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# Metabolism of Dihydrotachysterol and 5,6-*trans*-Cholecalciferol in the Chick and the Rat

By D. ERIC M. LAWSON and PHILLIP A. BELL*
*Dunn Nutritional Laboratory, University of Cambridge and Medical Research Council, Milton Road, Cambridge CB4 1XJ, U.K.*

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Dihydrotachysterol and 5,6-*trans*-cholecalciferol are biologically active analogues of cholecalciferol (vitamin D) with a similarity in steric structure to 1,25-dihydroxycholecalciferol, the active form of the vitamin. The question arises as to the nature of the active form of these analogues. High specific radioactivity $^{14}C$- and $^{3}H$-labelled forms of dihydrotachysterol and 5,6-*trans*-cholecalciferol and its 25-hydroxy derivative were synthesized and their metabolism was studied in chicks and rats. All these steroids were very rapidly metabolized compared with cholecalciferol; 20% of the dihydrotachysterol dose was excreted in bile in the first 24h, about 50% as a carboxylic acid derivative. Although polar metabolites were detected in tissues, no 1-hydroxy form was observed. Larger proportions of the parent steroid and its 25-hydroxy metabolite were detected in tissues compared with cholecalciferol, but no single metabolite was detected at the intracellular site of action of cholecalciferol. It is suggested that analogues of cholecalciferol will be biologically active if they possess a hydroxyl group in the same steric position as that at C-1 of cholecalciferol, with the greatest activity shown by those that also have a C-25 hydroxyl group. The implication of these findings for the chemical features necessary for binding to receptor proteins are briefly discussed.

After the chemical characterization and synthesis of cholecalciferol there has been a continuing search for biologically and clinically valuable analogues of this vitamin. Side-chain variations in this molecule are not only found in nature but are chemically easier to effect than are modifications of the steroid nucleus, and thus their biological activity has been studied in some detail. In animals but not in birds cholecalciferol and ergocalciferol have similar antirachitic activity but any further change in the ergocalciferol side chain, e.g. substituting an ethyl group for a methyl group at C-24, decreases the activity considerably. In addition antirachitic activity is absent from those compounds containing a hydroxyl group in the side chain other than at C-25 or those in which the side chain has been shortened by one or more carbon atoms (see review by Norman, 1968; Bontekoe *et al.*, 1970). Studies of the metabolism of radioactively labelled cholecalciferol have led to the finding that the compound has to be hydroxylated at C-1 and C-25 to give 1,25-dihydroxycholecalciferol (I) before it is effective in its target tissues (Fraser & Kodicek, 1970; Lawson *et al.*, 1971*a*; Gray *et al.*, 1971; Holick *et al.*, 1971; Norman *et al.*, 1971). Presumably therefore the requirement for an intact and unchanged side chain is not only to maintain the C-25 hydroxyl group in the correct steric position but also to allow its interaction with receptors unhindered by the presence of other substituents.

Other analogues of cholecalciferol have been produced by the isomerization of the A ring or by reduction of one or more of the three double bonds. Of these substances the hypercalcaemic activity of dihydrotachysterol makes it not only of biological interest but also of clinical value. The hypercalcaemic activity of dihydrotachysterol$_3$ (II, R = H) in chicks is about 5% of that of cholecalciferol (Hibberd & Norman, 1969), whereas its antirachitic activity is much less than 1% (Stohl & Farber, 1941). The therapeutic value of dihydrotachysterol in certain clinical conditions is a consequence of its hypercalcaemic activity, which is rapidly apparent and is of short half-life. With the discovery of 1,25-dihydroxycholecalciferol as the functionally active metabolite of cholecalciferol a chemical explanation for the therapeutic value of dihydrotachysterol could be given. Thus the steric rearrangement that the A ring of cholecalciferol undergoes in the reduction process to form dihydrotachysterol results in the C-3 hydroxyl group occupying approximately the same steric position as the C-1 hydroxyl group of 1,25-dihydroxycholecalciferol. Similarly the formation of 5,6-cholecalciferol (III, R = H) results in the C-3

* Present address: Tenovus Institute for Cancer Research, The Heath, Cardiff CF4 4XX, U.K.

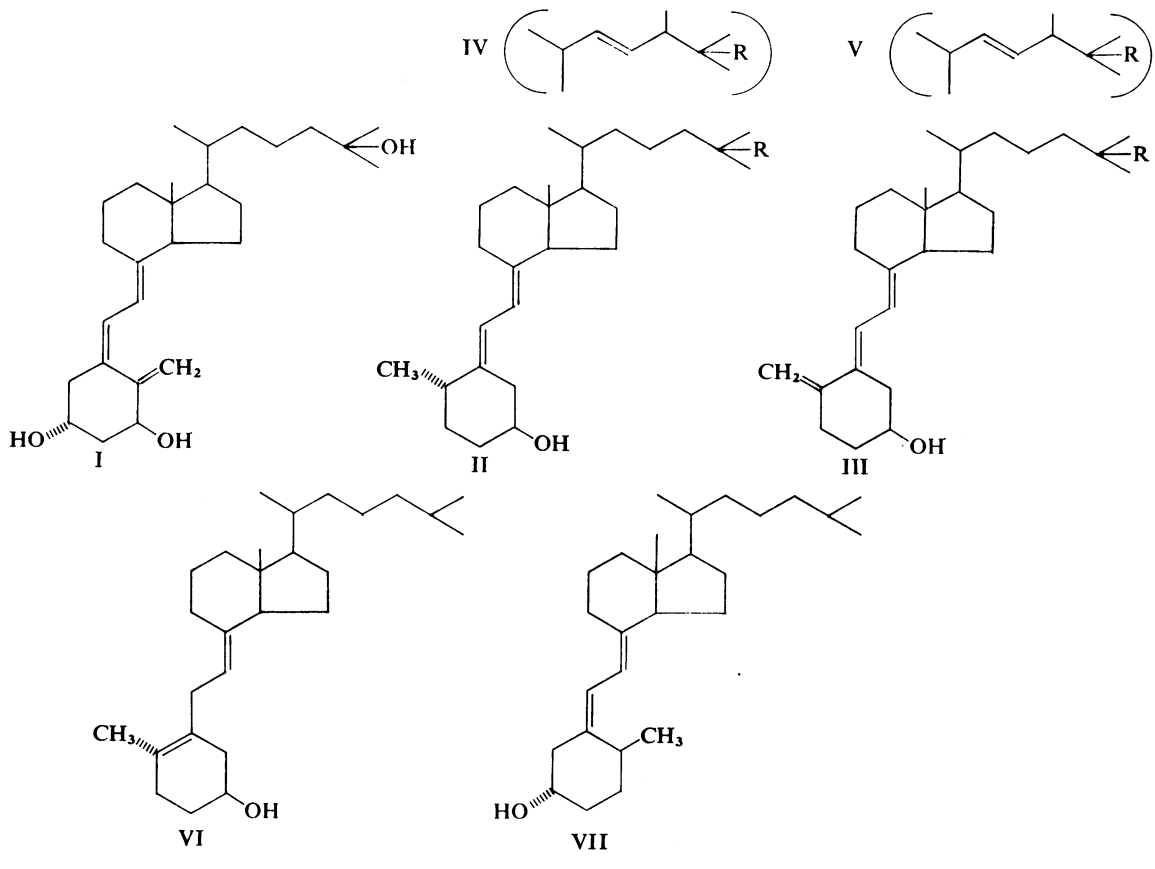


Fig. 1.

I, 1,25-Dihydroxycholecalciferol; II, R = H and R = OH, dihydrotachysterol$_3$ and 25-hydroxydihydrotachysterol$_3$ respectively; III, R = H and R = OH, 5,6-*trans*-cholecalciferol and 25-hydroxy-5,6-*trans*-cholecalciferol respectively; IV, R = H and R = OH, dihydrotachysterol$_2$ and 25-hydroxydihydrotachysterol$_2$ respectively; V, R = H and R = OH, 5,6-*trans*-ergocalciferol and 25-hydroxy-5,6-*trans*-ergocalciferol respectively; VI, dihydrocholecalciferol I; VII, dihydrocholecalciferol IV.

hydroxyl group occupying this same position (Fig. 1). Although comparisons have been made of the relative potency of dihydrotachysterol and cholecalciferol (de Man & Roborgh, 1958; Bosmann & Chen, 1966; Hibberd & Norman, 1969) few similar studies have been carried out with 5,6-*trans*-cholecalciferol (Holick *et al.*, 1972). It appears, though, that in both cases the relative potency is low, with the actual value depending on the particular metabolic effect of cholecalciferol being measured, and on the species involved.

Some studies of the metabolism of dihydrotachysterol in rats have been described (Hallick & DeLuca, 1972) and it has been shown that the biological activities of 5,6-*trans*-cholecalciferol are retained in anephric rats (Holick *et al.*, 1972). In the present paper we describe the synthesis of highly radioactive forms of these compounds and the metabolism of both substances in chicks and rats. Interest in these analogues stems from the advantages of establishing the structural features of the 1,25-dihydroxycholecalciferol essential for activity. Such information is necessary not only to have a full understanding of the interaction of this steroid with its receptors but also to aid the discovery of compounds that might inhibit the biological action of 1,25-dihydroxycholecalciferol.

## Experimental

### *Materials*

Tachysterol$_3$ (4-methyl-3,5-dinitrobenzoic acid) ester was a gift from Professor E. Havinga, University of Leiden, The Netherlands. Tachysterol$_2$ (4-methyl-3,5-dinitrobenzoic acid) ester, dihydrotachysterol$_2$ and dihydrotachysterol$_3$ were gifts from Philips–Duphar N.V., Amsterdam, The Netherlands. Free tachysterol$_2$ and tachysterol$_3$ were prepared from their esters by saponification by the method of Van de Vliervoet *et al.* (1956).

### *U.v. and i.r. spectroscopy*

U.v.-absorption spectra were recorded in ethanol by using a Unicam SP.800 recording spectrophotometer. The molar extinctions used were: dihydrotachysterol$_2$, dihydrotachysterol$_3$, 25-hydroxydihydrotachysterol$_3$, $\varepsilon_{251} = 39200$; 5,6-*trans*-cholecalciferol, 25-hydroxy-5, 6-*trans*-cholecalciferol, $\varepsilon_{273} = 24300$. I.r. spectra were recorded for solutions in carbon disulphide by using a Unicam SP.200 spectrophotometer.

### *Measurement of radioactivity*

Samples were assayed in a Packard Tri-Carb model 3375 liquid-scintillation spectrometer by the method of Lawson *et al.* (1971*b*). Corrections for quenching were made by using automatic external standardization together with correlation curves for $^3H$ and $^{14}C$ radioactivities, either individually or combined.

### *Chromatography*

(*a*) *Adsorption on silica-gel columns.* The radioactive chemicals described below and the serum and some tissue extracts were chromatographed on a column (50cm × 1.5cm) of silica-gel M.F.C. (Hopkin and Williams, Chadwell Heath, Essex, U.K.) deactivated with 8–10% (w/w) of water. The column was packed in light petroleum (b.p. 60–80°C) and eluted in the following sequence, 10ml fractions being collected: fractions 1–5, light petroleum; fractions 6–75, linear gradient from light petroleum to diethyl ether; fractions 76–95, diethyl ether; fractions 96–130, linear gradient from diethyl ether to 50% (v/v) methanol in diethyl ether. The column was then flushed with 200ml of methanol. Portions of the fractions were taken for measurement of radioactivity and u.v. absorbance.

Occasionally lipid extracts of tissues were chromatographed on short columns (10cm × 1cm) of untreated silica-gel M.F.C. as described by Lawson *et al.* (1969).

(*b*) *Thin-layer chromatography.* This was performed with 20cm-long plates spread with a layer of silica-gel G or $GF_{254}$ (E. Merck A.-G., Darmstadt, Germany) and activated at 110°C for 2h before use. The layers were 0.25 and 1.0mm thick for analytical and preparative purposes respectively. Appropriate marker substances were used in each case and detected by their absorption of u.v. light and with iodine or antimony trichloride. The plates were developed with the solvent systems mentioned in the text.

(*c*) *Gas–liquid chromatography.* This was performed with a Pye 104 instrument with a flame-ionization detector, on 1.54m (5ft) glass column packed with 3% OV-1 on 80–100 mesh Diatomite CQ. The column temperature was 220° or 240°C and the flow rate of the argon carrier was 50ml/min. Retention times were measured relative to 5α-cholestane (1.00).

(*d*) *Chromatography on Sephadex LH-20.* Some of the fractions obtained from the other chromatographic procedures were further fractionated on a column (60cm × 1.5cm) of Sephadex LH-20 (Pharmacia, Uppsala, Sweden), eluted with dichloromethane.

### *Animals*

Rhode Island Red × Light Sussex chicks were obtained from the National Institute for Research in Dairying (Shinfield, Reading, U.K.). They were fed on the diets and supplements as described by Lawson *et al.* (1969) and were used after 4 weeks, when they were vitamin D-deficient. Piebald weanling rats were raised on the diet of Numeroff *et al.* (1955). Unless otherwise stated the dosing material was administered intracardially in propylene glycol 16h before the animals were killed. In all experiments the animals were starved overnight before being killed.

### *Lipid extraction*

Suspensions of tissues and tissue fractions were extracted with chloroform–methanol by the method of Bligh & Dyer (1959), and the lipids in the chloroform phase were taken for chromatography or radioactivity measurements.

### *Reduction of tachysterol by lithium in liquid $NH_3$*

$NH_3$ gas was dried over KOH pellets and condensed in a flask cooled in a bath containing acetone and solid $CO_2$. When 50ml of $NH_3$ had been condensed, dry diethyl ether (25ml) was added, followed by a trace of lithium which gave a lasting blue coloration and confirmed the absence of water. Lithium wire (50mg) was added and the solution was stirred for 10min. After the addition of tachysterol$_3$ (50mg) in dry diethyl ether (10ml), stirring was continued for a further 5min, and then the mixture was decomposed by the addition of solid $NH_4Cl$ followed by the cautious addition of water. The mixture was allowed to warm to room temperature, and the ether phase was washed several times with water. After brief drying over anhydrous $Na_2SO_4$ the ether extract was evaporated to dryness. The crude product had $\lambda_{max.}$ 250nm and gave two unresolved peaks on g.l.c.

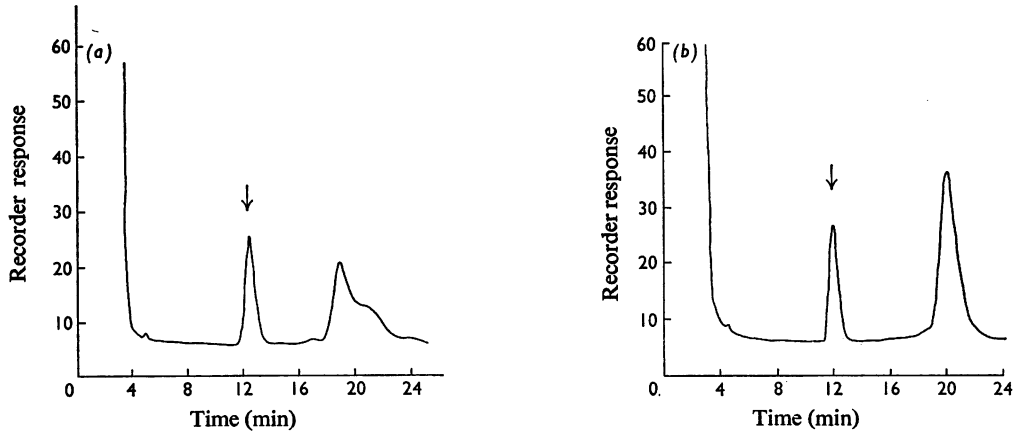


Fig 2. *Gas–liquid chromatograms of the products of tachysterol reduction by lithium in liquid $NH_3$*

(*a*) Crude products, (*b*) purified dihydrotachysterol$_3$. For details of the chromatography see the text. Arrow indicates 5$\alpha$-cholestane marker. The recorder response is in arbitrary units.

with retention times at 220°C relative to the 5$\alpha$-cholestane marker (1.00) of 1.71 and 1.91 (Fig. 2*a*). Authentic dihydrotachysterol$_3$ gave a peak at 1.91.

The components of the crude reaction mixture were separated and purified by preparative t.l.c. with chloroform as the developing solvent. Two. u.v.-absorbing bands were observed, with $R_F$ 0.37 and 0.26. A third component, with $R_F$ 0.33, overlapping with the band at $R_F$ 0.37, was detected after a narrow strip of the plate was sprayed with antimony trichloride in chloroform. Authentic dihydrotachysterol$_3$ ran with $R_F$ 0.37. By eluting the bands separately with diethyl ether–ethanol (9:1, v/v) and by repeated rechromatography in the same system, three components were obtained in a pure form, each giving only a single symmetrical peak on g.l.c.

The properties of the least polar compound, $R_F$ 0.37, were identical in all respects with those of authentic dihydrotachysterol$_3$ (II, R = H). The compound had $\lambda_{max.}$ 243, 251.5 and 261nm, g.l.c. relative retention times of 1.91 at 220°C or 1.72 at 240°C (Fig. 2*b*), and had an i.r. spectrum identical with that of an authentic sample, with a characteristic absorption band at 1060cm$^{-1}$ (equatorial OH).

The compound with $R_F$ 0.33 had g.l.c. retention times of 1.70 (220°C) and 1.57 (240°C). It showed no u.v. maxima above 215nm, and is therefore considered to be dihydrocholecalciferol I (Fig. 1, formula VI; Von Werder, 1939).

The most polar component, of $R_F$ 0.26 had g.l.c. retention times of 1.72 (220°C) and 1.57 (240°C). It showed $\lambda_{max.}$ 244, 252 and 262nm, and an i.r.-absorption band at 1060cm$^{-1}$ characteristic of an equatorial OH group. This compound is therefore considered to be dihydrocholecalciferol IV (Fig. 1, formula VII; Westerhof & Keverling Buisman, 1957).

Reduction of tachysterol$_2$ by the same procedure resulted in a similar range of products, and as with tachysterol$_3$, the least polar product isolated after t.l.c. was identical with authentic dihydrotachysterol$_2$ (IV, R = H), $\lambda_{max.}$ 243, 251.5 and 261nm, and g.l.c. relative retention time of 1.94 (240°C).

### [1,2-$^3H_2$]*Dihydrotachysterol*$_3$

This compound was prepared by the reduction method described above from [1,2-$^3H_2$]tachysterol$_3$ (74mg), obtained as a by-product in the preparation of [1,2-$^3H_2$]cholecalciferol (Lawson *et al.*, 1971*b*). The crude reduction product was purified by repeated t.l.c. until the product gave only a single peak on g.l.c. with a relative retention time of 1.72 (240°C) and showed no u.v. absorption in the range 205–215nm. The yield was 1.51mg of [1,2-$^3H_2$]dihydrotachysterol$_3$, of specific radioactivity 64.1mCi/mmol. The purity of this material was confirmed by co-chromatography on silicic acid with authentic dihydrotachysterol$_3$. A further batch of [1,2-$^3H_2$]dihydrotachysterol$_3$ was prepared by the same method; the specific radioactivity was 257.3mCi/mmol.

### [1-$^3H$]*Dihydrotachysterol*$_2$

Reduction of [1-$^3$H]tachysterol$_2$ (Pelc & Kodicek, 1971) with lithium in liquid $NH_3$, and work-up by the procedures detailed above, gave [1-$^3$H]dihydrotachysterol$_2$ (0.87mg of specific radioactivity 168mCi/mmol).

*[4-$^{14}C$]Dihydrotachysterol$_2$*

Reduction of [4-$^{14}$C]tachysterol$_2$ (Pelc & Kodicek, 1972) gave, after purification, [4-$^{14}$C]dihydrotachysterol$_2$ (0.20mg) of specific radioactivity 28.6mCi/mmol.

*25-Hydroxydihydrotachysterol$_3$*

The starting material for this preparation was 25-oxo-26-nortachysterol$_3$ (400mg), obtained as a by-product from the preparation of 25-oxo-26-norcholecalciferol (Bell & Scott, 1973). The starting material, in dry diethyl ether (50ml), was refluxed for 30min with the Grignard reagent prepared from magnesium (0.49g) and methyl iodide (2.98g) in dry diethyl ether (50ml). The complex was decomposed by the addition of saturated $NH_4Cl$, and the ether layer was separated, washed with water, briefly dried over anhydrous $Na_2SO_4$, and concentrated under reduced pressure.

The crude product showed $\lambda_{max.}$ 279nm, and gave a single spot on t.l.c. of considerably higher polarity than the starting material. The crude 25-hydroxytachysterol$_3$ was reduced without further purification by using lithium (200mg) in liquid $NH_3$ (50ml). After the usual extraction the product was purified by preparative t.l.c. with chloroform–methanol (9:1, v/v) as developing solvent. Repeated rechromatography eventually gave 25-hydroxydihydrotachysterol$_3$ (9.04mg; II, R = OH), $\lambda_{max.}$ 243, 251.5, 261nm, $\nu$ 1060cm$^{-1}$ (equatorial OH), and having a relative retention time on g.l.c. of 2.78 (240°C), identical with that predicted on the basis of the known retention time of tachysterol$_3$ and the increment found in the cholecalciferol series to result from addition of a 25-hydroxyl group.

*5,6-trans-[1,2-$^3H_2$]Cholecalciferol*

[1,2-$^3H_2$]Cholecalciferol (0.75mg) in light petroleum (b.p. 60–80°C; 20ml) was treated with a solution of iodine in light petroleum (0.01%, w/v; 0.1ml) and left in diffuse daylight for 1h. The solution was evaporated under $N_2$, and purified by t.l.c. with chloroform as the developing solvent. Two u.v.-absorbing bands were observed on the chromatogram, the more polar band corresponding to unchanged cholecalciferol. Elution of the less polar band with diethyl ether gave 5,6-*trans*[1,2-$^3$H]cholecalciferol (0.42mg), $\lambda_{max.}$ 273.5nm, of specific radioactivity 508mCi/mmol. The purity of this material was confirmed by co-chromatography of a portion with authentic 5,6-*trans*-cholecalciferol on a silicic acid column.

*5,6-trans-[4-$^{14}C$]Cholecalciferol*

Iodine isomerization of [4-$^{14}$C]cholecalciferol by the procedure detailed above gave 5,6-*trans*-[4-$^{14}$C]-cholecalciferol of specific radioactivity 32.3mCi/mmol.

*25-Hydroxy-5,6-trans-[26-$^{14}C$]cholecalciferol*

Iodine isomerization of 25-hydroxy[26-$^{14}$C]cholecalciferol by the procedure described above gave a product which was purified by preparative t.l.c. with chloroform–methanol (19:1, v/v) as developing solvent. The yield of 25-hydroxy-5,6-*trans*-[26-$^{14}$C]-cholecalciferol (III, R = OH) was 2.70mg, and the specific radioactivity was 62mCi/mmol. The compound had $\lambda_{max.}$ 274nm, and the bis-(trimethylsilyl) derivative of this compound gave a single peak on g.l.c. with a relative retention time of 4.54 (240°C), identical with the retention time predicted on the basis of the known retention time of the trimethylsilyl ether 5,6-*trans*-cholecalciferol and the increment to be expected for a 25-trimethylsilyloxy group.

Non-radioactive 25-hydroxy-5,6-*trans*-cholecalciferol was also prepared by the same procedure.

## Results

*Dihydrotachysterol*

The concentration of radioactivity in the tissues of rachitic chicks and rats after an intracardial dose of either 0.5 or 10.0$\mu$g of [1,2-$^3H_2$]dihydrotachysterol$_3$ is much lower than has been found with an equivalent dose of radioactive cholecalciferol. In general the tissues of the two species contained similar amounts of total radioactivity except for rat liver and kidney which contained significantly more radioactivity than chick liver and kidney (Table 1). No pattern could be discerned in the difference in the amounts of tissue radioactivity found with these two doses.

Chromatography of the tissue lipid extracts showed

Table 1. *Tissue radioactivity after administration of [1,2-$^3H_2$]dihydrotachysterol$_3$ to rachitic chicks and rats*

Vitamin D-deficient chicks and rats were each given 0.5 or 10$\mu$g of [1,2-$^3H_2$]dihydrotachysterol$_3$ and the tissues were removed after death 16h later. The results are expressed as percentages of the dose/g of fresh tissue or /ml of blood.

| | Tissue radioactivity (% of dose) | | | |
|---|---|---|---|---|
| | Chick | | Rat | |
| [1,2-$^3H_2$]-Dihydrotachysterol ($\mu$g) | 0.5 | 10.0 | 0.5 | 10.0 |
| Blood | 0.07 | 0.06 | 0.08 | 0.10 |
| Liver | 0.13 | 0.11 | 0.32 | 0.77 |
| Intestine | 0.08 | 0.09 | 0.10 | 0.11 |
| Kidney | 0.07 | 0.09 | 0.14 | 0.47 |
| Bone | 0.06 | 0.06 | 0.07 | 0.04 |
| Muscle | 0.10 | 0.06 | 0.05 | 0.04 |

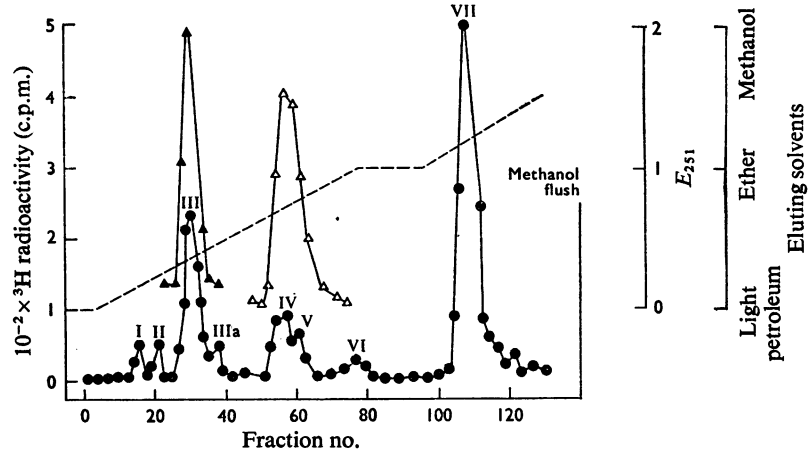


Fig. 3. *Silicic acid chromatography with gradient elution of the lipid extracts of blood of chicks dosed with* 0.5 *μg of* [1,2-$^3H_2$]-*dihydrotachysterol*$_3$

●, $^3H$; ▲, extinction at 251 nm due to dihydrotachysterol$_3$; △, extinction at 251 nm due to 25-hydroxydihydrotachysterol$_3$; ----, gradient shape with solvents shown. Fractions (10ml) were collected. For details see the text.

Table 2. *Chromatographic distribution of radioactivity from lipid extracts of chick and rat blood*

The lipid extracts from chick and rat blood (see the legend to Table 1), were chromatographed on a silica-gel column developed with a gradient of light petroleum–diethyl ether–methanol. Results are expressed as a percentage of the total radioactivity recovered (>90%) from the column. Dihydrotachysterol$_3$ and its 25-hydroxy metabolite co-chromatograph with peaks III and IV respectively.

| Chromatographic fraction | Distribution of radioactivity | | | |
|---|---|---|---|---|
| | Chick | | Rat | |
| [1,2-$^3H_2$]-Dihydrotachysterol (μg) | 0.5 | 10.0 | 0.5 | 10.0 |
| Peak I | 1.5 | 1.8 | 1.9 | 4.6 |
| Peak II | 2.2 | 2.6 | 0.3 | — |
| Peak III | 16.0 | 24.4 | 8.3 | 20.0 |
| Peak IIIa | 2.9 | — | 1.3 | — |
| Peak IV | 8.2 | 15.8 | 37.8 | 27.9 |
| Peak V | 3.0 | 6.9 | 13.0 | 12.7 |
| Peak VI | 3.9 | 5.5 | 3.8 | 1.6 |
| Peak VII | 51.3 | 29.6 | 15.8 | 24.1 |

that more metabolites are formed from dihydrotachysterol$_3$ than are observed after the administration of cholecalciferol. Fractionation of blood lipids from the chick and the rat on a column of silica gel with gradient elution showed essentially the same pattern in both animals, of seven major peaks of radioactivity (Fig. 3).

No attempt was made to identify all the metabolites of dihydrotachysterol$_3$ in blood and tissues with certainty, but it was established that in all the chromatographic systems used large proportions of the radioactivity co-chromatographed exactly with authentic standards. The resolution achieved with gradient elution from the silica-gel column allowed a more exact measurement (Fig. 3 and Table 2) than was achieved with the t.l.c. system. Thus peaks III and IV co-chromatographed exactly with authentic dihydrotachysterol$_3$ and 25-hydroxydihydrotachysterol$_3$ respectively. Peak I is most probably the ester form of dihydrotachysterol$_3$, since on saponification of this fraction and chromatography on silica gel the radioactivity was recovered in the dihydrotachysterol$_3$ region. Peak VII corresponded to the position of the dihydroxymetabolites of vitamin D observed in tissues after administration of the vitamin to animals. The nature of the radioactivity in the other peaks was not investigated. For routine purposes the tissue lipids were analysed by the t.l.c. system in which the developing solvent was ethyl acetate–heptane (1:1, v/v), and the radioactivity was proportioned according to the amounts found in the area of the authentic standards (Table 3). However, it should be emphasized that the radioactivity in the position of the monohydroxy compounds is primarily due to dihydrotachysterol and in the position of the dihydroxy compounds to the 25-hydroxy metabolite of this steroid.

The main radioactive component of the blood of rats that had received dihydrotachysterol$_3$ 18h previously was the 25-hydroxy metabolite. Decreasing the dose of the steroid raised the proportion of this metabolite found in blood, with consequent decrease

Table 3. *Chromatographic distribution of radioactivity from lipid extracts of various tissues from the rat and the chick*

The lipid extracts of the tissues of the chicks and rats dosed with 0.5$\mu$g of [1,2-$^3H_2$]dihydrotachysterol were chromatographed by t.l.c.; the identities of the components of each fraction have not been established. Results are expressed as percentages of recovered radioactivity (50–70%).

| | Radioactivity (% of recovered radioactivity) | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Mucosa | | Kidney | | Bone | | Liver | | Muscle | |
| Chromatographic fraction | Rat | Chick | Rat | Chick | Rat | Chick | Rat | Chick | Rat | Chick |
| Ester | 2.7 | 2.0 | 2.0 | 4.0 | 3.0 | 5.0 | 4.0 | 5.0 | 2.0 | 4.0 |
| Monohydroxy compounds | 25.5 | 25.3 | 21.3 | 35.3 | 30.7 | 53.6 | 50.9 | 36.1 | 23.7 | 49.8 |
| Dihydroxy compounds | 43.1 | 28.1 | 62.1 | 31.7 | 52.0 | 25.0 | 32.4 | 27.4 | 54.3 | 26.6 |
| Trihydroxy compounds | 23.0 | 39.1 | 12.5 | 30.4 | 12.0 | 12.5 | 9.3 | 26.1 | 15.4 | 14.3 |

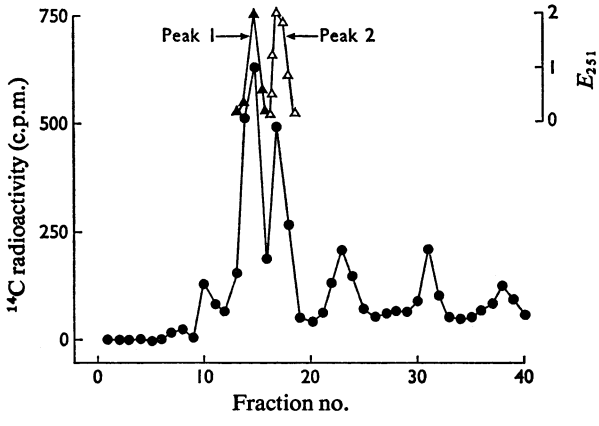


Fig. 4. *Distribution of $^{14}C$ after chromatography on Sephadex LH-20 of lipid extracts of intestines from vitamin D-deficient rats given* 0.15$\mu$g *of [4-$^{14}C$,1-$^3H$]dihydrotachysterol$_2$*

Eluting solvent was dichloromethane. Fractions 0–20 were 5ml and 21–40 were 10ml. ●, $^{14}$C; ▲, △, extinction at 251nm. Peak 1, dihydrotachysterol; peak 2, 25-hydroxydihydrotachysterol.

of the other metabolites (Table 2). In the chick, however, the major components were the more polar metabolites found in peak VII, and there was a significant increase in the proportion of this fraction as the dose of the steroid was decreased. The recovery of radioactivity from these columns was over 93%.

The proportion was measured of the various components found in the other tissues of the animals given 0.5$\mu$g of the radioactive steroid (Table 3). The major component in the rat tissues, as in blood, is 25-hydroxydihydrotachysterol$_3$. In the chick, however, the pattern is more varied. In bone and muscle, dihydrotachysterol$_3$ accounts for the majority of the radioactivity, whereas in the other tissues similar proportions are found of this steroid and of its more polar metabolites. The effect in the tissues of raising the dose of dihydrotachysterol$_3$ to 10$\mu$g per animal was to increase the proportion of this steroid still present after 24h.

The most polar fraction (peaks VI and VII) in chick and rat tissues co-chromatographs with the dihydroxy metabolites of cholecalciferol on columns of silica gel and on t.l.c. These metabolites of cholecalciferol can be effectively separated on Sephadex LH-20 columns (Lawson *et al.*, 1971*a*; Suda *et al.*, 1970). The application of this procedure to the dihydroxy metabolites of dihydrotachysterol$_3$ separated them into three fractions, none of which was in the same region as 1,25-dihydroxycholecalciferol. Hydroxylation at C-1 of [1-$^3$H]dihydrotachysterol results in a loss of 85% of the $^3$H radioactivity (Bell & Kodicek, 1970) and can be used as a sensitive test for 1-hydroxylation. Further groups of rachitic rats and chicks were dosed with 0.15$\mu$g of [4-$^{14}$C,1-$^3$H]-dihydrotachysterol$_2$ and the lipid extracts were chromatographed. The pattern of metabolites was similar to that shown in Table 3 with no indication of a change in $^3H/^{14}C$ ratio in any fraction. A further group of three rachitic rats received 10$\mu$g of the double-labelled dihydrotachysterol$_2$ and the lipid extracts were chromatographed. Fig. 4 shows the $^3$H and $^{14}$C elution profile of the eluate from a column of Sephadex LH-20.

As expected, therefore, from the steric structure of dihydrotachysterol, there is no formation of 1,25-dihydroxydihydrotachysterol$_3$ *in vivo*, but see the Discussion section. Such a finding is consistent with the observation that the activity of dihydrotachysterol is not affected by the removal of the kidneys. In other experiments we have shown that the pattern of metabolites in intestine, bone and blood was unchanged in anephric rats.

The question arises therefore as to the nature of the radioactivity in the intestinal nuclei which, from consideration of the findings with cholecalciferol, would be responsible for the biological activity of dihydrotachysterol. The intestine of rachitic chicks

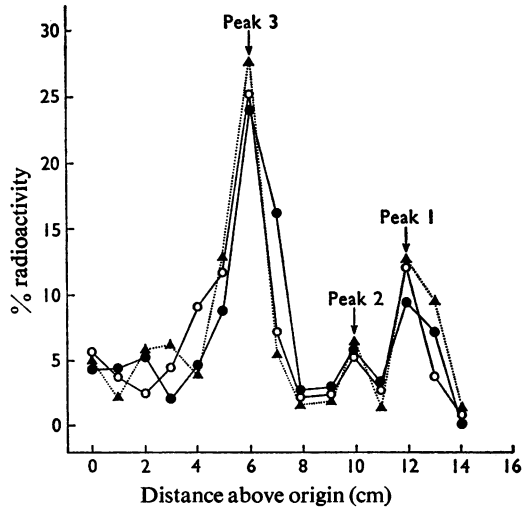


Fig. 5. *Thin-layer chromatogram of lipid extracts of intestinal cell fractions of vitamin D-deficient birds dosed with* 0.5$\mu$g *of* [1,2-$^3H_2$]*dihydrotachysterol*$_3$.

Developing solvent was ethyl acetate. For other details see the text. Position of authentic substances shown. Peak 1, dihydrotachysterol$_3$; Peak 2, 25-hydroxydihydrotachysterol$_3$; Peak 3, 1,25-dihydroxycholecalciferol. ○, ●, Homogenate; ○, nuclear debris; ▲, cytoplasmic.

Table 4. *Radioactivity in chick tissues after a dose of labelled* 5,6-*trans-cholecalciferol or* 25-*hydroxy*-5,6-*trans-cholecalciferol*

Rachitic chicks were dosed with 5$\mu$g of either 5,6-*trans*-[1,2-$^3H_2$]cholecalciferol or of 25-hydroxy-5,6-*trans*-[26-$^{14}$C]cholecalciferol. Radioactivity in the lipid extracts of the tissues was measured and expressed as percentage of dose/g of tissue or/ml of blood.

| Tissue | 5,6-*trans*-[$^3$H]-Cholecalciferol | 25-Hydroxy-5,6-*trans*-[$^3$H]cholecalciferol |
|---|---|---|
| Blood | 0.05 | 0.04 |
| Liver | 0.08 | 0.07 |
| Intestine | 0.03 | 0.03 |
| Bone | 0.03 | 0.02 |

dosed with 10$\mu$g of [1,2-$^3$H]dihydrotachysterol$_3$ was homogenized and fractionated into a nuclear-debris fraction and cytosol (Lawson *et al.*, 1971*b*). The lipids extracted from these two intracellular fractions and a portion of the total homogenate were chromatographed by t.l.c. The type and proportions of the metabolites found in cytosol and nuclear fraction did not differ either from each other or from that found in the total homogenate (Fig. 5).

*Biliary excretion of dihydrotachysterol*

The poor recoveries of radioactivity (about 50%) from the chromatographies of the lipid extracts of all the tissues, except blood, suggested the presence of a very polar metabolite of dihydrotachysterol. Lipid extracts of portions of liver and intestine from rachitic rats dosed with 0.5$\mu$g of dihydrotachysterol contained 1.6 and 1.1% of the dose respectively. The proportion of this radioactivity found in the unsaponifiable material of these tissues was 47.4% for the liver and 22.9% for the intestine. The radioactivity remaining in the saponification mixture in both cases became soluble in ether only after acidification of the mixture. This suggested that the radioactivity in this fraction was associated with a substance containing a carboxyl group. The material soluble in ether after acidification was chromatographed on a column of silica gel that had been activated at 120°C for 5 days. The column was developed with increasing proportions of acetone in benzene. Such a system has been of value in the separation of bile acids (Eneroth & Sjovall, 1969). The 8% (v/v) acetone in benzene fraction contained 67% of the recovered radioactivity, with a further 26% of the radioactivity in the 30% acetone in benzene fraction. The total recovery of radioactivity in this system was 80.4%. These results imply that the majority of the very polar radioactivity in tissues is associated with a substance containing one hydroxyl and one carboxyl group. Rachitic rats that had received 0.5$\mu$g of radioactive dihydrotachysterol excreted about 17% of the dose, of which about 10% was acidic in nature.

*Metabolism of* 5,6-*trans-cholecalciferol*

In common with dihydrotachysterol, very little radioactivity from either labelled 5,6-*trans*-cholecalciferol or labelled 25-hydroxy-5,6-*trans*-cholecalciferol was recovered in the tissues of rachitic chicks (Table 4). However, the chromatographic pattern was much simpler, with no evidence of a large number of metabolites, but instead the pattern more closely resembled that obtained with a dose of cholecalciferol. The major component in liver, intestine and bone was unchanged *trans*-cholecalciferol, whereas the 25-hydroxy metabolite was the main radioactive component in the blood (Table 5). All tissues contained a significant amount of a fraction with chromatographic properties similar to those of the dihydroxy metabolites of cholecalciferol. When the rachitic chicks were dosed with 25-hydroxy-5,6-*trans*-cholecalciferol the main component was this more polar fraction. As expected if 5,6-*trans*-cholecalciferol is active in anephric rats, the high proportion of polar radioactivity in the intestine did not contain a 1-hydroxylated component. This was shown by the administration of 0.125$\mu$g of 5,6-*trans*-[4-$^{14}$C,1,2-$^3$H]cholecalciferol to rachitic rats and

Table 5. *Chromatographic distribution of radioactivity from lipid extracts of tissues of chicks dosed with labelled 5,6-trans-cholecalciferol or 25-hydroxy-5,6-trans-cholecalciferol*

The lipid extracts of chicks dosed with labelled 5,6-*trans*-cholecalciferol (A) or 25-hydroxy-5,6-*trans*-cholecalciferol (B) (see the legend to Table 4) were fractionated by t.l.c. and results are expressed as percentage of the dose recovered. Radioactivity recovered from the plate was 50–70% of initial dose.

| | Radioactivity (% of dose recovered) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Blood | | Liver | | Intestine | | Bone | |
| Chromatographic fraction | A | B | A | B | A | B | A | B |
| Ester | 4.0 | — | 6.0 | — | — | — | 5.0 | — |
| 5,6-*trans*-Cholecalciferol | 24.4 | — | 56.6 | — | 39.5 | — | 55.0 | — |
| 25-Hydroxy-5,6-*trans*-cholecalciferol | 44.4 | 73.8 | 20.5 | 42.7 | 18.1 | 39.6 | 23.0 | 53.6 |
| Polar fraction | 13.2 | 18.1 | 9.0 | 21.6 | 33.3 | 42.3 | 9.4 | 13.2 |

chicks. The plasma and tissue lipids were obtained and chromatographed on several systems, but in no case was a fraction found with a lowered $^3H/^{14}C$ ratio. In other experiments pure intestinal nuclei were prepared, from rachitic birds that had received 5$\mu$g of 5,6-*trans*-[1,2-$^3$H]cholecalciferol. The lipids were extracted and chromatographed and found to be identical with the pattern found for the whole intestine.

## Discussion

Convincing evidence now exists from this and other studies (Hallick & DeLuca, 1972; Holick *et al.*, 1972) that dihydrotachysterol and 5,6-*trans*-cholecalciferol do not undergo hydroxylation at C-1 *in vivo*, despite the fact that the 25-hydroxy derivatives of both steroids will act as substrates for the 1-hydroxylase enzyme in incubations of kidney homogenates (D. R. Fraser, personal communication). Although we have found large quantities of polar metabolites of these analogues in the tissues examined of the rat and chick, no portion co-migrated with 1,25-dihydroxycholecalciferol in all chromatographic systems. Further, no metabolites of [4-$^{14}$C,1-$^3$H]-dihydrotachysterol$_2$ or 5,6-*trans*-[4-$^{14}$C,1,2-$^3$H]-cholecalciferol were detected with a lowered $^3H/^{14}C$ ratio, nor was there any evidence that the metabolism of dihydrotachysterol in anephric rats differed from that in intact animals. These findings agree with those of Hallick, & DeLuca (1972), who showed that 5,6-*trans*-cholecalciferol and dihydrotachysterol were as active in calcium absorption and bone mobilization in anephric rats as in sham-operated animals. The question arises therefore as to the metabolically active form of these analogues. Hallick & DeLuca (1972) did not find in rat bone any metabolite of dihydrotachysterol more polar than 25-hydroxy-dihydrotachysterol and concluded therefore that this metabolite was the active form in this tissue. They were unable to decide whether the active form in the intestine was 25-hydroxydihydrotachysterol, or a more polar metabolite, or both. In the present study polar metabolites were found in all tissues and in some cases in very high amounts. After a low dose of cholecalciferol only one of the polar metabolites, 1,25-dihydroxycholecalciferol, is found in the intestinal nuclei. However, with both these analogues a number of metabolites were present in the nuclear fraction in the same proportion as found in the whole tissues.

In common with the other steroid hormones 1,25-dihydroxycholecalciferol is concentrated in its target tissues, but of the metabolites of these analogues only the concentrations of dihydrotachysterol and its 25-hydroxy derivative in the tissues were equal to or greater than their plasma concentrations. Consequently it is to be expected that the affinity of the tissue receptors for these steroids will be much less than for 1,25-dihydroxycholecalciferol and of the same order as the affinity of these analogues with the plasma transporting proteins. It is possible that the dominant factor in the structure of the steroids influencing the magnitude of the affinity, particularly for the target tissue receptors, is a hydroxyl group at approximately the same steric position as that at C-1 of cholecalciferol. Hence the finding that the complete pattern of tissue steroids is also present in the intestinal nuclei. The low potency of these steroids and their rapid metabolism is also explicable on the basis of their relative binding affinities for the proteins which either transport or partake in the metabolism of cholecalciferol and its metabolites. We have shown (Preece *et al.*, 1972) that the association constants of 25-hydroxydihydrotachysterol$_3$ and 25-hydroxy-5,6-*trans*-cholecalciferol for rat plasma proteins is significantly lower than that for 25-hydroxycholecalciferol. Thus the plasma pool of these analogues will be much smaller than that of 25-hydroxycholecalciferol from an equivalent

amount of the parent steroid. On the other hand 1,25-dihydroxycholecalciferol is rapidly metabolized (Lawson & Emtage, 1974) and the enzymes or protein cofactors involved may take up the analogues and be responsible for their rapid metabolism, thus accounting for the well established short biological half-life of the dihydrotachysterols.

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