Metabolism of Vitamins D and A

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Recent advances in the elucidation of the metabolism of vitamins A and D have been reviewed. Retinol and retinoic acid are metabolized to CO_2 and other excretion products by three pathways. Both in vivo and in vitro evidence has been obtained for all three. One pathway, traced to the microsomal fraction, involves oxidative decarboxylation of the terminal carbon to CO_2 with TPNH and O_2 as requirements. Vitamin D is metabolized to 11 metabolites, which are readily separated on silicic acid columns. An ester of vitamin D with long-chain fatty acids represents one metabolite fraction. Quantitatively most important is another metabolite which has been isolated and identified as 25-hydroxycholecalciferol. It probably represents the metabolically active form of vitamin D. Other metabolites are as yet unidentified.

Within the last ten years, new and revolutionary findings have injected into the field of the metabolism of fat-soluble vitamins a renewed interest in finding a detailed understanding of the metabolism and function of these compounds at the molecular level. This discussion of recent developments regards only two fat-soluble vitamins, A and D.

VITAMIN D

Recent advances in the study of the metabolism of vitamin D have been made possible by modern techniques of isotopic labeling, various chromatographic methods, and new methods of identification with only minute quantities—mass spectrometry and nuclear magnetic resonance spectrometry.

Although attempts were made in the last decade (Kodicek, 1958), it was not possible to examine the metabolism of vitamin D extensively because the specific activity of the labeled vitamins was too low to permit experiments with physiologic doses. It was shown, however, that absorption of vitamin D requires the presence of bile; the primary excretion of the end products of vitamin D is in the feces via the bile; and vitamin D is not excreted intact but is metabolized (Kodicek, 1956). However, the old methods and nonphysiologic doses led to incorrect conclusions or assumptions, not enumerated here.

It was only after the synthesis of radioactive vitamins D_2 (Imrie *et al.*, 1967), D_3 (Neville and DeLuca, 1966), and D_4 (DeLuca *et al.*, 1968) of sufficient radioactivity that progress became rapid. The methods of synthesis of radioactive D_3 and D_4 are shown in Figures 1 and 2, respectively.

Cholesteryl benzoate labeled in the 1,2 positions was brominated allylically with *N N'*-dibromodimethylhydantoin and then dehydrohalogenated to yield the 7-dehydrocholesteryl benzoate, which on irradiation with ultraviolet light after hydrolysis yielded 1,2-^{3}H vitamin D_3. The 22, 23 ^{3}H vitamin D_4 was prepared by reaction of the ergosteryl acetate with maleic anhydride followed by reduction of the side chain with tritium using palladium black as the catalyst. The maleic anhydride was removed by sublimation at 220°C. and following hydrolysis, the 22, 23 ^{3}H vitamin D_4 was prepared in the usual fashion. A radioactive vitamin D_2 of high specific activity was also obtained by a biosynthetic route (Imrie *et al.*, 1967).

Using physiologic quantities of radioactive vitamin D, it was possible to detect metabolites of vitamin D that had been

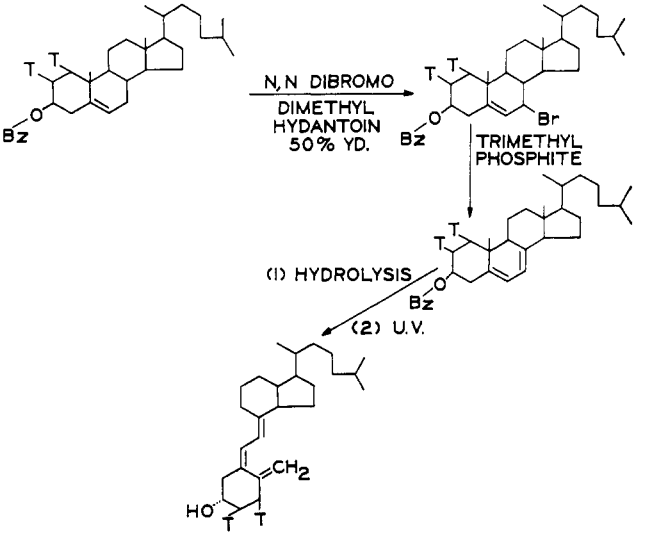

Figure 1. Synthesis of 1,2-^{3}H vitamin D_3 of high specific activity

26,000 d.m.p./I.U.

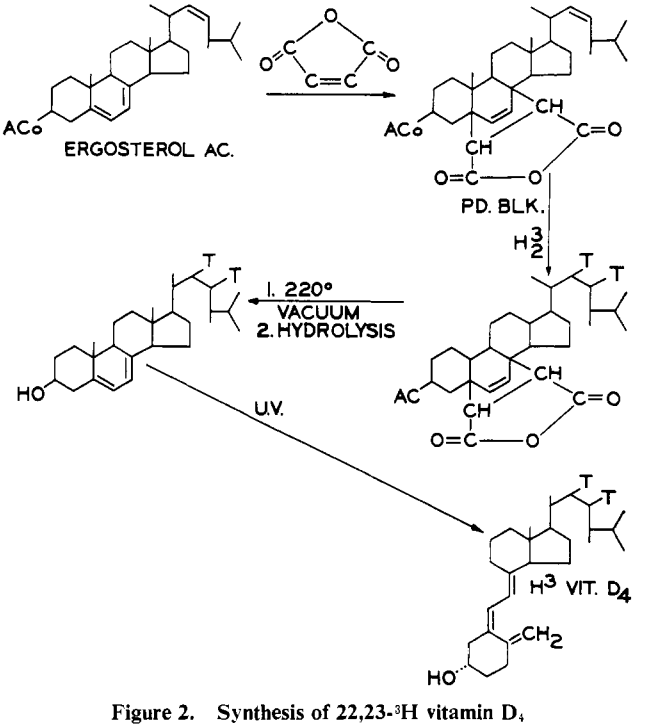

Figure 2. Synthesis of 22,23-^{3}H vitamin D_4

150,000 d.p.m./I.U.

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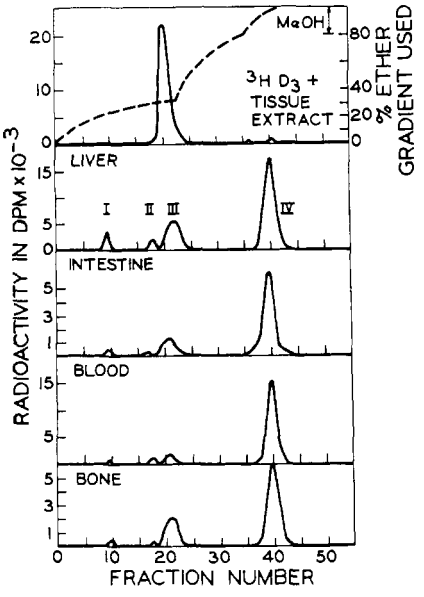

Figure 3. Silicic acid column chromatography of methanol-chloroform extracts of tissues from rats given 0.25 μg. of 1,2-^{3}H vitamin D$_3$ intravenously 12 hours previously

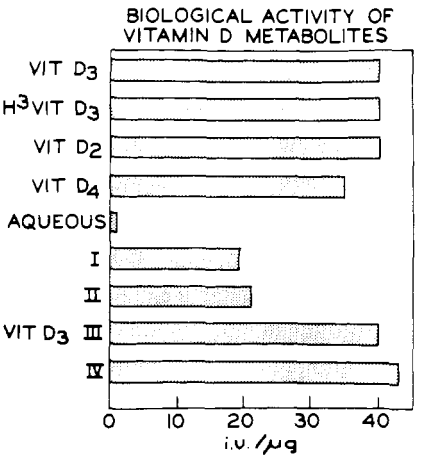

Figure 4. Biological activity of vitamin D$_3$ and its metabolite fractions

Biological activity assessed by line test assay method for vitamin D activity

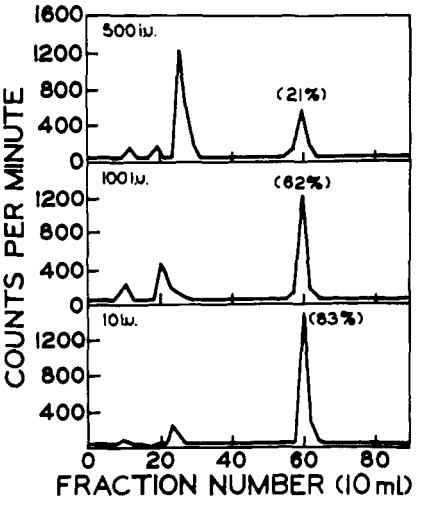

Figure 5. Effect of dose on distribution of ^{3}H among metabolite fractions after 1,2-^{3}H vitamin D$_3$

bypassed previously. After intravenous injection of radioactive vitamin D, tissues were removed and extracted by the methanol-chloroform procedure of Bligh and Dyer (1959). Some metabolic degradation products of vitamin D were readily apparent, in that some radioactivity was detected in the methanol-aqueous phase. These products were, however, biologically inactive and have not been studied further (Lund and DeLuca, 1966). However, chromatography of the $CHCl_3$ extract on silicic acid yielded four major radioactive components (Figure 3). Peak I was identified as an ester of vitamin D and long-chain fatty acids (Lund *et al.*, 1967), because on hydrolysis in base, the resulting radioactivity chromatographed as peak III which has been positively identified as unchanged vitamin D (Norman *et al.*, 1964). Furthermore, this substance gives full biological activity by the rickets cure test in rats (Lund *et al.*, 1966). Fraser and Kodicek (1965, 1968) also positively identified esters of vitamin D and provided evidence that the fatty acids are palmitic, linoleic, oleic, and steric. The ester fraction appears to have little physiologic significance in mammals, because their concentration remains small regardless of dose or time after dose (Lund *et al.*, 1966).

Peak II represents an unidentified metabolite which is approximately half as effective as vitamin D in curing rickets in rats. It does not appear to be either previtamin D or 5,6-*trans*-vitamin D. The quantity of this metabolite remains small regardless of dose or time after dose and is thus not considered of great significance. Peak III represents unchanged vitamin D$_3$ as determined by cochromatography and biological assay (Norman *et al.*, 1964). Of major interest is the peak IV metabolite fraction, since it probably represents the metabolically active form of the vitamin (DeLuca, 1967).

When bioassayed on the basis of radioactivity, this metabolite was at least as active as the parent vitamin in curing rickets in rats (Figure 4) (Lund and DeLuca, 1966). Furthermore, it produced all known physiologic effects of the vitamin, including bone mobilization and stimulation of intestinal transport of calcium. Surprisingly, however, it produced these effects more rapidly than vitamin D itself (Morii *et al.*, 1967).

When the dose of ^{3}H vitamin D$_3$ was reduced to physiologic doses—i.e., 10 I.U.—the proportion of radiaoctivity in this fraction greatly increased (Lund and DeLuca, 1966) (Figure 5). Efforts were also made to rule out artifactual production of this metabolite fraction. It soon became evident that the peak IV fraction was not a single radioactive substance and a more careful chromatographic procedure was developed (Ponchon and DeLuca, 1969) capable of separating peak IV into many fractions (Figure 6). When these fractions were biologically assayed for vitamin D activity, only that labeled as peak IV was biologically active.

The properties of the peak IV metabolite prompted an intensive effort in our laboratory to isolate and identify this metabolite. A method of isolation of the metabolite in pure form was devised (Blunt *et al.*, 1968a) (Figures 7 and 8). Hogs were chosen as the source of the material because preliminary experiments showed that large amounts of the metabolite could be induced in their blood by feeding large amounts of vitamin D (Blunt *et al.*, 1968a). Plasma was therefore obtained from four hogs fed for 26 days on 250,000 I.U. of D$_3$

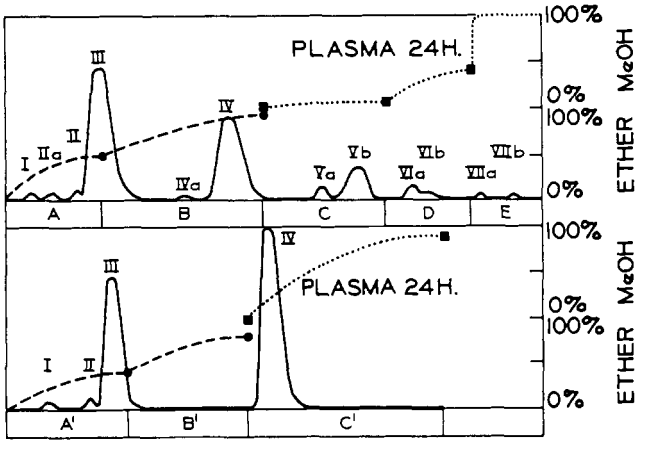

Figure 6. Chromatography of plasma extract on silicic acid using a refined gradient system

—— Radioactivity
- - - - - - - - - - Gradient
Vitamin D-deficient rats had received 0.25 μg. 1,2-^{3}H vitamin D$_3$ 24 hours before
A, *B*, *C*, etc. New system
A', *B'*, *C'*. Old chromatographic system

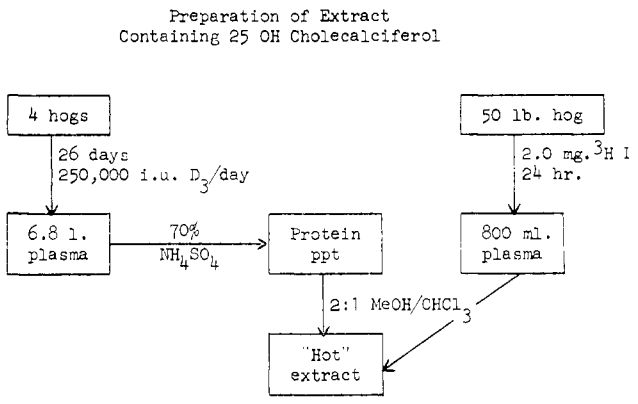

Figure 7. Preparation of extract for isolation of 25-hydroxycholecalciferol

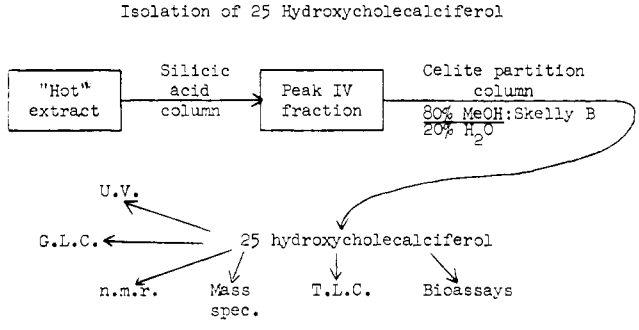

Figure 8. Flow diagram of isolation of 25-hydroxycholecalciferol from porcine plasma

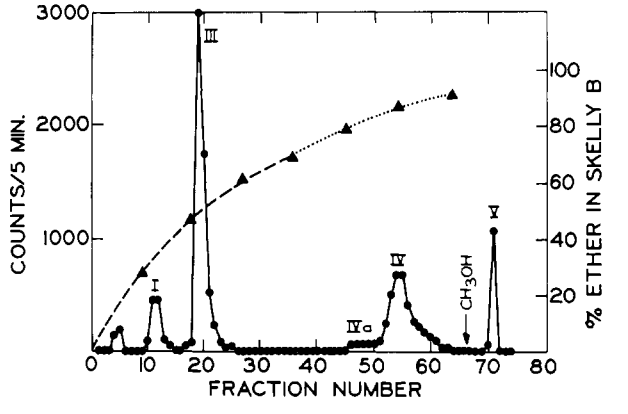

Figure 9. Silicic acid chromatography in isolation of 25-hydroxycholecalciferol from porcine plasma

per day. The protein of the plasma which binds the metabolite was precipitated out by means of 70% saturation with ammonium sulfate. The precipitate was then extracted with methanol and chloroform. A small pig was injected with ^3H vitamin D_3, the blood plasma was isolated and extracted, and the chloroform extract was combined with that obtained from the four pigs. The metabolite was isolated by silicic acid chromatography (Figure 9) followed by partition chromatography (Figure 10) to yield 1.3 mg. of pure metabolite (Blunt *et al.*, 1968a,b). The purity of the metabolite was established by TLC, GLC (Figure 11), and its ultraviolet spectrum (Figure 12). Its structure was unequivocally established as 25-hydroxycholecalciferol by a combination of techniques, including ultraviolet analysis, behavior on GLC to produce pyro and isopyro forms, mass spectra, and nuclear magnetic resonance spectra. Finally, it was synthesized by

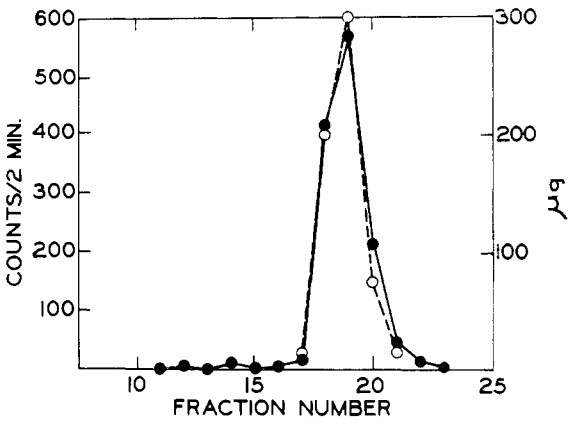

Figure 10. Partition chromatography of 25-hydroxycholecalciferol on Celite using 80% methanol, 20% water equilibrated with a petroleum fraction b.p. 65–67°C. ○ μg. 25 OH D_3 based on spectrum. ● Radioactivity

two independent methods (Blunt and DeLuca, 1969); the synthetic material is identical in all respects, including biological activity, to the isolated 25-hydroxycholecalciferol. Thus, its structure is firmly established (Figure 13).

The biological properties of 25-hydroxycholecalciferol provide strong evidence that it represents the metabolically active form of the vitamin (Blunt *et al.*, 1968). Table I shows that on a weight basis it is 1.4 times as active as cholecalciferol itself in curing rickets in rats. Results demonstrate a similar relationship in chicks. In a study of calcium transport by everted sacs of intestine, a 0.25-μg. intravenous dose of 25 OH D_3 produces a response in 3 hours while 6 to 10 hours are required for a similar response to vitamin D_3 (Figure 14). Likewise, a rise in serum calcium at the expense of bone occurs 4 hours after a 2.5-μg. dose of 25 OH D_3, while 12 hours is required for a similar response to 2.5 μg. of D_3 (Figure 15). Finally this substance induces bone resorption in culture at a concentration of 0.3 I.U. per ml., while as much as 500 I.U. per ml. of vitamin D_3 produces a questionable response (Trummel *et al.*, 1969). The resorption of "old" bone is considered an important physiologic action of vitamin D in vivo (DeLuca, 1967).

The 25-hydroxycholecalciferol is almost certain to be the metabolically active form of the vitamin, but only continued experiments with isolated systems will establish this important concept.

Finally the present study has shown that the nuclear membrane of intestinal mucosa accumulates a large amount of this metabolite, and in some way it initiates the transcription of a specific DNA into mRNA which codes for a calcium transport component (Stohs and DeLuca, 1967).

Table I. Comparative Effectiveness of 25-Hydroxycholecalciferol and Vitamin D_3 in the Cure of Rickets in Rats[a]

Type of Dosage[b]	25-OH D_3, I.U./μg.	Vit D, I.U./μg.
Oral (8)[b]	58 ± 5[c]	40 ± 4[c]
Oral (7)[b]	52 ± 3	38 ± 5
Intravenous (7)[b]	56 ± 2	41 ± 3

[a] Standard line test assay for vitamin D activity carried out as described in U. S. Pharmacopeia (1955).
[b] Number of rats per group.
[c] Standard deviation.

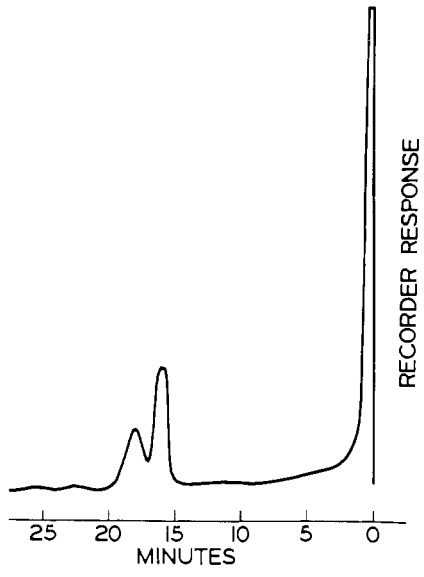

Figure 11. Gas-liquid chromatography of 25 - hydroxycholecalciferol demonstrating purity of isolated material from porcine plasma

Repeated attempts have not verified reports from another laboratory that the metabolite is found in the chromatin (Haussler *et al.*, 1968). Using high specific activity ^{3}H D_3 (160,000 d.p.m. per I.U.) or ^{3}H D_3 (26,000 d.p.m. per I.U.) which allows such an experiment to be carried out, no radioactivity could be found in intestinal chromatin isolated by the method of Marushige and Bonner (1966), a result which verifies our earlier failure to find radioactivity in deoxyribonucleoprotein isolated by the method of Zubay and Doty (1959).

The only other metabolite of vitamin D that has been reported is a sulfate ester in which the SO_4 is esterified to the 3-hydroxy group (Higaki *et al.*, 1965a,b). This has been shown to occur in homogenates of liver, using phosphoadenosylphosphosulfate as the sulfate donor. This substance is currently considered as an excretory product from massive amounts of vitamin D, but additional work must be carried out to establish its existence when physiologic amounts of vitamin D are given.

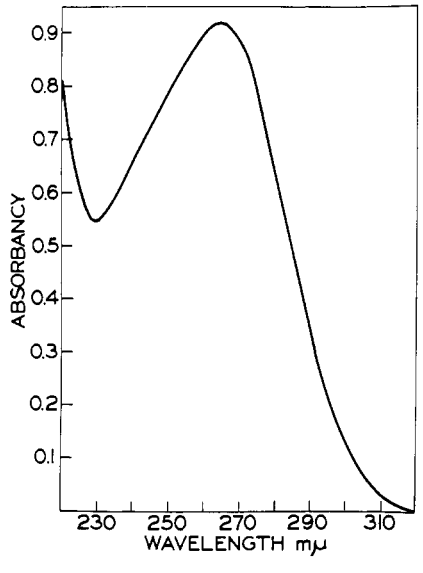

Figure 12. Ultraviolet spectrum of isolated 25-hydroxycholecalciferol in diethyl ether

Much work remains to be done on the metabolites of vitamin D. Many metabolites are as yet unidentified, and the enzymatic steps for virtually all reactions involving the vitamin D molecule also remain unknown. A start has been made, however, with the isolation and identification of the 25-hydroxycholecalciferol, which is probably the metabolically active form of vitamin D.

VITAMIN A

Virtually all the progress which has been made is the result of the synthesis of two specifically labeled vitamin A compounds by the Hoffman LaRoche group: 6,7-^{14}C retinoic

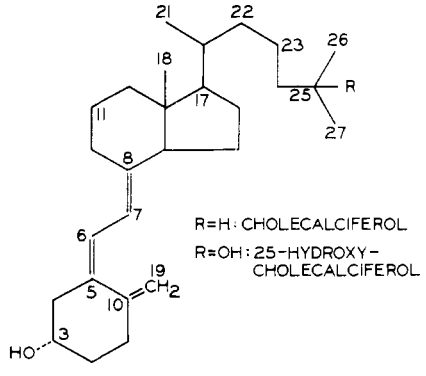

Figure 13. Structure of 25-hydroxycholecalciferol, believed to be metabolically active form of vitamin D_3

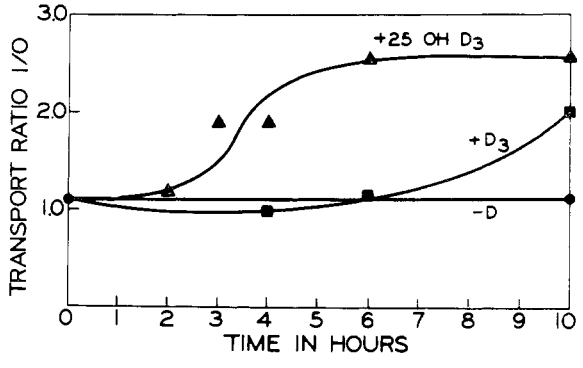

Figure 14. Response of calcium transport system of rats to 0.25 μg. intravenously of 25-hydroxycholecalciferol or vitamin D_3

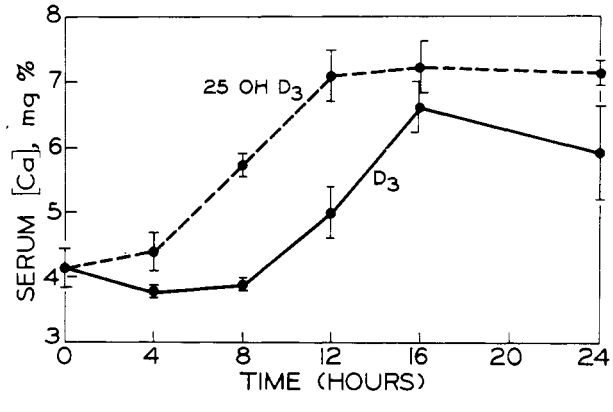

Figure 15. Serum calcium response of vitamin D-deficient rats on a low-calcium diet to 25-hydroxycholecalciferol or vitamin D_3

2.5 μg. of either compound in 0.02 ml. ethanol administered intravenously

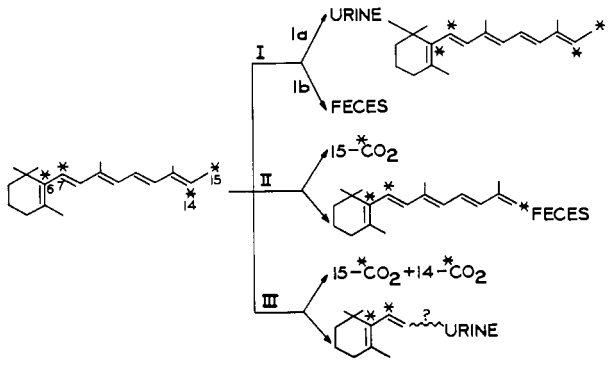

Figure 16. Proposed pathways of retinol or retinoic acid metabolism

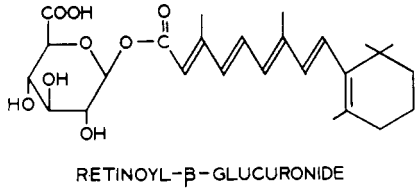

Figure 17. Retinoyl β-glucuronide

Table II. Recovery of ^{14}C from Rats Given Radioactive Retinoic Acid[a]

Fraction	Position of ^{14}C in Retinoic Acid (14.5 μg.) 6,7-^{14}C, % dose	14-^{14}C, % dose	15-^{14}C, % dose
Carbon dioxide	0.8 ± 0.4 (2)	18.9 ± 3.4 (3)	35.0 ± 4.5 (2)
Urine	38.0 ± 4.4 (4)	18.3 ± 0.3 (3)	19.9 ± 1.7 (5)
Feces	64.5 ± 1.3 (4)	61 (3)[b]	43.7 ± 3.0 (4)
Total recovery	103.3	98.2	98.6

[a] Means ± S.D. Number in parentheses indicates number of rats. [b] Values obtained were all low, because of bacterial decomposition of radioactive products in feces. A plot of the three values obtained *vs.* time lapse from completion of experiment to time of combustion of samples was linear and extrapolated to 61% recovery of dose at zero time.

acid and retinol, and 15-^{14}C retinoic acid and retinol. In addition, 14-^{14}C vitamin A compounds are available commercially (Tracerlab, Inc.).

These compounds have made possible some insight into the degradation of the vitamin A molecule (Roberts and DeLuca, 1967). If physiologic amounts of ^{14}C retinoic acid (14.5 μg. per rat) are administered intravenously and the pathways of the resulting ^{14}C excretion products are examined, the following data are obtained (Table II): From the 6,7-^{14}C positions, no $^{14}CO_2$ arises, whereas both 14-C and 15-C positions yield $^{14}CO_2$. As much as 30% of the dose of 15-^{14}C is excreted as CO_2 which is interpreted as an oxidative decarboxylative pathway. The remainder of the dose appears in the feces and urine. The 14 position yields less $^{14}CO_2$ but, nevertheless, represents an oxidative side-chain degradation. If the assumption is made that the urinary and fecal excretion products labeled with 15-^{14}C from retinoic acid represent intact molecule products, some analysis can be made in regard to the retinoic acid chain. From the recovery data, the following pathways of retinoic acid metabolism can be postulated to account for the excretion data (Figure 16). Pathway I represents intact side-chain excretion products, probably in large measure the retinoyl β-glucuronide isolated and identified by Olson and his coworkers (Dunagin *et al.*, 1965, 1966) (Figure 17). Olson and coworkers (Lippel and Olson, 1968) have reported that 90% or more of administered retinoic acid is excreted by this route. However, not more than 60% of injected retinoic acid has been identified as this product. Bile duct cannulated rats continue to metabolize 30% of 15-^{14}C retinoic acid to $^{14}CO_2$ (Roberts and DeLuca, 1968b), making it unlikely that retinoyl-β-glucuronide represents 90% of the excretory products of retinoic acid. A glucuronide of retinol is probably the major excretion form of retinol (Lippel and Olson, 1968), although its identification is incomplete at the present time. Only small amounts of retinoyl glucuronide are formed from retinol (Emerick *et al.*, 1967). The oxidatively decarboxylated retinoic acid yields a metabolite shorter by one carbon, as shown in pathway II (Figure 16), with this product appearing in the feces via the bile. A third pathway (III) involves oxidation of the side chain to some point between the 7 and 14 positions. This pathway is shown in Figure 16 as pathway III, yielding urinary products.

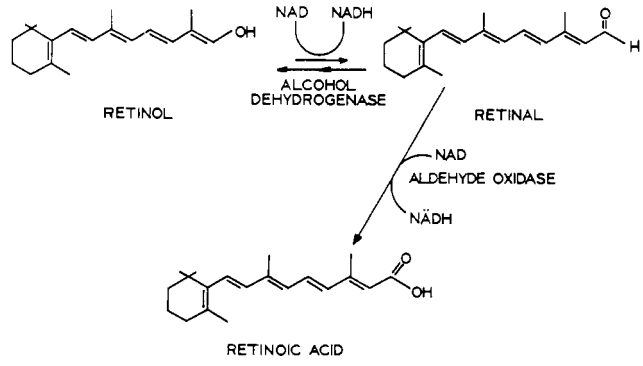

Figure 18. Conversion of retinol to retinoic acid

Table III shows that data similar to those obtained with retinoic acid can be obtained with retinyl acetate. Thus, the pathways for retinol metabolism and excretion are apparently similar. The metabolism of retinyl acetate is much slower than retinoic acid—for example, the ^{14}C recovery from 14.5 μg. of retinoic acid was complete in 48 hours, while that

Table III. Recovery of ^{14}C from Rats Given Radioactive Retinyl Acetate[a]

Fraction	Position of ^{14}C in Retinyl Acetate 6,7-^{14}C, (2.0 μg.) Recovery, % dose	Total,[b] %	2.0 μg. Recovery, % dose	Total,[b] %	(1.46 μg.) Recovery, % dose	Total,[b] %
Carbon dioxide	2.5 (2)	4.5	15 (2)	30.6	8.8 (2)	32.1
Urine	26.8 ± 2.8 (4)	48.2 ± 5.3	15.0 ± 2.0 (2)	30.6 ± 4.1	8.3 ± 0.8 (2)	30.3 ± 2.9
Feces	26.3 ± 5.0 (4)	47.3 ± 9.0	18.9 ± 2.6 (2)	38.6 ± 5.3	10.3 ± 2.5 (2)	37.6 ± 9.1
Total	55.6	100.0	48.9	100.0	27.4	100.00

[a] Values for urine and carbon dioxide represent radioactivity recovered after 4 days. Feces values represent 5-day collection to allow for time lag in elimination. [b] Per cent of total amount of radioactivity recovered in 4 days.

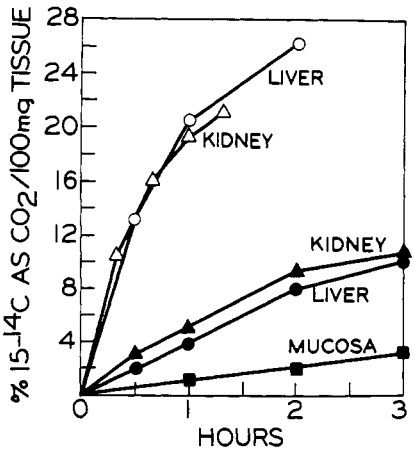

Figure 19. Rate of C-15 decarboxylation of retinoic acid by tissue slices

Each flask contained Krebs-Ringer phosphate and tissues from a retinol-deficient rat (●, ▲, ■) or Krebs-Ringer bicarbonate and tissue from a stock rat (○, △).

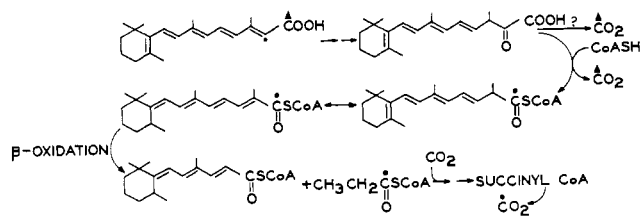

Figure 20. Proposed pathway of metabolism of 14-^{14}C retinoic acid in tissue slices

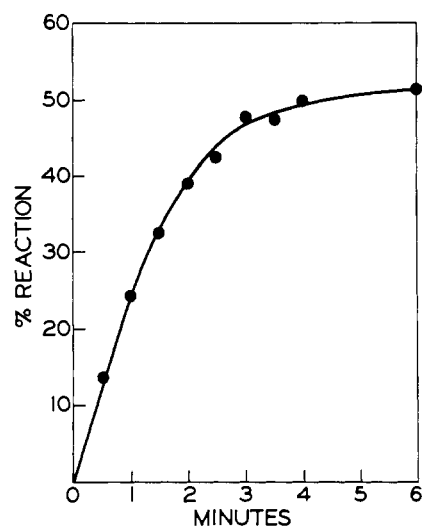

Figure 21. Rate of C-15 decarboxylation of retinoic acid by microsomes from rat liver

Reaction mixture as described in Table V

from 2.0 μg. of retinyl acetate was only 30% complete in 4 to 5 days. The pathway of retinol to retinoic acid is well known (Figure 18). Retinol is oxidized via a nonspecific alcohol dehydrogenase to retinal, requiring NAD (Futterman and Saslaw, 1961; Wald and Hubbard, 1950; Zachman and Olson, 1961). Retinal is also readily reduced to retinol by reversal of this reaction; thus the interconversion of retinol to retinal is not unexpected in vivo. Retinal is further oxidized to retinoic acid by the indicated reaction (Figure 18) (Dimitrovsky, 1961; Elder and Topper, 1962; Futterman, 1962; Mahadevan *et al.*, 1962). Furthermore, whether free retinoic acid is a significant intermediate in retinol metabolism is questionable, since only small amounts of retinoic acid as the glucuronide can be found in the bile after retinol administration (Dunagin *et al.*, 1964; Emerick *et al.*, 1967). That this reaction is irreversible in vivo is also well known, since retinoic acid cannot cure night blindness and reproductive failure due to retinol deficiency (Dowling and Wald, 1958, 1960; Thompson *et al.*, 1961a,b; Thompson, 1964). No storage of retinol in the liver results from even massive doses of retinoic acid (Arens and Van Dorp, 1964a,b; Sharman, 1949).

Table IV. Inhibitors of Retinoic Acid Metabolism to CO_2 by Kidney Slices[a]

Inhibitor	% Control $^{14}CO_2$ 15-^{14}C	14-^{14}C
$-O_2(N_2)$	46	..
+ malonate, $2 \times 10^{-2}M$	133	26
Fluoroacetate	98	18
Antimycin A 3, μg./ml.	110	22
DPPD $2 \times 10^{-7}M$	0	0

[a] Krebs-Ringer phosphate buffer used as medium. Incubations for 2 hours.

Table V. Requirements for Microsomal Oxidative Decarboxylation of Retinoic Acid[a]

Medium	% of Complete
Complete	100
− Fe^{2+}	59
− NADPH	12
− PP_i	65
− NADPH, Fe^{2+}, PP_i	12
− Microsomes	12
− NADPH, PP_i	12
− Fe^{2-}, PP_i	32

[a] Reaction mixture contained 3.6 mM of KCl, 5 mM $MgCl_2$, 5 mM phosphate buffer (pH 7.3); 88 mM NADPH, excess glucose 6-PO_4 dehydrogenase, 1 mM glucose-6-PO_4, 0.3 mM pyrophosphate (PP_i) and microsomes from liver.

Because of the irreversibility in vivo of retinoic acid to retinol, it has been postulated that an active metabolite of vitamin A must exist which is formed from either retinol or retinoic acid. A search for such a compound in several laboratories ensued with only 13-*cis*-retinoic acid (Zile *et al.*, 1967) and retinoyl glucuronide (Dunagin *et al.*, 1965, 1966) being positively identified as metabolites of retinoic acid. Many unidentified metabolites have been described, but no further information on these is available at the present time.

In this laboratory, work continued on the oxidative reactions of retinoic acid. It was possible to show that slices of liver and kidney can oxidatively decarboxylate retinoic acid in vitro (Figure 19) (Roberts and DeLuca, 1968a). Both the 15-^{14}C and 14-^{14}C labeled retinoic acids yielded $^{14}CO_2$. The production of $^{14}CO_2$ from 15-^{14}C retinoic acid was not inhibited by TCA cycle inhibitors (Table IV). The oxidation of 14-^{14}C retinoic acid was markedly inhibited by such things as fluoroacetate, malonate, or antimycin A. It is evident then that pathway II involves the scission of the retinoic acid side chain between the 7 and the 14 position. This piece of the side chain is then probably metabolized in some way via the TCA cycle (Figure 20).

The oxidative decarboxylation of the 15-^{14}C retinoic acid has now been traced to the microsomal fraction, as shown in Figure 21 (Roberts and DeLuca, 1968b). The reaction takes place in both liver and kidney microsomes and requires O_2, NADPH, Fe^{2+}, and a pyrophosphate moiety (Table V). The

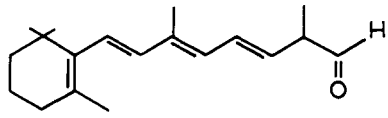

Figure 22. Proposed product of oxidative decarboxylation of retinoic acid

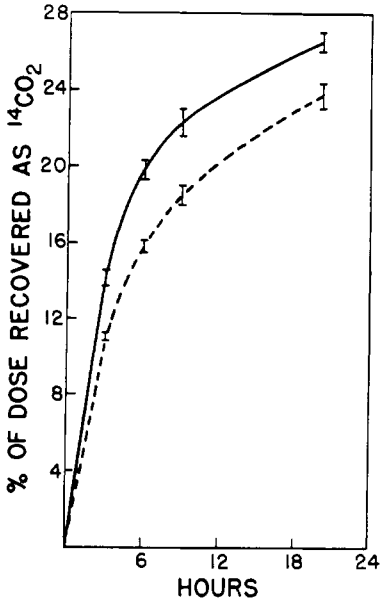

Figure 23. Effect of DPPD on in vivo decarboxylation of 15-^{14}C retinoic acid

Rats given 14.5-μg. intravenous injection of 15-^{14}C retinoic acid. One half the rats were fed a diet containing 0.01 to 0.05% DPPD and given an oral dose of 2 mg. DPPD on oil 3 hours before the experiment.
—— No DPPD
- - - +DPPD

pyrophosphate can be supplied by many nucleotides, including thiamine pyrophosphate, but is specific only for pyrophosphate. Boiled microsomes do not catalyze the reaction, while ascorbate plus boiled microsomes will. This reaction is inhibited by *p*-chloromercuribenzoate, $K_3Fe(CN)_6$, EDTA, phenozine methosulfate, and *N,N'*diphenyl-*p*-phenylenediamine (DPPD), but not by SKF-525 A, phenobarbital, and aminopyrine. In this regard the reaction resembles a lipid peroxidation system and probably involves a free radical mechanism. It does not appear to be a microsomal hydroxylation reaction. The reaction product has not yet been identified, although all evidence suggests that the first product is a 14-C aldehyde (Figure 22). Unfortunately, proof of this structure is lacking at the present time.

An attempt was made to assess the in vivo importance of the microsomal decarboxylation system by administering DPPD in vivo to rats and observing its effect on $^{14}CO_2$ production from 15-^{14}C retinoic acid (Roberts and DeLuca, 1969). The data show approximately a 20% inhibition by DPPD (Figure 23). Thus at least a portion of the in vivo decarboxylation of retinoic acid is related to the microsomal reaction.

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