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Absolute Stereochemical Configuration of Phytanyl (Dihydrophytyl) Groups in Lipids of *Halobacterium cutirubrum**

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ABSTRACT: The absolute stereochemical configuration of the phytanyl (3,7,11,15-tetramethylhexadecyl) groups in the lipids of *Halobacterium cutirubrum* was determined as follows. Conversion of the phytanyl groups to phytanic and pristanic acids yielded (+)-3,7,11,15-tetramethylhexadecanoic acid and (−)-2,6,10,14-tetramethylpentadecanoic acid, respectively, showing that C-3 in the phytanyl group has the D or *R* configuration. Reduction of the pristanic acid to pristane (2,6,10,14-tetramethylpentadecane) gave the *meso*-6*R*,10*S* isomer, identical with that obtained from phytol, showing that C-7 and C-11 in the phytanyl groups both have either the *R*,*R* or the *S*,*S* configuration. Barbier–Wieland degradation of the pristanic acid yielded a C_{18} ketone identical with (6*R*,10*R*)-14-trimethylpentadecanone-2, showing that C-7 and C-11 in the phytanyl groups have the *R* configuration.

These findings thus establish the absolute configuration of the bacterial phytanyl groups as (3*R*,7*R*,11*R*)-15-tetramethylhexadecyl. The bearing of these findings on the configuration of phytanyl derivatives from various sources is discussed.

The lipids of extremely halophilic bacteria have been found to be derivatives of a di-*O*-alkylglycerol ether (Sehgal *et al.*, 1962; Faure *et al.*, 1963, 1964; Kates *et al.*, 1963, 1966), the structure and configuration of which has been established as L-2,3-di-*O*-(3′,7′,11′,15′-tetramethylhexadecyl)glycerol (Kates *et al.*, 1965a,b). However, there still remained to be determined the configuration of the asymmetric carbon atoms 3, 7, and 11 in the tetramethylhexadecyl (phytanyl or dihydrophytyl) chains, and an investigation of this question was therefore undertaken. It was also of interest to compare the configuration of the bacterial phytanyl groups with that found for phytol from chlorophyll (Burrell *et al.*, 1959, 1966; Crabbe *et al.*, 1959) and the phytanyl side chains in α-tocopherol (Mayer *et al.*, 1963) and vitamin K_1 (Mayer *et al.*, 1964).

The problem of determining the configuration of the bacterial phytanyl groups could be conveniently divided into two parts: first, the configuration of C-3, and second, the configuration of C-7 and C-11.[1] The approach used to determine the configuration at C-3, was based on the fact that in monomethyl-branched long-chain carboxylic acids of the same configuration the molecular rotations alternate in sign and decrease sharply in absolute value as the methyl-branching group is moved along the chain from the 2 position (Abrahamsson *et al.*, 1963). Thus, in the D series, the 2-methylalkanoic acid has M_D −28°, the 3-methyl acid has +13°, the 4-methyl acid has −2°, and the 5- and 6-methyl acids have rotations close to zero,

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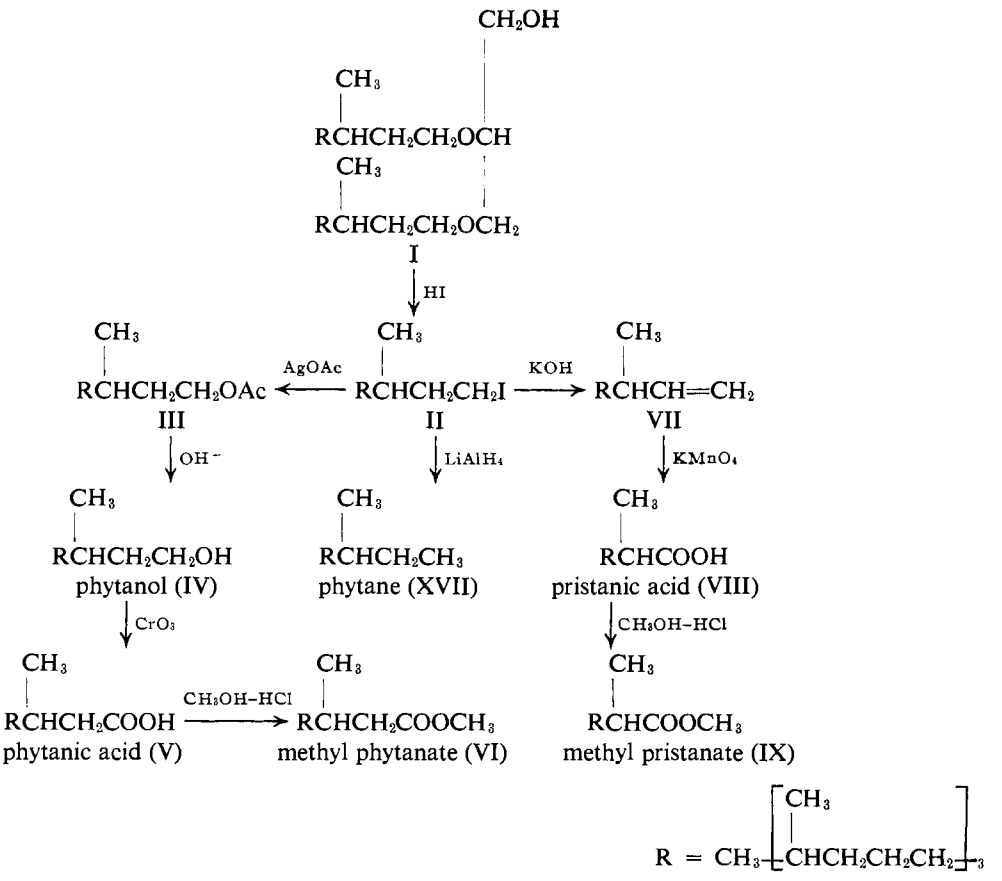
[1] The sequence rule outlined by Cahn (1964) is used throughout this paper for absolute configurations. It should be noted that the 7*R* and 11*R* carbon atoms in phytol become the 6*R* and 10*S* carbon atoms, respectively, in pristane, although the conversion of phytol to pristane does not involve reactions at these asymmetric centers.

Scheme I



as have also acids with methyl groups further down the chain; furthermore, these correlations are essentially independent of the over-all chain length of the acid. It follows that the rotation of a multibranched acid will be determined by the configuration of the asymmetric center(s) closest to the carboxyl group (either the 2- or 3-carbon atom), the contributions from asymmetric centers further down the chain being negligible.

The bacterial phytanyl groups were converted to phytanic (3,7,11,15-tetramethylhexadecanoic) acid and to pristanic (2,6,10,14-tetramethylpentadecanoic) acid by the reactions shown in Scheme I. Di-*O*-phytanylglycerol (I) obtained after hydrolysis of the lipids of *Halobacterium cutirubrum* (Kates *et al.*, 1965a) was cleaved with HI, and the resulting phytanyl iodide (II) was converted, *via* phytanyl acetate (III), to phytanol (IV) which was oxidized to phytanic acid (V). Phytanyl iodide (II) was also dehydrohalogenated to phytene-1 (VII) which was then oxidized to yield pristanic acid (VIII). Both acids have been shown to consist of a single stereoisomer by gas–liquid partition chromatography on an open-tubular column with polyester coating (Ackman and Hansen, 1967). The phytanic acid had M_D +11°, a value close to that of synthetic D-3-methylnonadecanoic acid (Ställberg-Stenhagen (1954)), and the pristanic acid methyl ester (IX) had M_D −37°, almost identical with that of synthetic methyl D-2-methyloctadecanoate (Ställberg, 1958) (Table I).

These results show that the phytanic and the pristanic acids both belong to the D series, thus establishing the *R* configuration of C-3 in the bacterial phytanyl groups. It might be argued that the asymmetric centers at C-7 and C-11 in the phytanic acid and C-6 and C-10 in pristanic acid could influence the rotation of these acids and thus affect the above assignment of configuration. However, the influence of these centers can only be relatively slight, since the phytol-derived phytanic and pristanic acids in which C-3 or C-2, respectively, is racemic have very low rotations (Table I).

Further confirmation of the *R* configuration of C-3 in the phytanyl side chains was obtained by comparison of the molecular rotations of phytanol (IV), pristanol (XIV), and phytanyl iodide (II) with corresponding analogous long-chain compounds of known configuration (Table I)

The approach used to determine the configurations at C-7 and C-11 was to degrade the bacterial phytanyl group to the C_{18} ketone, 6,10,14-trimethylpentadecanone-2 (XII), the two diastereomers of this ketone (the 6*R*,10*R*, and 6*S*,10*R* isomers) having been synthesized previously and their optical rotations determined (Burrell *et al.*, 1959). For purposes of the

TABLE I: Molecular Rotation and Configuration of Phytanyl Derivatives and Related Compounds.

Compound	Source	$[\alpha]_D^{22}$[a] (deg)	M_D[a]	Known or Assigned Configuration
1-Iodo-3,7,11,15-tetramethylhexadecane (II)	*H. cutirubrum*	−6.1	−25	3*R*,7*R*,11*R*
D-1-Iodo-3-methylnonadecane	Synthetic[b]	−6.9	−28	3*R*
3,7,11,15-Tetramethylhexadecanol-1 (IV)	*H. cutirubrum*	+2.4	+7.2	3*R*,7*R*,11*R*
Dihydrophytol	Phytol	−0.8	−2	3*RS*,7*R*,11*R*
D-3-Methylnonadecanol	Synthetic[b]	+2.2	+6.7	3*R*
3,7,11,15-Tetramethylhexadecanoic acid (V)	*H. cutirubrum*	+3.5	+11	3*R*,7*R*,11*R*
D-3-Methylnonadecanoic acid	Synthetic[b]	+4.20	+13	3*R*
Phytanic acid	Phytol	−0.84	−2.6	3*RS*,7*R*,11*R*
3,7,11,15-Tetramethyl-1-hexadecene (VII)	*H. cutirubrum*	−4.8	−13	3*R*,7*R*,11*R*
Methyl 2,6,10,14-tetramethylpentadecanoate (IX)	*H. cutirubrum*	−11.8	−37	2*R*,6*R*,10*R*
Methyl D-2-methyloctadecanoate	Synthetic[c]	−12.1	−38	2*R*
Methyl pristanate	Phytol	−0.74	−2.3	2*RS*,6*R*,10*R*
6,10,14-Trimethylpentadecanone-2 (XII)	*H. cutirubrum*	+0.61	+1.6	6*R*,10*R*
6,10,14-Trimethylpentadecanone-2 (XII)	Phytol	+0.66	+1.8	6*R*,10*R*
2,6,10,14-Tetramethylpentadecanol-1 (XIV)	*H. cutirubrum*	+3.2	+9.1	2*R*,6*R*,10*R*
Pristanol	Phytol	+0.18[d]	+0.5[d]	2*RS*,6*R*,10*R*
2,6,10,14-Tetramethylpentadecane (XVI)	*H. cutirubrum*	0.0	0	6*R*,10*S*
Pristane	Phytol	0.0	0	6*R*,10*S*
3,7,11,15-Tetramethylhexadecane (XVII)	*H. cutirubrum*	+3.9	+11	3*R*,7*R*,11*R*

[a] In chloroform, unless otherwise noted. [b] Ställberg-Stenhagen (1954). [c] Ställberg (1958). [d] As pure liquid.

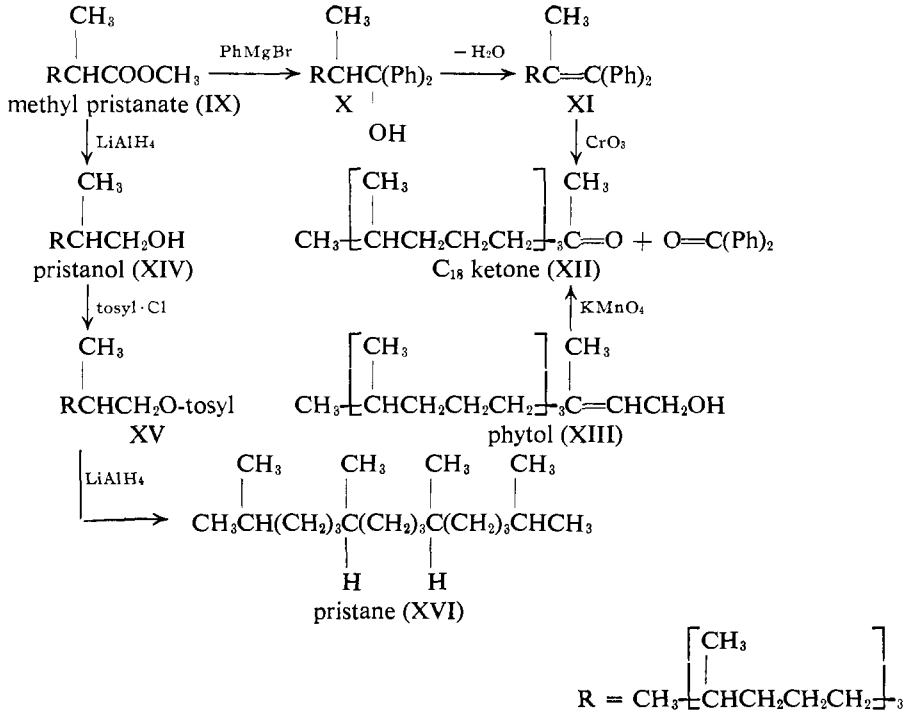
present study, the 6*R*,10*R* stereoisomer was prepared by permanganate oxidation of phytol (XIII), which is known to have the 7*R*,11*R* configuration (Burrell *et al.*, 1959, 1966; Crabbe *et al.*, 1959). Conversion of the bacterial phytanyl group to the C_{18} ketone was achieved by Barbier–Wieland degradation of methyl pristanate (IX), as shown in Scheme II. The ketone was isolated from the reaction mixture as the bisulfite addition compound and proved to have a specific rotation identical with that of the 6*R*,10*R* isomer derived from phytol and isolated in the same way (Table I).

This finding thus shows that C-7 and C-11 in the phytanyl group both have the *R* configuration. However, further confirmation of this assignment was sought by converting the phytanyl group to the symmetrical C_{19} hydrocarbon, pristane (XVI) (2,6,10,14-tetramethylpentadecane), and determining whether the product obtained was the *meso*-6*R*,10*S* isomer or one of the 6*R*,10*R* enantiomers. The sequence of reactions involved was as follows (see Scheme II). Methyl pristanate (IX) was reduced with lithium aluminium hydride to pristanol (XIV), which was then converted to pristane (XVI) by reduction of the *p*-toluenesulfonate (XV) with lithium aluminium hydride. The pristane obtained had no measurable rotation and was therefore the *meso*-6*R*,10*S* isomer; furthermore, it was identical with the *meso*-6*R*,10*S* isomer of pristane obtained from phytol (XIII) by the same sequence of reactions (*cf.* Burrell *et al.*, 1959).

These results confirmed that C-7 and C-11 have the same configuration, namely the *R* configuration. The bacterial phytanyl chain is therefore established as being (3*R*,7*R*,11*R*)-15-tetramethylhexadecyl. It is noteworthy that the *R* configuration of carbons atoms 3, 7, and 11 in the bacterial phytanyl chains is the same as that for the corresponding asymmetric centers in α-tocopherol (Mayer *et al.*, 1963), and that C-7 and C-11 have the same configuration as the corresponding asymmetric centers in the phytyl chain of chlorophyll (Burrell *et al.*, 1959, 1966; Crabbe *et al.*, 1959) and vitamin K (Mayer *et al.*, 1964). The enzyme(s) involved in reduction of the double bonds in the isoprenoid precursors of the bacterial phytanyl groups would thus appear to have the same stereospecificity as corresponding enzymes in plants and animals.

The present findings have a bearing on the configuration of pristanic and phytanic acids isolated from various natural sources. These two acids have been identified in butterfat (Sonneveld *et al.*, 1962; Hansen and Morrison, 1964; Hansen *et al.*, 1965), human and animal tissues (Avigan, 1966), and marine fish oil (Gupta and Peters, 1966), and phytanic acid has been found in ox plasma (Duncan and Garton, 1963; Lough, 1964), ox and sheep perinephric fat (Hansen, 1965a–c), rumen bacteria (Hansen, 1966), and humans with Refsum's syndrome (Klenk and Kahlke, 1963; Hansen, 1965d). Since phytanic and pristanic acids in the above organisms are probably derived ultimately from the phytol of chlorophyll (Steinberg *et al.*, 1966;

Scheme II



Mize *et al.*, 1966; Patton and Benson, 1966; Hansen, 1966; Hansen *et al.*, 1965, 1966; Stoffel and Kahlke, 1965), it follows that C-7 and C-11 in the phytanic acid (and C-6 and C-10 of pristanic acid) should have the *R* configuration, as in natural phytol. Furthermore, since it is the asymmetric center closest to the carboxyl group (C-3 or C-2 in phytanic and pristanic acids, respectively) that determines the optical activity of branched-chain acids, the stereochemical configuration of this center in phytanic and pristanic acids from the above sources should be readily ascertained from the sign and magnitude of their optical rotations.

Although the data in the literature on phytanic and pristanic acids either lack optical rotatory measurements or give rotatory dispersion values, there are a few examples where comparison with the present results is possible. Hansen and Shorland (1953) and Sonneveld *et al.* (1962) reported a value for $[\alpha]_D$ of $+1°$ for phytanic acid in butterfat, and Hansen (1965a) found $[\alpha]_D$ $+0.3°$ for phytanic acid from ox fat, indicating that in these samples, both the *S,R,R* and *R,R,R* isomers are present with the latter isomer predominating (*cf.* Ackman and Hansen, 1967). For the phytanic acid from ox plasma, however, Lough (1964) has recorded a value of $[\alpha]_{500}$ $-3.8°$, which clearly establishes the configuration at C-3 as *S*, an assignment opposite to that of the same center in the bacterial phytanic acid (Table I). The occurrence of the *S,R,R* isomer alone in a phytanic acid of animal origin is unusual, in view of the recent findings of Ackman and Hansen (1967), using samples of our bacterial phytanic and pristanic acids as reference standards, that both the *R,R,R* and *S,R,R* isomers occur in animals, but that the *R,R,R* isomers are usually predominant in terrestial mammals, while the *S,R,R* isomers are predominant in marine life.

Pristanic and phytanic acids have also been identified in petroleum (Cason and Graham, 1965) and shale from the Green River formation (*ca.* 60×10^6 years; Eglinton *et al.*, 1966). The corresponding hydrocarbons, pristane and phytane, respectively, have also been identified in various geological material, such as the Green River shale (Cummins and Robinson, 1964; Eglinton *et al.*, 1966), petroleum (Bendoraitis *et al.*, 1962), and coal tar (Kochloefl *et al.*, 1963); these hydrocarbons have also been detected recently in extraterrestial material such as the Orgueil meteorite (Murphy and Nagy, 1966; Breger, 1966). If sufficient quantities of these isoprenoid acids and hydrocarbons could be obtained from geological material, stereochemical comparison with the stereoisomers of phytanic and pristanic acids and of phytane and pristane obtained here should greatly aid in establishing the biological or nonbiological origin of the geological material. Such comparisons would also help to determine whether the geological material is of plant or bacterial (halophile) origin.

Experimental and Results Section

Physical Measurements. Infrared spectra were measured either on thin films of the substances (oils) between

two sodium chloride plates or on their solutions in chloroform or carbon tetrachloride in 0.5-mm cells, using a Perkin-Elmer Model 21 double-beam spectrometer with sodium chloride optics.

Optical rotations were measured at 22° at 589 (sodium D line), 578, 546, 436, and 365 mμ on chloroform solutions of the substances or on undiluted oils, using a Perkin-Elmer polarimeter, Model 141, with digital readout.

Chromatography. Rapid thin layer chromatographic analyses for purity determinations and monitoring of columns were carried out on 7.5 × 2.5 cm microscope slides coated with silica gel "plain" (Research Specialties Co., Richmond, Calif.). They were developed in wide-mouthed screw-capped jars lined with filter paper and the spots were visualized by spraying with 40% H_2SO_4 followed by charring on an open flame. The R_F values quoted are mean values of at least three independent determinations on microplates with an error of ±0.05. For preparative separations of up to 100 mg of material, 20 × 20 cm glass plates with a 0.6-mm-thick layer of silica gel "plain" (previously washed with chloroform–methanol, 1:1) were used. They were developed in ordinary rectangular jars lined with filter paper, and bands were visualized under ultraviolet light of short and long wavelengths; components were eluted from the silica gel with ethyl ether or mixtures of ethyl ether and methanol.

Unless otherwise stated, column chromatography was performed on Bio-Rad or Unisil silicic acid (200–325 mesh) using 30:1 weight ratios of silicic acid to the substance applied. All solvents were pure and anhydrous, except chloroform which contained 0.75% of alcohol. The boiling range of the petroleum ether used was 30–60°.

Gas–liquid partition chromatography was carried out on 4-ft glass columns (4-mm i.d.) of 10% Apiezon L on Gas-Chrom P at 198° and 28 psi, or 10% butanediol succinate polyester on Gas-Chrom A at 180° and 20 psi, using a Pye Argon chromatograph with strontium-90 ionization detector.

2,3-Di-O-(3′,7′,11′,15′-tetramethylhexadecyl)glycerol (Diphytanylglycerol) (I). This glyceryl diether was prepared from total acetone-insoluble lipids (1.2 g) of *H. cutirubrum* by methanolysis in boiling 2.5% methanolic HCl (100 ml) for 4 hr, as described elsewhere (Kates *et al.*, 1965a, 1966). The product obtained (750 mg) showed one major spot on thin layer chromatography (R_F 0.40 in chloroform–ether, 20:1, 0.55 in chloroform–ether, 9:1) corresponding to the diphytanylglycerol (I), a minor fast-moving spot (chiefly pigments and hydrocarbons), and a trace spot of monophytanylglycerol at the origin. This preparation was used in the next step without further purification.

1-Iodo-3,7,11,15-tetramethylhexadecane (II). A. FROM *H. cutirubrum.* The crude 2,3-di-*O*-(3′,7′,11′,15′-tetramethylhexadecyl)glycerol (I, 736 mg) was refluxed for 18 hr in 50 ml of 47% hydriodic acid with magnetic stirring. The reaction mixture was then diluted with an equal volume of water and extracted with ethyl ether. The organic layer was washed with water, saturated aqueous sodium hydrogen carbonate, 5% sodium thiosulfate, and again with water, dried over sodium sulfate, and evaporated to dryness. The yield of the crude iodide was 850 mg (92%). After purification by preparative thin layer chromatography using petroleum ether as solvent, the iodide showed only one spot on thin layer chromatography (R_F 0.65 in petroleum ether), and gave a single peak on gas–liquid partition chromatography (retention relative to octadecyl iodide, 0.75, on butanediol succinate polyester at 180°). The product had the following specific rotations (*c* 5.2, chloroform): $[\alpha]_{589}^{22}$ −6.1°, $[\alpha]_{578}^{22}$ −6.2°, $[\alpha]_{546}^{22}$ −7.1°, $[\alpha]_{436}^{22}$ −13.1°, and $[\alpha]_{365}^{22}$ −23.5°. Its infrared spectrum (liquid film) showed the following absorption bands: OH (absent), CH_2 and CH_3 (2960, 2930, 2870, 1465, 1380, and 740 cm^{-1}), and $C(CH_3)_2$ (1380–1365 and 1180 cm^{-1}).

B. FROM PHYTOL. Dihydrophytyl iodide was prepared by reaction of dihydrophytol (prepared from phytol by catalytic hydrogenation) with 47% HI, as described previously (Kates *et al.*, 1965a); it was purified as described above and had the same physical properties as the bacterial phytanyl iodide, except for optical rotation, $[\alpha]_D$ −0.75° (*c* 3.9, chloroform).

3,7,11,15-Tetramethyl-1-hexadecanol (Phytanol) (IV). A. FROM *H. cutirubrum.* A solution of the phytanyl iodide (II, 750 mg) in glacial acetic acid (50 ml) was stirred and heated under reflux with powdered silver acetate (3.0 g) for 20 hr. The mixture was centrifuged free of silver salts, and the supernatant was concentrated under reduced pressure to a small volume. The residue was diluted with 100 ml of ethyl ether, and the ether solution was washed successively with water, a saturated solution of sodium bicarbonate (until neutral), 5% sodium thiosulfate solution, and finally water. The ether solution was dried over sodium sulfate and brought to dryness under reduced pressure; the residual oil was dissolved in petroleum ether and the solution was cleared by centrifugation and evaporated to dryness under reduced pressure, yielding the phytanyl acetate (III, 620 mg, 99% yield); R_F 0.60 on thin layer chromatography in chloroform–ether (9:1); retention relative to methyl stearate on gas–liquid partition chromatography on Apiezon L at 197°, 1.13, and on butanediol succinate polyester at 180°, 1.03.

Without further purification, the phytanyl acetate (III, 620 mg) was saponified in 0.7 N sodium hydroxide in 90% methanol (50 ml) under reflux for 2 hr. Extraction with petroleum ether yielded an oil (539 mg), which was purified by chromatography on a column of silicic acid (25 g). Elution with petroleum ether (150 ml) and benzene (100 ml) removed some nonpolar material, after which the phytanol (IV) was eluted with chloroform (250 ml); yield, 369 mg (68% over-all from iodide). The product had R_F 0.40 on thin layer chromatography in chloroform–ether (9:1), and R_F 0.20 in chloroform; on gas–liquid partition chromatography it had a retention relative to octadecanol of 0.84 on butanediolsuccinate polyester at 180°, and 0.87 on Apiezon L at 198°; it had the following specific

rotations in chloroform solution (*c* 5.4): 589 mμ, +2.54°; 578 mμ, +2.80°; 546 mμ, +3.13°; and 436 mμ, +5.25°.

Anal. Calcd for $C_{20}H_{42}O$ (298.5): C, 80.46; H, 14.18. Found: C, 80.23; H, 14.33.

The alcohol IV showed the following absorption bands in the infrared region (liquid film): OH (3340 cm^{-1}, br), CH_2 and CH_3 (2960, 2930, 2880, 1465, 1380, and 735 cm^{-1}), $C(CH_3)_2$ (1380–1370 cm^{-1}, doublet), and primary alcoholic CO (1055 cm^{-1}).

B. FROM PHYTOL. Dihydrophytol was prepared by catalytic hydrogenation of phytol (derived from chlorophyll; Mann Research Laboratories) and purified by chromatography on a column of silicic acid, as described previously (Kates *et al.*, 1965b). It had the same physical properties as the bacterial phytanol (IV), except that it had $[\alpha]_D^{22}$ −0.4° (*c* 4.0, chloroform).

3,7,11,15-Tetramethylhexadecanoic (Phytanic) Acid (V) and Its Methyl Ester VI. A. FROM *H. cutirubrum.* To a solution of 49 mg of phytanol (IV) in 3 ml of glacial acetic acid–acetone mixture (1:2) was added dropwise with stirring a solution of 40 mg of CrO_3 in 0.05 ml of water over a period of 10 min at 22°. After further stirring at 40–50° for 30 min, water (30 ml) and solid sodium bisulfite were added to remove the excess oxidant; the mixture was strongly acidified with 15 ml of 10% sulfuric acid and extracted with several portions of ethyl ether. The combined extracts were washed with water until neutral, then diluted with benzene and brought to dryness under reduced pressure. The residual crude phytanic acid (50 mg, contaminated with traces of unreacted phytanol and phytanyl acetate) was purified by preparative thin layer chromatography with chloroform–ethyl ether (3:1) as solvent (R_F of phytanic acid, phytanol, and phytanyl acetate, 0.45, 0.55, and 0.90, respectively). The phytanic acid (V) obtained (40 mg, 76% yield) had the following specific rotations in chloroform (*c* 3.3): 589 mμ, +3.46°; 578 mμ, +3.98°; 546 mμ, +4.55°; and 436 mμ, +7.43°.

The purified phytanic acid was converted to the methyl ester VI by heating under reflux in 2.5% methanolic HCl (4.5 ml) for 1 hr, adding 0.5 ml of water, and extracting the ester with several portions of petroleum ether, using the apparatus described previously (Kates, 1964). The methyl ester VI obtained on evaporation of the solvent showed a single spot with the following R_F values on thin layer chromatography: 0.47 in benzene and 0.90 in chloroform–ether (3:1). It showed a major peak (96–97%) with retention 0.73 relative to methyl stearate on butanediol succinate polyester, and 0.79 on Apiezon L. A small amount (3–4%) of methyl pristanate was the only impurity present; this probably arose from traces of phytene-1 present in the phytanol preparation. The methyl ester VI had the following specific rotations in chloroform–methanol (1:2): 589 mμ, +3.78°; 578 mμ, +4.19°; 546 mμ, +4.55°; and 436 mμ, +6.94°.

Anal. Calcd for $C_{21}H_{42}O_2$ (326.6): C, 77.24; H, 12.96. Found: C, 77.14; H, 12.67.

The methyl ester VI showed the following absorption bands in the infrared (liquid film): OH (absent), CH_2 and CH_3 (2960, 2935, 2880, 1465, 1438, and 735 cm^{-1}), $C(CH_3)_2$ (1380–1370 doublet, 1170, and shoulder at 1150 cm^{-1}), ester C=O (1738 cm^{-1}), and ester CO (1195 cm^{-1}).

B. FROM PHYTOL. Phytanic acid was prepared from dihydrophytol (derived from phytol) by oxidation with CrO_3, as described previously (Kates *et al.*, 1965a). Its physical properties were identical with those of the bacterial phytanic acid except that it had $[\alpha]_D^{22}$ −0.84° (*c* 4.2, chloroform–methanol, 1:2) and α_D^{22} −0.13° (oil); its methyl ester had $[\alpha]_D^{22}$ −0.6° (*c* 4.0, chloroform–methanol, 1:2) and α_D −0.22 (oil).

3,7,11,15-Tetramethyl-1-hexadecene (1-Phytene) (VII). A. FROM *H. cutirubrum.* A solution of the 1-iodo-3,7,11,15-tetramethylhexadecane (II, 500 mg) in dry benzene (10 ml) was heated under reflux with 2 g of finely powdered potassium hydroxide for 3 hr with stirring. A further 2-g portion of powdered potassium hydroxide and 10 ml of dry benzene were added and the refluxing was continued for 17 hr. The cooled reaction mixture was diluted with ethyl ether and water and acidified with 10% sulfuric acid. The organic layer was washed with water, diluted with benzene, and evaporated under reduced pressure. The residual crude phytene-1 (VII, 352 mg), containing about 15% of unreacted iodide (II), was heated under reflux in glacial acetic acid (20 ml) with silver acetate (500 mg) for 18 hr, and then saponified in methanolic NaOH, as described above, to convert the iodide to phytanyl acetate and then to phytanol. The resulting mixture of phytene and phytanol was then separated by preparative thin layer chromatography using petroleum ether as solvent, yielding 210 mg (72% yield, corrected for unreacted iodide) of pure phytene-1 (VII), which had R_F 0.90 on thin layer chromatography in petroleum ether. On gas–liquid partition chromatography, it had a retention relative to octadecane of 1.10 on butanediol succinate polyester at 180°, and 0.86 on Apiezon L at 198°; its specific rotations in chloroform (*c* 6.7) were: 589 mμ, −4.97°; 578 mμ, −4.85°; 546 mμ, −5.50°; and 436 mμ, −9.78°.

Anal. Calcd for $C_{20}H_{40}$ (280.5): C, 85.63; H, 14.37. Found: C, 85.51; H, 14.60.

The phytene-1 (VII) showed the following absorption bands in the infrared region (in carbon tetrachloride solution): OH (absent), CH_2 and CH_3 (2960, 2930, 2870, 1465, 1380, and 735 cm^{-1}), $C(CH_3)_2$ (1380–1370 doublet, 1175, and shoulder at 1160 cm^{-1}), and vinyl group (3080, 1830, 1640, 1420, 995, and 915 cm^{-1}); absorption bands for *cis* or *trans* double bonds were absent.

B. FROM PHYTOL. Phytene-1 was prepared from phytol-derived phytanyl iodide as described for the corresponding bacterial substance, or from phytol-derived phytanyl bromide as described previously (Kates *et al.*, 1965b). The product was purified by thin layer chromatography as described above, and had properties identical with those of the bacterial phytene except that it had $[\alpha]_D$ −0.5° (*c* 6.3, chloroform).

2,6,10,14-Tetramethylpentadecanoic (Pristanic) Acid (VIII) and Its Methyl Ester IX. A. FROM *H. cutirubrum.* To a solution of 210 mg of phytene-1 (VII) in 4 ml of glacial acetic acid was added 0.4 g of powdered potassium permanganate, with stirring and cooling below 20° over a period of 10 min. After stirring for an additional 40 min at 25°, the mixture was acidified with 5 ml of 10% sulfuric acid, decolorized by addition of solid sodium metabisulfite, diluted with 10 ml of water, and extracted with ethyl ether. The organic layer was washed with water, diluted with benzene, and concentrated under reduced pressure. The residual oil was dissolved in 40 ml of 0.7 N NaOH in methanol–water (9:1) and neutral material (containing mostly the C_{18} ketone) was extracted with petroleum ether. After acidification of the mixture with 6 N sulfuric acid, the acidic product was extracted with petroleum ether; yield of pristanic acid (VIII), 115 mg (52%). The product showed one spot on thin layer chromatography with R_F 0.50 in chloroform–ether (3:1). With some preparations difficulties in removal of neutral material were encountered; in such cases the reaction mixture was fractionated by preparative thin layer chromatography using chloroform–ether (9:1) as solvent.

The pristanic acid was converted to the methyl ester IX as described above for phytanic acid, and the ester was finally purified by preparative thin layer chromatography with chloroform as solvent (R_F *ca.* 0.5). It showed a single spot on thin layer chromatography with the following R_F values on microslides: 0.47 in benzene and 0.80 in chloroform-ether (9:1). On gas–liquid partition chromatography it showed a single peak with retention relative to methyl stearate of 0.47 on butanediol succinate polyester at 180°, and 0.496 on Apiezon L at 197°. Ester IX had the following specific rotations, in chloroform (*c* 5.5): 589 mμ, −11.8°; 578 mμ, −12.2°; 564 mμ, −13.8°; and 436 mμ, −23.5°; in chloroform–methanol (1:2; *c* 2.3), $[\alpha]_D$ −12.1°.

Anal. Calcd for $C_{20}H_{40}O_2$ (312.5): C, 76.86; H, 12.90. Found: C, 76.90; H, 12.70.

Methyl pristanate (IX) had the following absorption bands in the infrared region (liquid film): OH (absent), CH_2 and CH_3 (2950, 2920, 2860, 1465, 1438, 1380, and 735 cm^{-1}), $C(CH_3)_2$ (1380–1370 doublet, 1170, and shoulder at 1150 cm^{-1}), ester C=O (1740 cm^{-1}), and ester CO (1200 cm^{-1}).

Methyl pristanate was also prepared by Barbier–Wieland degradation of methyl phytanate as described elsewhere (Gupta and Peters, 1966), but the over-all yield was only 33%.

B. FROM PHYTOL. Pristanic acid and its methyl ester were prepared as described for the corresponding bacterial compounds, except that the phytene-1 used as starting material was derived from phytol. The products obtained had properties identical with those of the respective bacterial compounds except that the acid had $[\alpha]_D$ −1.3° (*c* 5, chloroform–methanol, 1:2); the methyl ester had $[\alpha]_D$ −0.74 (*c* 5.4, chloroform) and −0.32° (neat).

Anal. Calcd for $C_{20}H_{40}O_2$ (312.5): C, 76.86; H, 12.90. Found: C, 76.31; H, 12.80.

1,1-Diphenyl-2,6,10,14-tetramethyl-1-pentadecanol (Diphenylpristanol) (X). A. FROM *H. cutirubrum.* To a solution of phenylmagnesium bromide prepared from 120 mg of magnesium and 1 g of bromobenzene in 8 ml of anhydrous ethyl ether was added dropwise a solution of 120 mg of methyl pristanate (IX) in 15 ml of anhydrous ether, over a period of 30 min. The mixture was heated under reflux for 3 hr, then cooled and diluted with a solution of 2 g of ammonium chloride in 15 ml of water, and the product was extracted with ethyl ether. The ether extract was washed with water until neutral, then diluted with benzene and concentrated under reduced pressure, yielding 340 mg of diphenylpristanol (X). The product had R_F 0.60 in benzene and showed the following absorption bands in the infrared (liquid film): OH (3500 cm^{-1}, br), CH_2 and CH_3 (2960, 2930, 2880, 1465, and 1380–1370, doublet), phenyl groups (3030–3060, 1955, 1880, 1800, 1600, 1450, 765, 745, 738, and 695 cm^{-1}), and tertiary alcoholic CO (1155 cm^{-1}).

1,1-Diphenyl-2,6,10,14-tetramethyl-1-pentadecene (Diphenylpristene) (XI). A solution of diphenylpristanol (X, 340 mg) in dry benzene (15 ml) was refluxed with *p*-toluenesulfonic acid (120 mg) for 2 hr. The cooled mixture was diluted with petroleum ether and washed with water, a saturated solution of sodium bicarbonate, and again with water. The benzene solution was concentrated under reduced pressure and the residual oil was purified by preparative thin layer chromatography with petroleum ether as solvent, yielding the pure diphenylpristene (XI, 138 mg). The product had R_F 0.90 in benzene and 0.25 in petroleum ether (microslide plate) and showed the following absorption bands in the infrared: OH (absent), CH_2 and CH_3 (2960, 2940, 2880, 1465, and 1380), and phenyl groups (3060–3030, 1950, 1880, 1800, 1600, 1495, 1445, 765, and 700 cm^{-1}). Compound XI had the following specific rotations in chloroform (*c* 4.3): 589 mμ, −3.75°; 578 mμ, −3.95°; 546 mμ, −4.64; 436 mμ, −9.23°; 365 mμ, −18.2°.

6,10,14-Trimethylpentadecan-2-one (C_{18} Ketone) (XII). A. FROM *H. cutirubrum.* To a solution of diphenylpristene (XI, 138 mg) in 4.5 ml of acetone–glacial acetic acid (2:1) was added dropwise a solution of 80 mg of chromium trioxide in 0.1 ml of water over a period of 5–10 min, and the mixture was stirred for 1 hr at 50–55°. Since a considerable amount of starting material remained unreacted, another 80-mg portion of chromium trioxide in 0.1 ml of water was added and stirring was continued for 30 min at 50–55°. The reaction mixture was diluted with 10% sulfuric acid, sodium metabisulfite was added to destroy excess oxidant, and the products were extracted with petroleum ether. Separation of the products by preparative thin layer chromatography with chloroform as solvent yielded 35 mg of ketone fraction (R_F 0.40) and 90 mg of unreacted diphenylpristene (R_F 0.75). The latter was again subjected to oxidation, as described, and the products were separated by thin layer chromatog-

raphy; this procedure was repeated once more, yielding a total of 65 mg of ketone fraction (XII plus benzophenone).

The desired C_{18} ketone XII was isolated by formation of its bisulfite adduct, as follows. To a solution of the ketone mixture in 1 ml of methanol was added 5 ml of an aqueous methanolic bisulfite solution (5 g of sodium metabisulfite in 8.5 ml of water, diluted with 7 ml of methanol, and 2 ml of water was added to dissolve any precipitate), and the mixture was shaken and left at room temperature for 30 min. On centrifugation of the mixture, the bisulfite adduct collected at the surface; the methanol–water phase was removed carefully with a Pasteur pipet, and the adduct was washed twice with 0.5 ml of the aqueous methanolic bisulfite solution. The ketone was regenerated by dissolving the adduct in 6 ml of methanol–chloroform (2:1), acidifying with 1.6 ml of 6 N sulfuric acid, and adding 2 ml each of chloroform and water. The mixture was centrifuged, and the chloroform (lower) phase was withdrawn and brought to dryness under a stream of nitrogen; the residual ketone was finally purified again by preparative thin layer chromatography with chloroform–petroleum ether (1:1) as solvent (R_F 0.3). The C_{18} ketone XII obtained (25 mg, 28% yield) showed only one spot on thin layer chromatography in chloroform (R_F 0.35) or chloroform–ether (10:1, R_F 0.60) and had a retention relative to heptadecanone-2 of 0.65 on butanediol succinate polyester at 180°; it still, however, contained 4–5% of benzophenone (relative retention 2.20) and had the following specific rotations in chloroform (*c* 3.9), which were very similar to those of the ketone obtained from phytol (see below): 589 mμ, +0.61°; 578 mμ, +0.99°; 546 mμ, +1.17°; and 436 mμ, +1.94°.

Anal. Calcd for $C_{18}H_{36}O$ (268.5): C, 80.54; H, 13.52. Found: C, 80.72; H, 13.40.

The infrared spectrum of compound XII showed the following absorption bands (liquid film): OH (absent), CH_2, CH_3, and $C(CH_3)_2$ (2950, 2925, 2870, 1465, 1380–1370 doublet, 1170, and 735 cm^{-1}), and keto C=O (1725 cm^{-1}).

B. From phytol. To a solution (500 mg) of phytol in 8 ml of glacial acetic acid was added with stirring 0.9 g of powdered potassium permanganate during 10 min. The mixture was stirred at room temperature for a further 20 min and then diluted with 10% sulfuric acid and decolorized by addition of solid sodium metabisulfite. The product was extracted with petroleum ether, and the residual oil obtained on evaporation of the solvent was fractionated on a column of 14 g of silicic acid, the following fractions (50 ml) being collected: fractions 1–3, benzene; fractions 4–10, benzene–chloroform (1:1). The ketone XII was eluted in fractions 1–3, but only the latter two yielded the chromatographically pure product (209 mg); fraction 1 gave 98 mg of product contaminated with nonpolar material.

The ketone XII derived from phytol had chromatographic properties and infrared spectrum identical with those of the bacterial ketone but the specific rotations of the former in chloroform (*c* 10.1) were: 589 mμ, +0.73°; 578 mμ, +0.76°; 546 mμ, +0.91°; 436 mμ, +1.85°; and 365 mμ, +4.16°. When the ketone was mixed with benzophenone in equimolecular proportion and purified by bisulfite adduct formation and thin layer chromatography as described above, or when the C_{18} ketone was prepared by Barbier–Wieland degradation of methyl pristanate derived from phytol and purified as described above, the product had similar specific rotations in chloroform (*c* 2.4) to those of the bacterial ketone: 589 mμ, +0.66°; 578 mμ, +0.91°; 546 mμ, +1.07°; and 436 mμ, +1.77°.

Anal. Calcd for $C_{18}H_{36}O$ (268.5): C, 80.54; H, 13.52. Found: C, 80.54; H, 13.45.

2,6,10,14-Tetramethyl-1-pentadecanol (Pristanol) (XIV). A. From *H. cutirubrum.* A solution of methyl pristanate (94 mg) in anhydrous ethyl ether (2 ml) was added dropwise to lithium aluminium hydride (0.1 g) in 20 ml of ether, and the mixture was refluxed for 2 hr. The excess hydride was destroyed by addition of methanol, and the mixture was acidified with 6 N HCl, diluted with water, and extracted with ether. The ether extract was washed with water and evaporated under reduced pressure (benzene was added to aid in removal of water) to an oil which was purified by preparative thin layer chromatography using chloroform–ether (20:1) as solvent. The pure pristanol (XIV) obtained (72 mg, 84%) had R_F 0.42 in chloroform–ether (9:1), and $[\alpha]_D$ +3.2° (*c* 3.6, chloroform). Its infrared spectrum (film) showed the expected bands for OH (3340 cm^{-1}), CH_2 and CH_3 (2960, 2930, 2880, 1465, 1380, and 735 cm^{-1}), $C(CH_3)_2$ (1380–1370 cm^{-1}), and alcoholic CO (1035 cm^{-1}) groups.

B. From phytol. Reduction of 5.8 g of methyl pristanate, derived from phytol, with 2 g of lithium aluminium hydride in 60 ml of ethyl ether as described above gave 5.0 g (97%) of fairly pure pristanol, a small part of which was further purified by thin layer chromatography as described above. The product was identical in all respects, except for optical rotation (α_D +0.18°, neat), with the bacterial pristanol.

2,6,10,14-Tetramethylpentadecane (Pristane) (XVI). A. From *H. cutirubrum.* A mixture of 70 mg of pristanol (XIV) and 85 mg of *p*-toluenesulfonyl chloride in 3 ml of anhydrous pyridine was kept at 25° for 48 hr. The mixture was poured into ice water, acidified with 10% sulfuric acid, and extracted with ether. The crude *p*-toluenesulfonate XV obtained after evaporation of the solvent was reduced in anhydrous ethyl ether (20 ml) with lithium aluminium hydride (0.1 g) under reflux for 2 hr. The excess hydride was destroyed with methanol, and the mixture was acidified with 10% sulfuric acid and extracted with ethyl ether. The crude pristane (XVI) obtained on evaporation of the solvent was purified by preparative thin layer chromatography with petroleum ether as solvent (R_F 0.9); the product obtained (44 mg, 67% yield based on the pristanol) was found by gas–liquid partition chromatography to contain about 80% of pristane together with several minor hydrocarbon impurities. Final purification was achieved by preparative gas–

liquid partition chromatography (Aerograph Autoprep) on a column of 15% SE-31 (0.25 in. × 2 ft) at 215° with a helium flow rate of 100 cc/min; the 2,6,10,14-tetramethylpentadecane (XVI) obtained (11 mg) gave a single peak on gas–liquid partition chromatography, with retentions relative to octadecane of 0.62 on butanediol succinate polyester at 180°, and 0.63 on Apiezon L at 198°. It had no measurable optical activity in chloroform solution (*c* 5.6), showing that it was the *meso*-6*R*,10*S* isomer. The infrared spectrum of pristane (XVI) showed the following bands: OH (absent), CH_2 and CH_3 (2960, 2930, 2880, 1465, and 1385 cm^{-1}), terminal isopropyl (1380–1370 doublet, 1170 cm^{-1}, and shoulder at 1150 cm^{-1}), and $(CH_2)_3$ (732 cm^{-1}).

B. FROM PHYTOL. The *p*-toluenesulfonate XV was prepared from 3.0 g of pristanol (XIV) derived from phytol, by reaction with 4.2 g of *p*-toluenesulfonyl chloride in 30 ml of anhydrous pyridine, as described above. The crude product was fractionated on a column of silicic acid (50 g), eluted as follows (50-ml fractions collected): fractions 1–3, petroleum ether; fractions 4–6, petroleum ether–benzene (3:1), fractions 7–9, petroleum ether–benzene (1:1), and fractions 10–11, benzene. The pure *p*-toluenesulfonate XV was eluted in fractions 6–8; yield, 3.7 g (80%); α_D −0.05° (neat); R_F on thin layer chromatography, 0.50 (benzene).

Anal. Calcd for $C_{26}H_{46}O_3S$ (438.6): C, 71.19; H, 10.57. Found: C, 71.29; H, 10.46.

The *p*-toluenesulfonate XV showed the following absorption bands in the infrared region (liquid film): OH (absent), CH_2 and CH_3 (2965, 2940, 2880, and 1465 cm^{-1}), aryl (1600, 1500, 830, 815, and 790 cm^{-1}), and *p*-toluenesulfonate (1367, 1190, 1180, 1100, 965, 758, and 665 cm^{-1}).

The *p*-toluenesulfonate XV (3.2 g) was reduced with lithium aluminum hydride (1.5 g) in ethyl ether (30 ml) under reflux for 2 hr. The crude pristane was isolated from the reaction mixture as described for the bacterial product and was purified by distillation at 0.5 mm in a microdistillation apparatus. The pristane (XVI) obtained was chromatographically pure and had physical properties (α_D (neat oil) 0.0°) identical with those of the bacterial pristane.

3,7,11,15-Tetramethylhexadecane (Phytane) (XVII). A. FROM *H. cutirubrum*. Phytanyl iodide (II, 122 mg) was reduced with lithium aluminum hydride (0.7 g) in anhydrous ethyl ether (40 ml) under reflux for 20 hr. After decomposition of the excess hydride with methanol and acidification of the mixture with 6 N HCl, the product was extracted with ethyl ether. The crude material obtained was purified by preparative thin layer chromatography using petroleum ether as solvent (R_F 0.9); yield of pure phytane (XVII), 56 mg (66%). The phytane obtained gave a single peak on gas–liquid partition chromatography with retentions relative to octadecane of 0.95 on butanediol succinate polyester at 180°, and 0.96 on Apiezon L at 198°; it had $[\alpha]_D$ +3.9° (*c* 2.8, chloroform); its infrared spectrum (liquid film) showed the following characteristic bands: OH (absent), CH_2 and CH_3 (2965, 2940, 2885, 1465, 1380, and 735 cm^{-1}), $C(CH_3)_2$ (1380–1370 doublet and 1170 cm^{-1} with shoulder at 1150 cm^{-1}).

Anal. Calcd for $C_{20}H_{42}$ (282.5): C, 85.02; H, 14.98. Found: C, 85.17; H, 14.71.

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Subcellular Location of Vitamin D and Its Metabolites in Intestinal Mucosa after a 10-IU Dose*

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ABSTRACT: The intracellular localization of ^3H from 10 IU of [^3H]vitamin D in rat or chick intestinal mucosa indicates that a major site of accumulation is the nuclear membrane. When the outer membrane of the nucleus is fragmented or stripped off with citric acid or Triton X-100, 60–80% of the nuclear radioactivity from [^3H]vitamin D can be removed. Of the ^3H from [^3H]-vitamin D associated with the nuclei, 80% is in the form of a polar metabolite(s) which is known to be biologically active.

The nuclear receptor sites for vitamin D and/or its metabolites can be saturated by the administration of large doses of the vitamin, dihydrotachysterol-2, but not 7-dehydrocholesterol.

There is little doubt that a lag exists between the time of vitamin D administration and the subsequent increase in the intestinal transport of calcium or the mobilization of mineral from bone (DeLuca, 1967). At least a portion of this time lag can be explained by some induction process which mediates the action of the vitamin (DeLuca, 1967). Thus actinomycin D, which inhibits DNA-directed synthesis of RNA,[1] has repeatedly been shown to block completely the physiologic responses to vitamin D (Eisenstein and Passavoy, 1964; Zull *et al.*, 1965, 1966a; Schachter and Kowarski, 1965; Norman, 1965, 1966; Harrison and Harrison, 1966). Other RNA and protein synthesis inhibitors such as puromycin and 5-fluororotic acid have given partial inhibition of vitamin D responses (Zull *et al.*, 1966a). It has also recently been demonstrated that vitamin D, when administered to vitamin D deficient rats, induces a two- to threefold stimulation of [^3H]orotic acid incorporation into nuclear ribonucleic acid (nRNA) of intestinal mucosa. This stimulation is completely blocked by actinomycin D (Zull *et al.*, 1966b; Stohs *et al.*, 1967), indicating the formation of mRNA (Reich, 1964). Therefore, the present knowledge of the mechanism of vitamin D action suggests that the physiologic expression of the vitamin and/or its metabolite(s) involves the transcription of specific genetic information into mRNA. The subsequent translation of this mRNA into functional protein components results in the maintenance of the $[Ca^{2+}]\cdot[HPO_4^{2-}]$ product of the blood at an appropriate level ensuring deposition of mineral into newly formed bone collagen (DeLuca, 1967).

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[1] Abbreviation used: TMK, 0.05 M Tris-Cl (pH 7.4), 0.005 M $MgCl_2$, and 0.025 M KCl.