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Synthesis of [1,2-$^3H_2$]Cholecalciferol and Metabolism of [4-^{14}C,1,2-$^3H_2$]- and [4-^{14}C,1-^{3}H]-Cholecalciferol in Rachitic Rats and Chicks

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[1,2-$^3H_2$]Cholecalciferol has been synthesized with a specific radioactivity of 508mCi/mmol by using tristriphenylphosphinerhodium chloride, the homogeneous hydrogen catalyst. With doses of 125ng (5i.u.) of [4-^{14}C,1-$^3H_2$]cholecalciferol the tissue distribution in rachitic rats of cholecalciferol and its metabolites (25-hydroxycholecalciferol and peak P material) was similar to that found in chicken with 500ng doses of the double-labelled vitamin. The only exceptions were rat kidney, with a very high concentration of vitamin D, and rat blood, with a higher proportion of peak P material, containing a substance formed from vitamin D with the loss of hydrogen from C-1. Substance P formed from [4-^{14}C,1,2-$^3H_2$]cholecalciferol retained 36% of ^{3}H, the amount expected from its distribution between C-1 and C-2, the ^{3}H at C-1 being lost. 25-Hydroxycholecalciferol does not seem to have any specific intracellular localization within the intestine of rachitic chicks. The ^{3}H-deficient substance P was present in the intestine and bone 1h after a dose of vitamin D and 30min after 25-hydroxycholecalciferol. There was very little 25-hydroxycholecalciferol in intestine at any time-interval, but bone and blood continued to take it up over the 8h experimental period. It is suggested that the intestinal ^{3}H-deficient substance P originates from outside this tissue. The polar metabolite found in blood and which has retained its ^{3}H at C-1 is not a precursor of the intestinal ^{3}H-deficient substance P.

It is now known that cholecalciferol (vitamin D_3) undergoes several metabolic reactions in the body, including the formation of esters (Fraser & Kodicek, 1968*a*,*b*,*c*; Fraser, 1969) and of the glucuronide of a metabolite (Bell & Kodicek, 1969). In addition there is a sequence of metabolic events leading to the formation of a compound that is active in the biochemical reactions necessary for the effective intestinal transfer of Ca^{2+}. The first step in cholecalciferol intermediary metabolism is its conversion in the liver into 25-hydroxycholecalciferol (Horsting & DeLuca, 1969), which is released into the blood. It has been further shown that the 25-hydroxycholecalciferol is then converted into a polar fraction peak P, a component of which is formed by a metabolic reaction involving the loss of hydrogen (shown by using ^{3}H) from C-1 (Lawson, Wilson & Kodicek, 1969*a*,*b*). It has been suggested that this reaction might involve the introduction of an oxygen function in C-1 with a possible formation of a ketone (Lawson *et al.* 1969*b*; Bell & Kodicek, 1970; Kodicek, 1970). This fraction is present in a number of tissues, with the intestine containing the highest concentration, and is found in the nuclei of intestinal cells as practically the only vitamin D metabolite. The portion of peak P that has lost its hydrogen (as ^{3}H) we wish to call the ^{3}H-deficient substance P until its exact chemical nature has been elucidated, to distinguish it from other polar compounds that are different only in that they have not lost their ^{3}H label at C-1. The ^{3}H-deficient substance P given intravenously was three times more biologically active than cholecalciferol in increasing the intestinal ^{45}Ca transfer into the blood of rachitic chicks and is the most potent compound with vitamin D activity so far known (Kodicek, Lawson & Wilson, 1970).

Since the loss of ^{3}H in the [1-^{3}H]cholecalciferol prepared by the method of Callow, Kodicek & Thompson (1966) resulted in the formation of a metabolite practically without any radioactivity, it was considered necessary to prepare another tritiated form of cholecalciferol, with high specific radioactivity, which could be used in metabolic studies *in vivo* but still retaining its label. [1,2-$^3H_2$]-Cholecalciferol was the material of choice, since it would allow us to confirm the results obtained with [1-^{3}H]cholecalciferol, namely the loss of

hydrogen at C-1 without any loss at C-2. This was made possible by the determination of the stereospecific ^3H distribution of the [1,2-$^3H_2$]cholecalciferol, which indicated that 64% of the ^3H was at C-1 and the rest at C-2 (Bell & Kodicek, 1970).

Existing synthetic routes for [1,2-$^3H_2$]cholecalciferol have a number of limitations. Preparation by heterogeneous catalytic tritiation of cholesta-1,4-dien-3-one to the 4-en-3-one, the initial step in the synthesis used by Neville & DeLuca (1966), is subject to poor yields of the desired partially reduced product. Higher yields in the tritiation procedure may be obtained by reduction of 5α-cholest-1-en-3-one to 5α-cholestan-3-one, as in the synthesis of [1α-^3H]cholecalciferol (Callow *et al.* 1966), but are offset by the decrease in overall yield caused by the additional steps necessary for conversion into cholest-4-en-3-one.

The reports by Birch & Walker (1966) and by Djerassi & Gutzwiller (1966) of the high yields of steroid 4-en-3-ones obtained from 1,4-dien-3-ones by using the homogeneous hydrogenation catalyst tristriphenylphosphinerhodium chloride suggested a new approach, which has been utilized in the preparation of [1,2-$^3H_2$]cholecalciferol reported here.

By using this [1,2-$^3H_2$]cholecalciferol, the tissue distribution of vitamin D metabolites in the rat has been shown to be similar to that found for the chick. Information has also been sought as to the site of synthesis of substance P and to the existence of possible intermediates.

EXPERIMENTAL

[1,2-$^3H_2$]*Cholest-4-en-3-one.* Cholesta-1,4-dien-3-one (1.5g) together with tristriphenylphosphinerhodium chloride (0.7g, prepared by the method of Birch & Walker, 1966) in benzene (75ml) was hydrogenated at atmospheric pressure. After 4–5h uptake of H_2 stopped. The solution was evaporated to dryness and the residue refluxed with light petroleum (b.p. 60–80°C) (50ml). The solution was filtered while hot and the residue washed with three 20ml portions of hot light petroleum (b.p. 60–80°C) and one 20ml portion of diethyl ether. Filtrate and washings were combined and evaporated to dryness, giving 1.5g of cholest-4-en-3-one, showing only one spot when chromatographed on t.l.c. plates of silica gel G (E. Merck A.-G., Darmstadt, Germany) with chloroform, identical with authentic cholest-4-en-3-one (R_F 0.3).

Repetition of the above procedure with cholest-1,4-dien-3-one (2g), rhodium catalyst (0.5g) and ^3H gas (58Ci/mmol, 350Ci) gave a crude hydrogenation product in which 67% of the radioactivity was present as cholest-4-en-3-one. Chromatography of this material on Florisil (200g) and elution with light petroleum (b.p. 60–80°C)–benzene (3:1, v/v) gave successively cholestan-3-one (50Ci), a mixture of cholestan-3-one and cholest-4-en-3-one (100Ci, containing 56% cholest-4-en-3-one), and cholest-4-en-3-one (111Ci). The impure fractions were rechromatographed on a second Florisil column, when further separation was achieved.

[1,2-$^3H_2$]*Cholesterol.* [1,2-$^3H_2$]Cholest-4-en-3-one (111Ci) was converted into [1,2-$^3H_2$]cholesterol via the enol acetate by the method of Belleau & Gallagher (1951). The product was purified via the digitonide, and finally by preparative t.l.c. on 1mm-thick layers of silica gel GF_{254}, developing with cyclohexane–ethyl acetate (3:2, v/v). Crystallization of the product from methanol gave 111.8mg of cholesterol monohydrate (specific radioactivity 59.7Ci/mmol). The radiochemical purity was >99%, determined by t.l.c. on silica gel G containing 4% (w/w) of $AgNO_3$, and developing with chloroform–acetone (49:1, v/v).

[1,2-$^3H_2$]*Cholesta-5,7-dien-3β-yl benzoate.* A 90mg sample of [1,2-$^3H_2$]cholesterol was benzoylated and then converted into the 5,7-diene by the method of Bernstein, Binoir, Dorfman, Sax & Subba Row (1949). Dilution with carrier and crystallization from acetone–methanol and from acetone gave 184mg (2.5Ci) of crystalline product, of specific radioactivity 6.7Ci/mmol. This material had a radiochemical purity of 64%, as determined by t.l.c. on silica gel G containing 20% (w/w) of $AgNO_3$, and developing with light petroleum (b.p. 60–80°C)–toluene (1:1, v/v).

[1,2-$^3H_2$]*Cholecalciferol.* [1,2-$^3H_2$]Cholesta-5,7-dien-3β-yl benzoate was diluted with carrier to give a final specific radioactivity of approx. 0.5Ci/mmol. A 100mg portion of this material was saponified by refluxing for 1h with a solution of NaOH (50mg) in 90% (v/v) methanol (5ml) and benzene (20ml). Water was added and the benzene layer was washed with water until neutral. The benzene was dried over Na_2SO_4 and evaporated to dryness under N_2. The residue was dissolved in ether.

The ether solution was transferred to a cylindrical irradiation vessel surrounding a quartz thimble, which in turn held a smaller concentric quartz thimble containing a 100W medium-pressure mercury arc lamp. The space between the thimbles contained 0.4% $NaNO_3$, continuously circulated via a cooling coil. The entire apparatus was placed in an ice bath. Agitation of the provitamin solution was maintained by a stream of dry O_2-free N_2, dispersed through a sintered-glass disc in the base of the irradiation vessel. The solution was irradiated for 12min, then evaporated to dryness on a rotary evaporator below 5°C. The residual oil was dissolved in chloroform (1ml) and applied as a streak to two 20cm×20cm silica gel G t.l.c. plates spread with a layer 2mm thick of silica gel GF_{254}. The plates were developed twice in chloroform and viewed under u.v. light. The least polar band, consisting of precholecalciferol, was scraped off and eluted with ether. The ether extract was evaporated to dryness, and the residue was dissolved in benzene (10ml) and methanol (1ml) and refluxed under N_2 for 2h. After evaporation of the solvent the residue, in chloroform (0.5ml), was again chromatographed on a silica gel GF_{254} t.l.c. plate (20cm×20cm, 2mm-thick layer). The plate was developed twice in chloroform and viewed under u.v. light. Two bands were visible, corresponding to precholecalciferol and cholecalciferol. Elution of the more polar band with diethyl ether gave 15.0mg of [1,2-$^3H_2$]-cholecalciferol of specific radioactivity 508mCi/mmol and $\lambda_{max.}$ 265nm.

Elution of the less polar band from the second chromatogram gave 5.9mCi of [1,2-$^3H_2$]precholecalciferol; elution

of the successively more polar bands from the first chromatogram afforded respectively 32 mCi of [1,2-$^{3}H_2$]-tachysterol$_3$ and 34.2 mCi of unchanged [1,2-$^{3}H_2$]cholesta-5,7-dien-3β-ol.

The vitamin was stored as a dilute solution (<1 mCi/ml) at 0°C until required.

[4-^{14}C]Cholecalciferol (21.2 mCi/mmol) was obtained from N. V. Phillips–Duphar, Petten, The Netherlands.

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 vitamin D-deficient diet and supplements as described by Lawson *et al.* (1969*b*) and were used after 4 weeks, when they had developed rickets. Piebald weanling rats were raised on the rachitogenic diet of Numerof, Sassaman, Rodgers & Schaefer (1955). After 2–3 weeks, the dosing material was administered intracardially in propylene glycol 16 h before the animals were killed, unless otherwise stated. In all experiments the animals were starved overnight before being killed. The tissues were removed from the animals in a cold-room at 4°C and then either washed in cold 0.9% NaCl before tissue fractionation or frozen for determinations on the whole tissue.

Tissue fractionations. The mitochondrial, microsomal and cytoplasmic fractions were obtained by a modified method of Schneider & Hogeboom (1950) as described by Wilson, Lawson & Kodicek (1967). The nuclear debris fractions and the pure nuclei were prepared as before (Lawson *et al.* 1969*b*).

Extraction of intracellular fractions and tissues. Suspensions of tissues and intracellular 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.

Chromatography. The lipid extracts were chromatographed either (*a*) on columns of silica gel M.F.C. (Hopkin and Williams Ltd., Chadwell Heath, Essex, U.K.) as described by Lawson *et al.* (1969*b*), or (*b*) by t.l.c. on silica gel G with ethyl acetate–heptane (1:1, v/v) as developing solvent. The silica gel was scraped off the plate in bands and the radioactivity eluted with diethyl ether–ethanol (3:1, v/v). Cholecalciferol and 25-hydroxycholecalciferol were added as markers and were detected with iodine or antimony trichloride. The R_F values in this solvent system were: cholecalciferol, 0.57; 25-hydroxycholecalciferol, 0.34; substance P, 0.10.

G.l.c. was performed with glass columns 120 cm × 6 mm (outer diam.) of 3.8% UC-W98 on 80–100 mesh Diatoport S in a F & M model 402 gas chromatograph. The column outlet was connected directly to a fraction collector (Packard Instruments Ltd., Wembley, Middx., U.K.). The eluent from the chromatograph was collected for 0.5 min intervals in glass tubes loosely packed with glass wool wetted with scintillator. After collection, the glass wool was extruded into counting vials and the tube rinsed with 10 ml of scintillator. The injection block temperature was 220°C, the column temperature 220°C and the argon carrier gas flow rate 50 ml/min.

Chemical determinations. Protein was determined by the method of Lowry, Rosebrough, Farr & Randall (1951). DNA was determined by the method of Burton (1956).

Measurements of radioactivity. Lipid samples were assayed in a Packard Tri-Carb model 3375 automatic liquid-scintillation spectrometer. Quenching was corrected for by using automatic external standardization and correlation curves for ^{14}C and ^{3}H, either individually or combined.

RESULTS AND DISCUSSION

[1,2-$^{3}H_2$]*Cholecalciferol.* Preliminary reductions of cholesta-1,4-dien-3-one with $^{1}H_2$ indicated that, in the hydrogenation time used, no reduction of the Δ^4-bond was to be expected, results confirmed by other workers (Birch & Walker, 1966; Djerassi & Gutzwiller, 1966). It was therefore surprising to find a considerable amount of 5α-cholestan-3-one present in the products of reduction when $^{3}H_2$ was used. This appears to indicate a substantial isotope effect, which, however, needs further confirmation and study. From results of Djerassi & Gutzwiller (1966) it had been hoped that the distribution of ^{3}H in the product would be exclusively 1α,2α-. However, it has been shown (Bell & Kodicek, 1970) that the distribution of ^{3}H in the [1,2-$^{3}H_2$]cholesterol, and consequently also in the [1,2-$^{3}H_2$]cholecalciferol derived from the cholesterol, is 36% in 2α-+2β-, 47–49% in 1α- and 15–17% in 1β-position.

Analysis of the tritiated vitamin by t.l.c. showed that 98% of the radioactivity could be recovered from the region corresponding to vitamin D. Analysis by collection of successive timed samples from g.l.c. of the vitamin showed that the double peak characteristic of the pyro and isopyro derivatives of the vitamin was present, and contained 95% of the recovered radioactivity (Fig. 1).

Tissue distribution of vitamin D metabolites in the rat. The low accumulation of vitamin D and its metabolites in the target tissues of rachitic rats re-

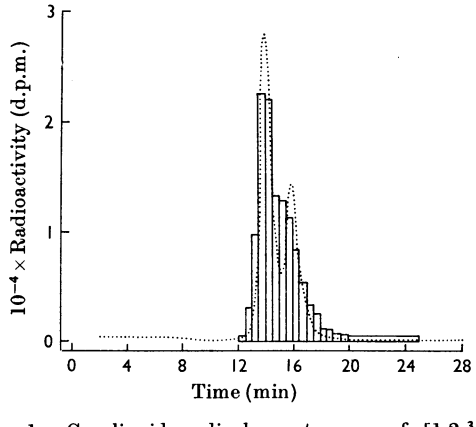

Fig. 1. Gas–liquid radiochromatogram of [1,2-$^{3}H_2$]-cholecalciferol. ····, Superimposed tracing of authentic cholecalciferol detected consecutively by flame ionization. For details of the chromatography see the text.

Table 1. *Distribution of radioactivity in lipid extracts of rat tissue after a dose of double-labelled cholecalciferol*

Four rachitic rats were each given 125ng of [4-^{14}C,-1,2-$^{3}H_2$]cholecalciferol (Expt. 1, $^{3}H/^{14}C$ ratio 23:1) and the tissues were removed 16h later. The results are expressed as percentages of the dose/ml or g of pooled fresh tissue.

	Total ^{14}C (d.p.m.)	Distribution (% of ^{14}C)	$^{3}H/^{14}C$ ratio
Blood	2100	0.89	19.2
Intestinal mucosa	530	0.20	16.6
Liver	2470	0.45	20.4
Kidney	6360	5.58	18.3
Bones	260	0.21	19.6
Muscle	720	0.16	22.7

ported before with high doses (Kodicek, Cruickshank & Ashby, 1960) was seen again even with doses of 125ng of [4-^{14}C,1,2-$^{3}H_2$]cholecalciferol (Table 1). The only notable feature was the high concentration of radioactivity recovered in the kidney, after intravenous administration of the labelled vitamin, a finding that was not observed in the chick. Comparison of the $^{3}H/^{14}C$ ratios of the dosing solution with those of the total radioactivity in tissues examined showed them to be decreased in all cases except muscle. However, the $^{3}H/^{14}C$ ratio of the blood of rats was lowered, in contrast with the finding of the chick.

The extracts from the tissues of the rats that had received one of the double-labelled preparations were chromatographed by either the silicic acid column method or the t.l.c. system with ethyl acetate–heptane (1:1, v/v) as the developing solvent. The proportions of the various metabolites in intestine and blood of rats under a variety of conditions are shown in Tables 2 and 3 and Fig. 2.

In the case of chicks administration of low doses, i.e. less than 1μg of labelled cholecalciferol, is essential to reveal characteristic patterns of metabolite excretion (Haussler & Norman, 1967), since under these circumstances the vitamin accounts for only a minor proportion of the radioactivity in all tissues, the rest appearing as more polar metabolites (Lawson *et al.* 1969*b*). A similar situation appears to occur in rats, where, however, the size of the dose had to be lowered even further, i.e. to 125ng of cholecalciferol. Thus after the intraperitoneal administration of 500ng of [4-^{14}C, 1-^{3}H]cholecalciferol to rachitic rats (Expt. 2.2) the major component in both intestine and blood was still the unchanged vitamin.

To ascertain whether the pattern of metabolites at this dosage was affected by the dietary intake of calcium, rachitic rats were transferred to the

Table 2. *Chromatographic distribution of the radioactivity from the lipid extract of rat intestine and blood*

Vitamin D-deficient rats (five) were given 125ng of either [4-^{14}C,1-^{3}H]cholecalciferol (Expt. 2.1, $^{3}H/^{14}C$ ratio 2.4:1; Expt. 2.5, $^{3}H/^{14}C$ ratio 4.1:1) or [4-^{14}C,1,2-$^{3}H_2$]cholecalciferol (Expt. 1, $^{3}H/^{14}C$ ratio 23:1). Lipid extracts of the pooled tissues were chromatographed on silica-gel columns (Expt. 2.5) or on the t.l.c. system (Expts. 1 and 2.1). Results are expressed as percentages of the ^{14}C recovered from the chromatogram.

	Intestine						Blood					
Total ^{14}C chromatographed (d.p.m.) ...	Expt. 1 500		Expt. 2.1 550		Expt. 2.5 300		Expt. 1 1000		Expt. 2.1 750		Expt. 2.5 820	
	% of ^{14}C	$^{3}H/^{14}C$ ratio	% of ^{14}C	$^{3}H/^{14}C$ ratio	% of ^{14}C	$^{3}H/^{14}C$ ratio	% of ^{14}C	$^{3}H/^{14}C$ ratio	% of ^{14}C	$^{3}H/^{14}C$ ratio	% of ^{14}C	$^{3}H/^{14}C$ ratio
Cholecalciferol ester	—	—	—	—	—	6.5	—	—	—	—	—	—
Cholecalciferol	30.3	21.9	21.4	2.4	33.5	4.1	17.6	22.2	27.9	2.4	24.3	3.7
25-Hydroxycholecalciferol	18.8	20.8	20.0	2.4	16.2	4.2	59.5	20.8	57.7	2.2	54.7	3.7
Peak P	34.4	13.2	45.6	1.6	36.5	1.1	5.2	11.9	5.8	1.6	13.1	2.7

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

The extracts were prepared from the tissues of rachitic rats treated as described in the legends to Tables 1 and 2 and Fig. 2 and the lipid extracts were chromatographed by the t.l.c. system. Results are expressed as percentages of the ^{14}C radioactivity recovered from the chromatogram.

	Liver				Kidney				Bone		Muscle	
	Expt. 1 1000		Expt. 2.2 400		Expt. 1 1260		Expt. 2.1 7000		Expt. 1 250		Expt. 1 700	
Total ^{14}C chromatographed (d.p.m.) ...	% of ^{14}C	^{3}H/^{14}C ratio	% of ^{14}C	^{3}H/^{14}C ratio	% of ^{14}C	^{3}H/^{14}C ratio	% of ^{14}C	^{3}H/^{14}C ratio	% of ^{14}C	^{3}H/^{14}C ratio	% of ^{14}C	^{3}H/^{14}C ratio
Cholecalciferol ester	—	—	3.2	—	—	—	—	—	—	—	—	—
Cholecalciferol	67.6	22.3	77.5	4.4	70.0	21.3	86.5	2.4	31.3	22.2	40.8	24.0
25-Hydroxycholecalciferol	14.7	19.7	5.7	4.4	6.0	20.2	6.0	—	48.3	20.7	44.9	20.3
Peak P	5.7	14.8	4.3	4.8	5.6	16.1	1.0	—	10.0	9.2	5.1	15.6

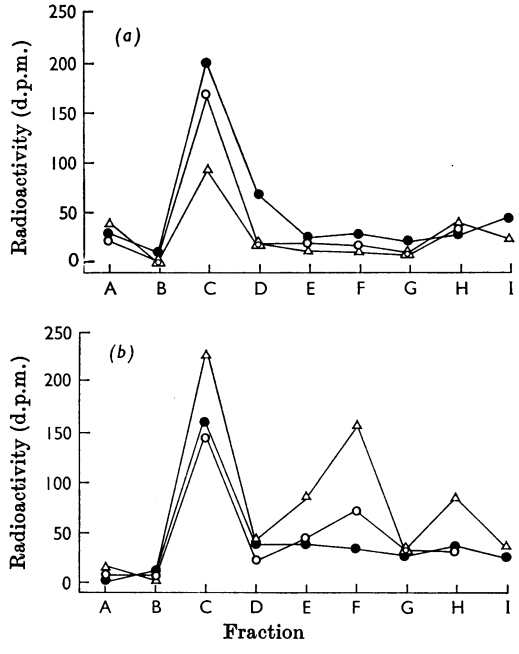

Fig. 2. Chromatographic distribution of ^{14}C from the lipid extracts of intestine (*a*) and blood (*b*) from vitamin D-deficient rats after a 500ng dose of [4-^{14}C,1-^{3}H]-cholecalciferol. ○, Expt. 2.2, ^{3}H/^{14}C ratio 4.7, intraperitoneal dose; ●, Expt. 2.3, ^{3}H/^{14}C ratio 4.9, intraperitoneal dose; △, Expt. 2.4, ^{3}H/^{14}C ratio 4.9, intracardial dose. Eluting solvents were successive 50ml portions of diethyl ether–light petroleum (b.p. 40–60°C) (1:19, v/v) (fractions A and B), diethyl ether–light petroleum (1:1, v/v) (fractions C and D), diethyl ether–light petroleum (3:1, v/v) (fraction E), diethyl ether (fractions F and G), methanol–diethyl ether (1:19, v/v) (fraction H) and methanol (fraction I). Components of the fractions are: A, cholecalciferol ester; C and D, cholecalciferol; E and F, 25-hydroxycholecalciferol; H, peak P.

normal-calcium vitamin D-deficient diet of DeLuca, Guroff, Steenbock, Reiser & Mannatt (1961) to lower the serum Ca^{2+} concentration (Expt. 2.3). Again there were no major peaks of radioactivity other than the vitamin. There was, however, a small peak of radioactivity more polar than peak P (fraction I). In Expt. 2.4 the labelled cholecalciferol was administered intracardially so as to achieve more closely a pulse dose. In this case a peak of radioactivity from the lipid extract of intestine was eluted from the silicic acid column with methanol–diethyl ether (1:19, v/v), which showed a slightly, but not significantly, lowered specific radioactivity ratio (fraction H, ratio 3.9). In blood, however, the fraction H corresponding to peak P did not show a lowered ratio, being 4.8. In this experiment (Expt. 2.4) 25-hydroxycholecalciferol appeared as a

distinct component in blood (fractions E and F). However, the latter compound became the major metabolite in blood when the dose, given intracardially, was lowered to 125ng (Table 2, Expts. 1, 2.1 and 2.5). It will be seen that there was an additional peak of radioactivity in the intestine attributable to peak P, and which showed, for the first time in rats, a significantly lowered specific radioactivity ratio. The radioactivity in this area of the chromatogram accounted for one-third to one-half of the total radioactivity recovered. In contrast with results with the chick, there was a small proportion of radioactivity as peak P with a lowered specific-radioactivity ratio in the plasma of rachitic rats.

In the rats given the 125ng of labelled cholecalciferol, peak P material with a lowered $^3H/^{14}C$ ratio was present in all other tissues examined, but it formed only a small portion of the total radioactivity (1–10%) (Table 3).

Tables 2 and 3 show that the specific radioactivity ratio in Expts. 2.1, 2.2 and 2.5, in which 125ng of [4-^{14}C,1-^{3}H]cholecalciferol had been administered, did not decrease to such low values as to indicate a total loss of ^3H at C-1. This has been interpreted (Lawson *et al.* 1969*b*) as being due to the presence in extracts of total tissues of two substances of similar polarity, but one retaining ^3H at C-1 and one without ^3H at this position. A similar situation appears to have occurred when [4-^{14}C,1,2-$^3H_2$]cholecalciferol was given (Expt. 1). Since only 64% of the ^3H in this labelled compound was at C-1 (Bell & Kodicek, 1970) the theoretical minimum ratio that could have been observed on loss of ^3H at C-1 in both α- and β-positions was 8.3. In most tissues the $^3H/^{14}C$ ratio decreased to a smaller extent with the possible exception of lipid extracts of bones, where the ratio was 9.2.

As mentioned above, liver, kidney, bones and muscle extracts of rats contained only a low proportion of metabolites more polar than 25-hydroxycholecalciferol as compared with the chick. Further, in the liver after a dose of 125ng of cholecalciferol the polar peak P, though not increased in total radioactivity, had a greatly decreased $^3H/^{14}C$ ratio (Table 3). In rat kidney the major proportion of radioactivity was in the form of cholecalciferol, whereas in bones and muscle the 25-hydroxycholecalciferol was in highest concentration.

Intracellular location of 25-hydroxycholecalciferol. Table 4 gives a typical result obtained from studies on chicks designed to show the intracellular localization of 25-hydroxycholecalciferol. It seems that this metabolite is not confined to any particular cell compartment, although the mitochondria showed a slight accumulation. The proportion of 25-hydroxycholecalciferol in the microsomal fraction was lower here than has been observed in other similar experiments. There was virtually no vitamin D detected in the cytoplasm. Although the nuclear-debris fraction did not show a specially high accumulation of peak P material, Lawson *et al.* (1969*b*) have shown that it occurs in highest concentration in pure nuclei. The cytoplasmic fraction is the only other cell compartment to contain appreciable quantities of peak P material.

Time-course of appearance of peak P. The pattern of metabolites in rachitic chick intestine, bone, blood and liver over the 8h after a dose of [4-^{14}C,1,2-$^3H_2$]-cholecalciferol ($^3H/^{14}C$ ratio 22.1) is shown in Fig. 3.

Bone and the intestinal nuclear-debris fraction contained the ^3H-deficient substance P already 1h after the dose, and it was present in the intestinal supernatant fraction from 2h onwards. By means of the more exacting separation achieved by t.l.c. it has been possible to determine the presence of small amounts of the ^3H-deficient substance P in chick blood, a finding first indicated by the column chromatographic procedures used by Lawson *et al.* (1969*a*,*b*). Thus small amounts of this metabolite were detected 2h after dosing with labelled cholecalciferol, rising by 8h to 1.6% of the total radio-

Table 4. *Intracellular distribution of vitamin D and metabolites in the chick intestine after a dose of* [4-^{14}C,1,2-$^3H_2$]*cholecalciferol*

Vitamin D-deficient chicks (four) were given a 500ng dose of [4-^{14}C,1,2-$^3H_2$]cholecalciferol and killed after 16h. Results are expressed as percentages of the dose recovered in the homogenate and also as a specific radioactivity.

	Distribution (% of ^{14}C)	Specific radioactivity (d.p.m./mg of protein)		
		Cholecalciferol	25-Hydroxycholecalciferol	Peak P
Homogenate	—	0.083	0.16	0.52
Nuclear debris	28.2	0.17	0.18	0.44
Mitochondrial	14.0	0.21	0.27	0.34
Microsomal	9.8	0.12	0.01	0.37
Cytoplasmic	47.9	0.06	0.15	0.68

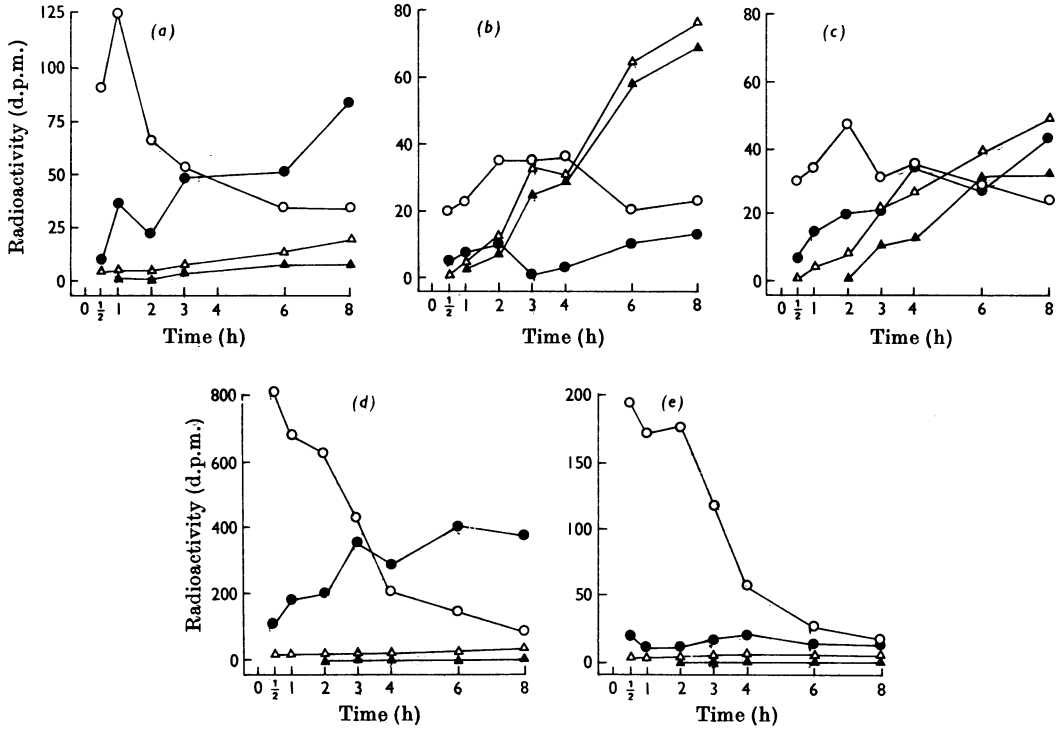

Fig. 3. Time-course of appearance of radioactive components from lipid extracts of (*a*) bone, (*b*) intestinal nuclear-debris fraction, (*c*) intestinal supernatant fraction, (*d*) blood and (*e*) liver of rachitic chicks after a 500ng dose of [4-^{14}C,1-^{3}H]cholecalciferol. ○, Cholecalciferol; ●, 25-hydroxycholecalciferol; △, peak P; ▲, ^{3}H-deficient substance P (for calculation see Lawson *et al*, 1969*b*).

activity in blood. The maximum concentration of the ^{3}H-deficient substance P was reached in the intestinal nuclear-debris fraction and the residual intestinal extract (intestinal supernatant fraction) and in bone 6h after the dose, amounting to 39, 37 and 8% respectively. It constituted almost the entire fraction eluted as peak P, in agreement with previous findings (Lawson *et al.* 1969*b*).

25-Hydroxycholecalciferol continued to accumulate in blood and bone up to 8h. However, in the liver, although 25-hydroxycholecalciferol accounted for an increasing proportion of the total radioactivity the actual concentration of this metabolite reached a maximum value by 4h. This is presumably a consequence of the liver secreting 25-hydroxycholecalciferol into the blood (Horsting & DeLuca, 1969). In the two intestinal fractions the 25-hydroxycholecalciferol accounted for up to 20% of the dose at 30min, but in the nuclear-debris fraction this proportion fell with time to values of 1–11%, in line with previous results obtained with [4-^{14}C,1-^{3}H]-cholecalciferol (Lawson *et al.* 1969*b*), whereas in the intestinal supernatant it rose only slightly up to 4h. Bone continued to accumulate 25-hydroxycholecalciferol during the time of this experiment. The amount of vitamin D in all the tissues examined declined throughout the 8h experimental period.

It is apparent from these results that none of the tissues examined concentrates 25-hydroxycholecalciferol from blood, i.e. that there is no mechanism in the tissues for enriching with this metabolite beyond the concentration encountered in blood. This supports the view that 25-hydroxycholecalciferol is not the final compound responsible for vitamin D activity. The ^{3}H-deficient substance P, however, which is at least twice as potent as 25-hydroxycholecalciferol in intestinal Ca^{2+}-transporting activity, is accumulated in the tissues with particular preference for the target organs. This is in accord with the view that the ^{3}H-deficient substance P with its specific tissue and nuclear localization is the active form or very closely related to it.

Metabolism of 25-hydroxy[4-^{14}C,1-^{3}H]*cholecalciferol in chicks.* It has been shown previously that 25-hydroxycholecalciferol is converted *in vivo* into peak P containing the ^{3}H-deficient substance P

Table 5. *Chromatographic distribution of the radioactivity from blood and liver lipid extracts at time-intervals after a dose of 25-hydroxy*[4-^{14}C,1-^{3}H]-*cholecalciferol*

Two or three vitamin D-deficient chicks at each time-interval each received a 250 ng dose of 25-hydroxy[4-^{14}C,1-^{3}H]cholecalciferol (^{3}H/^{14}C ratio 2.6:1). Lipid extracts were chromatographed by the t.l.c. system. Results are expressed as d.p.m./ml or g of pooled fresh tissue or as percentages of the ^{14}C recovered from the chromatogram.

	Blood					Liver				
		25-Hydroxycholecalciferol		Peak P			25-Hydroxycholecalciferol		Peak P	
Time (h)	Total radioactivity (d.p.m./ml)	% of ^{14}C	^{3}H/^{14}C ratio	% of ^{14}C	^{3}H/^{14}C ratio	Total radioactivity (d.p.m./g)	% of ^{14}C	^{3}H/^{14}C ratio	% of ^{14}C	^{3}H/^{14}C ratio
½	187.3	94.1	2.6	2	<0.1	87.7	60.0	2.6	13.4	2.4
1	157.5	92.8	2.6	3	1.4	72.3	65.3	2.5	17.7	1.7
2	124.2	91.8	2.6	3.5	0.9	71.7	69.1	2.5	11.1	1.5
4	58.5	82.2	2.7	17.0	1.8	—	—	—	—	—

Table 6. *Chromatographic distribution of the radioactivity from lipid extracts of intestinal and kidney supernatant fractions at time intervals after a dose of 25-hydroxy*[4-^{14}C,1-^{3}H]*cholecalciferol*

Two or three vitamin D-deficient chicks at each time-interval each received 250 ng of 25-hydroxy[4-^{14}C,1-^{3}H]cholecalciferol (^{3}H/^{14}C ratio 2.6:1). The supernatant fraction here is that portion of the tissue homogenate not sedimented after centrifugation at 700*g* for 10 min. Lipid extracts were chromatographed by the t.l.c. system. Results are expressed as d.p.m./g of original pooled fresh tissue or as percentages of the ^{14}C recovered from the chromatogram.

	Intestinal supernatant						Kidney supernatant					
			25-Hydroxy-cholecalciferol		Peak P				25-Hydroxy-cholecalciferol		Peak P	
Time (h)	Total radioactivity (d.p.m./g)	Ester area % of ^{14}C	% of ^{14}C	^{3}H/^{14}C ratio	% of ^{14}C	^{3}H/^{14}C ratio	Total radioactivity (d.p.m./g.)	Ester area % of ^{14}C	% of ^{14}C	^{3}H/^{14}C ratio	% of ^{14}C	^{3}H/^{14}C ratio
½	11.7	12	55.2	2.6	16.1	0.70	46.5	0	77.1	2.5	13.3	—
1	—	—	—	—	—	—	33.7	0	68.8	2.5	20.5	2.2
2	12.6	14	41.0	2.6	32.8	0.43	21.2	0	2.2	2.2	10.0	2.7

(Lawson *et al.* 1969*b*). To extend this observation the pattern of metabolites in various tissues has been studied at short time-intervals after an intracardial dose of 25-hydroxy[4-^{14}C,1-^{3}H]cholecalciferol to vitamin D-deficient chicks (Tables 5 and 6). In blood over 90% of the radioactivity was present as 25-hydroxycholecalciferol up to 2h, falling to 82% by 4h. The ^{3}H-deficient substance P was present after 30min in blood, as indicated by the lowered ratio, although it should be appreciated that the low amount of radioactivity does not allow for optimum accuracy in these measurements. At 4h the proportion of the ^{3}H-deficient substance P had risen significantly. In the liver 60–70% of the tissue radioactivity was due to unchanged 25-hydroxycholecalciferol. However, the ^{3}H-deficient substance P was only detected after 1h. The intestinal mucosa was fractionated into a nuclear-debris fraction and a supernatant fraction that contained the mitochondria, microsomes and the cell cytoplasm. The nuclear-debris fraction had very little radioactivity, amounting to only 1.0% of the dose. However, its $^{3}H/^{14}C$ ratio was very low, being 0.45 after 30min and 0.1 after 2h. In the supernatant fraction the total radioactivity was 2% of the dose, of which 25-hydroxycholecalciferol accounted for about half. Again the ^{3}H-deficient substance P was present from 30min onwards.

The kidney nuclear-debris fraction contained only a trace of radioactivity at any of the time-intervals examined, although all the extracts showed the $^{3}H/^{14}C$ ratio to be decreased. The kidney supernatant fraction contained about 3% of the dose, with from 70–80% accountable as 25-hydroxycholecalciferol; the polar metabolites amounted to 10–20% of recovered ^{14}C radioactivity, but at these early time-intervals the ^{3}H-deficient substance P could not be detected.

These and other results lead us to the conclusion that the intestine may not be the site of formation of the ^{3}H-deficient substance P. Thus small amounts of it are present in blood from very early time-intervals after doses of either cholecalciferol or 25-hydroxycholecalciferol. That very little was present in blood at these early time-intervals is to be expected, since the target tissues such as intestine and bone would be so avid for this material. Further, there is no specific accumulation of the 25-hydroxycholecalciferol at any of the intracellular sites at which hydroxylation reactions usually take place, and which might be expected to occur if this were the site of the conversion. Further, the presence of large amounts of peak P material in the intestinal cytoplasm and its accumulation in the nuclei resembles the findings obtained on the intracellular distribution of the steroid hormones in their target tissues.

The presence of a polar metabolite in all tissues, and in particular blood, which has retained ^{3}H at C-1, raises the possibility that this is an intermediate between 25-hydroxycholecalciferol and the ^{3}H-deficient substance P. This polar metabolite, which does not show a decreased $^{3}H/^{14}C$ ratio, was prepared from the blood of pigs that had been dosed with [4-^{14}C,1-^{3}H]cholecalciferol. The polar fraction of the blood lipid extract was purified by gradient elution chromatography (Lawson *et al.* 1969*b*) and by the t.l.c. system until it was free of labelled 25-hydroxycholecalciferol. The ^{3}H-deficient substance P was not detectable in the dosing solution, but, with the accuracy of the radioactive measurements, could have amounted to 4–5%. Each of two vitamin D-deficient chicks recovered an intracardial injection of 250ng of [4-^{14}C,1-^{3}H]peak P ($^{3}H/^{14}C$ ratio 3.4:1) derived from blood and were killed 16h later. Measurement of the radioactivity of the lipid extracts of the blood showed that it contained 2.2% of the dose substance. The intestine contained only traces of ^{14}C radioactivity, amounting to 0.15% of the injected material. If the peak P material derived from blood had been the precursor of the ^{3}H-deficient substance P in the intestine a much larger concentration would be expected, since intracardial injections of 125ng of the ^{3}H-deficient substance P caused an accumulation in the intestine of the order of 1.2% (Kodicek *et al.* 1970).

We suggest, therefore, that the peak P material from blood that has not lost its ^{3}H is not a precursor of the ^{3}H-deficient substance P in the intestine, despite the fact that the intestinal extract showed a lower $^{3}H/^{14}C$ ratio. Our interpretation of this result is that this is due to the presence of undetectable traces of the ^{3}H-deficient metabolite in the dosing material, since the presence of 3% of the ^{3}H-deficient material in the dosing solution would have given rise to this concentration of peak P in the intestine if the latter tissue had concentrated the circulating ^{3}H-deficient material.

Cousins, DeLuca, Suda, Chen & Tanaka (1970) reported the existence of two metabolites in intestinal nuclei, called by them peaks V and VI. The main radioactive component of nuclei a few hours after a dose of labelled 25-hydroxycholecalciferol was peak V, which, they claimed, was formed via peak VI. By using their column system we found that peak V from intestinal extracts has a lowered $^{3}H/^{14}C$ ratio and is identical with our peak P, whereas peak VI has retained all the ^{3}H at C-1. The chromatographic properties in two systems indicate that peak V and the ^{3}H-deficient substance P are identical and that peak VI and blood peak P are very similar. The interrelation between these metabolites of vitamin D discussed here and those reported by Cousins *et al.* (1970) will only be fully understood when their chemical structure and the

tissue responsible for their synthesis is known. Suda *et al.* (1970) reported the isolation and identification of a polar metabolite, 21,25-dihydroxycholecalciferol, from blood that is more active than cholecalciferol in the utilization of bone mineral, but less active in the cure of rickets and in intestinal Ca^{2+} transport. It is thus possible that several specific active forms exist that act at different sites of some target organs.

Note added in proof. It has now been shown that the tritium-deficient substance derived from 25-hydroxy-[1-^{3}H]cholecalciferol and found in various tissues is produced only by the kidney (Fraser & Kodicek, 1970).

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