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Vitamin D: new findings on its metabolism and its role in calcium nutrition

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The problems involved in elucidating the role of vitamin D in calcium metabolism have become more defined in the latter half of the 1960's as a consequence of two factors. One was the use of inhibitors of protein synthesis which, it is believed, has allowed the delineation of the area of cell metabolism controlled by the vitamin. The other factor was the availability of radioactively labelled cholecalciferol of sufficiently high specific activity to enable one to follow the metabolism of tracer quantities of the vitamin.

As a consequence of these two techniques it appears that the biochemical changes associated with vitamin D deficiency and reported before 1964 are all secondary consequences of an initial action of the vitamin. This applies not only to the well established changes such as the decreased alkaline phosphatase activity (Heymann, 1933), decreased citrate content of bone, blood and other tissues (Dickens, 1941) but also the more recently discovered changes such as the increased permeability of kidney mitochondria to calcium (Engstrom & DeLuca, 1964) and the altered turnover of intestinal phospholipids (Thompson & DeLuca, 1964; Lawson, 1969, Kowarski & Schachter, 1969).

The biological activity of various isomers and derivatives of vitamin D has been studied so as to define more clearly those features of the molecule essential for full activity. Until recently it was thought that there were only three essential features.

(1) *A free hydroxyl group at C-3*. Replacement of this group with a keto (Windaus & Buchholz, 1938), a sulphydryl (Bernstein & Sax, 1951) or a halogen (Bernstein, Oleson, Ritter & Sax, 1949) destroyed the vitamin D activity of the molecule.

(2) *An intact side-chain preferably of the cholesterol type*. Thus, replacement of the side-chain with an oxygen atom abolished biological activity. However, some variation can be tolerated without losing all activity: the most well-known example being ergocalciferol which has 100% activity in the rat but only 10% in the chick (Chen & Bosmann, 1964). The biological activity in the rat for other calciferols

being 22,23-dihydroergocalciferol, 50–75% (McDonald, 1936); sitocalciferol, 10% (Grab, 1936); stigmacalciferol, 5% (Rosenberg, 1945).

(3) *Three conjugated double bonds at C*10–19, *C*5–6 *and C*7–8. Thus, isomers of vitamin D, either structural such as iso-vitamin D, tachysterol and isotachysterol, or geometric such as 5,6-*trans*-vitamin D have only a fraction of the activity of the 5,6-*cis*-form of vitamin D (E. M. Cruickshank and E. Kodicek, private communication). The various hydrogenated products of vitamin D have insignificant amounts of activity with the one notable exception of dihydrotachysterol (Westerhof & Buismann, 1957). This latter steroid has proved of clinical value particularly in the treatment of hyperparathyroidism (Fourman & Royer, 1968). However, these three features of the vitamin D molecule are the only ones which readily lend themselves to chemical manipulations. As a consequence of the metabolic studies with the labelled vitamin it has been shown that the intact side-chain is necessary for an hydroxyl group at C-25 and that for the expression of complete biological activity a further oxygen atom is inserted into ring A, probably at C-1.

With regard to the biological function of vitamin D it is now well established that the vitamin has a direct action on bone (Carlsson, 1952), intestine (Nicolaysen & Eeg-Larsen, 1953), kidney (Harrison & Harrison, 1941) and clinicians would also include muscle (Smith & Stern, 1967). The function of vitamin D in bone is two-fold, being required for the proper mineralization of bone, i.e. calcium acquisition and, in addition, for the mobilization of calcium into blood. At present it is still not possible to demonstrate an effect of vitamin D on the in vitro uptake of calcium by bone or cartilage cells. However, physiological doses of vitamin D will induce a mobilization of bone mineral (Carlsson, 1952). The balance of calcium between the plasma and bone is maintained by vitamin D, calcitonin and parathyroid hormone, with the latter hormone only able to act in the presence of vitamin D (Harrison, Harrison & Park, 1958). These two effects, of calcium mobilization and acquisition, are quite different since the histological lesions produced by calcium deficiency are distinct from those of rickets and, conversely, calcium infusions to rachitic animals and osteomalacic patients do not fully lead to normal calcification of osteoid tissue (Fourman & Royer, 1968).

The other major target site of vitamin D is the small intestine. It seems to be agreed that calcium is absorbed more efficiently in the presence of vitamin D, but whether it is an effect on the active transport or the facilitated diffusion of the calcium is unclear. According to Wasserman & Taylor (1969) the vitamin acts on two or possibly three stages in the absorption process. The movement from intestinal lumen to the mucosal cell interior is increased, the size of the cellular pool of calcium being absorbed is also increased and the transfer of the calcium across the cell serosal surface to the mucosal undercoats is also improved.

There is general agreement that the action of vitamin D on the kidney is to increase the reabsorption of tubular phosphate (Harrison & Harrison, 1941) and although a similar effect on the intestinal absorption of phosphate can be demonstrated it is generally accepted that this is secondary to the effect on calcium. With regard to muscle, clinicians are convinced that there is a direct effect of vitamin D

on skeletal muscle but this aspect of vitamin D function has received little attention from biochemists and physiologists.

It is now well established that there is a lag in the action of vitamin D for every known physiological response to the vitamin (Norman, 1968). This was first appreciated by Irving (1944) who showed that a definite time had to elapse following the administration of the vitamin before an effect on the skeleton could be observed. Later, Lindquist (1952), who studied the effect of 0·375 μg vitamin D on ^{45}Ca metabolism in rats, observed a maximal effect on intestinal calcium absorption after 4 h, on serum calcium levels after 6 h and on deposition of calcium in bone after 36–48 h. Norman (1966*a*) found that the duration of the lag and the magnitude of response is proportional to the size of the dose of vitamin D. Consequently, the current approach to understanding the function of vitamin D is to relate any biochemical change to the time-lag between the administration of the vitamin and the physiological response under study. The prevailing view is that in rachitic animals the first biochemical process to respond to vitamin D is the one controlled direct by the vitamin.

Control of protein biosynthesis by vitamin D

Eisenstein & Passavoy (1964) began the present phase of biochemical investigations on the function of vitamin D when they demonstrated that actinomycin D inhibited the hypercalcaemic effect of the vitamin. Later, Norman (1966*a*) and Zull, Czarnowska-Misztal & DeLuca (1966) extended this observation and showed that actinomycin D and other inhibitors of protein synthesis blocked the action of vitamin D on all known physiological responses. They established that the inhibition was not a toxic response nor did it alter the metabolism of labelled vitamin D. The inhibition of intestinal calcium absorption in the chick could only be observed when the actinomycin D was given within 2 h of 2·5 μg of cholecalciferol. When the inhibitor was given 5–8 h after this dose of vitamin D_3, the maximum response of the vitamin on calcium absorption was still observed 24 h after the administration of the vitamin. These experiments with actinomycin D strongly suggest either that vitamin D acts at some particular stage in protein synthesis, most probably connected with transcription or, alternatively, that vitamin D activity can only be expressed with protein synthesis continuing. Support for the former view came with the demonstration that vitamin D increases the incorporation of orotic acid into RNA (Stohs, Zull & DeLuca, 1967; Norman, 1966*b*; Lawson, Wilson, Barker & Kodicek, 1969). Table 1 shows the stimulation by 125 μg of cholecalciferol of the orotic acid incorporation into chick intestinal *n*-RNA. This is one of the earliest effects of vitamin D being observed 20 min after an intracardial dose of the vitamin. Since there is no increase in either the RNA of the whole tissue or the nuclear fraction, the effect of the vitamin must be on the turnover of the *n*-RNA (Lawson, Wilson, Barker *et al.* 1969). Attempts have been made to localize this action more exactly. Lawson, Wilson, Barker *et al.* (1969) were unable to demonstrate an effect of vitamin D on the DNA-dependent RNA polymerase activity of intestinal nuclei, or on the intestinal

Table 1. *Effect of cholecalciferol on the incorporation of* [5-^{3}*H*]*orotic acid into n-RNA from vitamin D-deficient chicks*

	Specific radioactivity of RNA (disintegrations/min per mg)	
Expt no.	Vitamin-deficient birds	Cholecalciferol-injected birds
1	4100	7 500
2	9900	12 000
3	6000	31 000

In each experiment 125 μg of cholecalciferol in propylene glycol was injected intracardially into each of three deficient chicks. A further three birds received only propylene glycol. After 40 min the birds were given an intraperitoneal injection of [5-^{3}H]orotic acid and killed 20 min later. The RNA was extracted from the isolated nuclei of the intestines. (From the *Biochem. J.* 1969, **115**, 263.)

pool of nucleotides and their precursors. However, vitamin D has recently been shown to increase the template capacity of the DNA from the intestine of rachitic chicks (Hallick & DeLuca, 1969). This last result seems to be in contradiction with the polymerase assays since both systems are examining essentially the same reaction; this point still awaits clarification. In addition, future investigations will have to consider such points as the fate of the *n*-RNA which is affected by the vitamin since it is known that most of the *n*-RNA does not in fact leave the nucleus (Harris, 1968). Further, the effect on the turnover of the *n*-RNA is greater than would be expected if only a single protein or protein system for calcium absorption is being synthesized in response to the vitamin. Other points upon which future investigations will concentrate are whether the vitamin acts to control DNA template capacity in direct combination with a protein or whether it acts by controlling the synthesis of a protein which itself switches on the genome responsible for calcium absorption. It should also be remembered that the crucial experiment has yet to be carried out showing that after the administration of vitamin D a protein is synthesized which is involved in calcium transport. However, Wasserman & Taylor (1969) have demonstrated the existence of a protein in intestinal mucosa which has a high affinity for calcium and appears in rachitic animals only after a physiological dose of vitamin D. An unequivocal demonstration of an involvement of this protein in the calcium transporting mechanism of the mucosal cell is still awaited.

Metabolism of vitamin D

Because of the low requirements of animals for vitamin D and of the very small concentrations found in the target tissues it was essential that radioactively labelled vitamin D of high specific radioactivity be used for the study of the metabolism of this vitamin. Such a specific radioactivity can only be achieved, of course, with tritium as the label. A search began about 10 years ago (Callow, Kodicek & Thompson, 1966) for such a molecule which had to have the following properties. (1) The reactions by which it is synthesized should be readily carried out in high yield. The present procedure, used in this laboratory for the preparation of [1-^{3}H] cholecalciferol requires twelve steps from cholecalciferol. Although each step can be

carried out with a good yield the total yield is only 10–15%. (2) The label should be in a part of the molecule not subjected to enzyme attack. (3) The tritium should not undergo chemical exchange with the hydrogen of the body water.

Callow *et al.* (1966) prepared several tritiated forms of cholecalciferol, in particular [6,7-$^3H_2$]-, [3α-^{3}H]-, [24,25,26,27-$^3H_9$]-, and [G-^{3}H]- but all of these preparations showed significant exchange of the ^{3}H with the hydrogen of the body water. Finally, [1α-^{3}H]cholecalciferol was synthesized and found to be the most radiochemically stable preparation of all of those examined. In this laboratory we have had available first, [1-^{3}H]cholecalciferol (Callow *et al.* 1966, Wilson, Lawson & Kodicek, 1967) and later, [1,2-$^3H_2$]cholecalciferol (Lawson, Pelc, Bell, Wilson & Kodicek, 1971) whereas the American workers have used almost entirely [1,2-$^3H_2$] material (Neville & DeLuca, 1966). None of these preparations loses significant quantities of tritium by chemical exchange.

The early work with labelled vitamin D showed that the lipid soluble radioactivity in the tissues was almost entirely due to the vitamin (Kodicek & Ashby, 1960). Even a thousandfold difference in the dose to rachitic rats from 500 μg to 0·5 μg did not give a different pattern of metabolites (Lawson *et al.* 1971). However, there is a cut-off point at which the pattern of metabolism changes and this phenomenon is seen most clearly after an intracardial dose of the vitamin.

From the chromatogram shown in Fig. 1 it can be seen that even at dose levels of 0·5 μg the major part of the rat intestinal radioactivity is due to unchanged cholecalciferol whether the dose is administered intracardially or intraperitoneally. Again the radioactivity in blood lipids after an intraperitoneal dose is primarily due to

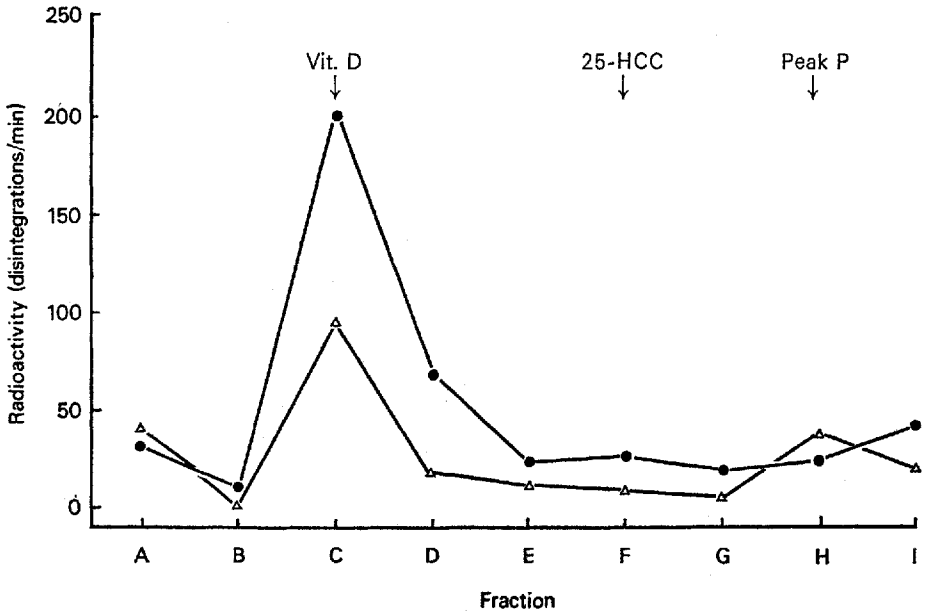

Fig. 1. Batch elution chromatography of the lipid extracts of intestines from vitamin D-deficient rats after a dose of 0·5 μg of [4-^{14}C]cholecalciferol 16 h before death. ●———●, intraperitoneal dose; △———△, intracardial dose; Vit. D, cholecalciferol; 25-HCC, 25-hydroxycholecalciferol; Peak P, polar chromatographic fraction.

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cholecalciferol, but after an intracardial dose the chromatograms of the blood radioactivity show a second component in approximately similar quantities to the vitamin (Fig. 2). The main constituent of this second fraction has been identi-

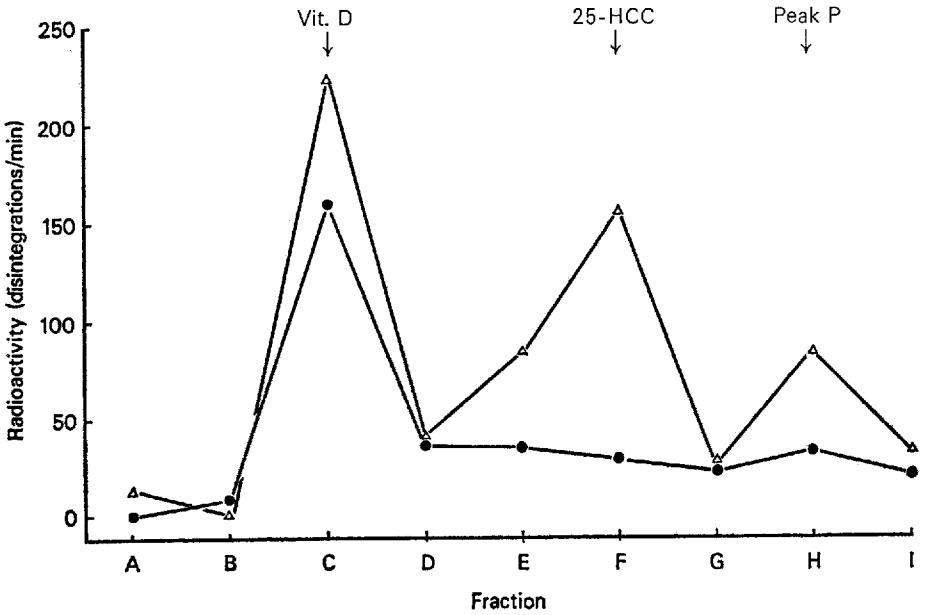

Fig. 2. Batch elution chromatography of the lipid extracts of blood from vitamin D-deficient rats after a dose of 0·5 μg of [4-^{14}C]cholecalciferol 16 h before death. ●———●, intraperitoneal dose; △———△, intracardial dose; Vit. D, cholecalciferol; 25-HCC, 25-hydroxycholecalciferol; Peak P, polar chromatographic fraction.

fied as 25-hydroxycholecalciferol (Blunt, DeLuca & Schnoes, 1968). This latter substance has about 40% more biological activity than cholecalciferol (Blunt, Tanaka & DeLuca, 1969) and for some time was thought by these authors (Blunt *et al.* 1969) to be the active molecule in the target tissue.

On reduction of the dose of labelled cholecalciferol to 0·125 μg, the major radioactive component of blood becomes 25-hydroxycholecalciferol (Fig. 3) with cholecalciferol accounting for only a small proportion. At even lower doses of the vitamin there is no further change in the pattern of blood metabolites (Lawson *et al.* 1971).

Although Stohs & DeLuca (1967) reported that the intestinal nuclei contained the highest concentration of vitamin D metabolites, we were unable to confirm this using the [1-^{3}H]cholecalciferol (Lawson, Wilson & Kodicek, 1969*a*,*b*). However, at this point [4-^{14}C]cholecalciferol of sufficiently high specific radioactivity became available and the specific accumulation of ^{14}C in the intestinal nuclei was confirmed.

To investigate the loss of the label from the [1-^{3}H]cholecalciferol used in our studies, a series of experiments was carried out in which the two isotopes together were administered intracardially to rachitic chicks. In our studies, chicks are routinely used since the change in the metabolic pattern of metabolites, mentioned

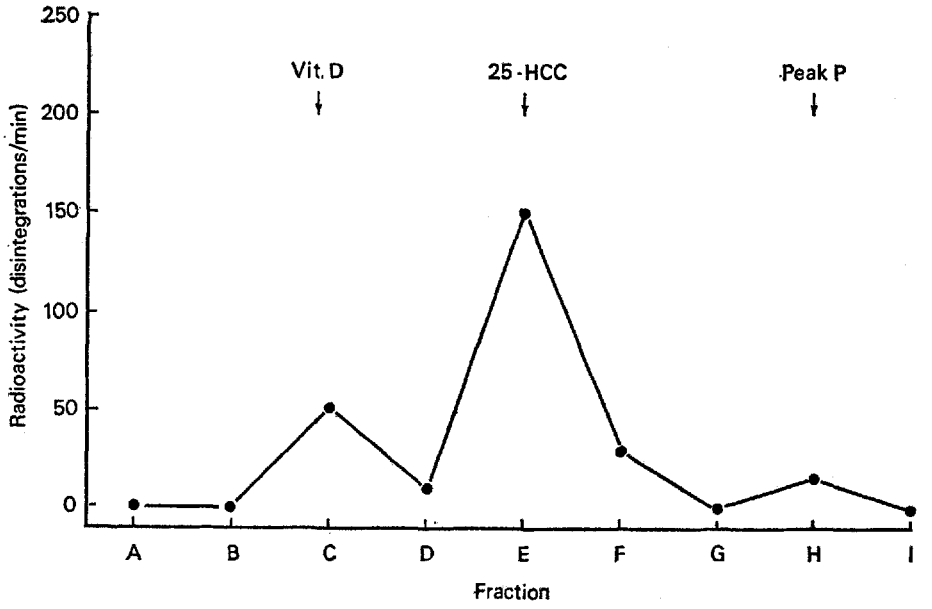

Fig. 3. Batch elution chromatography of the lipid extracts of blood from vitamin D-deficient rats after an intracardial dose of 0·12 μg of [4-^{14}C] cholecalciferol 16 h before death. Vit. D, cholecalciferol; 25-HCC, 25-hydroxycholecalciferol; Peak P, polar chromatographic fraction.

above, occurs at about 1·25 μg (Haussler & Norman, 1967) instead of 0·125 μg as in the rat and, consequently, higher doses of labelled vitamin can be used. Further, intracellular fractions are more readily prepared from chick intestine as it contains less mucus than rat intestine.

The chromatogram of the chick blood radioactivity (Fig. 4) shows that a similar pattern of metabolites exists in the chick blood at this dose level (0·5 μg) as in rat

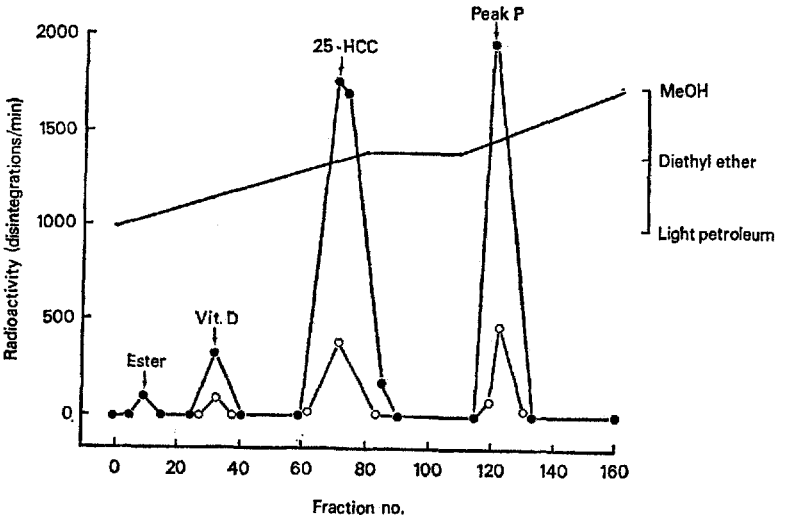

Fig. 4. Gradient-elution chromatography of the lipid extract of the blood of vitamin D-deficient chicks after a dose of 0·5 μg of [4-^{14}C, 1-^{3}H] cholecalciferol 16 h before death. ●———●, ^{3}H radioactivity; ○———○, ^{14}C radioactivity. Vit. D, cholecalciferol; 25-HCC, 25-hydroxycholecalciferol; Peak P, polar chromatographic fraction. (From the *Biochem. J.* 1969, **115**, 269.)

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blood after 0·125 μg of labelled vitamin. All the radioactive peaks contain the same proportion of ^{14}C and ^{3}H. However, the chromatogram of the intestinal lipids (Fig. 5) shows the presence of a peak more polar even than 25-hydroxycholecalciferol

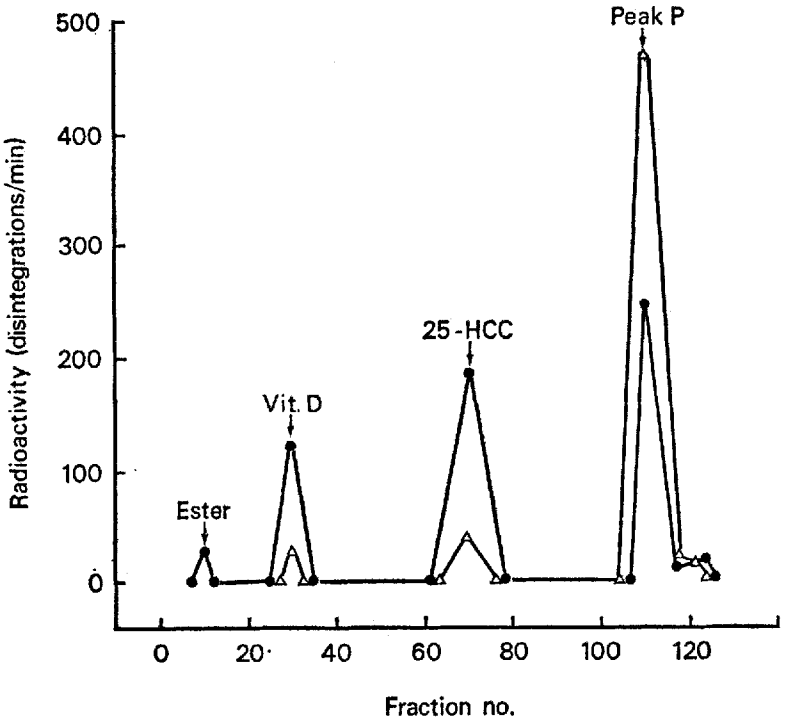

Fig. 5. Gradient-elution chromatography of the lipid extract of the intestines of vitamin D-deficient chicks after an intracardial dose of 0·5 μg of [4-^{14}C, 1-^{3}H] cholecalciferol 16 h before death. ●———●, ^{3}H radioactivity; ○———○, ^{14}C radioactivity; Vit. D, cholecalciferol; 25-HCC, 25-hydroxycholecalciferol; Peak P, polar chromatographic fraction. (From the *Biochem. J.* 1969, **115**, 269.)

which contains very little ^{3}H but which, in terms of ^{14}C, accounts for the major part of the radioactivity (60%). The small quantity of 25-hydroxycholecalciferol present in the intestine of rachitic chicks 16–18 h after dosing contains the same proportion of ^{3}H:^{14}C as the cholecalciferol present in the intestine and in the dosing solution. The polar chromatographic fraction from the intestinal lipids has been called peak P and is quite distinct from that seen in blood although it is eluted at the same point in the chromatogram since the tritium content is so different. Chromatography of the lipids from pure intestinal nuclei showed that 97% of the ^{14}C is due to peak P and the ^{3}H:^{14}C ratio of this fraction was very low (Lawson, Wilson & Kodicek, 1969*b*).

This polar chromatographic fraction which has lost a hydrogen from C-1 is present in varying proportions in bone, kidney, liver and muscle. Thin-layer chromatography of the blood lipids of chicks shows that peak P with a lowered ^{3}H:^{14}C ratio accounts for a small proportion of the radioactivity.

A major difference between the rat and chick is that rat blood contains a much higher proportion of peak P with the lowered ^{3}H:^{14}C ratio.

A full discussion of this loss of tritium has been given before (Lawson, Wilson & Kodicek, 1969*b*). Briefly, the most straightforward, but not the only, explanation is that the loss of tritium is due to the insertion of an oxygen function into the molecule at or adjacent to C-1. This additional oxygen group would account for the increased polarity of this metabolite. The intestinal intracellular distribution of vitamin D and its metabolites, and the chromatogram of intestinal nuclear radioactivity suggest that the biologically important compound is that which has lost its ^{3}H. A consequence of this metabolic loss of ^{3}H is that this metabolite can be recognized and measured even in the presence of a larger quantity of other metabolites. So, various studies of all types can be carried out on this specific metabolite simply by comparing the ^{3}H:^{14}C ratios in the chromatographic peaks. Thus double-labelled 25-hydroxycholecalciferol was prepared and shown to be a precursor of peak P (Lawson, Wilson & Kodicek, 1969*b*). At present it does not appear that any component of peak P of blood, which has retained all its ^{3}H, is a precursor of the tritium-deficient substance in the intestine. It has also been established that intestinal and bone peak P appear 1 h after a dose of cholecalciferol which is well before any effect on intestinal calcium transport can be observed. However, the metabolite which has lost a portion of the ^{3}H has only been detected in blood 2 h after a dose of cholecalciferol and can be readily detected up to 4 h.

Bioassay of peak P

Because of the minute quantities of this metabolite which are present in the intestine (<3 ng/g of tissue) it has not been possible to obtain sufficient of the intestinal peak P to carry out either classical chemical analysis or the standard bioassays. However, about 5 μg of the tritium-deficient substance was obtained from about 450 chicks and the effect of graded doses of this material on the intestinal absorption of ^{45}Ca was compared with both cholecalciferol and 25-hydroxycholecalciferol (Table 2). There was 15% increase in the effect of 25-hydroxycholecalciferol over that of the parent vitamin. As little as 0·025 ng (1 i.u.) of peak P has a measurable response on ^{45}Ca absorption, the mean relative potency being 2·9 times greater than cholecalciferol. Consequently, peak P is the most potent substance possessing vitamin D activity (Kodicek, Lawson & Wilson, 1970).

One interesting feature of this assay was the failure to observe any increase in the serum calcium levels of the peak P dosed birds although both the 250 ng and 500 ng levels of cholecalciferol and 25-hydroxycholecalciferol had restored these levels to normal. This means that peak P was, at the most, no more effective in raising the serum calcium levels than an equivalent dose of cholecalciferol and 25-hydroxycholecalciferol. Although a number of possible explanations could be advanced for this observation the most intriguing is the possibility that the calcium mobilization action of vitamin D is not mediated through peak P.

Table 2. *The relative potency of vitamin D_3, 25-hydroxycholecalciferol and peak P on intestinal absorption of ^{45}Ca*

	(Means of five determinations)* Serum ^{45}Ca levels (counts/min per ml of serum)		
Dose level (ng)	Cholecalciferol	25-Hydroxy-cholecalciferol	Peak P
25	—	—	2800
62·5	2200	2600	3100
125	2500	3000	4500
250	3700	4400	—
500	4900	—	—
Relative biological potency (RBP)	100	115	290
95% Fiducial Limits	—	43<RBP<168	150<RBP<540

Graded doses of the three steroids were given intracardially to groups of five rachitic chicks. After 24 h, a solution of 1 μCi ^{45}Ca in 0·25 ml of water was given orally and the blood collected 1 h later. Peak P, polar chromatographic fraction from intestinal lipids.
*The value for the rachitic control group was 1900 counts/min per ml of serum.

Biosynthesis of peak P

The rate of appearance of the polar metabolites of vitamin D is consistent with their formation from 25-hydroxycholecalciferol taking place in the target organs (Lawson *et al.* 1971). However, Fraser & Kodicek (1970) were unable to show the formation of peak P from double-labelled 25-hydroxycholecalciferol using homogenates, slices, everted sacs or isolated cells of the intestine.

A search was therefore made for the tissue responsible for the conversion of 25-hydroxycholecalciferol into a polar substance with a concomitant loss of ^{3}H. After testing homogenates of several tissues including liver, adrenals, parathyroid, ultimobranchial body and epiphysis, a successful conversion was only observed with homogenates of kidney. It was also shown that, in bilaterally nephrectomized rats, peak P was not present either in plasma or intestinal mucosa following an intracardial dose of 125 μg of [4-^{14}C, 1-^{3}H]cholecalciferol. It thus appears that the kidneys alone are responsible for the formation of the polar substance formed with a metabolic loss of hydrogen from C-1. However, the chemical relationship between this substance and that found in the intestinal nuclei has still to be established. This finding on the site of synthesis of peak P, although unexpected, is not inconsistent with the pathogenesis of some diseases involving calcium metabolism. For example, in several kinds of kidney malfunction, bone lesions develop which are indistinguishable from rickets or osteomalacia. Examples of such diseases are chronic renal failure, Fanconi syndrome and cystinosis. In these and other cases it may well be that the unfortunate bone changes are a consequence of the inability of the kidney to synthesize peak P.

This new metabolite of cholecalciferol may also be of value in understanding the pathology of patients who are either unable to utilize cholecalciferol (vitamin D-resistant rickets of various types) or who seem to be hypersensitive to the vitamin (sarcoidosis). Recent investigations in four such patients did not show an abnormal metabolism of 25-hydroxycholecalciferol, but the metabolism of the more polar

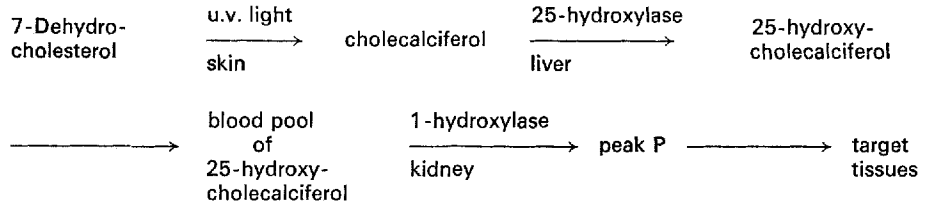

Fig. 6. Biosynthesis of peak P (polar chromatographic fraction from kidney lipids).

compounds was not examined (Park, DeLuca, Rios, Suda, Ruskin & Delea, 1970).

The biosynthesis of peak P is shown diagrammatically in Fig. 6. Calcium homeostasis is really dependent upon a source of ultraviolet radiation rather than on an accessory food factor. As a consequence of the studies reviewed above it now appears that, in the formation of the active agent of vitamin D from 7-dehydrocholesterol, passage through three tissues is necessary. The skin is essential for the conversion of the provitamin to cholecalciferol, whereas only the liver is said to be able to form the 25-hydroxyderivative (Horsting & DeLuca, 1969). This metabolite is rapidly secreted into the plasma, where its turnover is slower than that of cholecalciferol (Mawer, Lumb & Stanbury, 1969). The proportion of cholecalciferol and 25-hydroxycholecalciferol in blood is dependent upon the level of cholecalciferol to which the body is exposed from either of its two sources. At the higher levels of intake and production, cholecalciferol accounts for the major proportion of the biological activity of blood. However, at those levels barely adequate to satisfy the animals requirements, the blood activity is primarily due to 25-hydroxycholecalciferol. The third tissue essential for the expression of the biological activity of vitamin D is the kidney which seems to be solely responsible for the conversion of the 25-hydroxycholecalciferol into the tritium-deficient substance. (Fraser & Kodicek, 1970). A specific control over the production of peak P must exist since only finite amounts are found in the target tissues.

This final step in the biosynthetic sequence means that vitamin D, in the form of its metabolite at present known as peak P, should be reclassified as a hormone since it fulfils all the necessary criteria. Thus, it is secreted in small amounts by only one tissue and is carried by the plasma to its target tissues with the whole process being under a fine control. The role of 25-hydroxycholecalciferol may be central to the, as yet untouched, problem of the control of the levels of peak P.

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