ml. of benzene–light petroleum and eluted with this solvent. The first 16–17 ml. contained all the 'ester' radioactivity. Although vitamin D is said to be isomerized to isotachysterol on floridin earth (Irmscher, Wirts & Daehne, 1959), the vitamin D esters were little adsorbed on this material and were identical with untreated esters on thin-layer chromatography. It was thus assumed that the esters had not been altered by this treatment.

Standard esters plus 2000 counts/min. of extracted 'ester' radioactivity were chromatographed in two dimensions, and the spots were made visible by spraying with antimony trichloride in chloroform at room temperature. Cholesteryl esters did not stain unless the plate was heated. However, because of the overload of these and other lipids, trailing of the vitamin D esters occurred, although they were still visible as discrete spots. These were lifted off individually, each was treated with 5 N-hydrochloric acid to remove the antimony trichloride and eluted with diethyl ether, and the radioactivity was then measured.

The results are shown in Table 2. Radioactivity was found mainly in four ester areas: palmitate, stearate, oleate and linoleate. Seven chromatograms were run with liver extracts, and the pattern of radioactivity was quite repeatable with little difference between the groups of rats given the two diets. Practically no radioactivity was present in the vitamin D spot, but when either the vitamin D fraction from the floridin-earth column or the saponified-ester fraction was applied to the two-dimensional system, radioactivity appeared only in the vitamin D spot. Standard vitamin D arachidonate remained at the origin and thus was removed with the silver nitrate-impregnated strip. Up to 10% of the radioactivity of the ester fraction was also found at the origin and was probably arachidonate, but because it could not be identified by this method it was not included in the calculation of results. On comparing the distribution of radioactivity it was noted that the proportion in saturated fatty acid esters was higher in kidney than in liver, but that the proportion in the unsaturated esters was lower.

From these results it was concluded that vitamin D esters are indeed found in these organs and that there is a difference in their composition between liver and kidney.

Ester synthesis from low doses of vitamin D. Some experiments *in vivo* on vitamin D metabolism are subjected to the criticism that large, non-physiological quantities are used to overcome difficulties in detecting trace amounts of the vitamin in tissues. It may be in this case that vitamin D esterification is a detoxication response to the high dose of 500 μg.

Table 2. *Percentage distribution of radioactivity in vitamin D esters identified by two-dimensional thin-layer chromatography*

Diet 1 is that of DeLuca *et al.* (1961); diet 2 is that of Steenbock & Black (1925). Results are means ± S.D.; those for liver on diet 1 are for three chromatograms, those for liver on diet 2 are for four chromatograms, and those for kidneys are for three chromatograms, two on diet 1 and one on diet 2.

	Liver			
Vitamin D ester	Diet 1	Diet 2	Mean	Kidneys
Palmitate	37 ± 5	40 ± 3	39	49 ± 6
Stearate	19 ± 6	24 ± 6	22	31 ± 4
Linoleate	20 ± 3	11 ± 6	16	4 ± 1
Oleate	11 ± 2	14 ± 2	13	3 ± 2
Myristate	3 ± 1	6 ± 3	5	9 ± 3
Other*	10 ± 5	5 ± 1	7	4 ± 1

* Linolenate, laurate, acetate and unidentified.

Table 3. *R_F and R_M values of vitamin D esters*

Mean R_F values are shown ± S.D., with the number of estimations in brackets. The systems used were: system I: 15–16 cm. thin-layer chromatography on silica gel G impregnated with aq. 5% (w/v) $AgNO_3$ and reactivated at 110°; developing solvent, diethyl ether–hexane (1:4, v/v); system II: 15–16 cm. thin-layer chromatography on silica gel G impregnated with 1% liquid paraffin (B.P.) in light petroleum; developing solvent, acetic acid.

$$R_M = \log[(1/R_F)-1]$$

Ergocalciferol ester	System I		System II	
	R_F	R_M	R_F	R_M
Stearate	0·74±0·04 (18)	−0·454	0·28±0·06 (18)	+0·410
Palmitate			0·34±0·05 (16)	+0·288
Myristate			0·41±0·06 (14)	+0·158
Laurate			0·48±0·06 (11)	+0·035
Acetate	0·58±0·03 (16)	−0·140	0·71±0·04 (18)	−0·389
Oleate	0·63±0·04 (17)	−0·231	0·37±0·04 (14)	+0·231
Linoleate	0·45±0·06 (17)	+0·087	0·45±0·03 (13)	+0·087
Linolenate	0·19±0·07 (9)	+0·630	0·52±0·02 (10)	−0·035
Free ergocalciferol	0·07±0·02 (14)	+1·123	0·75±0·04 (15)	−0·477

(20000 i.u.). To investigate this possibility, rats were given, by the same method as before, 10 μg. (400 i.u.) of [1-^{3}H]cholecalciferol. Tissues were collected after 24 hr., extracted with diethyl ether and chromatographed with chloroform on thin layers, and the radioactivity was measured along the plate. The results are included in Table 1 and show the presence of ester in liver and kidney. In this experiment the relative amount of vitamin D in kidney is 6–7 times higher than with the 500 μg. dose and may be an effect of its functional localization (Kodicek, Darmady & Stranack, 1961), observed when the amount of vitamin D available is low.

Theoretical aspects of ester separation. The relative chromatographic behaviour of the vitamin D esters is observed to follow the normal pattern dependent on the length and degree of unsaturation of the variable acyl side chain. When the R_F values (Table 3) for the reversed-phase partition system are plotted against the number of carbon atoms or the number of double bonds in the side chain, linear functions relate chemical structure to chromatographic separation. By means of a parameter R_M, equal to $\log[(1/R_F)-1]$, introduced by Bate-Smith & Westall (1950) for paper chromatography, this relationship can be made quantitative. The mean ΔR_M is for each additional C_2 group 0·125 and for each double bond 0·133, illustrating the near coincidence of a saturated ester with the mono-unsaturated C_2-longer homologue (e.g. palmitate and oleate). If this R_M increment is constant, as has been shown for other compounds on paper chromatography (Marcinkiewicz, Green & McHale, 1963), then the position of other esters in the series can be predicted. This is likely to be successful only when the structural change is some distance from a chemically functional group in the vitamin D moiety. Intramolecular interaction may produce R_M deviations such as is seen with vitamin D acetate, the first member of the ester series, which does not fit the linear relation of the higher homologous esters.

Table 4. *Comparison of percentage compositions of rat liver esters*

The values are based on those quoted in the literature: for cholesteryl ester by Getz, Bartley, Stirpe, Notton & Renshaw (1961); for retinyl ester by Futterman & Andrews (1964*b*); for vitamin D ester by Fraser & Kodicek (1965).

Acyl group	Cholesteryl ester	Retinyl ester	Vitamin D ester
$C_{14:0}$	0·58	0·3	5
$C_{16:0}$	21·2	69·0*	39*
$C_{16:1}$	5·47	2·0	?
$C_{18:0}$	12·25	15·1	22
$C_{18:1}$	27·05*	5·9	13
$C_{18:2}$	17·96	1·4	16
$C_{18:3}$	0·25	?	
$C_{20:4}$	7·55	?	7
Others	7·69	6·3	

* Major component.

DISCUSSION

The success of these experiments in locating the small quantity of vitamin D ester synthesized in rats can be attributed to the high specific radioactivity of the labelled vitamin and the greater sensitivity of thin-layer chromatography than the classical reversed-phase paper chromatography of vitamin D of Kodicek & Ashby (1954). It is noteworthy that previous experiments (Kodicek, 1957,

1958) seeking vitamin D esters in rats were set up to determine whether vitamin D is deposited in the liver mainly in the ester form, as is the case with vitamin A. In this regard they showed the dissimilarity between the two vitamins.

Since the quantity of vitamin D ester is quite low it is of interest to conjecture on its significance in the rat. It may be that esterification is a detoxication mechanism whereby some of the large dose of 500 μg. is temporarily deposited in an inactive form. However, when 10 μg. of cholecalciferol is given, a similar percentage of the dose is esterified, indicating that this process takes place regardless of the quantity present. If this were so there might be a non-specific esterifying enzyme acting on vitamin D. Two enzymes either of which might be involved are the synthetic esterases for cholesterol and retinol. Both are specific for their respective substrates and produce a characteristic pattern of ester composition in liver, with oleate being the major component for cholesterol and palmitate for retinol (Table 4). The pattern for liver vitamin D esters, however, is dissimilar to both. This may be evidence for a specific vitamin D synthetic esterase or for esterification by known enzymes with the variation in ester composition merely a reflection of the range of fatty acids available.

Should there be a specific vitamin D synthetic esterase it is possible that the ester has a functional significance, perhaps in the transport or storage of the vitamin. If the ester has a role in transport it might be expected to be found in the small-intestinal mucosa playing a part in vitamin D absorption. None, or very little, could be located at this site in the present experiments, but these were carried out 24 hr. after dosing and no information was available for earlier periods when most of the vitamin D was being absorbed. Other work (Fraser & Kodicek, 1966) has found that the intestine is a site of vitamin D esterification, in agreement with Bell (1966).

It has long been known that vitamin D in salt-water fish is esterified; e.g., 70% of vitamin D in cod liver occurs as ester (Hickman, 1937). Esterification here presumably makes storage of the very large amounts of vitamin D found in some fish possible. In rats the amount of vitamin D that is esterified, in contrast to vitamin A, is so small that it seems unlikely that this represents an efficient storage form. In fact the apparent inefficiency of esterification in the rat may be a protective mechanism. Thus the rapid destruction of orally or parenterally received vitamin D and its poor esterification together would protect the animal against hypervitaminosis D. It is possible that under natural environmental conditions the ultraviolet-light-induced conversion of 7-dehydrocholesterol may supply vitamin D in excess of requirements and perhaps in excess of any storage capacity.

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